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The regulation of allergy and asthma

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Summary: Allergic diseases and asthma are caused by exaggerated T-helper 2 (Th2)-biased immune responses in genetically susceptible individuals. Tolerance to allergens is a mechanism that normally prevents such responses, but the specific immunological events that mediate tolerance in this setting are poorly understood. A number of recent studies indicate that regulatory T cells (Tregs) play an important role in controlling such Th2-biased responses. Tregs involved in regulating allergy and asthma consist of a family of related types of T cells, including natural CD25⁺ Tregs as well as inducible forms of antigen-specific adaptive Tregs. Impaired expansion of natural and/or adaptive Tregs is hypothesized to lead to the development of allergy and asthma, and treatment to induce allergen-specific Tregs could provide curative therapies for these problems.

Keywords: airway hyperresponsiveness, allergy, asthma, regulatory T cells, tolerance

Introduction

Asthma is an immunological disease that has increased dramatically in prevalence over the past two decades. In industrialized countries, the prevalence of asthma has nearly doubled since 1980, such that in the United States and other Westernized countries, one in five to ten individuals is affected. As a result, asthma has reached epidemic proportions, and current healthcare expenditures for asthma in industrialized countries are enormous.

Asthma and allergy are inflammatory diseases, caused by dysregulated immune responses in the respiratory mucosa. It is believed that overzealous T-helper 2 (Th2)-driven responses result in the development of asthma. Thus, CD4⁺ T cells producing Th2 cytokines play a prominent role in the lungs of asthmatic subjects (1), particularly because interleukin-4 (IL-4) and IL-13 enhance immunoglobulin E (IgE) production, IL-4, IL-9, and IL-10 enhance mast cell growth, IL-5 enhances eosinophil accumulation, and IL-9 and IL-13 directly enhance mucus hypersecretion and airway hyperreactivity (AHR) (2, 3).

Although it is clear that Th2-driven immune responses critically regulate the development of asthma, the particular immunological mechanisms that develop *in vivo* to prevent

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Th2-driven inflammation and that prevent allergy and asthma symptoms from developing in non-allergic and non-asthmatic individuals are not clear. Allergy and asthma are complex genetic traits, in which environmental factors, such as exposure to allergens, infections, and air pollution, interact with genetic factors to influence its development. Although all individuals are exposed to the allergens and other sensitizing agents in the environment, allergic disease and asthma develop only in some of the exposed individuals, suggesting that both environmental and genetic factors play important roles in determining protection against the development of pathogenic Th2 responses. While the genetic and immunological mechanisms that protect against the development of allergic and asthmatic inflammatory responses are only beginning to be understood, there is growing evidence that regulatory T-cell (Treg) development plays an important role in the protection against asthma.

Understanding protective immune responses in allergy and asthma

Non-allergic individuals develop tolerance to allergens that protects against allergy and asthma, as manifested by a lack of clinical symptoms in these individuals on exposure to allergen. Initially, investigators attributed the lack of symptoms in non-allergic individuals to an absence of allergen-specific immune responses, due either to lack of sufficient previous exposure to allergen or to favorable immune response genes that prevented recognition of allergens (immunological ignorance). However, linkage studies indicate that major histocompatibility complex (MHC) and immune responsiveness control responses to only a very limited number of allergenic epitopes, while allergic individuals tend to develop allergies to a very broad range of antigens. Furthermore, it is now clear that non-allergic individuals are exposed to a large number of allergens in quantities sufficient to induce immune responses, which appear to protect against rather than induce the development of allergic symptoms (4). The precise mechanisms of tolerance to allergens are still poorly understood, although our understanding has evolved extensively over the past several years.

Hygiene hypothesis

A wide variety of epidemiological observations regarding the effect of environmental factors on the development of asthma and allergy have provided important insight into the protective immune responses that occur in non-allergic individuals.

For example, the observation that the prevalence of asthma and allergies has increased dramatically over the past two decades while certain environmental exposures (e.g. exposure to infectious diseases) have decreased suggests that certain infections may reduce the risk of developing asthma and allergy, presumably by enhancing the development of protective immunity against allergens. These observations are the basis for the 'hygiene hypothesis', which suggests that improved hygiene in industrialized societies, with improved public health measures and the use of vaccines and antibiotics, has reduced the incidence of infections that would normally stimulate the immune system in some imprecisely understood way that mitigates against asthma and allergies (5). Additional observations that support the hygiene hypothesis include observations showing that children from large families, i.e. having older siblings, have a reduced risk of developing asthma (6). In a similar way, children placed in daycare settings in the first year of life (with presumed exposure to infectious agents) have a reduced risk of developing asthma (7). Furthermore, exposure to farm animals early in life reduces the likelihood of developing asthma and allergic rhinitis (8). Several investigators suggest that exposure to bacterial endotoxin, presumably from farm animal manure, may be important in producing this protective effect, and others have shown that extensive exposure to cats or dogs and their epidermal allergens may replicate the protective effects of farm animal exposure (9). Additional studies demonstrate that early childhood exposure to antibiotics, which may alter gastrointestinal flora, and exposure to intestinal endotoxin [reduced Toll-like receptor (TLR) signaling] is associated with an increased incidence of allergy (10, 11). This idea is consistent with the observation that oral tolerance cannot be induced in germ-free mice (12). Furthermore, elimination of commensal intestinal microflora and TLR signaling with broad spectrum antibiotics also prevented oral tolerance from developing, enhanced allergic sensitization (13), and resulted in susceptibility to intestinal inflammation (14). Toll-like receptor signaling may not only involve microorganisms, as high molecular mass hyaluronan, a constituent of extracellular matrix, can signal through TLR2 and TLR4 and provide anti-inflammatory effects in the lung, although hyaluronan fragments, generated during lung injury, signal through the same TLRs and can exacerbate lung inflammation (15).

These observations suggest that TLR signaling by commensal bacteria or by extracellular matrix components, under normal steady-state conditions, is required for the maintenance of intestinal and pulmonary epithelial cell homeostasis and possibly for the induction of some forms of Tregs.

Moreover, these observations demonstrate that there is a fundamental relationship between environmental immune stimulation and the development of disease. Under some circumstances and in some individuals, environmental stimulation leads to the development of allergy and asthma, but under other conditions in other individuals, environmental stimulation leads to immune responses that protect against or prevent asthma and allergy. Examination of these environmental factors should provide important clues regarding the immunological mechanisms that protect against asthma and allergy.

What is the nature of the protective response?

Because Th1 cells crossregulate Th2 cells in some systems, allergen-specific Th1 cells have been assumed to regulate allergic disease and asthma. T-helper 1 cells inhibit the development and proliferation of Th2 cells (16), and IgE production is reciprocally regulated by IL-4 and interferon- γ (IFN- γ), suggesting that protection from allergy is due to the development of inhibitory allergen-specific Th1 cells. Allergen-specific T-cell clones derived from the peripheral blood of non-allergic individuals have been shown to produce Th1 cytokines (17, 18). In addition, individuals with strong delayed-type hypersensitivity responses to *Mycobacteria tuberculosis* after immunization with bacille Calmette-Guérin are less likely to have allergies and asthma (19), suggesting that environmental exposure to *Mycobacteria* may expand Th1 cells and protect against allergy and asthma. Furthermore, neonates who subsequently develop allergic disease have been shown to have cord blood mononuclear cells with a reduced capacity to produce IFN- γ (20), due either to environmental exposure or to genetic predisposition. Similarly, patients with multiple sclerosis, an autoimmune disease characterized by the overproduction of Th1 cytokines in myelin-specific T cells and by an increased capacity of monocytes to produce IL-12, were protected from the development of allergy (19, 21, 22). Finally, administration of IL-12 intratracheally (23) or of IL-12 plus IL-18 (24) inhibited the development of allergic disease and AHR, a cardinal feature of asthma.

While these studies indicate that allergen-specific Th1 cells may function as Tregs in allergic disease, other observations suggest that pure Th1 responses may actually exacerbate allergic disease and asthma. Thus, allergen-specific T cells in the peripheral blood and in the lungs of patients with asthma also produce IFN- γ , which appears to actually contribute to the severity of the disease (25, 26). Moreover, classical Th1 cells, when adoptively transferred into mice, do not counterbalance effector Th2 cells but rather codominantly generate inflammatory responses in the lungs (27). Furthermore, in contrast to the

significant inflammation found in the lungs of patients with asthma, the airways of non-allergic subjects are characterized by the absence of inflammatory changes, including the absence of Th1 cells. This finding suggests that other regulatory mechanisms, distinct from the Th1-biased immune responses, are also involved in suppressing inflammation and protecting against allergic disease and asthma. This idea is consistent with the fact that the prevalence of both allergic diseases and autoimmune Th1-biased diseases, such as type 1 diabetes, multiple sclerosis, and inflammatory bowel disease, have increased substantially in past decades, suggesting that changing environmental factors have inhibited regulatory mechanisms that could inhibit both pathological Th2 and Th1 responses. Alternatively, it is possible that IFN- γ is sometimes produced in combination with suppressive cytokines such as IL-10 by Tregs (as discussed later) (28), which might explain how IFN- γ in some instances might be effective in inhibiting asthma and allergy.

Investigating regulatory immune mechanisms in allergy and asthma

To more clearly understand and characterize the nature of regulatory immune responses that protect against asthma and allergy, investigators have recently employed three distinct approaches. In each of these approaches, insight provided by epidemiological studies has guided the experiments. The first approach has been to examine animal models of tolerance to exogenous antigens, assuming that antigenic tolerance is an immunological mechanism that occurs in non-allergic non-asthmatic individuals. The second approach has been to study non-allergic individuals who have been exposed to allergens, and to characterize the allergen-specific protective immune responses that occur in such individuals. Finally, the third approach has been to examine the immunological mechanisms by which allergen immunotherapy (immunization with allergen, as in allergy shots) clinically cures patients, presumably by inducing a form of antigen-specific protective immunity. While protection against allergy and asthma may involve multiple mechanisms, experimental models utilizing these three approaches indicate that Tregs play a very important role in inhibiting the development of allergy and asthma.

Murine models of tolerance

In animal models, immune tolerance to self- or exogenous antigens/allergens has been shown to be mediated by several mechanisms, including clonal deletion, anergy, immune deviation, and immune suppression by Tregs. Neither clonal

deletion nor anergy has been studied extensively in the context of allergic disease and asthma. It is likely, however, that clonal deletion of some allergen-specific clones occurs over time in asymptomatic individuals, particularly when allergen exposure is chronic or with exposure to large quantities of allergen that perhaps occurs on exposure to allergens in foods. In contrast, when the level of antigen exposure is relatively low, the development either of immune deviation or of Tregs, rather than deletion, is thought to be favored (29). Immune deviation or split tolerance is defined as the occurrence of one form of immunity but not another, for example, when humoral but not cellular immunity occurs or when IgE but not IgG is produced. Immune deviation has been explained by the development of subsets of Th cells (e.g. Th1 versus Th2 cells), but, as discussed above, the development of Th1 responses cannot fully explain protective immunity against allergy and asthma. However, Treg development may mediate tolerance to allergens. Tregs may directly prevent the activation and function of non-regulatory effector T cells (for example, either Th1 or Th2 effector cells), or Tregs may inhibit antigen presentation by dendritic cells (DCs) to effector T cells.

The study of Tregs has advanced markedly over the last few years, driven by a few key findings and improvements in the technology for identifying and isolating Tregs. Several subtypes of Tregs have been described, including those that develop centrally in the thymus, specific for auto/self-antigens (also called natural CD25⁺ Tregs) and those that develop in the periphery on exposure to exogenous antigen/allergens (also called adaptive Tregs). The precise relationship between natural and adaptive subsets of Tregs is not yet certain.

Thymus-generated natural Tregs (nTregs)

Natural Tregs develop in the thymus and constitute 5–10% of the CD4⁺ T cells in the periphery, both in mice and in humans. Natural Tregs constitutively express the CD25 (IL-2 receptor) molecule, as well as cytotoxic T-lymphocyte antigen-4 (CTLA-4), glucocorticoid-induced tumor necrosis factor receptor (GITR), and the transcription factor forkhead box protein 3 (Foxp3) (30), as discussed in several other reviews in this volume of *Immunological Reviews*. Although the expression of CD25 has been used in many studies to specifically identify nTregs in naïve mice, the value of CD25 as a marker of Tregs is limited because CD25 is highly expressed (as are CTLA-4 and GITR) on activated CD4⁺ T cells, virtually eliminating the value of CD25 as a marker for Tregs in settings of immune activation. In contrast, the expression of Foxp3 is highly

restricted to a subset of $\alpha\beta$ T-cell receptor (TCR) T cells, and, irrespective of CD25 expression, expression correlates closely with suppressor activity (31), primarily in mice and less clearly in humans. Studies in mice suggest that Foxp3, which binds to DNA, localizes to the nucleus, acts as a transcriptional repressor (32), and functions as a Treg lineage specification factor (31, 33, 34), at least in mice.

Natural Tregs develop in the thymus presumably through mechanisms that involve TCR–ligand interactions, as well as additional poorly understood signals, distinct from those required for non-regulatory T-cell selection (35). The TCR repertoire and specificity of Tregs are overlapping with those of non-regulatory T cells, and a large proportion of the TCRs of Tregs are self-reactive (36). The process of positive selection in the thymus for Tregs does not appear to involve the AIRE (autoimmune regulator) gene, which induces the transcription in the thymic medullary epithelium of a large set of genes encoding tissue-specific proteins. Rather, AIRE is thought to primarily induce clonal deletion of self-reactive thymocytes and not positive selection of Tregs (37). A factor that may be critically involved in positive selection of nTregs in the thymus is thymic stromal lymphopoietin (TSLP), which is highly expressed in Hassall's corpuscles in the thymic medulla and which activates DCs to induce thymocyte expression of Foxp3 and expansion of Foxp3-expressing thymocytes (38). Whether this mechanism involving TSLP that results in the development of natural CD25⁺ Tregs is also involved in the development of adaptive Tregs in the periphery is not known.

Suppression of allergic responses

In the regulation of allergy in murine models, CD4⁺ T cells (both CD25⁺ and CD25[−] subpopulations) have been shown to be effective in suppressing the production of antigen-specific IgE, in mice bearing monoclonal populations of B and T lymphocytes specific for influenza virus hemagglutinin and ovalbumin (OVA), respectively (39). The regulatory CD4⁺ T-cell population was absent when the transgenic mice were backcrossed to recombination-activating gene (RAG)^{−/−} mice, suggesting that the Tregs had TCRs that were distinct from the monoclonal T cells and therefore were presumably not (OVA) antigen specific. Similarly, CD25⁺ cells isolated from OVA-specific TCR transgenic DO11.10 mice (not backcrossed to RAG^{−/−} mice) were effective in inhibiting allergen-induced AHR (40). These natural CD25⁺ Tregs are absent in DO11.10 mice backcrossed to RAG^{−/−} mice, presumably because natural CD25⁺ cells utilize non-transgenic TCRs (41).

Therefore, the antigen specificity of the 'natural' CD25⁺ T cells and the precise mechanisms of suppression are not clear. The CD25⁺ T cells may affect antigen presentation by DCs, as depletion of CD25⁺ T cells in a mouse model of asthma resulted in an increase in allergen-induced AHR and inflammation, as well as in an increase in the number of activated pulmonary myeloid DCs (42). Similarly, in a system involving chronic infection with a gastrointestinal nematode, *Heligmosomoides polygyrus*, the number of CD25⁺Foxp3⁺ T cells was greatly increased, resulting in a reduction in airway inflammation, although AHR was not examined (43). This study suggests that helminth infections provide an environmental signal that enhances the development of nTregs, which inhibit allergy and asthma. As with many other studies of CD25⁺ Tregs, the specific mechanisms by which suppression occurred were not clear, as CD25⁺ cells from both wildtype and IL-10-deficient mice infected with *H. polygyrus* could transfer the suppression. The inhibitory effect appeared to be mediated by activated CD25⁺ T cells that inhibited the allergic response in an antigen non-specific manner (44). It is possible therefore that helminth infections, by increasing the frequency of CD25⁺ cells, would be able to prevent both allergic and autoimmune diseases.

Exogenous antigen-specific adaptive Tregs

In contrast to the allergen non-specific effects of natural CD25⁺Foxp3⁺ Tregs, allergen-specific adaptive Tregs are thought to limit immune responses to allergens and prevent allergic disease in an antigen-specific fashion. While it is clear that nTregs are positively selected in the thymus by encounter with self-antigens, it is likely that allergen-specific adaptive Tregs expand after encounter with exogenous allergens (e.g. after exposure to allergens in food or plant pollens) in the periphery rather than in the thymus. This finding suggests that adaptive Tregs develop through pathways distinct from CD25⁺ nTregs, presumably from non-regulatory CD25⁻ T-cell precursors. Alternatively, it is possible that adaptive Tregs derive from the same dedicated Treg lineage as do nTregs, possibly by differentiation in the thymus, but then expand in the periphery after encounter with allergen. Because adaptive Tregs do not have clear-cut markers other than Foxp3 that make them easy to identify and isolate, the specific characteristics and pathways by which they develop are difficult to study. However, in humans who have mutations in the Foxp3 gene and have the IPEX syndrome (immune dysregulation, polyendocrinopathy, X-linked syndrome), the development of severe autoimmune disease as well as food allergy and eczema suggests that Tregs expressing Foxp3 play an

important role both in regulating autoimmunity and in regulating allergic responses to exogenous allergens. Therefore, natural and adaptive Tregs may be related at the very least by the expression of Foxp3. The development of allergy, therefore, may be due to insufficient development of allergen-specific Tregs expressing Foxp3 (45). Because antigen-specific Tregs can be induced upon exposure or immunization with antigen in both mice and humans, further discussion of antigen-specific Tregs in this review will be deferred to the section on immunotherapies.

Natural killer T (NKT) cells regulate immune responses

Another cell type that expresses CD25 and which has regulatory activity is natural killer T cells (NKT) constitute a small subset of lymphocytes that express markers of typical natural killer cells and conventional T cells. Natural killer T cells are either CD4⁺ or CD4⁻CD8⁻, and a small population of human NKT cells is CD8⁺. NKT cells express $\alpha\beta$ TCRs, but many NKT cells express a conserved or invariant TCR α chain. In mice, the invariant TCR is called V α 14J α 18 (also known as J α 281), which usually associates with V β 8.2, while in humans, NKT cells express V α 24J α 18 (also known as J α Q), which associates with V β 11 (46, 47). Natural killer T cells respond to glycolipid antigens presented by the MHC class I-like non-polymorphic molecule CD1d, by rapidly producing large quantities of cytokines such as IL-4, IL-13, IL-10, and IFN- γ (46). This capacity to rapidly produce cytokines suggests that NKT cells are part of the innate immune system and regulate the development of adaptive immunity. Natural killer T cells with the invariant TCR (iNKT cells) can be rapidly activated by α -GalactosylCeramide (α -GalCer), a naturally occurring glycolipid in marine sponges that can be presented by CD1d, resulting in the rapid secretion of both IFN- γ and IL-4. Overexpression of the V α 14-J α 18 TCR α chain in mice results in 100 times more IL-4 production by CD4⁺ T cells upon mitogen stimulation *in vitro*, 10 times more IL-4 production *in vivo* with anti-CD3 stimulation, and increased IgE and IgG1 production (48). These observations suggest that NKT cells promote Th2 responses. However, the precise mechanisms by which NKT cells are activated to produce these cytokines and effects, and the precise endogenous or exogenous glycolipids to which iNKT cells respond, are not clear. NKT cells express inhibitory receptors such as Ly49, which are killer immunoglobulin-like receptors that bind to MHC class I molecules and inhibit cytolytic activity in NK cells. It is thought that these receptors may also inhibit the function of NKT cells (49).

NKT cells in tolerance

Like natural CD25⁺ T cells, NKT cells have also been shown to possess important suppressor cell activity. Immune tolerance to corneal transplants cannot occur in J α 18^{-/-} mice, which lack the α chain of the invariant TCR of NKT cells and therefore lack iNKT cells (50). The inhibitory effects appear to be related to anterior chamber-associated immune deviation, which mediates tolerance to antigen placed into the anterior chamber of the eye (51). This process depends on IL-10 production and the induction of CD8⁺ Tregs (52), suggesting that NKT cells might be able to mediate antigen-specific tolerance by inducing antigen-specific Tregs.

In type 1 diabetes, NKT cells also appear to play a prominent role in mediating tolerance. The absolute number of NKT cells in non-obese diabetic mice appears to be reduced, and increasing the number of NKT cells or transfer of iNKT cells to mice with diabetes reduces disease progression (53–55), suggesting that NKT cells can prevent the development of type 1 diabetes. In a study of identical human twins discordant for diabetes, investigators showed that the diabetic subjects had a much lower frequency of the double negative NKT cells compared with the non-diabetic sibling (56), although this finding has been controversial (57). The few NKT cells that remained in patients with diabetes produced IFN- γ but not IL-4, whereas NKT cells in disease-free individuals produced both IFN- γ and IL-4. Finally, tolerance to and survival of rat xenogeneic pancreatic islet grafts in mice required the presence of iNKT cells (58), indicating that iNKT cells can suppress cytotoxic inflammatory responses in the pancreatic islets. Similarly, in mice with experimental autoimmune encephalitis (EAE), iNKT cells can prevent disease, and transfer of iNKT cells could correct the autoimmune inflammatory response (59). In humans, patients with multiple sclerosis appear to have a deficiency of NKT cells (60), consistent with the idea that iNKT cells suppress inflammation and autoreactive T cells.

In allograft transplantation models, NKT cells also play a critical role following immunomodulatory therapy. In a model of cardiac allograft transplantation that requires immunological conditioning regimens, tolerance to the cardiac transplant required NKT cells and did not occur in CD1d^{-/-} or J α 18^{-/-} mice (61, 62). Furthermore, non-myeloablative total lymphoid irradiation conditioning prior to bone marrow transplantation greatly reduced graft-versus-host disease (GVHD) in murine models. In this murine model, the reduction in GVHD was associated with an increase in iNKT cells producing IL-4, and severe GVHD occurred when NKT cells were removed from the graft bone marrow (63). Moreover, in

humans a similar phenomenon appears to occur, where conditioning with total lymphoid irradiation prior to bone marrow transplantation increases the number of NKT cells in the recipient and greatly reduces GVHD (64). The NKT cells in the recipient appear to produce IL-4 and suppress alloreactive T cells. The increase in NKT cell numbers is thought to be due to the resistance of NKT cells to radiation-induced apoptosis (65). Thus, iNKT cells function as Tregs, or possibly induce Tregs that suppress inflammation.

NKT cells in disease

By rapidly producing large quantities of cytokines, iNKT cells can not only inhibit immune responses but also have the capacity to enhance immunity. As such, there are a number of situations in which iNKT cells play a significant role in amplifying adaptive immunity. During infection with *Borrelia burgdorferi* (66), *Cryptococcus neoformans* (67), *Plasmodium falciparum* (68), *Trypanosoma cruzi* (69), and *Leishmania major* (70), the presence of NKT cells greatly augments host defense. Similarly, in cancer, iNKT cells can enhance anti-tumor responses (71). However, this proinflammatory effect of iNKT cells, when it occurs under certain aberrant circumstances, might result in unwanted inflammation and pathology, for example by exacerbating allergic asthma.

NKT cells and asthma

Because NKT cells produce large quantities of IL-4, the role of NKT cells was examined in the development of AHR. Surprisingly, in mice deficient of NKT cells, AHR could not be induced after sensitization and challenge with ovalbumin or ragweed allergen (72–74), even though normal Th2 responses and normal IgE responses on immunization developed in these mice (75). The important role of iNKT cells in asthma is supported by the observation that administration of α -GalCer 24 h prior to challenge with OVA inhibited allergen-induced AHR (76–78), presumably because strong iNKT cell agonists such as α -GalCer induce iNKT cell anergy (79), which prevents their subsequent role in inducing AHR. The critical role of NKT cells may be surprising, as other studies demonstrated that in β 2 microglobulin-deficient mice, which lack expression of all class I molecules including CD1d and therefore lack both CD8 cells and NKT cells, AHR could be induced (80). It is possible, however, that other problems in the β 2 microglobulin-deficient mice [e.g. lack of CD8 cells or the presence of increased numbers of CD1d-independent NKT cells (81)] might enhance the development of AHR in these mice.

The specific role of NKT cells in the development of asthma is complex. Although the development of Th2-driven responses to allergens is an important factor in the development of asthma (consistent with the fact that 80% of individuals with asthma have environmental allergies), iNKT cells may provide a lung-specific mechanism that allows asthma to develop in allergic individuals. Only about a third of all individuals who develop sensitization to environmental allergens develop asthma (82), suggesting that local effects in the lung are required, such as the presence of iNKT cells, which allows some of the allergic individuals to then develop asthma. In this process, iNKT cells may in some way license Th2 cells to induce AHR or may enhance Th2 cell development. This idea is supported by the finding that co-administration α -GalCer, which directly activates iNKT cells, along with protein antigen, sensitizes mice to these proteins, thereby enhancing the subsequent development of AHR induced by challenge with protein (83, 84). Alternatively, iNKT cells might induce AHR by responding directly to endogenous glycolipids in the lung environment, or to exogenous glycolipids in environmental allergens, such as in cypress pollen (85). Activated iNKT cells might then be capable of directly inducing AHR in the absence of Th2 cells. Thus, iNKT cells may function in several different ways, either in conjunction with or independent of Th2 cells, in enhancing the development of AHR and asthma.

Therefore, the role of iNKT cells *in vivo* in the development of asthma may depend upon the activation state of the iNKT cell, as well as on the interplay between iNKT cells and conventional CD4⁺ T cells. We suggest, however, that because NKT cells are required in murine models of asthma, the aberrant activation of iNKT cells may be critically important in causing the clinical syndrome of asthma in humans. If so, then the control of asthma symptoms may require either preventing the activation of iNKT cells or suppressing iNKT cell function, for example by Tregs. Further study of iNKT cell function, activation requirements, and response to Tregs, particularly in humans, is necessary to fully understand their role in asthma.

Human studies: examination of Tregs in non-allergic individuals

The understanding of mechanisms that control asthma and allergy has been considerably advanced by the study of immune responses in both allergic and non-allergic subjects. While the specific types of experiments that can be performed in humans are limited compared with experiments performed in mice, performance of studies in human systems are

ultimately essential to establish which specific immunological mechanisms occur in humans and to direct further exploratory studies in mice.

Immunological studies in non-allergic individuals have demonstrated the presence of allergen-specific antibody of the IgG, but not the IgE, isotype in most non-allergic individuals, indicating that 'non-allergic' individuals are not 'ignorant' of allergens, but rather that they do indeed respond to environmental exposure to allergens. Thus, both allergic and non-allergic individuals respond to environmental allergen exposure, but with different forms of immunity (immune deviation) such that non-allergic individuals remain asymptomatic. The basis for the different forms of immunity was initially hypothesized to be due to the possibility that non-allergic individuals responded to different epitopes of allergens than did allergic individuals. However, while the data are limited, it is now generally accepted that the antigenic epitopes recognized by T cells from both allergic and non-allergic individuals are indeed identical (86, 87). This idea is consistent with mouse models of Th1/Th2 cell differentiation, in which both Th1 and Th2 cells with distinct cytokine profiles and functions can be generated from common precursor cells recognizing a specific antigenic epitope with specific TCRs.

The specific allergen epitopes recognized by T cells from allergic and non-allergic individuals require further examination. Past studies identifying T-cell epitopes for allergens have been performed with little attention to MHC restriction (88) and without analysis of TCR utilization. Because MHC class II molecules determine the T-cell epitopes and because the diversity of human class II MHC polymorphisms complicates this analysis, more definitive results await future examination of allergen-specific T cells and their TCR utilization, perhaps with class II MHC tetramers, which identify T cells and their specific TCRs through binding of specific class II MHC antigen-peptide complexes. Although T cells from both allergic and non-allergic individuals with identical MHC class II tissue types should theoretically recognize identical allergen epitopes (these epitopes are based on optimal antigen peptide binding to class II antigens), it is feasible that, in some instances, T cells from allergic and non-allergic individuals will recognize distinct allergen peptides, possibly by preferential utilization of different MHC molecules. A similar distinction has been suggested for IgE antibody binding epitopes, in which antibody from allergic individuals binds to 'linear' (denatured) food allergen epitopes, while antibody from individuals who more rapidly outgrow food allergy recognize 'conformational' epitopes on native proteins (89).

In addition to immune deviation, other specific tolerance mechanisms may occur in non-allergic individuals, including anergy, deletion, or suppression by Tregs. These latter mechanisms, however, are difficult to study in humans because anergic cells, Tregs, and deleted cells are difficult to detect in the absence of specific markers (e.g. transgenic TCRs, as utilized in mouse models) and therefore difficult to evaluate. Human alveolar macrophages, the most abundant phagocytic cell in the lung, have been shown to actively tolerize or anergize CD4⁺ T cells (90). This finding suggests that alveolar macrophages, which express very low levels of costimulatory molecules, may induce a form of anergy T cells in the lungs that effectively limits immune responses in the pulmonary compartment. The anergy induced with alveolar macrophages may be similar to that induced with immature DCs, which also express low levels of costimulatory molecules and which induce anergy or the development of Tregs (91). Currently however, the basis for the different forms of immunity in allergic and non-allergic individuals is thought to be primarily due to differences at the level of Tregs rather than anergy or differences at the level of antigen epitope recognition.

Natural CD25⁺ Tregs in human allergy

The fact that non-allergic individuals develop IgG but not IgE antibody to allergens suggests that immunity in non-allergic individuals is characterized by immune deviation away from 'Th2 responses' and towards 'Th1 responses', which might induce blocking IgG antibodies. However, because pure Th1 responses have been shown to be proinflammatory (27), interest has turned to tolerance, perhaps as a form of immune deviation mediated by Tregs, as a mechanism that prevents the development of symptoms in non-allergic individuals.

Several studies have examined the role of the natural CD4⁺CD25⁺ T-cell population in regulating allergic disease. In healthy non-allergic subjects, the proliferative response of T cells from these individuals to cows' milk antigen is essentially absent. However, depletion of CD25⁺ T cells resulted in significant T-cell proliferation, suggesting that CD25⁺ T cells normally suppress the responses to dietary antigens (92). In other studies, children who had outgrown cows' milk sensitivity had higher frequencies of circulating CD4⁺CD25⁺ T cells and decreased *in vitro* proliferative responses to bovine β -lactoglobulin (a milk protein) in peripheral blood mononuclear cells compared with children who maintained clinically active milk sensitivity (93). In this study, cows' milk sensitivity was defined as having symptoms of bloody stools, diarrhea, failure to thrive, and anemia (non-type 1

hypersensitivity). Excluded from this study were children with milk-induced anaphylaxis with milk-specific IgE (type 1 hypersensitivity), as the investigators wished to study milk induced-colitis rather than type 1 IgE-mediated allergic hypersensitivity to milk. Nevertheless, the reduced proliferative response to milk (β -lactoglobulin) in children who had outgrown milk-induced gastrointestinal symptoms could be reversed by removal of the CD25⁺ cells from the cultured peripheral blood cells, but this increase in T-cell proliferation did not occur in allergic individuals. These results indicate that in cows' milk-induced colitis, CD25⁺ inhibitory Tregs become evident after cows' milk challenge in allergic children who become tolerant to cows' milk.

CD25⁺ Tregs may also suppress allergic responses to inhaled allergens (e.g. cat allergen and grass pollen) and appear to also regulate type 1-mediated hypersensitivity responses. However, CD25⁺ T cells from allergic donors may be defective, as CD25⁺ T cells from non-allergic donors but not from allergic donors suppressed proliferation and IL-5 secretion by their own allergen-stimulated CD4⁺CD25⁻ T cells (94). The loss in inhibitory activity of CD25⁺ T cells from allergic donors was most pronounced during symptomatic periods, when pollen counts were highest. Similarly, CD25⁺ T cells from both allergic and non-allergic individuals potently suppressed T-cell proliferation and Th2 cytokine production in response to birch allergen outside of the pollen season; however, during the birch pollen season, CD25⁺ T cells from allergic patients but not from non-allergic controls were defective in downregulating birch-pollen induced IL-13 and IL-5 production (95). These studies suggest that the natural CD25⁺ T-cell population includes T cells that recognize allergens, or that following activation, antigen non-specific CD25⁺ T cells can inhibit antigen-specific responses. Alternatively, antigen-specific adaptive Tregs expressing CD25 are present in this population and can inhibit allergen-specific responses (see below).

In patients with atopic dermatitis, a disease associated with asthma and allergic rhinitis, the number of CD4⁺CD25⁺Foxp3⁺ Tregs appears, paradoxically, to be elevated (96). This result is surprising, because patients with IPEX syndrome (Foxp3 deficiency) have no CD25⁺ cells and develop severe atopic dermatitis and food allergy, suggesting that CD25⁺ Tregs might be deficient in atopic dermatitis. The CD25⁺ Tregs in patients with atopic dermatitis were present in a higher frequency than in non-atopic and asthmatic subjects and had normal suppressive function when mixed with CD4⁺CD25⁺ cells (96). However, when stimulated with staphylococcal enterotoxin B superantigen, which may be present at high levels on the skin of patients with atopic dermatitis, the CD25⁺ Tregs lost their suppressive function, suggesting that environmental factors inhibited the function

of CD25⁺ Tregs in these patients. Alternatively, it may be that the increase in the number of natural CD25⁺ cells in patients with atopic dermatitis is irrelevant, that allergen-specific adaptive Tregs are of greater importance than natural CD25⁺ Tregs in controlling atopic dermatitis, and that these adaptive Tregs are deficient in patients with atopic dermatitis.

Allergen-specific Tregs in non-allergic individuals

Recent analysis of non-allergic individuals demonstrated that T cells from non-allergic individuals responding to allergen produced inhibitory cytokines such as IL-10, and that the amount of IL-10 produced was reduced in allergic individuals (97). For example, non-allergic bee keepers who had been stung multiple times had T cells that were anergic to venom antigens, due to the strong production of IL-10 (98). Furthermore, when peripheral blood cells from healthy non-allergic individuals were stimulated and expanded with aero-allergens and then analyzed for the frequency of cytokine-producing cells detected using cytokine secretion assay kits (Miltenyi Biotec, Auburn, CA, USA), IL-10-producing allergen-activated T cells were present at a higher frequency in non-allergic individuals. However, the allergen-activated IL-10-producing cells expressed CTLA-4, CD25, programmed death-1 (PD-1), transforming growth factor- β receptors I and II, and suppressed antigen-specific proliferation of CD4⁺ T cells. The suppressor activity of these cells was reduced by neutralization of IL-10 or TGF- β , or by blocking CTLA-4 or PD-1. Therefore, these allergen-activated IL-10-producing cells had many properties of natural CD25⁺ Tregs (expression of Foxp3 was not assessed). Because these Tregs may have been expanded as bystander-activated cells, it is not yet clear whether these IL-10-producing cells are natural CD25⁺ Tregs or allergen-specific adaptive Tregs.

It is clear that patients with allergy have a higher frequency of allergen-specific Th cells producing IL-4 and IL-13 (97). As observed in a large cohort of allergic and non-allergic children, increased IL-4, IL-5, and IL-13 were associated with allergy, and IL-10 was associated with negative allergy skin tests (99). Thus, allergic disease appears to result from reduced development of Tregs (adaptive allergen-specific or possibly natural CD25⁺), from enhanced development of Th2-driven immune responses, or both.

Regulation by hepatitis A virus (HAV) and T-cell immunoglobulin mucin-1 (TIM-1)

The specific mechanisms by which Tregs develop to inhibit allergy and asthma are not clear. What is clear, however, is that the mechanisms responsible for enhancing the

development of regulatory mechanisms must be less common today than 20 years ago, because the prevalence of asthma and allergy is much higher today than 20 years ago. It has been hypothesized that infectious diseases or alterations in commensal microflora in the intestinal tract and reduced TLR signaling (11) can enhance such regulatory mechanisms (hygiene hypothesis), but data for specific infectious pathogens or microorganisms responsible for these changes are very limited. There are strong data associating only a few infectious diseases with protection against allergy and asthma: HAV (100, 101), *Salmonella* (102), and possibly helminths (103). Initially, because the transmission of HAV and *Salmonella* occurs via fecal oral routes, it was assumed that infection with HAV or *Salmonella* is merely a marker for poor hygiene, and that poor hygiene is responsible for the protective effect associated with these gastrointestinal infections. Recent identification, however, of the receptor for HAV as TIM-1, which is preferentially expressed on Th2 cells, strongly suggests that HAV can directly affect T-cell differentiation and possibly reduce Th2 cell development and enhance Treg development.

The TIM gene family was positionally cloned using a congenic mouse model of asthma, in a locus that regulated AHR and IL-4 production, α pr (T-cell airway phenotype regulator) (104). T-cell immunoglobulin mucin-1 (TIM-1) is preferentially expressed on Th2 cells (104) and provides a strong positive costimulatory signal for T cells (105). In contrast, TIM-3 is preferentially expressed on Th1 cells (106) and provides a strong negative signal to T cells (107). TIM-1 is genetically polymorphic both in mice and in humans and has been shown to be an important atopy susceptibility gene in humans (108–110), particularly in subjects with evidence of past HAV infection. These observations suggest that when HAV interacts with specific polymorphic variants of TIM-1, the development of allergy and asthma is suppressed. The specific mechanisms by which this might occur are not yet known, but they may involve enhanced Treg development or enhanced deletion of allergen-specific Th2 cells.

Examination of the mechanisms of allergen immunotherapy for allergic diseases

The third approach in the examination of immunological mechanisms that protect against asthma and allergy is to examine the mechanisms by which allergen immunotherapy functions to limit allergic disease and asthma. Conventional allergen immunotherapy has been used in patients for the treatment of allergic disease for nearly 100 years, and when performed correctly, this therapy has been shown to be very

effective in controlling the symptoms of allergy and asthma (111, 112). Conventional allergen immunotherapy is performed by the subcutaneous administration of increasing doses of allergen over a 3–5 year period. Because the clinical benefit of allergen immunotherapy persists for years after the shots are discontinued, allergen immunotherapy is thought to alter the underlying immunological processes and in many instances effect cure, particularly in patients with bee venom allergy (111, 112).

The specific mechanisms by which allergen immunotherapy reduces allergic symptoms have been studied for decades. Initially, the major focus of study was on the induction of blocking antibody, presumably due to a shift in the cytokine profile of Th cells (113). In 1980, the induction of CD8⁺ suppressor T cells was proposed (114). However, as our understanding of Th cell subsets evolved over the last two decades, the focus of study has shifted to allergen-specific Th1 cells producing IFN- γ (115), and more recently to CD25⁺ Tregs (116) or allergen-specific CD4⁺ Tregs producing IL-10 (98, 116–120).

Induction of antigen-specific Tregs

The pace of study of human allergen-specific Tregs has quickened over the past 3 years, generally as more data have been generated in murine systems. In mouse models, antigen-specific adaptive Tregs have been induced by a number of immunization strategies. The induction of tolerance by intravenous or oral administration of antigen resulted in the development of antigen-specific Tregs (121) or suppressive Th3 cells (122), although Foxp3 expression was not examined in these early studies. In addition, administration of peptide subcutaneously (123) or with osmotic pumps (124), administration of antigen into the respiratory tract (125, 126), or administration of antigen with the adjuvant heat-killed *Listeria monocytogenes* (28, 127) induced the development of antigen-specific Tregs that expressed Foxp3 from CD4⁺CD25⁻ cells (28, 124–126). Some of these Tregs expressed IL-10 (125), while others expressed TGF- β (126) or both IL-10 and IFN- γ (28), suggesting that a spectrum of antigen-specific Tregs may exist with a wide range of cytokine profiles. Many of these antigen-specific Tregs have been shown to inhibit the development of allergen-induced AHR (28, 125, 126).

These studies indicate that immunization or exposure to antigen can indeed induce the development of a spectrum of allergen-specific Tregs that presumably arise from non-regulatory T cells upon immunization, at least in mice.

These adaptive Tregs appear to be related to natural CD25⁺ Tregs, in that they express Foxp3 and IL-10 or TGF- β . However, these adaptive Tregs have characteristics distinct from natural CD25⁺ Tregs (e.g. lack of CTLA-4 expression, derive from CD25⁻T cells, and proliferate to specific exogenous antigen). In addition, these allergen-specific Tregs have a spectrum of cytokine profiles and characteristics, including those that produce IL-10 (IL-4 initially), express GATA-3, the master regulator for Th2 polarization, and Foxp3 (Th2 regulatory cells), those that express IL-10, IFN- γ , T-bet, and Foxp3 (Th1 regulatory cells) (28, 125), and those that express TGF- β and Foxp3 (126). All of these have potent inhibitory activity and blocked the development of AHR in murine models of asthma. Thus, several types of adaptive Tregs that express Foxp3 can inhibit the development of Th2-driven inflammation and AHR in an antigen-specific fashion.

The development of antigen-specific Tregs involves specific subsets of mature DCs. Although immature DCs have been suggested to induce the development of Tregs in some systems (128), in the respiratory tract, mature myeloid CD8 α ⁻ DCs expressing high levels of costimulatory molecules, including inducible costimulator (ICOS)-ligand, and that transiently produced IL-10, were shown to induce the development of Th2 regulatory cells (129). DC production of IL-10 and expression of ICOS-ligand were required, because neutralization of IL-10 and blockade of ICOS or ICOS-ligand blocked the development of the Tregs. In contrast, mature CD8 α ⁺ DCs, activated with heat-killed *L. monocytogenes*, producing both IL-10 and IL-12, were shown to induce the development of Th1 regulatory cells (28). In this case, both IL-10 and IL-12 were required, as DCs incapable of producing either cytokine could not induce the Tregs. In other studies, plasmacytoid DCs were shown to be responsible for respiratory tolerance and to induce T cells with inhibitory capacity, although these T cells were not further characterized (131). The role of plasmacytoid DCs in inducing Treg development is consistent with studies in humans, showing that CD40 ligand-activated DC2 cells can induce the development of human Tregs and CD8⁺ Tregs (132, 133).

Antigen-specific adaptive Tregs expressing Foxp3 may be related to TR1 cells, which are antigen specific and develop *in vitro* in the presence of IL-10 (134) or in the presence of vitamin D3 and the corticosteroid dexamethasone (135). However, TR1 cells do not express Foxp3 (136). Furthermore, in some systems, treatment of mice with corticosteroids prevents the development of antigen-specific Tregs, suggesting that corticosteroids can block

the function of DCs that induce Tregs (130). Although corticosteroids can clearly inhibit allergic inflammation when used therapeutically, corticosteroid therapy at certain doses may have the potential to worsen allergic diseases by preventing the development of protective immunity and Tregs. The non-specific immunosuppressive effect of corticosteroids and their effects on Tregs may be related to the observation that other immunosuppressive agents, e.g. calcineurin inhibitors, used in transplantation significantly reduced the number of CD4⁺CD25⁺ Tregs, thereby preventing eventual tolerance induction (137). In contrast, *in vitro* treatment with corticosteroids can induce Foxp3 expression in non-regulatory human T cells, but only twofold (138), and can enhance IL-10 production, but also only twofold (139). The suppressor activity of such Foxp3⁺IL-10⁺ T cells was modest.

Additional mechanisms have been shown to increase Foxp3 expression in T cells. Exposure to TGF- β (140, 141) and blockade of IL-6 receptor (142) enhances Foxp3 expression, but the precise role of such Foxp3⁺ cells *in vivo* is not clear. It is also not clear if the expression of Foxp3 is due to the expansion of a small population of Foxp3⁺ T cells that might contaminate the population or due to a true conversion of non-regulatory T cells into Tregs. The induction of Tregs in the periphery is unlikely to involve TSLP, which induces Foxp3 expression in Tregs in the thymus (38), because expression of TSLP in the lungs and the skin has been shown to result in the development of allergic airway inflammation (143, 144) and atopic dermatitis (145), respectively. Although meticulous analyses of mouse T cells *in vitro* and *in vivo* show no upregulation of Foxp3 after activation of non-regulatory T cells (31, 146), under certain circumstances on encounter with allergen outside of the thymus naïve CD25⁻ cells, those presumably without regulatory cell activity develop into allergen-specific adaptive Tregs producing Foxp3. The precise mechanisms by which this occurs in the periphery, however, remain to be elucidated.

Mechanisms by which Tregs suppress allergy and asthma

Precisely how allergen-specific Tregs suppress Th2-driven responses is also not yet clear, and it is likely that several distinct mechanisms are important. First, it is thought that Tregs localize to sites of inflammation, suggesting that Tregs can inhibit the function of Th2 (and Th1) effector cells at mucosal surfaces. Second, the production of IL-10 and/or TGF- β is likely to play a very important role in their capacity

to directly inhibit airway inflammation and AHR. Production of IL-10 and TGF- β is associated with peripheral T-cell tolerance and Tregs, which are induced by mucosal exposure to antigen. Oral tolerance has been used in animal models to block the development of both Th1-mediated (147–152) and Th2-mediated disease (153, 154). It is known that the application of IL-10 into the lungs using replication-deficient adenovirus vectors in a murine model of AHR abrogates both the cellular and physiological recall responses *in vivo*, and conversely, removal of IL-10 using knockout systems exacerbates airway inflammation (155, 156). Although some studies suggest that IL-10 may exacerbate allergen-induced airway responses (157), it appears that IL-10-producing cells, such as Tr1 cells (158) or OVA-specific cells engineered to produce IL-10 (159), critically down-modulate Th2-driven responses and AHR. Similarly, TGF- β production by OVA-specific T cells engineered to produce TGF- β (160) and those expressing cell surface TGF- β (126) abolished Th2-driven AHR. In other systems, blockade of TGF- β signaling resulted in enhanced antigen-induced airway inflammation (161) or in a reduction in the capacity of Tregs to inhibit Th2-driven inflammation (162, 163). Because TGF- β also regulates Th1-driven autoimmune diseases, such as EAE, uveoretinitis, and collagen-induced arthritis (148–152, 164), TGF- β plays an important inhibitory role both in Th2- and in Th1-driven inflammation.

The suppressive effects of IL-10 and TGF- β may be due to direct effects on effector T cells or possibly by direct cell-to-cell contact of suppressors with effector T cells. Alternatively, IL-10 may inhibit effector T cells by effects on antigen-presenting cells, as increased IL-10 levels have been associated with increased monocyte production of indoleamine 2,3 dioxygenase, which catabolizes tryptophan and inhibits T-cell function (165). IL-10 and TGF- β might inhibit DC function by reducing expression of costimulatory molecules and increasing production of IL-12 (42). Alternatively, TGF- β may directly affect Th2 cells by inhibiting GATA-3 expression and reducing Th2 cytokine production (166). Antigen-specific Tregs in general do not express inhibitory molecules such as CTLA-4 or PD-1, and therefore these inhibitory pathways are not likely to be involved in suppression. However, the inhibitory effects of some antigen-specific Tregs can be blocked by blockade of the ICOS–ICOS-ligand pathway (28, 125), suggesting that direct contact through ICOS–ICOS-ligand may be important. Further study of allergen-specific Tregs is required to clearly understand the mechanisms of suppression.

Improving the immunological effects of allergen immunotherapy

Another approach to understanding the biology of Tregs is to examine newer methods of allergen immunotherapy, which may more efficiently induce Tregs. Traditional immunotherapy, while conferring clinical benefits and improving the course of allergic diseases and asthma (167–169), is inefficient and associated with adverse side effects, including anaphylaxis. Furthermore, because sustained benefit requires prolonged administration over a period of several years, a number of approaches have been taken recently to greatly enhance the efficiency of immunotherapy regimens in humans. Firstly, the use of purer allergen preparations, including recombinant allergen proteins, may increase the safety and specificity of immunotherapy as well as improve the diagnosis of specific allergy (170). This strategy is dependent, however, on a clear understanding of the important allergenic epitopes that induce Tregs, and approaches that utilize pure single recombinant allergens, which do not induce Tregs, may not be clinically effective. Secondly, to limit the allergenicity of whole allergens, investigators have examined peptide-based allergen preparations, which do not bind IgE and therefore do not activate mast cells but which reduce both Th1- and Th2-cytokine synthesis, while increasing levels of IL-10 (171, 172). The beneficial effects, however, of peptide immunotherapy appear not to depend on enhancing the development of CD4⁺CD25⁺ Tregs, although allergen-specific adaptive Tregs were not examined (173). A third strategy is to administer the doses orally, which may reduce adverse reactions (174) and activate a different type of Treg that might secrete more TGF- β (125). Clinical trials in humans with allergy have shown that oral immunotherapy and sublingual/swallow immunotherapy with allergen are safe and effective in reducing allergy symptoms, are associated with an increase in specific IgG and a decrease in IgE (175–177), and can change the course of allergic disease (178). A fourth strategy, which may improve the safety of allergen immunotherapy is to administer anti-IgE-monoclonal antibody, omalizumab, concomitantly with allergen immunotherapy (179, 180). Such a combination may reduce allergic reactions associated with immunotherapy and improve the rapidity by which immunotherapy induces Tregs.

A fifth strategy to improve the efficiency by which immunotherapy induces Tregs is to use adjuvants.

Treatment of mice with mycobacterium induced allergen-specific Tregs producing IL-10 and TGF- β , which protected against airway inflammation (181). Similarly, the use of just one dose of heat-killed *L. monocytogenes* as adjuvant induced allergen-specific Tregs producing IFN- γ and IL-10, which protected against airway inflammation and AHR in mice (182, 183) and against food allergy in dogs (184). The combination of IFN- γ and IL-10 produced by the Tregs induced with heat-killed *L. monocytogenes* as adjuvant may be particularly effective in reducing allergic inflammation (185). TLR8 agonists (186) and TLR9 agonists (187, 188) may also be effective in reducing AHR. The adjuvant closest to clinical application in humans is immunostimulatory CpG motifs, which are present in bacterial DNA but suppressed in vertebrates, and which activate TLR9 (189). Trials in humans showed that CpG-allergen (ragweed) conjugates possesses greatly reduced allergenicity, and reports of the clinical efficacy are highly encouraging (190, 191). Because the beneficial effects of the CpG-allergen conjugates appear to last well beyond the treatment period (which generally includes only six injections), it is possible that the CpG-allergen conjugates induce some form of Tregs, which may produce IL-10 and IFN- γ , although this has not been formally studied. In any case, adjuvants such as CpG or heat-killed *L. monocytogenes* appear to greatly enhance the efficiency and efficacy of allergen immunotherapy to provide safe and long-lasting control of allergic diseases.

Conclusions

Allergen-specific tolerance protects against asthma and allergic diseases. Both genetic and immunological factors influence the development of tolerance, which is mediated by deletion or anergy of allergen-specific cells, or by the development of immune deviation and/or of Tregs. Several forms of Tregs are effective in limiting allergic inflammation and asthma, including nTregs and adaptive Tregs (Fig. 1). Immunotherapies that enhance the development of allergen-specific Tregs may provide safe, specific, and long-lasting control of allergic diseases and asthma. Further understanding of the mechanisms involved to induce these cells is necessary to utilize these cells more efficiently in therapeutic strategies.

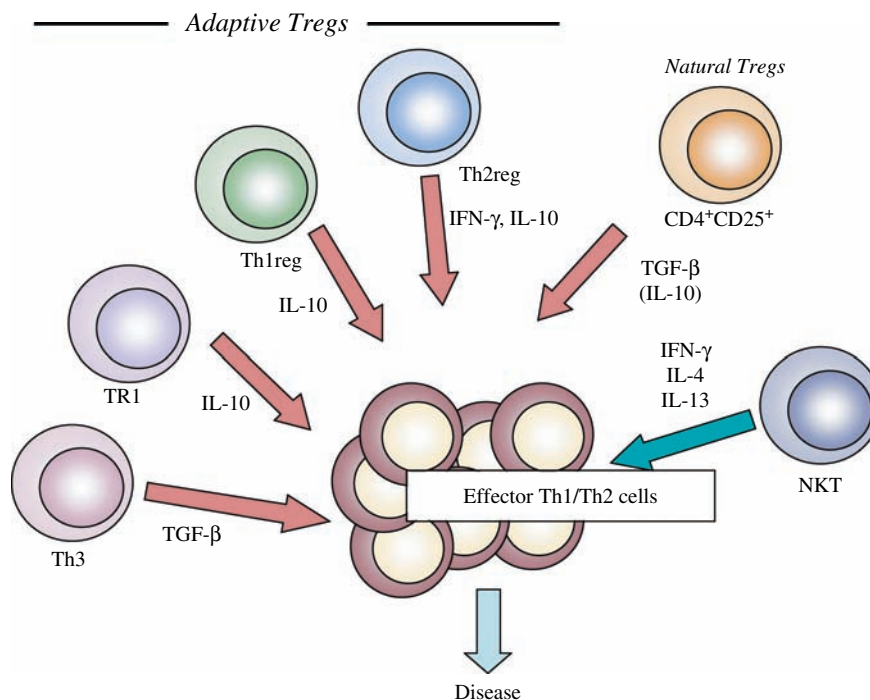


Fig. 1. Regulatory T cells (Tregs) in allergy and asthma. There is a family of Tregs, including natural Tregs and adaptive Tregs, that influences the development of allergy and asthma. Adaptive, antigen-specific

Tregs include T-helper 3 (Th3) cells, TR1 cells, Th1 regulatory cells (Th1reg), and Th2 regulatory cells (Th2reg). Natural killer T (NKT) cells also affect these diseases and enhance the development of asthma.

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