

Mechanisms of Allergen-Specific Immunotherapy: T-Regulatory Cells and More

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T-cell tolerance is characterized by the functional inactivation of the cell to antigen encounter while remaining alive for an extended period in an unresponsive state. In recognition of the importance of immunologic tolerance, the 1960 Nobel Prize in Physiology and Medicine was awarded to Medawar [1] for discovering that skin allografts in mice and chicken can be accepted if they are preinoculated during embryonic development with allogeneic lymphoid cells, and to Burnet [2,3] for first proposing that exposure to antigens before the development of immune response specifically abrogates the capacity to respond to that antigen in later life. During the past decade, this area of immunologic research has gained much attention and popularity. Overall, studies on T-cell unresponsiveness suggest that anergy, tolerance, and active suppression are not entirely distinct but rather represent linked mechanisms that may involve the same molecular events. The term *anergy* was first coined by Von Pirquet [4] in 1908 to describe the loss of delayed-type hypersensitivity to tuberculin in individuals infected with measles virus. The term has been clinically accepted to describe negative tuberculin skin test results in conditions in which they are expected to be positive. In 1980, *anergy* was redefined to describe the specific inactivation of B cells in mice by high doses of antigen [5]. It was subsequently used for T cells to describe a phenomenon in which antigen presentation to T-cell clones in the absence of professional antigen-presenting cells (APC) induced a

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hyporesponsive state characterized by subdued interleukin (IL)-2 production and proliferation on restimulation [6].

In early studies, which serve as the basis for the definition of anergy/tolerance, functional unresponsiveness was analyzed through nonsophisticated assays such as IL-2 measurement and the determination of total IgG production. In addition, until recently antigens used in mouse models contained high amounts of impurities, such as lipopolysaccharides and other innate immune response-triggering substances, which may influence the outcome of experiments. Although some biochemical steps overlap with anergy, activation-induced cell death induced through the triggering of death receptors and caspase activation represents a distinct physiologic response [7,8]. Target cells do not survive in an unresponsive state but are deleted through apoptosis.

Why exposure to allergens leads to atopic disorders in some individuals but not others is still not understood. However, a strong interaction between environmental and genetic factors is clearly involved. During allergic inflammation, four cardinal events involving memory/effector T cells and other effector cells, such as mast cells, eosinophils, and basophils, can be classified as (1) activation, (2) organ-selective homing, (3) prolonged survival and reactivation inside the allergic organs, (4) and effector functions [9,10]. T cells are activated by aero-allergens, food antigens, autoantigens, and bacterial superantigens in allergic inflammation [11,12]. They are under the influence of skin-, lung-, or nose-related chemokine networks and they show organ-selective homing [13–15]. A prolonged survival of the inflammatory cells and strong interaction with resident cells of the allergic organ and consequent reactivation are observed in the sub-epithelial tissues [16,17]. T cells play important effector roles in atopic dermatitis and asthma through the induction of hyper IgE production, eosinophil survival, and mucus hyperproduction (Fig. 1) [11,17,18]. In addition, activated T cells induce apoptosis of bronchial epithelial cells and keratinocytes as major tissue injury events [19–22]. Peripheral T-cell tolerance to allergens can overcome the pathologic events in allergic inflammation because they all require T-cell activation.

The initial event responsible for the development of allergic diseases is the generation of allergen-specific CD4⁺ T helper cells. The current view is that under the influence of IL-4, naïve T cells activated by APC differentiate into T helper 2 (Th2) cells [23–25]. Once generated, effector Th2 cells produce IL-4, IL-5, and IL-13 and exert several regulatory and effector functions. These cytokines induce production of allergen-specific IgE by B cells, development and recruitment of eosinophils, production of mucus, and contraction of smooth muscles [23,24,26]. Furthermore, the degranulation of basophils and mast cells by IgE-mediated cross-linking of receptors is the key event in type I hypersensitivity, which may lead to chronic allergic inflammation. Although Th2 cells are responsible for the development of allergic diseases, T helper 1 (Th1) cells may contribute to chronicity and effector phase in allergic diseases [19–22,27,28]. Distinct Th1 and Th2 subpopulations of T cells counter-regulate each other and play a role in distinct diseases [23,24]. In addition, a further subtype of T cells that have immunosuppressive function and cytokine profiles distinct from either

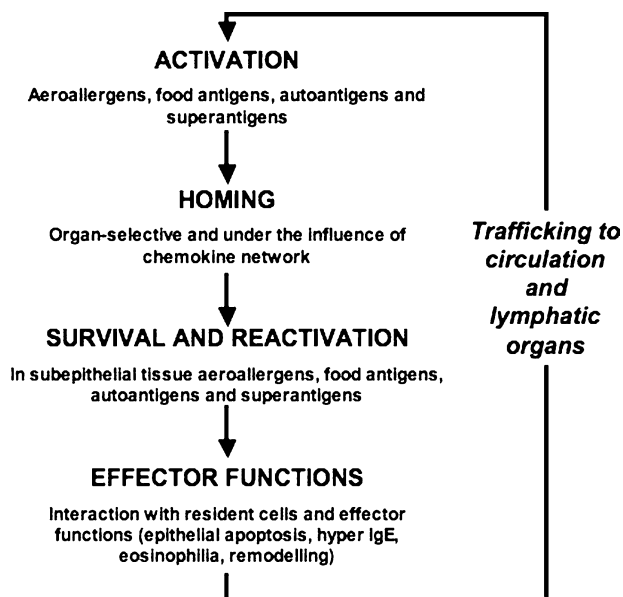


Fig. 1. The four sequential processes characterizing allergic inflammation. Various antigens or yet unidentified factors activate T cells; these cells then undergo organ-selective homing according to the influence of organ-related chemokine networks. T cells within subepithelial tissues show increased survival and are continuously stimulated. These activated T cells then trigger effector functions, including apoptosis, hyper IgE, and eosinophilia. Increasing allergen-specific Treg-cell numbers may abrogate all of these events and lead to a healthy immune response against allergens.

Th1 and Th2 cells, termed *regulatory/suppressor T cells (Treg)*, has been described [29–34]. In addition to Th1 cells, Treg cells are able to inhibit the development of allergic Th2 responses [35] and play a major role in allergen-specific immunotherapy [32,36]. This article examines allergen-specific peripheral tolerance mechanisms in humans and discusses novel methods of T-cell suppression.

Peripheral T-cell tolerance to allergens in healthy immune response and allergen-specific immunotherapy

The symptoms of IgE-mediated allergic reactions, such as rhinitis, conjunctivitis, and asthma, can be alleviated through temporary suppression of inflammatory mediators and immune cells using agents such as antihistamines, antileukotrienes, β_2 -adrenergic receptor agonists, and corticosteroids [37–40]. However, the only long-term solution is allergen-specific immunotherapy, which specifically restores normal immunity to allergens. Allergen-SIT is most efficiently used in allergy to insect venoms and allergic rhinitis [41–45]. In 1911, the original report of Noon [46] suggested that grass pollen extracts used for immunotherapy of hay fever induced a toxin that caused allergic symptoms. Researchers suggested that

in response to injection of pollen extract, antitoxins develop and prevent the development of disease. Neutralizing antibodies have been shown to generate during allergen-specific immunotherapy [47,48]. Despite being used in clinical practice for nearly a century, the underlying immunologic mechanisms of allergen-specific immunotherapy are slowly being elucidated [36,49–54]. A rise in allergen-blocking IgG antibodies, particularly of the IgG4 class which supposedly block allergen and IgE-facilitated antigen presentation [55–57]; the generation of IgE-modulating CD8⁺ T cells [58]; and a reduction in the numbers of mast cells and eosinophils, including the release of inflammatory mediators [59–61], were shown to be associated with successful allergen-specific immunotherapy. Furthermore, allergen-specific immunotherapy was found to be associated with a decrease in IL-4 and IL-5 production by CD4⁺ T cells [45,62,63]. Also, a shift from a Th2 cytokine pattern toward increased interferon (IFN)- γ production in allergen-specific immunotherapy of allergy to bee venom, wasp venom, grass pollen, and house dust mite (HDM) has been observed [62,64]. The induction of a tolerant state in peripheral T cells, however, seems to represent the crucial step in allergen-specific immunotherapy (Fig. 2) [32,36,49,52]. Peripheral T-cell tolerance is characterized mainly by suppressed proliferative and cytokine responses against allergens and their isolated T-cell recognition sites (epitopes) [49]. T-cell tolerance is initiated by the autocrine action of IL-10, which is increasingly produced by antigen-specific T cells [32,52]. Tolerized T cells can be reactivated to produce either distinct Th1 or Th2 cytokine patterns, depending on the cytokines present in the tissue microenvironment, leading to either a successful or unsuccessful outcome in allergen-specific immunotherapy [49].

Most studies examined cultures of peripheral blood mononuclear cells (PBMCs). Whether these events reflect the changes in the immune response in the tissues is of interest. T-cell responses after grass pollen immunotherapy have been examined in nasal mucosal and skin tissue. Increased numbers of IL-10 mRNA-expressing cells were shown after allergen-specific immunotherapy with grass pollen during the pollen season. However, in contrast to the findings in peripheral blood, IL-10 was not increased in nonatopic subjects exposed during the pollen season. In addition, the studies showed reduced accumulation of T cells in skin and nose after allergen challenge but no decrease in T cell numbers during pollen season. Increased Th1 activity was shown in the skin and nasal mucosa [65–67], whereas increases in IFN- γ observed after allergen challenge outside the pollen season correlated with the clinical improvement [68]. During the summer pollen season, increases in the levels of IFN- γ and IL-5 were observed, with the ratio in favor of IFN- γ [69]. However, that modulation of peripheral immune responses is apparently pivotal for the effects of allergen-specific immunotherapy. Local tissue responses do not necessarily reflect peripheral tolerance and are dependent on several mechanisms, such as cell apoptosis, migration, homing, and survival signals, which are highly dependent on natural allergen exposure and environmental factors [10].

Individuals who are healthy and those who have allergies exhibit all three subsets of CD4⁺ T cells, but in different proportions. In healthy individuals,

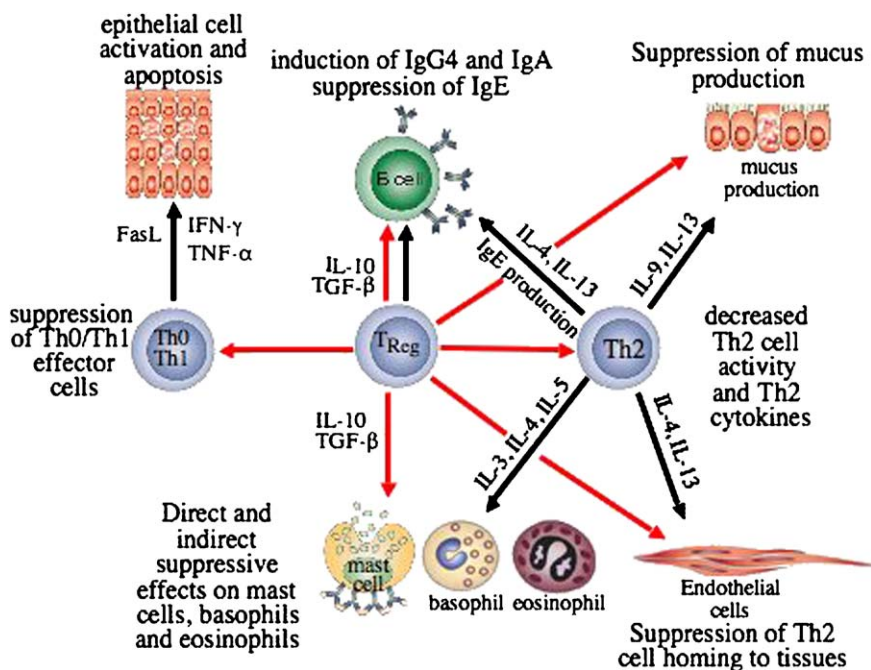


Fig. 2. Peripheral tolerance mechanisms in allergen-specific immunotherapy and healthy immune response to allergens. Immune deviation toward Treg-cell response is an essential step in allergen-specific immunotherapy and natural allergen exposure of individuals who are nonallergic. Treg cells use multiple suppressor factors that influence the final outcome of allergen-specific immunotherapy. IL-10 and TGF- β induce IgG4 and IgA, respectively, from B cells as noninflammatory Ig isotypes, and suppress IgE production. These two cytokines directly or indirectly suppress effector cells of allergic inflammation, such as mast cells, basophils, and eosinophils. In addition, Th2 cells, which are dominated by Treg cells, can no longer induce IgE through IL-4 and IL-13 and cannot provide cytokines such as IL-3, IL-4, IL-5, IL-9, and IL-13, which are required for the differentiation, survival, and activity of mast cells, basophils, and eosinophils and mucus-producing cells. In addition, migration of the inflammatory cells to the affected organs is controlled by Th2 cell-related chemokines and adhesion factors, which is also inhibited directly or indirectly by Treg cells and their cytokines. Moreover, suppression of the induction of Th0/Th1 cells abrogates tissue injury mechanisms, such as apoptosis of keratinocytes and bronchial epithelial cells, through interferon- γ , TNF- β , and Fas-ligand (FasL). The gray line designates suppression and the black line stimulation.

type 1 T regulatory (Tr1) cells represent the dominant subset for common environmental allergens, whereas a high frequency of allergen-specific IL-4 secreting (Th2) cells is found in individuals who are allergic [70]. Hence, a change in the dominant subset may lead to either the development of allergy or recovery from the disease. Peripheral tolerance to allergens involves multiple suppressive factors, such as IL-10, TGF- β , cytotoxic T-lymphocyte antigen-4 (CTLA-4), and programmed death-1 (PD-1) [70]. Accordingly, allergen-specific peripheral T-cell suppression mediated by IL-10 and TGF- β and other suppressive factors and a deviation toward a Treg-cell response were observed in normal immunity as key

events in the healthy immune response to mucosal antigens. The analysis of other cytokines of the IL-10 family, such as IL-19, IL-20, IL-22, IL-24, and IL-26, showed that suppressor capacity for allergen/antigen-stimulated T cells is only a function of IL-10 in this family [71].

Unlike in mucosal allergies, no increase in TGF- β production was observed during specific immunotherapy in venom allergy. Differences in the control mechanisms that regulate the immune response to venoms and aeroallergens might be caused by different routes of natural allergen exposure and the induction of chronic events of allergic inflammation, leading to tissue injury and remodeling in the latter case. In humans, increasing evidence suggests that Treg cells play a major role in the inhibition of allergic disorders. Reports have shown that IL-10 levels in the bronchoalveolar-lavage fluid of patients who have asthma are lower than in healthy controls, and T cells in children who have asthma also produce less IL-10 mRNA than do T cells in healthy children [72,73]. Although some reports imply a role for TGF- β in the pathogenesis of asthma, particularly in remodeling of injured lung tissue in humans [74], a recent report indicates that the increased allergic inflammation observed after blocking CTLA-4 is clearly associated with decreased TGF- β levels in the bronchoalveolar-lavage fluid of mice [75].

Peripheral T-cell tolerance to allergens is associated with regulation of antibody isotypes and suppression of effector cells

The serum levels of specific IgE and IgG4 antibodies delineate allergic and normal immunity to allergen. Although peripheral tolerance has been shown in specific T cells, the capacity of B cells to produce specific IgE and IgG4 antibodies was not abolished during specific immunotherapy [49]. In fact, specific serum levels of both isotypes increased during the early phase of treatment. However, the increase in antigen-specific IgG4 was more pronounced and the ratio of specific IgE to IgG4 decreased by 10 to 100 fold. Also, the in vitro production of phospholipase A₂-specific IgE and IgG4 antibodies in PBMCs changed concomitantly with the serum levels of specific isotypes. A similar change in specific isotype ratio was observed in specific immunotherapy of various allergies. Moreover, the production of IL-10, which is induced and increases during allergen-specific immunotherapy, seems to counter-regulate antigen-specific IgE and IgG4 antibody synthesis [32]. IL-10 is a potent suppressor of total and allergen-specific IgE, while simultaneously increasing IgG4 production [32,76]. Thus, IL-10 not only generates tolerance in T cells but also regulates specific isotype formation and skews the specific response from an IgE- to an IgG4-dominated phenotype (see Fig. 2). The healthy immune response to *Dermatophagoides pteronyssinus* (Der p 1) showed increased specific IgA and IgG4, small amounts of IgG1, and almost undetectable IgE antibodies in serum [36]. Although HDM-specific immunotherapy did not significantly change specific IgE levels after 70 days of treatment, a significant increase in specific IgA, IgG1, and IgG4 was observed [36]. The increase of specific IgA and IgG4 in

serum coincides with increased TGF- β and IL-10, respectively. These coincident increases may account for the role of IgA, TGF- β , IgG4, and IL-10 in peripheral mucosal immune responses to allergens in healthy individuals [32,77].

As early as the 1930s, Cooke and colleagues [47] suggested the induction of blocking antibodies through specific immunotherapy. Lichtenstein and colleagues [48] assigned these blocking antibodies to the IgG subclass. Research focusing on the subclasses of IgG antibodies suggested that IgG4 in particular captures the allergen before reaching the effector cell-bound IgE, thereby preventing the activation of mast cells and basophils. In fact, many studies showed increases in specific IgG4 levels in association with clinical improvement [78,79]. In the case of venom allergy, the rise of antivenom IgG correlates, at least at the onset of desensitization, with protection achieved through the treatment [80,81]. The concept of blocking antibodies has recently been reevaluated. Blocking antibodies seem to inhibit not only allergen-induced release of inflammatory mediators from basophils and mast cells but also IgE-facilitated allergen presentation to T cells, and prevent allergen-induced boost of memory IgE production during high allergen exposure during pollen season. Grass pollen immunotherapy has been shown to induce allergen-specific, IL-10-dependent protective IgG4 responses [82]. The data established an absolute association between IgG4-dependent blocking of IgE-binding to B cells in patients who underwent immunotherapy, and a trend toward a correlation with clinical efficacy. Measuring the blocking activity of allergen-specific IgG rather than its crude levels in sera seems to be preferable. This preference can explain the lack of correlation between antibody concentration and degree of clinical improvement. However, IgG4 antibodies can be viewed as having the ability to modulate the immune response to allergens and thus the potential to influence the clinical response. In a study using well-defined recombinant allergen mixtures, all treated subjects developed strong allergen-specific IgG1 and IgG4 antibody responses [83]. Some patients were not sensitized to *Phleum pratense* (Phl p 5) but nevertheless developed strong IgG antibody responses to that allergen. Researchers have suggested that subjects who do not have specific IgE against a particular allergen do not experience a significant IgG4 response [84], but recent studies do not support this view and are consistent with induction of a tolerant immune response together with induction of allergen-specific IgG4 [83].

An early effect of allergen-specific immunotherapy is an efficient modulation of the thresholds for mast cell and basophil degranulation. Although a definite decrease in IgE antibody levels and IgE-mediated skin sensitivity normally requires several years of allergen-specific immunotherapy, most patients are protected against bee stings already at an early stage of bee venom-specific immunotherapy. This early sensitivity occurs because effector cells of allergic inflammation, such as mast cells, basophils, and eosinophils, require T-cell cytokines for priming, survival, and activity [85, 86], but these cytokines are not efficiently provided by suppressed Th2 cells and generated Treg cells (see Fig. 2). In addition, IL-10 was shown to reduce proinflammatory cytokine release from mast cells [87]. IL-10 also down-regulates eosinophil function and activity and

suppresses IL-5 production by human resting Th0 and Th2 cells [88]. Moreover, IL-10 inhibits endogenous granulocyte-macrophage colony-stimulating factor production and CD40 expression by activated eosinophils and enhances eosinophil cell death [89].

Peripheral T-cell tolerance by inhibition of T-cell costimulation

Inhibition of T-cell costimulatory molecules at the cell surface or through their intracellular signal transduction has repeatedly been reported to play an important role in T-cell tolerance [90–92]. T cells can be anergized in experimental animal models that bypass costimulatory signals [6,93,94]. The interaction between B7 and CD28 may determine whether a T-cell response develops. Blocking antibodies to B7-2 inhibits the development of specific IgE, pulmonary eosinophilia, and airway hyperresponsiveness in mice [95]. CTLA-4, a molecule on activated T cells, seems to act as an endogenous inhibitor of T-cell activation. CTLA-4-Ig, a soluble fusion-protein construct, is also effective in blocking airway hyperresponsiveness in mice [96]. Anti-CD28, anti-B7-2, and CTLA-4-Ig also block the proliferative response of T cells to allergen [97]. Furthermore, cytokine production by memory/effector T cells, particularly those of the Th2 subset, is highly dependent on costimulation through the ICOS/B7RP1 (ICOS-ligand) pathway. Blockage [98] or genetic disruption [99] of this costimulatory pathway markedly reduced allergen-induced asthma in mice. Thus, blockage of B7-2/CD28 and ICOS/B7RP1 interaction may be a promising approach for treating allergic disease in humans.

One mechanism of direct T-cell suppression by IL-10 is through inhibition of CD28 costimulation. IL-10 inhibits the proliferative T-cell response in PBMCs to various antigens, and the superantigen staphylococcal enterotoxin B [100]. However, IL-10 does not affect the proliferative responses of T cells stimulated with anti-CD3 monoclonal antibody (mAb). Analysis of the activity of T-cell receptors (TCRs) on T cells showed the essential requirement for costimulation in T-cell activation and its relation to the number of triggered TCRs [100]. IL-10 inhibited the T-cell proliferation within the range of triggered TCRs that require T-cell costimulation. T cells, which were stimulated with varying concentrations of anti-CD3, and a constant amount of anti-CD28 showed that low numbers of triggered TCRs required CD28 costimulation. Thus, IL-10 suppressed only those T cells that had low numbers of triggered TCRs and required CD28 for proliferation [100].

Stimulation of CD28 by B7 surface molecules leads to tyrosine phosphorylation of CD28. Ligation of IL-10 receptor (IL-10R) on CD28 stimulation inhibits tyrosine phosphorylation of CD28, as detected after 10 minutes [100,101]. The inhibitory effect of IL-10 on CD28 seemed to be specific for the CD28 pathway because IL-10 did not affect ZAP-70 tyrosine phosphorylation stimulated by CD3 cross-linking. As a consecutive event for signal transduction, the

association of CD28 with the phosphatidylinositol 3-kinase (PI3-K) p85 molecule was inhibited by IL-10. This inhibition can be blocked specifically by preventing the binding of IL-10 to its receptor with an anti-IL-10R mAb. Binding of PI3-K to CD28 occurs through direct interaction between SH2 domain motifs of p85 PI3-K and a (p)YXXM motif in the cytoplasmic part [102]. This binding leads to a downstream signaling cascade in the transcription of various T-cell activation genes.

Although no clinical studies have related inhibition of costimulation to the specific treatment of allergy and asthma, it remains a fruitful approach to the treatment of autoimmunity and allergy.

T-regulatory cells

Since the mid-1990s, the concept of T-cell-mediated immune suppression has been strongly explored. Many types of suppressor T cells have been described in several systems, and their biology has been the subject of intensive investigation. Although many aspects of the mechanisms through which suppressor cells exert their effects have not been elucidated, it is well established that Treg cells suppress immune responses through cell-to-cell interactions or the production of suppressor cytokines (Table 1) [31,32,52,103].

Type-1 T regulatory cells

Tr1 cells are defined by their ability to produce high levels of IL-10 and TGF- β [31,103]. Tr1 cells specific for various antigens arise *in vivo*, but may also

Table 1
Regulatory/suppressor cells and their subsets

Regulatory/suppressor cells	Suppressor mechanism ^a
T cells	
Tr1	IL-10, TGF- β , CTLA-4, PD-1
Th3	TGF- β
CD4 ⁺ CD25 ⁺ Treg	IL-10, TGF- β , CTLA-4, PD-1, GITR
CD8 ⁺ CD25 ⁺ CD28 ⁻ Treg	Same as CD4 ⁺ CD25 ⁺
CD4 ⁻ CD8 ⁻ Treg	Induction of apoptosis
TCR- γ/δ Treg	IL-10, TGF- β
B-cell subset	IL-10
DC subset	IL-10
NK-cell subset ^b	IL-10
Macrophages	IL-10, TGF- β
Resident tissue cells ^b	IL-10, TGF- β

Abbreviations: DC, dendritic cell; NK, natural killer.

^a Multiple other suppressive mechanisms may exist.

^b NK cells and resident tissue cells are included in the table because these cells express suppressive cytokines.

differentiate from naïve $CD4^+$ T cells. Tr1 cells have a low proliferative capacity, which can be overcome by IL-15 [104]. Tr1 cells suppress naïve and memory Th1 and Th2 responses through the production of IL-10 and TGF- β [103]. The use of Tr1 cells in identifying novel targets for the development of new therapeutic agents, and as a cellular therapy to modulate peripheral tolerance in allergy and autoimmunity, can be foreseen [52,105,106].

In vitro generation of Tr1 cells through stimulating naïve $CD4^+$ T cells in the presence of IL-10, IFN- α , or a combination of IL-4 and IL-10 has been reported [31,103]. To overcome problems in the cytokine profiles of Tr1 cells, a combination of vitamin D₃ and dexamethasone was shown to induce human and mouse naïve $CD4^+$ T cells to differentiate into Tr1 cells in vitro [107]. In contrast to the in vitro-derived $CD4^+$ T cells, these cells produced only IL-10 and no IL-5 and IFN- γ , retained strong proliferative capacity, and prevented central nervous system inflammation in an IL-10-dependent manner. Clear evidence now exists that IL-10- or TGF- β -producing Tr1 cells are generated in vivo in humans during the early course of allergen-specific immunotherapy, suggesting that high and increasing doses of allergens induce Tr1 cells in humans [32,36,108].

Regulatory/suppressor T-cell clones have been induced through oral feeding of low doses of antigen in a TCR-transgenic experimental encephalitis model [29,109]. $CD4^+$ T-cell clones isolated from mesenteric lymph nodes in orally tolerated animals produced high levels of TGF- β and variable amounts of IL-4 and IL-10 on activation with appropriate antigen or anti-CD3 antibody [29]. These cells functioned in vivo to suppress encephalitis induction with myelin basic protein and were designated *Th3 cells*. TGF- β and IL-10 seemed critical because treatment with neutralizing antibodies abrogated the disease-protective effects of these cells. These Treg cells also exerted bystander immune suppression in vitro. The immunologic features of Th3 and Tr1 cells suggest that they can be of the same T-cell subset.

CD4⁺ CD25⁺ regulatory T cells

Clear evidence from various animal models and human studies shows an active mechanism of immune suppression whereby a distinct subset of T cells inhibits the activation of conventional T cells in the periphery [110–113]. This Treg cell population was determined to be $CD4^+$ $CD25^+$ T cells. $CD4^+$ $CD25^+$ T cells constitute 5% to 10% of peripheral $CD4^+$ T cells and express the IL-2 receptor α chain (CD25) [110] and the characteristic transcription factor FoxP3 [114]. They can prevent the development of autoimmunity, indicating that the normal immune system contains a population of professional Treg cells. Elimination of $CD4^+$ $CD25^+$ Treg cells leads to spontaneous development of various autoimmune diseases, such as gastritis or thyroiditis, in genetically susceptible hosts. In mice, these cells have been shown to express $CD45RB^{\text{low}}$ [30]. The $CD38^-CD25^+CD4^+CD45RB^{\text{low}}$ subpopulation contains T cells that respond to recall antigens and produce high levels of cytokines in response to polyclonal stimulation. In contrast, the $CD38^+$ cells within this subpopulation do not proliferate.

erate or produce detectable levels of cytokines, and inhibit anti-CD3–induced proliferation by the CD38[−] population [115].

Other regulatory T cells

Researchers have proposed that, in addition to CD4⁺ T cells, CD8⁺ Treg cells may have a role in oral tolerance [116,117]. Recent efforts to generate suppressor cell lines in vitro resulted in a population of CD8⁺ CD28[−] T cells, restricted by allogeneic class I HLA antigens, that were able to prevent up-regulation of B7 molecules induced by Th cells on APCs [118]. This outcome resulted in the suppression of CD4⁺ T cells in an HLA-nonrestricted fashion [118]. The magnitude of a CD8⁺ T-cell–mediated immune response to an acute viral infection is also subject to control by CD4⁺ CD25⁺ Treg cells. If natural Treg are depleted with specific anti-CD25 antibody before infection with virus, the resultant CD8⁺ T-cell response is significantly enhanced, suggesting that controlling suppressor effects at vaccination could result in more effective immunity [119].

Double-negative (CD4[−] CD8[−]) TCR $\alpha\beta$ ⁺ Treg cells that mediate tolerance in several experimental autoimmune diseases have been described [120]. These double-negative T cells are specific for class I major histocompatibility complex molecules. The suppressive effect of these cells on the proliferation and cytotoxic activity of CD8⁺ T cells with the same antigen specificity was not mediated by cytokines, but was instead attributed to Fas-mediated apoptosis of alloreactive T cells [121].

γ/δ T cells with regulatory functions have also been described. A population of γ/δ Treg cells with a cytokine profile reminiscent of Tr1 cell clones has been isolated from tumor-infiltrating lymphocytes [122]. These Treg cells could play a role in inhibiting immune responses to tumors [123]. Studies have also shown that aerosol delivery of protein antigens resulted in the differentiation of γ/δ T cells with regulatory functions [122]. Induction of tolerance through various doses of ovalbumin (OVA) has been shown to be abrogated in mice lacking TCR δ [124]. In contrast, TCR γ/δ -deficient mice have the same degree of IgE-specific unresponsiveness after aerosol priming and immunization with OVA [125].

Other regulatory cell types

One study has recently proposed a regulatory role for IL-10–secreting B cells [126]. These B cells prevented the development of arthritis, and their suppressive effect was particularly IL-10–dependent because the B cells isolated from IL-10–deficient mice did not protect from arthritis.

Some indications exist that dendritic cells (DCs) can induce peripheral T-cell tolerance and that a regulatory DC subset may exist. Pulmonary DCs from mice exposed to respiratory antigen transiently produce IL-10 [127]. These phenotypically mature pulmonary DCs, which were B7^{hi}, stimulated the development of

CD4⁺ Tr1-like cells that also produced high amounts of IL-10. Adoptive transfer of pulmonary DCs from IL-10^{+/-} but not IL-10^{-/-} mice exposed to respiratory antigen induced antigen-specific unresponsiveness in recipient mice. In accordance with these findings, IL-10 inhibited the development of fully mature DCs, which induced a state of alloantigen-specific anergy in CD4⁺ T cells [128]. These studies show that IL-10 production by DCs is critical for the induction of tolerance, and that phenotypically mature regulatory DCs may exist under certain circumstances.

Cells such as natural killer cells, epithelial cells, macrophages, and glial cells have been shown to express suppressor cytokines such as IL-10 and TGF- β . Although they have not been coined as professional regulatory cells, they may efficiently contribute to the generation and maintenance of a regulatory/suppressor-type immune response [129–134]. The expression of suppressor cytokines in resident tissue cells may also contribute to this process.

Mechanisms of regulatory T-cell generation

Two major hypotheses concern the generation of Treg cells. One suggests that Treg cells emerge from the thymus as a distinct subset of mature T cells with defined functions [110,112]. However, several studies have shown that Treg cells may differentiate from naïve T cells in the periphery when they encounter antigens in high concentrations [31,107,135]. Numerous studies suggest that thymic differentiation accounts for Treg cells that are specific for self-peptides and are devoted to the control of autoimmune responses, whereas peripheral differentiation may be required for environmental antigen-specific T cells for which an undesired immune response results in pathology. DCs not only control immunity but also maintain peripheral tolerance, two complementary functions that would ensure the integrity of the organism in an environment full of pathogens and allergens. The tolerogenic function of DCs depends on certain maturation stages and subsets of different ontogeny, and can be influenced by immunomodulatory agents. The differentiation of thymus-derived Treg cells does not depend on interaction with specialized DCs [136], whereas several studies support a role for DCs in the induction of Tr1 cells.

Immature DCs control peripheral tolerance through inducing Tr1-cell differentiation [34]. Related to prevention and development of asthma, airway DCs control the pulmonary immune response and determine tolerance and immunity to newly encountered antigens. Immature DCs are distributed throughout the lungs, capture allergens, and migrate to the T-cell area of mediastinal lymph nodes within 12 hours [137]. They express a partially mature phenotype with an intermediate array of costimulatory molecules and induce T-cell tolerance [138]. Antigen presentation by partially mature airway DCs that express IL-10 induce the formation of Tr1-like cells that inhibit subsequent inflammatory responses [127]. Moreover, depletion and adoptive transfer of pulmonary plasmacytoid DCs

have shown that these cells have an important role in protection from allergen sensitization and asthma development in mice [139]. Although molecular mechanisms of Treg-cell generation have not been elucidated, some existing therapies for allergic diseases, such as treatment with glucocorticoids and β_2 -agonists, might function to promote the numbers and function of IL-10-secreting Tr1-like cells [140,141].

Suppression mechanisms of regulatory T cells

Much uncertainty remains about the mechanisms of action of Treg cells. Initial studies have shown that Treg cells act as suppressor T cells, which inhibit the activation of effector cells and prevent inflammation in models of chronic infection, organ transplantation, and autoimmunity [29,31,142]. Treg cells have their suppressive functions only at certain stages of inflammation. Both Tr1 cells and $CD4^+ CD25^+$ Treg cells, which are highly capable of preventing allergen-induced activation and proliferation of effector T cells, cannot suppress the effector functions of preactivated T cells. This outcome was shown in a study of atopic dermatitis, where neither type of Treg cell could inhibit the apoptosis of keratinocytes induced by preactivated Th1 cells [143].

Most studies have failed to find a soluble factor as a suppressive mechanism of $CD4^+ CD25^+$ Treg cells. The suppression of antigen-induced proliferation of $CD4^+$ T cells was dramatically reduced after coculture with activated Treg clones that had been separated from the responding T cells by a Transwell (Corning Costar, Cambridge, MA) insert [144]. However, in Transwell membrane cultures that separate suppressor cells and target cells, the distance between two populations is approximately 2 mm, which may influence the concentration of suppressor cytokines. Membrane-bound TGF- β might be one of the mechanisms of suppression of $CD4^+ CD25^+$ Treg cells [145]. The suppressive effects of Tr1 cells were abrogated by the addition of neutralizing mAb directed against TGF- β and IL-10, implicating a role for suppressive cytokines in the mechanism of immune suppression in vitro and in vivo in different settings and different autoimmune and allergy models (Table 2) [31,36,100,145,146]. This suppression is a hallmark of Tr1 clones, because OVA-specific Th1 or Th2 clones derived from the same mice have no suppressive effects but rather enhanced OVA-induced proliferation of naive $CD4^+$ T cells [35].

One group of $CD4^+ CD25^+$ Treg cells originates from the thymus as a distinctive subset [110,112,147]. Thymectomy at a very early stage of development induces various autoimmune diseases in genetically susceptible animals [148,149]. Furthermore, induction of autoimmune diseases in an immunodeficient animal model was prevented by adoptively transferred $CD4^+$ T cells or $CD4^+ CD8^-$ thymocytes isolated from normal syngeneic animals. In a rat model, $CD4^+$ Treg cells were found to be of the $CD45^{RC\text{low}}$ phenotype and to produce IL-2 and IL-4 but not IFN- γ on in vitro stimulation [148]. IL-4 and TGF- β are critical in preventing autoimmunity because neutralization of either of these cy-

Table 2
Mechanisms of action of IL-10 and TGF-β during allergen-SIT

IL-10	TGF-β
Suppresses allergen-specific IgE	Suppresses allergen-specific IgE
Induces allergen-specific IgG4	Induces allergen-specific IgA ^a
Blocks CD28 signal transduction pathway, suppresses allergen-specific Th1 and Th2 cells	Suppresses allergen-specific Th1 and Th2 cells
Inhibits DC maturation, leading to reduced class II major histocompatibility complex and costimulatory ligand expression, induces IL-10-producing DCs	Down-regulates FcεRI expression on Langerhans cells
Reduces release of proinflammatory cytokines by mast cells	Associated with CTLA-4 expression on T cells
Suppresses eosinophils, mast cells, and basophils ^a	Suppresses eosinophils, mast cells and basophils ^a
Suppresses endothelial cell expression of adhesion molecules and decreased inflammatory cell transmigration ^a	Suppresses endothelial cell expression of adhesion molecules and decreased inflammatory cell transmigration ^a
Induces IL-10 and TGF-β in T cells	Induces FoxP3 in T cells

^a Most mechanisms have been shown in several studies, however, some are still under debate and their mechanisms remain to be further investigated.

tokines abrogates the protective response. In another study, CD4⁺ CD25⁺ Treg cells from thymus were shown to exert their suppressive function through the inhibition of IL-2Rα-chain in target T cells, induced by the combined activity of CTLA-4 and membrane TGF-β1 [150].

Studies of this activated CD4⁺ T-cell subpopulation have shown that they do not proliferate on normal TCR-mediated stimulation, but they do suppress proliferation of other T cells. TCR stimulation was required for these cells to suppress other T cells. Such suppression, however, was not restricted to T cells specific for the same antigen. CD4⁺ CD25⁺ T cells are the only lymphocyte subpopulation in mice and humans that express CTLA-4 constitutively. The expression apparently correlates with the suppressor function of CTLA-4. The addition of anti-CTLA-4 antibody or its fragment of antigen binding reverses suppression in cocultures of CD4⁺ CD25⁺ and CD4⁺ CD25⁻ T cells [151]. Similarly, treating mice that were recipients of CD4⁺ CD45RB^{low} T cells with these agents abrogated the suppression of inflammatory bowel disease [152]. These studies indicate that signals resulting from the engagement of CTLA-4 with its ligands, CD80 and CD86, are required for the induction of suppressor activity. Under some circumstances, the engagement of CTLA-4 on the CD4⁺ CD25⁺ Treg cells with specific antibody or with CD80/CD86 might lead to inhibition of the TCR-derived signals that are required for the induction of suppressor activity.

PD-1 is an immunoreceptor tyrosine-based inhibitory motif (ITIM)-containing receptor expressed on T-cell activation. PD-1-deleted mice develop autoimmune diseases, suggesting an inhibitory role for PD-1 in immune responses [153]. Members of the B7 family, PD-L1 and PD-L2, are ligands for PD-1. PD-1: PD-L engagement on murine CD4 and CD8 T cells results in inhibition of pro-

liferation and cytokine production. T cells stimulated with anti-CD3/PD-L1 display dramatically decreased proliferation and IL-2 production [154]. PD-1:PD-L interactions inhibit IL-2 production even in the presence of costimulation. Thus, after prolonged activation, the PD-1:PD-L inhibitory pathway dominates. Exogenous IL-2 is always able to overcome PD-L1-mediated inhibition, indicating that cells maintain IL-2 responsiveness.

Glucocorticoid-induced tumor necrosis factor receptor family-related gene (TNFRSF18, GITR) is expressed by CD4⁺ CD25⁺ alloantigen-specific and naturally occurring circulating Treg cells [155,156]. Stimulation of CD4⁺ CD25⁺ Treg cells through GITR breaks immunologic self-tolerance [156]. GITR is up-regulated in CD4⁺ CD25⁻ T cells after TCR stimulation and also functions as a survival signal for activated cells [157]. In addition, CD103 ($\alpha_E\beta_7$ integrin) and CD122 (β chain of IL-2 receptor) are highly expressed on CD4⁺ CD25⁺ Treg cells, which correlates with their suppressive activity [158,159].

The X-linked forkhead/winged helix transcription factor FoxP3 is characteristic for CD4⁺ CD25⁺ Treg cells [160,161]. It is highly expressed in CD4⁺ CD25⁺ but not CD4⁺ CD25⁻ T cells [160,161]. It acts as a silencer of cytokine gene promoters and programs the development and function of CD4⁺ CD25⁺ Treg cells [160–163]. Mutations in the FoxP3 gene in humans leads to a severe immune dysregulation known as *IPEX syndrome* [164]. In addition, the absence of FoxP3⁺ CD4⁺ CD25⁺ Treg cells in inflammatory and lesional skin suggests a mechanism for the development of eczematous lesions in atopic dermatitis observed in IPEX syndrome [143].

The failure of Treg cells to proliferate after TCR stimulation in vitro suggests that they are naturally anergic. However, Treg cells expressing a transgenic TCR were shown to proliferate and accumulate locally in response to transgenically expressed tissue antigen, whereas their CD25⁻ counterparts are depleted at such sites [165].

Because the concept of professional suppressor cells is regaining interest in the immunologic community, how the manipulation of regulatory/suppressor T cells might be used clinically must now be considered. Sakaguchi and Sakaguchi [166] showed for first time that depletion of regulatory T cells from mice led to the development of autoimmune disease. Cotransfer of CD4⁺ CD25⁺ Treg cells together with effector, disease-causing, T cells prevented mice from developing multiple experimentally induced autoimmune diseases such as colitis, gastritis, type 1 diabetes, and thyroiditis [167–170]. Because tumor antigens are an important group of autoantigens, the depletion of Treg cells should result in an enhanced immune response to tumor vaccines. Several studies have shown that the antibody-mediated depletion of CD25⁺ T cells facilitates the induction of tumor immunity [171,172].

Further studies are needed to show whether in vivo generation or adoptive transfer of Treg cells or their related suppressive cytokines may clinically change the course of allergy and asthma. Small molecular weight compounds that may generate Treg cells or increase their suppressive properties are an important target for use in not only allergy and asthma but also transplantation and autoimmunity.

Role of histamine receptor 2 in peripheral tolerance to allergens

As a small molecular weight monoamine that binds to four different G-protein-coupled receptors, histamine has recently been shown to regulate several essential events in the immune response [173,174]. Histamine receptor 2 (HR2) is coupled to adenylate cyclase, and studies in different species and several human cells have shown that inhibition of characteristic features of the cells primarily by cyclic adenosine monophosphate formation dominates in HR2-dependent effects of histamine [175]. Histamine enhances Th1-type responses by triggering the histamine receptor HR1, whereas Th1- and Th2-type responses are both negatively regulated by HR2. Human Th1 cells predominantly express HR1 and Th2 cells mostly express HR2, resulting in their differential regulation by histamine [38]. Histamine induces the production of IL-10 by DCs [176]. In addition, histamine induces IL-10 production by Th2 cells [177] and enhances the suppressive activity of TGF- β on T cells [178]. All three of these effects are mediated through HR2, which is highly expressed on Th2 cells and suppresses IL-4 and IL-13 production and T-cell proliferation [38]. These recent findings suggest that HR2 may represent an essential receptor that participates in peripheral tolerance or active suppression of inflammatory/immune responses. Histamine also regulates antibody isotypes, including IgE [38]. A high amount of allergen-specific IgE is induced in HR1-deleted mice. In contrast, deletion of HR2 leads to significantly lower amounts of allergen-specific IgE, probably caused by direct effect on B cells and indirect effect through T cells.

A double-blind, placebo-controlled trial analyzed the long-term protection from honeybee stings through terfenadine premedication during rush immunotherapy with honeybee venom [179]. After an average of 3 years, 41 patients were re-exposed to honeybee stings. Surprisingly, none of the 20 patients who had been given HR1-antihistamine premedication, but 6 of 21 who had been given placebo, had a systemic allergic reaction to the re-exposure through either a field sting or a sting challenge. This highly significant difference suggests that antihistamine premedication during the initial dose-increase phase may have enhanced the long-term efficacy of immunotherapy. Expression of HR1 on T lymphocytes is strongly reduced during ultrarush immunotherapy, which may lead to a dominant expression and function of tolerance-inducing HR2 [180]. Administration of antihistamines decreases the HR1/H2R expression ratio, which may enhance the suppressive effect of histamine on T cells. Further studies are required to substantiate these promising findings that support the use of antihistamine pretreatment in all patients undergoing venom-specific immunotherapy.

Summary

Peripheral T-cell tolerance is the key immunologic mechanism in the healthy immune response to self and noninfectious, nonself antigens. This phenomenon

is clinically well documented in allergy, autoimmunity, transplantation, cancer, and infection. Changes in the fine balance between allergen-specific Treg cells and Th2 or Th1 cells are crucial in the development and treatment of allergic diseases. Strong evidence supports the role of Treg cells or immunosuppressive cytokines as a mechanism through which allergen-specific immunotherapy and healthy immune response to allergens are mediated (see Fig. 2).

In addition to the treatment of established allergy, prophylactic approaches must be considered before initial sensitization occurs. Allergen-specific Treg cells may in turn dampen the Th1 and Th2 cells and cytokines, ensuring a well-balanced immune response. Enhancement of the number and activity of Treg cells could be an obvious goal for the treatment of many diseases related to dysregulation of the immune response. Small molecular weight compounds that may generate Treg cells or increase their suppressive properties are an important target for use in not only allergy and asthma but also transplantation and autoimmunity. Treg cells may not always be responsible for a healthy immune response; several studies have shown that they may be responsible for the chronicity of infections and tumor tolerance. Treg-cell populations have proven difficult but not impossible to grow, expand, and clone in vitro. A crucial area for future study is the identification of drugs, cytokines, or costimulatory molecules that induce the growth while preserving the suppressor function of Treg cells. Applying current knowledge of Treg cells and related mechanisms of peripheral tolerance may lead to more rational and safer approaches to the prevention and cure of allergic disease in the near future.

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