

Mucosal immunity and allergic responses: lack of regulation and/or lack of microbial stimulation?

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Summary: Allergic hyperreactivity is defined as an exaggerated immune response [typically immunoglobulin E (IgE) but also non-IgE mediated] toward harmless antigenic stimuli. The prevalence of allergic disease has increased dramatically during the last 20 years, especially in developed countries. Both genetic and environmental factors contribute to susceptibility to allergy. Evidence has emerged supporting the hypothesis that a reduction in antigenic stimulation brought about by widespread vaccination, improvements in standards of hygiene, and extensive use of antibiotics has contributed to the dysregulation of T-helper 2 cell (Th2) type responsiveness that typifies allergy. Regulation of the inherently Th2-biased mucosal immune response is crucial both to the maintenance of homeostasis at this strategic defensive barrier and to the prevention of allergic disease. The ability of Th1 responses to counter-regulate Th2 reactivity is well characterized. More recently, interest has centered on regulatory T cells, which can suppress both Th1 and Th2 cells through the secretion of immunosuppressive cytokines such as interleukin-10 and transforming growth factor- β . In this review, we discuss the basic cellular mechanisms of allergic diseases at mucosal surfaces, focusing on allergic responses to food, before examining newer work that suggests the induction of allergic hyperreactivity is due to a deficient immuno-regulatory network, a lack of microbial stimulation, or both.

Cellular mechanisms of allergic diseases

Hypersensitivity reactions (both allergic and non-allergic) are exaggerated responses triggered by low doses of innocuous antigens. Although the production of immunoglobulin E (IgE) is often considered the hallmark of an allergic response, allergic hypersensitivity reactions can also be non-IgE mediated (1) (Fig. 1). Atopic individuals have a hereditary predisposition to produce IgE antibodies against common allergens and typically have one or more atopic diseases (i.e. allergic rhinitis, asthma, and atopic eczema). These disorders affect 10–15% of the western population, and their prevalence has doubled in the last two decades. The cellular mechanisms governing allergic hyperreactivity are not fully understood; both genetic and environmental factors play a role.

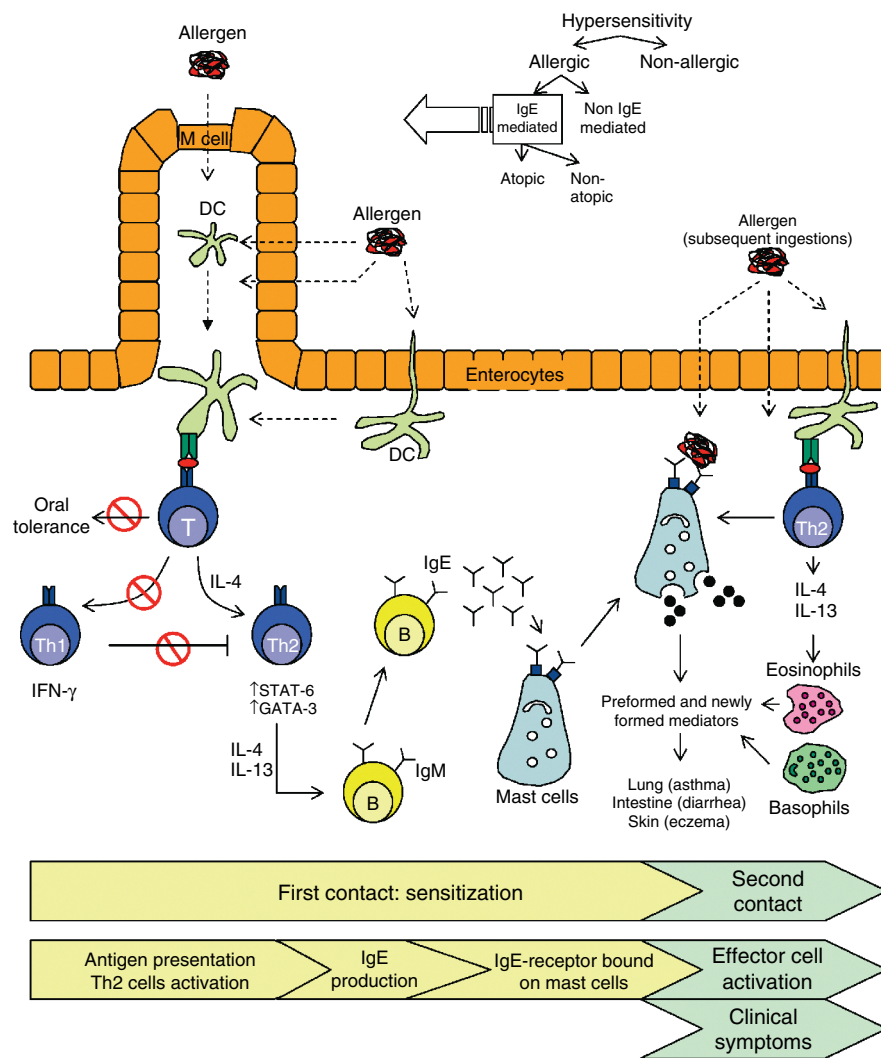


Fig. 1. Cellular mechanisms of allergic responses to food. In healthy individuals, ingestion of innocuous antigens leads to systemic non-responsiveness (oral tolerance). In allergic patients, oral tolerance is not induced. In the T helper 2 cell (Th2)-biased mucosal cytokine microenvironment, antigen-specific Th2 cells are generated when antigen fragments are presented to naïve T cells by antigen-presenting cells, mainly dendritic cells (DCs). Once activated, Th2 cells produce interleukin-4 (IL-4) and IL-13, which promote immunoglobulin E (IgE) production by B cells. IgE binds to its receptor on allergic effector cells (mast cells). Upon subsequent ingestion, antigen presentation leads to a rapid T-cell activation and the secretion of Th2 cytokines, triggering mediator release by eosinophils and basophils. At the same time, antigenic fragments may interact directly with receptor-bound IgE on mast cells and aggregation of receptors triggers the release of the preformed and newly formed chemical mediators (particularly histamine) responsible for clinical symptoms. STAT 6, signal transducer and activator of transcription 6.

Immediately after birth, the mucosal surfaces of the gastrointestinal, respiratory, and urogenital tracts are assailed by a large variety of microorganisms and environmental antigens. Epithelial cells form a physical barrier to protect the body from invasion by potentially pathogenic microorganisms. Numerous mechanisms of defense reinforce the epithelial barrier; among these the production of secretory IgA (S-IgA) seems to be particularly important (2). In the gut, dietary proteins may be taken up by specialized epithelial cells (M cells) (3), enterocytes (4), or dendritic cells (DCs) (5), depending on their nature and size, before being processed and presented as fragments to naïve T cells in the gut-associated lymphoid tissue (GALT) (Fig. 1). Depending on the allergenicity of the fragments and the cells present in the surrounding microenvironment, naïve T-helper cells (Th0 cells) differentiate into particular types of helper T cells. These helper T-cell subsets are classified based on their cytokine profile. Th1 cells are characterized by their production of

interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α). In contrast, Th2 cells are characterized by the production of interleukin-4 (IL-4), IL-5, IL-9, and IL-13 (6). Healthy individuals exhibit a transient proliferative response to food antigens, but non-responsiveness (oral tolerance) is ultimately induced (reviewed in 7). Non-responsiveness is not induced in atopic individuals, and instead, an exaggerated Th2 response to specific dietary antigen(s) ensues. Th2 polarization at mucosal surfaces is apparently regulated by the same factors present at other sites. It is generally accepted that early IL-4 expression during an immune response is critical for the development of Th2 cells (8), whereas the early expression of IL-12 and IL-18 by antigen-presenting cells (APCs) favors the development of Th1 cells (9). IL-4 is produced at low levels by naïve Th0 cells but at high levels by natural killer (NK) cells, basophils, mast cells, and eosinophils (10), all of which play a role in the development of allergic diseases. IL-13 secreted by NKT cells also contributes to the Th2 polarization

of Th0 cells (11–13). IL-4 and IL-13 released into the microenvironment interact with their receptors on T cells, triggering a biochemical cascade which leads ultimately to the activation of the transcription factors signal transducer and activator of transcription 6 (STAT6, 14) and GATA-3 (15). GATA-3 inhibits the production of IFN- γ , increases the activation of the IL-4 promoter (16), and also regulates IL-5 and IL-13 expression (17, 18). IL-4 and IL-13 then promote the production of IgE by B cells (19, 20), which binds to its receptors on mast cells (21). Upon subsequent ingestion (or, in the airways, inhalation), allergenic fragments interact directly with receptor-bound IgE on mast cells, and aggregation of receptors promotes mediator release (histamine, leukotriene, chemokines, etc.) (22), leading to allergic symptoms (23). Antigen presentation by APCs to antigen-specific Th2 cells also leads to rapid T-cell activation and Th2-type cytokine secretion (Fig. 1).

Food allergy

Up to 8% of children less than 3 years of age and approximately 2% of the adult population experience food-induced allergic disorders. Most recognized food hypersensitivities are IgE mediated, and they are more prevalent in atopic patients. Allergic reactions may occur several minutes to hours after contact with the offending allergens, and patients present with a variety of clinical symptoms including abdominal pain, nausea, flatulence, diarrhea, and vomiting. A number of factors can affect the development of food allergy, including diet and culture, route of exposure, processing, cooking, digestion, and the allergen itself. Ninety percent of food-related hypersensitivity reactions can be attributed to eight major types of food: milk, eggs, fish and crustaceans, peanuts, soybeans, tree nuts, and wheat (24). The precise properties that render a food molecule allergenic are unknown, but generally food allergens are proteins with at least two IgE-binding sites (epitopes) that are highly stable and resistant to food processing and digestive enzymes in the gut (24, 25). The number of epitopes, their location on the quaternary structure of a protein, and their amino acid sequence may contribute to the overall allergenicity, because a single amino acid substitution within an epitope can abolish IgE binding (26, 27).

Allergic diseases: lack of regulation?

The increasing prevalence of allergic diseases in the developed world parallels the reduction in infectious diseases that has resulted from widespread vaccination, the use of antibiotics, and high hygiene standards (28, 29). These observations led

to the formulation of the 'hygiene hypothesis', which suggested that microbial infections induce Th1-biased immune responsiveness, which, in turn, protects the host from Th2-biased allergic diseases (30). There are, however, several problems with this hypothesis as originally proposed. The first is that while the prevalence of diseases related to Th2-biased immune dysregulation has been increasing, so too has the prevalence of Th1-biased diseases like inflammatory bowel diseases (31). Second, parasitic helminth infections that induce a polarized Th2-biased response are associated with protection from the development of clinical manifestations of atopy (32, 33). These observations suggest that microbe-induced protection against atopy is not simply the result of immune deviation. Because the cytokines secreted by Th1 cells cross-regulate Th2 cells (6) at least partially (34), it was assumed that Th1 cells had a beneficial role in allergic disease and asthma. However, direct examination of the role of antigen-specific Th1 effector cells in asthma demonstrated that although Th1 cells were able to reduce mucus production and airway eosinophilia, they failed to inhibit Th2 cell-induced airway hyperreactivity (35). This finding suggests that other forms of immunity might be effective in downregulating both inflammation and allergic diseases and asthma. Wills-Karp *et al.* (28) have proposed an alternative paradigm, which suggests that all types of microbial stimulation (both Th1 and Th2 polarizing) induce regulatory cells that control immune responsiveness through the production of immunosuppressive cytokines [IL-10 and transforming growth factor- β (TGF- β)] (28).

Regulatory T cells

The term regulatory T cells (Tregs) refers to cells that actively control or suppress the function of other cells, generally in an inhibitory fashion. The phenotypic and functional characterization of the cells involved in immune regulation has recently experienced a resurgence, and a number of different types of Tregs have been described (36). As discussed in detail below, some of these represent 'natural' thymus-derived lineages and some appear to be induced by antigen exposure. The precise interrelationships of the cells in these two broad subsets is not yet clear; it is clear, however, that they play a major role in preventing allergic disease. Natural CD4⁺CD25⁺ Tregs were first described by Sakaguchi *et al.* (37). These authors showed that the transfer of T cells depleted of CD4⁺CD25⁺ T cells into athymic nude mice led to a variety of spontaneous autoimmune diseases, while the reconstitution of CD4⁺CD25⁺ cells after transfer of CD4⁺CD25⁻ cells prevented these diseases (37). About 2 years later, Groux *et al.* (38) reported

that CD4⁺CD25⁺ Tregs suppress antigen-induced airway eosinophilia via their influence on the development of the Th2 phenotype. In addition, an interesting clinical study showed that children who outgrew their allergy to cow's milk proteins had higher frequencies of circulating CD4⁺CD25⁺ T cells and decreased *in vitro* proliferative responses to bovine β -lactoglobulin (a major cow's milk allergen) in peripheral blood mononuclear cells (PBMCs) compared with children who maintained clinically active allergy (54). This finding suggests that, in children, tolerance to cow's milk proteins correlates with the presence of circulating CD4⁺CD25⁺ Tregs capable of suppressing potential effector cells. However, the lower numbers of Tregs in allergic children cannot be the sole explanation for their symptoms. Ling *et al.* (55) reported that CD4⁺CD25⁺ T cells from non-atopic donors suppressed proliferation and IL-5 production by their own allergen-stimulated CD4⁺CD25⁻ T cells, whereas the inhibition of proliferation by CD4⁺CD25⁺ T cells from atopic donors was significantly reduced. This study suggests that the inadequacy in Treg function in atopic individuals may be related to a deficiency in the suppressive activity of CD4⁺CD25⁺ T cells rather than insufficient numbers of Tregs.

IL-10-secreting Tr1 cells have also been shown to prevent the induction of allergic diseases. Cottrez *et al.* (41) observed that the co-transfer of Tr1 cells and the model allergen ovalbumin inhibited serum ovalbumin-specific IgE. Th1-like Tregs producing both IFN- γ and IL-10 have also recently been described (56). These Tregs develop from CD4⁺CD25⁻ T cells and express Foxp3 in conjunction with T-bet, a marker of Th1 cells (57). Using an animal model of airway hyper-reactivity, Akbari *et al.* (58) demonstrated that the adoptive transfer of Th1-like Tregs reduced the development of airway inflammation. The same group reported in 2002 that a subset of Tregs producing IL-10, some IL-4, but no IFN- γ (Th2-like) was able to block the development of airway inflammation (58).

The recent work suggests that the concept of Tregs is much more complicated than it would appear from earlier studies. The activation of naïve T cells by DCs seems to generate a large spectrum of T cells (Th1 or Th2 polarized) with and without regulatory activity. The factors driving the acquisition of regulatory activity in the periphery are not yet understood, but it has been hypothesized that CD4⁺CD25⁺ Tregs may educate CD4⁺CD25⁻ T cells to become suppressor cells via cell – cell contact (50). In addition, it is not yet clear if Th1-like and Th2-like Tregs are derived from Th1 and Th2 effector cells, respectively, or from precursor cells during Th1 and Th2 differentiation. In contrast, Tr1 cells (CD4⁺CD25⁻ Tregs)

have a unique profile of cytokine production that is distinct from Th0, Th1, or Th2 cells suggesting that they may be derived from a non-related Th1 or Th2 cell precursor. However, Th1-like, Th2-like, and Tr1 Tregs all suppress the proliferation and the differentiation of both Th1 and Th2 type effector cells through the production of suppressive cytokines (Fig. 2). New insights on the effects of Tregs provided by recent studies notwithstanding, many questions on the role of Tregs in preventing allergic diseases remain to be elucidated. Prominent among these questions are the mechanisms by which Tregs are stimulated *in vivo*, their mode of action, and their regulation.

DCs and the induction of Tregs

There is increasing evidence that DCs play a role in the differentiation and expansion of Tregs *in vitro* and *in vivo*. DCs are bone marrow-derived APCs; small numbers of DCs can be found in most tissues of the body including the GALT (reviewed in 59). DCs in the periphery are mostly immature and are characterized by their low-level expression of cell-surface major histocompatibility complex (MHC) class II and co-stimulatory molecules (CD80, CD86, and CD40). After antigen uptake and migration to secondary lymphoid organs, immature DCs acquire a mature phenotype and can present antigen to naïve T cells. DCs capture self-antigens derived from dead tissues as well as pathogenic and non-pathogenic microbes and environmental proteins at mucosal surfaces. Presentation of both innocuous and self-antigens by DCs does not typically lead to immune hypersensitivity, suggesting that DCs play an important role in the control of self-tolerance and regulation of immune responses to the environment. At least some of the mechanisms by which antigen presentation by DCs regulate tolerance have been described; these include the deletion of self-reactive T cells, the induction of non-responsiveness by immature DCs expressing low levels of costimulatory molecules, and the expansion of Tregs (60). In humans, two major subpopulations of DCs can be identified on the basis of CD11c expression: CD11c^{hi} 'myeloid' DCs and CD11c^{lo} or - 'plasmacytoid' or 'lymphoid' DCs (61). In the mouse, all DCs described thus far are CD11c⁺ and can be divided into three major types. These include plasmacytoid DCs characterized by B220 and Gr1 expression and IFN- α production, CD11b⁺CD8 α ⁻ myeloid DCs, and CD8 α ⁺ lymphoid DCs (59, 62, 63). These DC subsets are functionally distinct (64) and are distributed differently in lymphoid organs (65). Activated DCs influence the generation of polarized Th1 and Th2 responses by their production of IL-12 (66) or IL-4 (67), respectively. Earlier work linked particular DC

subsets with Th1 or Th2 differentiation (68). Subsequent work, however, has emphasized the plasticity of DCs (69).

It was first suggested that Tregs are induced by immature DCs (70, 71). Increasing evidence supports the concept that Tregs may be stimulated *in vivo* by specific subsets of DCs (Fig. 2). In either scenario, IL-10 produced by DCs seems to play a critical role (72). Two different subsets of DCs exhibiting plasmacytoid morphology have been shown to induce Tr1-type Tregs *in vitro* (73) and *in vivo* (74) in an IL-10 dependent fashion. Th1-type Tregs have been found to be induced *in vitro* and *in vivo* by pulmonary CD8 α^+ DCs after treatment with heat-killed *L. monocytogenes* (56), whereas Th2-type Tregs were shown to be induced by pulmonary CD8 α^- DCs secreting IL-10 (58). The resulting Tregs produced IL-10 and blocked the development of asthma in mice (58). The pool of CD4 $^+$ CD25 $^+$ Tregs can be expanded *in vivo* by mature DCs secreting IL-10 (56, 75) or by immature DCs in presence of ovalbumin conjugated with anti-DEC 205 (76). Taken together, these studies suggest that particular types of Tregs are induced and stimulated by specific subsets of activated or immature DCs.

Allergic diseases: lack of microbial stimulation?

The reduction of Th1 responsiveness as a consequence of improved hygiene and the widespread use of vaccination cannot fully explain the high prevalence of allergic diseases in developed countries. Holt (77) has proposed that it is not pathogens but microorganisms from the environment and the normal commensal flora of the gastrointestinal tract that play a prominent role in the prevention of allergic sensitization. Whereas the molecular pathways by which microbes activate the innate immune system to induce protective immunity have been well characterized, it is less clear how microbial signaling pathways may affect the pathogenesis of allergic diseases. Pattern-recognition receptors (PRRs) on APCs are activated by their recognition of microbial pathogen-associated molecular patterns (78). To date, the Toll-like receptors (TLRs) represent the best-characterized PRRs. More than 10 mammalian TLRs and many of their ligands have been identified (78, 79). In general, TLR2 signals in response to a wide range of lipopeptides, peptidoglycans, and other products from Gram-positive bacteria in co-operation with TLR1 or TLR6, TLR4 binds to endotoxins [lipopolysaccharides (LPS)] produced by most Gram-negative bacteria, TLR3 recognizes double-stranded RNA, TLR5 signals in response to bacterial flagellins, and TLR9 recognizes specific hypomethylated (CpG) DNA sequences. The fact that all TLRs signal via shared adapter molecules does not seem to limit the variety of

immunological responses TLR signaling can induce (80). Although the link between TLR signaling and allergy is far from being elucidated, several new studies report protection from allergic diseases induced by bacteria signaling through TLR4, TLR2, and TLR9.

TLR4

Several epidemiological studies report an inverse association between endotoxin exposure and atopy rates (81, 82), although this trend remains controversial (83). Children raised on farms with livestock had less atopy and asthma than non-farming children (84), which was attributed to exposure to higher levels of endotoxins (81). The protective effect of endotoxin has also been observed among non-farming children; house-dust endotoxin levels have been found at higher levels in non-sensitized infants' homes (82). The protection against allergen sensitization by indoor endotoxin exposure early in life may be explained by increased proportions of Th1-type T cells in non-sensitized infants with no effect on Th2-type cytokines (82). Although the effect of endotoxins has been studied mostly in children, some studies reported that endotoxin can also protect against atopy later in childhood or in adulthood. A new trend of thought suggests that endotoxins may even reverse (as opposed to protect against) atopic diseases, but this hypothesis is not supported yet by epidemiological studies (85). A recent study examined a role for TLR4 signaling in atopy using explanted mucosa from atopic and non-atopic children cultured *ex vivo* with LPS (86). LPS stimulation prevented allergen-induced Th2-type inflammation and upregulated Th1 type cytokines in explants from atopic children. TLR4 can be expressed on T cells, particularly on CD4 $^+$ CD25 $^+$ T cells (87), and its expression is increased by LPS (86). These TLR4 $^+$ regulatory cells produce IL-10 suggesting the possibility that at mucosal surfaces, TLR4 signaling by the commensal flora is important for the induction of Tregs (86). In addition, several studies have demonstrated that atopy and asthma are associated with a genetic polymorphism for TLR4 (88, 89) as well as polymorphism for CD14, the endotoxin receptor on monocytes and other inflammatory cells (90). TLR4 polymorphisms have been shown to be associated with decreased LPS-induced IL-12 (p70) and IL-10 (89), which are important for the downregulation of the Th2 response.

Animal studies have reported findings consistent with the epidemiological work. Using a model of airway hyper-reactivity, Watanabe *et al.* (91) demonstrated that endotoxin-free ovalbumin was far more effective than endotoxin-contaminated commercial protein in stimulating

IgE production and respiratory dysfunction. However, the level of LPS exposure seems to determine the type of immune response. Low levels of inhaled LPS induce a Th2-type response specific to inhaled antigens, whereas high levels of LPS result in a Th1 response in a mouse model of allergic sensitization (92). In a murine model of allergy, activation of TLR4 by λ -carrageenan leads to decreased levels of both ovalbumin-specific IgE and histamine release upon systemic challenge with ovalbumin (93). More recently, Velasco et al. (94) reported that the treatment of mice with the TLR4 agonist Lipid A during allergen sensitization or challenge decreased the allergen-induced pulmonary recruitment of eosinophils. Treatment with Lipid A, prior to sensitization, is more efficient as shown by decreased pulmonary eosinophilia as well as lower levels of IL-13 in bronchoalveolar lavage fluid, lower total serum IgE, and lower airway hyperresponsiveness (94). Our group has recently described an important role for TLR4 signaling in the induction of food allergy using a murine model of peanut hypersensitivity (95). We observed elevated plasma histamine levels and anaphylactic symptoms in three different strains of mice lacking functional TLR4 compared with controls. TLR4 wildtype mice exhibited allergic symptoms and a Th2-type response similar to TLR4 mutant mice when the bacterial flora was reduced by antibiotic treatment. However, no allergic symptoms were observed in TLR4 wildtype mice with a restored intestinal microbiota at the time

of sensitization with peanut, confirming that the commensal flora was the source of the TLR4 ligand (95). Similar results were observed when susceptibility to allergy was evaluated in TLR4 knockout mice (95) (Fig. 3). Taken together, these data suggest that TLR4 signaling by the intestinal microbiota or by biochemical products like λ -carrageenan prevent allergic reactions. Whether the ability to influence susceptibility to allergy is unique to innate immune signaling by LPS on Gram-negative bacteria or extends to TLR ligands expressed by other components of the intestinal microbiota (Gram-positive bacteria, fungi, etc.) remains to be determined.

TLR2

The relationship between TLR2 and allergic responses is controversial. Using a murine model of asthma, Redecke et al. (96) observed that two subcutaneous treatments with ovalbumin plus Pam3Cys, a synthetic ligand of TLR2, before intranasal rechallenge with ovalbumin, aggravated asthma by increasing both Th2 cytokine production and the recruitment of eosinophils to the lungs. The effects of Pam3Cys were attributed to its ability to induce Th2 cytokine production by bone marrow-derived DCs treated *in vitro* (96) and was confirmed in *in vitro* studies performed with human DCs treated with TLR agonists (97–99). However, this study was performed with commercial ovalbumin preparations known to be contaminated with endotoxins (91, 100).

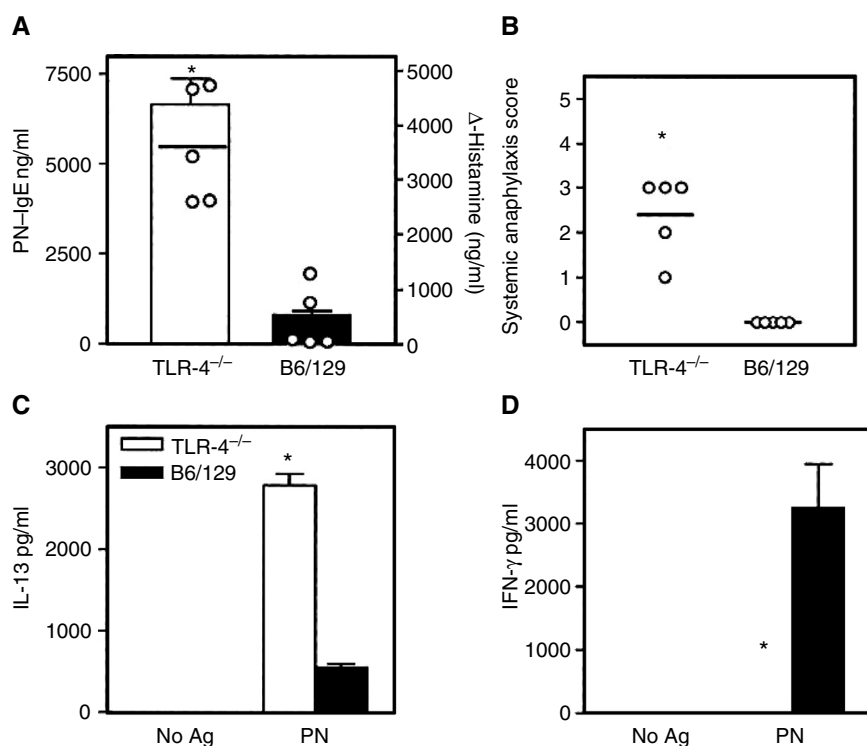


Fig. 3. Effect of Toll-like receptor 4 (TLR4) deficiency on the allergic response to peanut (PN) in mice. B6/129 TLR4^{-/-} mice and their wildtype controls were sensitized three times (days 0, 14, 21) with the PN allergen Ara h 1 and cholera toxin before challenge on day 28 with two doses of Ara h 1 given 30 min apart. Anaphylactic symptoms (B) were evaluated for each mouse 30 min after the last challenge. Histamine released in plasma during challenge is expressed as the difference between histamine levels before and after challenge (Δ -histamine) for each mouse (A). In correlation with anaphylactic symptoms (B), TLR4^{-/-} mice produced higher levels of PN-specific immunoglobulin E (IgE) and released higher amounts of histamine than TLR4 wildtype controls (A). After *ex vivo* restimulation with Ara h 1, splenocytes isolated from TLR4^{-/-} mice produced high levels of IL-13 (Th2-type cytokine) (C) and little interferon- γ (IFN- γ) (Th1-type cytokine) (D) when compared with TLR4 wildtype controls. Reprinted with permission from Bashir et al. (95), © 2004, The American Association of Immunologists.

Because endotoxins at low levels can prime for a Th2 type response in a model of asthma (92), the Th2 response observed by Redecke *et al.* (96) may be explained at least partially by endotoxin contamination of the ovalbumin. In contrast, stimulation of TLR2 with PamCys or peptidoglycan, a natural TLR2 ligand (78), decreased pulmonary recruitment of eosinophils, total serum IgE, and airway hyperresponsiveness in a murine model of asthma using an endotoxin-free preparation of ovalbumin (< 0.01 EU/ml) as antigen (94). However, a recent epidemiological study revealed that polymorphisms in genes encoding TLR2 modify the frequency of asthma and allergies (101). Taken together, these data suggest that further studies are needed to determine the effect of TLR2 signaling on allergic diseases.

TLR9

The effects of CpG oligodeoxynucleotides (ODNs), a well-defined TLR9 ligand (102), on allergic responses and asthma has been well studied during the last 10 years (reviewed in 103). Synthetic ODNs [CpG ODNs and ISS ODNs (synthetic immunostimulatory sequence)] have been found to prevent (104, 105) and treat asthma in different murine models (106–109). Co-administration of CpG ODNs during sensitization with peanut and cholera toxin also abrogated anaphylactic symptoms and serum peanut-specific IgE in our model of peanut allergy induced in TLR4-deficient mice (95). Moreover, both systemic (106, 107, 109) and mucosal (108, 110) administration of allergen and synthetic CpG ODNs are effective at reversing established allergic sensitization. This effect is partly mediated by IFN- γ , because the administration of CpG to IFN- γ knockout mice failed to inhibit eosinophil recruitment, a hallmark of the asthmatic response (105). CpG ODNs act as potent Th1-polarizing adjuvants (111) by activating NK cells to produce IFN- γ and stimulating DCs to express IL-12 and IL-18 mRNA (112). Increasing evidence suggests that CpG ODNs protect against Th2-mediated allergic diseases by augmenting the Th1 response and restoring the Th1 – Th2 balance. The specificity of the Th1 response stimulated by CpG ODNs remains controversial, because both antigen-dependent (110) and antigen-independent responses (113) have been reported to protect against allergic disease. However, CpG ODNs also provide protection against allergic diseases by inhibiting eosinophilia (through the reduction of IL-5) (110), inhibiting the accumulation of peribronchial mast cells (via the reduction of IL-4 and IL-9) (114), modulating costimulatory molecule expression (B7.1 and B7.2) in lung tissues (109), and downregulating the antigen-specific Th2-biased response

mediated by plasmacytoid DCs in nasal mucosa (115). Interestingly, intraperitoneal treatment with CpG ODNs one day before challenge with ovalbumin in intranasally sensitized mice induced indoleamine 2,3-dioxygenase (IDO) in both lung epithelial cells and APCs (macrophages and DCs) (116); the enzymatic activity of IDO has been associated with the suppressive activity of DCs and inhibition of T-cell proliferation (117). In addition, high levels of TGF- β were found in bronchoalveolar lavage fluids of CpG-treated mice (106), suggesting that the protective effect of CpG ODNs may be partially mediated through induction of Tregs and/or regulatory DCs.

MyD88

Myeloid differentiation factor 88 (MyD88) is important for signaling by all TLRs characterized thus far [with the possible exception of TLR3 (78, 118)]. Recent findings reveal that signaling through the MyD88 pathway is essential to maintain intestinal epithelial homeostasis (119). At the same time, TLR signaling using MyD88 as an adapter molecule has also been found to play a crucial role in the activation of the adaptive immune response (120). MyD88-dependent TLR activation seems to release the suppressive activity of Tregs and allow for the induction of a productive immune response (121, 122). Schnare *et al.* (120) reported that MyD88 knockout mice could not mount a Th1 type response to immunization with Freund's adjuvant but developed a normal Th2 response to vaccination with alum, suggesting that signaling through MyD88 controls the Th1 (but not the Th2) response. However, recent findings have demonstrated that in addition to providing positive signals for Th1 cells, mouse DCs activated by TLR ligands using a MyD88-dependent pathway provide a potent negative signal that prevents the development of Th2 cells (123). This negative signal is apparently reversible, since stimulation of DCs by flagellin drives MyD88-dependent Th2 immunity in mice (124). Moreover, MyD88^{-/-} mice have high levels of circulating IgE, suggesting a complex role for the MyD88 pathway in the control of the aberrant Th2 response that characterizes allergic hyperactivity (120). Taken together, these data suggest that signaling through TLR and MyD88 controls the Th1 response by actively inhibiting the Th2 response. The apparent Th2-biased 'default' of immune responsiveness in the absence of MyD88 signaling is reminiscent of that seen at the neonatal stage and might reflect the influence of innate immune signaling on the maturation of the immune system (125). At this point, the role of MyD88 in the development of allergic

diseases remains speculative, since no work has been performed in animal models of allergic disease or asthma.

Can Tregs stimulated by microbial signals prevent and treat allergic diseases?

There is increasing evidence that Tregs fail to be adequately stimulated in allergic patients. At the same time, several studies support the hypothesis that a decrease of immune stimulation by microbial products (both from the environment and from the gut flora) has led to an increased prevalence of allergic disease. Therefore, prophylactic and therapeutic approaches based on the stimulation of immunoregulatory networks by microbial agents have become attractive. The modulation of the intestinal microflora by microorganisms having beneficial effects on human health (called 'probiotics') and treatment with helminthic parasites (or their products) are two promising approaches currently being examined.

Effect of probiotics on allergic diseases

In humans, microbial colonization of the intestine begins just after birth. However, development of the normal flora is a gradual process, which is initially determined by factors such as composition of the maternal gut flora, environment, and possibly genetic influences. Additional variables, such as the degree of hygiene, mode of delivery (natural or caesarean), use of antibiotics and other medications can all have a substantial effect on microbial colonization and development of normal gut flora in infants. The evidence that the intestinal microflora is closely linked to the development of allergic diseases is supported by several studies performed in mice (126) and in humans comparing the bacterial composition of the intestinal microflora of atopic and non-atopic children. Atopic children have more clostridia (127, 128) and bacteroides (129) and tend to have fewer bifidobacteria (127–129) and enterococci (128) than healthy children; this trend is maintained at older ages (130). In addition, in earlier studies, it was shown that the higher prevalence of allergies in infants fed standard formulas compared with breast-fed infants (131) correlated with lower frequencies of bifidobacteria in their feces (132). Furthermore, while the composition of the adult intestinal microflora is quite stable over time, the intestinal microflora of infants is constantly changing (133), and its composition has been reported to be modulated after oral administration of specific bacterial strains called probiotics (134, 135).

Probiotics are defined as 'a live microbial feed supplement that is beneficial to health' (135). A probiotic effect can be induced by the ingestion of viable microorganisms, either in the form of specific preparations such as powder, tablets or capsules, or through yogurts and other fermented foods. Lactobacilli and bifidobacteria are the most common probiotic species, but several other microbes exhibiting probiotic properties are under investigation, including *Escherichia coli*, propionibacteria, enterococci, and yeast (136, 137). Beneficial effects of probiotics have been reported in the treatment of inflammatory bowel diseases, infection with *Helicobacter pylori*, travelers' diarrhea, and antibiotic-associated diarrhea (reviewed in 138). Oral administration of probiotics has also been reported to have beneficial effects on prevention and treatment of allergic diseases (139). A clinical trial performed in Finland enrolling 159 pregnant women reported that the administration of *Lactobacillus* GG prenatally to mothers for 2 weeks and postnatally to babies for 6 months halved the subsequent occurrence of eczema in 2-year-old at-risk infants (140), and this effect extended beyond infancy (141). In agreement with these findings, some probiotic strains have been shown to prevent allergic disease by promoting oral tolerance induction in murine models (126, 142). Treatment with probiotics has been reported to be efficient for the treatment of eczema in children ranging from 1 to 13 years (143) and in infants (mean of 4.6 months of age) (144). The mechanisms by which probiotic bacteria exert their beneficial effects remain unclear, but some hypotheses have begun to emerge from the literature.

Probiotics may decrease allergic diseases by promoting immunosuppressive cytokines (TGF- β) in the milk of lactating mothers (145), stimulating IL-10 production in atopic children (146), increasing the production of IFN- γ in infants experiencing cow's milk allergy (147), and inhibiting Th2 type cytokine production by PBMCs in allergic patients (148). In addition, based on *in vitro* studies, some probiotic strains have been found to stimulate DCs and modulate their cytokine profiles, as evidenced by the increased production of IL-10 (149–152). Interestingly, DCs producing high levels of IL-10 have been found to stimulate the differentiation of Tregs producing IL-10 (74), suggesting that probiotics, through the modulation of DC phenotype and cytokine production, may stimulate the production of Tregs. Increased numbers of CD4⁺ T cells in the ileal epithelium of humans treated with *Lactobacillus reuteri* have been reported, but their potential regulatory activity has not been investigated (153). Based on *in vitro* studies, von der Weid *et al.* (154) reported a strong stimulation of immunosuppressive cytokines (IL-10 and

TGF- β) by CD4⁺ T cells stimulated by one strain of *Lactobacillus paracasei*, suggesting an ability of some probiotics to stimulate Tregs, but further studies are needed to support this hypothesis.

Effects of helminth infections on allergic diseases

Although parasitic helminth infections cause marked morbidity in much of the developing world and are associated with a strong Th2-type response (155–158), they have been associated, somewhat unexpectedly, with a low prevalence of asthma and allergic diseases. Early studies reported tantalizing links between the suppression of allergic hyperreactivity by helminth infection and its reversal by treatment with antihelminthics (159). However, support for Wills-Karp's (28) model of microbe-induced immunoregulation has only been obtained recently. Yazdanbakhsh and colleagues (160) evaluated various immunological parameters to explain the reduced skin test reactivity to a ubiquitous allergen (house dust mites) observed in helminth-infected children in Gabon. Only high-level production of *Schistosoma* antigen-specific IL-10 significantly correlated with reduced skin test reactivity. Other work has confirmed a reduced risk of atopy in helminth-infected children, despite high levels of parasite-induced total IgE (161). In a murine model, we have found that enteric infection can act as an adjuvant to prime for a Th2-biased response to a typically tolerogenic form of dietary antigen (162). However, helminth infection protected against the anaphylactic symptoms and antigen-specific IgE induced in a model of food allergy. Helminth-dependent protection against allergy was abrogated when the helminth-infected, allergen-sensitized mice were treated with neutralizing antibodies to IL-10 (33). The unexpected identification of Th2 responses without atopy has sparked a flurry of speculation on the mechanisms by which helminth infection might protect against allergy (32, 160, 163). The explanation most commonly proposed has been that helminth infection induces T cells with immunoregulatory function capable of suppressing allergic hyperreactivity. Recent reports have begun to provide evidence for immunoregulatory T cells in helminth-infected individuals (164, 165). IL-10 is important for the polarization of the Th2 response and development of resistance against parasites (166, 167) and is produced by both B (168) and T cells (167) during parasitic infection. Infection-induced IL-10 seems to block the release of IL-12 by DCs in response to CD40 ligation leading to an impaired Th1-type response (167). IL-10-secreting Tregs may therefore be involved in both the suppression of Th1 responses required for Th2 polarization and protection against allergy. CD4⁺CD25⁺Foxp3⁺ natural Tregs are expanded in parasite-

infected mice and are more effective than CD4⁺CD25⁺ T cells from non-infected mice at suppressing T-cell proliferation (167). However CD4⁺CD25[−] T cells from infected mice also suppress IL-12 production by DCs, suggesting that Th2 cells and Tregs act in concert to suppress the Th1 response and induce Th2 polarization (167). The mechanisms by which helminths activate innate immunity and elicit immunoregulatory cells are not yet clear. Some evidence suggests that, like Gram-positive bacteria, helminths signal the innate immune system via TLR2. *In vitro*, lysophosphatidylserine from the

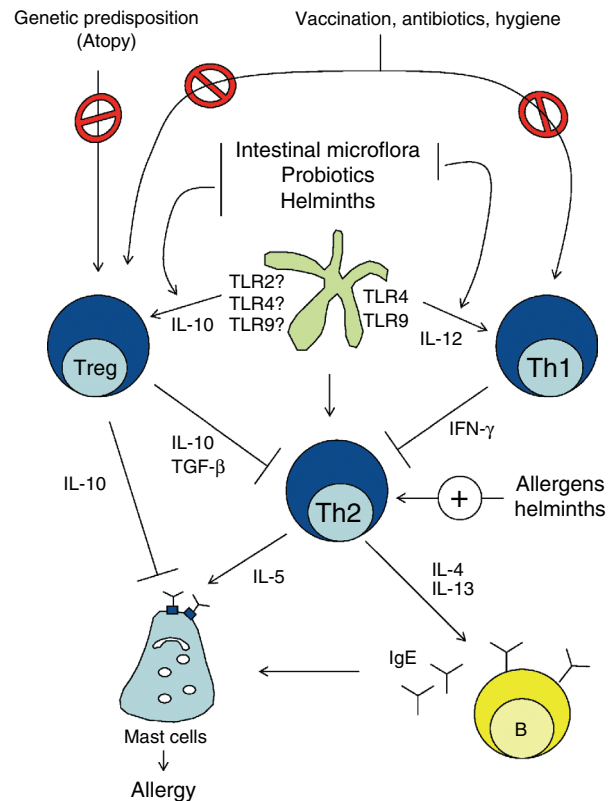


Fig. 4. Factors controlling T helper 2 cell (Th2) activity. Allergen-specific Th2 cells are downregulated by both Th1 cells and T regulatory cells (Tregs). Th1 cells act through the production of interferon- γ (IFN- γ), whereas suppression by Tregs is mediated principally through interleukin-10 (IL-10) and transforming growth factor- β (TGF- β). The reduction of infectious diseases brought about by widespread vaccination, use of antibiotics, and high hygiene standards have resulted in both decreased activation of Th1 cells and insufficient induction of immunoregulatory networks. In addition, Tregs fail to be adequately stimulated in atopic individuals with a genetic predisposition to allergy. Taken together, these observations may help to explain why the prevalence of Th2-biased diseases like allergies has been increasing for the past 20 years. The manipulation of the intestinal microflora and treatment with probiotic bacteria are promising tools for the modulation of both Th1 cells and Tregs. Th1 cells may be induced through innate immune system signaling via Toll-like receptor 4 (TLR4) and TLR9, while the activation of the IL-10-secreting dendritic cells (DCs), which drive Tregs, may be mediated through TLR2. Like allergens, helminth infections induce a polarized Th2 response. However, helminth infections also induce IL-10-secreting Tregs, which prevent the development of atopy.

helminthic parasite *Schistosoma mansoni* activates TLR2-bearing DCs to induce potent IL-10-secreting Tregs (169). Indeed, a growing number of studies have shown that microbial lipids from a diverse array of pathogens play an immunomodulatory role by signaling the innate immune system via TLR2 (reviewed in 170).

Conclusions

The cellular mechanisms regulating allergic diseases and an explanation for their increasing prevalence in developed countries are far from being elucidated. Nevertheless, the available evidence suggests that widespread vaccination and

improved hygiene have contributed to a reduction in the stimuli that counterbalance the Th2-type response that can lead to allergic hyperreactivity. Recent studies emphasize an important role for Tregs in the control of allergic diseases. However, the reasons why Tregs appear to be less frequent and/or less efficient in atopic patients are not yet fully understood. A better understanding of both the influence of the intestinal microflora on the immune system as well as the mechanisms by which particular micro-organisms, such as helminths and probiotic bacteria, stimulate immunoregulatory responses may lead to promising tools to restore homeostasis at mucosal surfaces and to prevent and protect against allergies (Fig. 4).

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