Mechanisms of allergic diseases

Can we produce true tolerance in patients with food allergy?

M. Cecilia Berin, PhD,[a,b] and Lloyd Mayer, MD[b]   *New York, NY*

Immune tolerance is defined as nonresponsiveness of the adaptive immune system to antigens. Immune mechanisms preventing inappropriate immune reactivity to innocuous antigens include deletion of reactive lymphocytes and generation of regulatory T (Treg) cells. The normal response to food antigens is the generation of antigen-specific Treg cells. In patients with food allergy, the dominant immune response is a T_H2-skewed T-cell response and the generation of food-specific IgE antibodies from B cells. It is not known whether a failure of the Treg cell response is behind this inappropriate immune response, but interventions that boost the Treg cell response, such as mucosal immunotherapy, might lead to a restoration of immune tolerance to foods. Tolerance has been notoriously difficult to restore in animal disease models, but limited data from human trials suggest that tolerance (sustained nonresponsiveness) can be re-established in a subset of patients. Furthermore, studies on the natural history of food allergy indicate that spontaneous development of tolerance to foods over time is not uncommon. The current challenge is to understand the mechanisms responsible for restoration of natural or induced tolerance so that interventions can be developed to more successfully induce tolerance in the majority of patients with food allergy. (J Allergy Clin Immunol 2013;131:14-22.)

Key words: Oral tolerance, food allergy, mucosal immunology, immunotherapy, regulatory T

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Abbreviations used	
CT:	Cholera toxin
CTLA-4:	Cytotoxic T lymphocyte–associated antigen 4
DBPCFC:	Double-blind, placebo-controlled food challenge
DC:	Dendritic cell
FoxP3:	Forkhead box protein 3
iTreg:	Induced regulatory T
nTreg:	Natural regulatory T
OIT:	Oral immunotherapy
SCIT:	Subcutaneous immunotherapy
SLIT:	Sublingual immunotherapy
Treg:	Regulatory T

Immune tolerance is defined as a nonresponsiveness of the adaptive immune system to an antigen and can be mediated either by deletion or inactivation of antigen-specific lymphocytes or deviation of antigen-specific T lymphocytes into **regulatory T (Treg) cells**. Immune tolerance is the basis of nonresponsiveness to self-antigens, and disruption of normal tolerance pathways leads to autoimmunity. In addition to discriminating self-antigens from non–self-antigens, the immune system must discriminate harmful non–self-antigens from innocuous antigens, such as those derived from food or the commensal flora. There is some overlap between immune mechanisms responsible for tolerance to self-antigens and innocuous non–self-antigens, which can also be mediated by deletion, anergy, or generation of antigen-specific Treg cells.

Removal of autoreactive lymphocytes is a process that occurs in the thymus and bone marrow and is known as central tolerance. Receiving a strong signal through the lymphocyte receptor at this early stage of lymphocyte development leads to apoptosis of the cell. The thymus has a specialized population of medullary epithelial cells that express a wide range of peripheral tissue antigens under the control of the transcription factor autoimmune regulator *(AIRE).*[1] Mutations in *AIRE* lead to autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (APECED) in human subjects, showing the importance of this pathway in tolerance to self-antigens.[2,3] The thymus is also the origin of a population of Treg cells that express the transcription factor forkhead box protein 3 *(FoxP3)* and are termed natural regulatory T (nTreg) cells. These are distinct from another population of regulatory CD4^{+} T cells that are induced in the periphery and also express FoxP3 termed induced regulatory T (iTreg) cells. iTreg cells will be discussed at a later point in this review. Deletion of autoreactive T cells during development in the thymus is incomplete, and nTreg cells are involved in the suppression of autoreactive effector T cells in the periphery. Human subjects and mice lacking Treg cells caused by mutations in the FoxP3 gene have severe autoimmunity, which is known as immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) in human subjects. Ablation of FoxP3^{+} Treg cells, even in adulthood, leads to rapid onset of autoimmunity in mice, showing that continued presence of FoxP3^{+} Treg cells is necessary for maintenance of self-tolerance.[4]

The paradigm of deletion of antigen-specific lymphocytes and generation of Treg cells also applies to tolerance induced in

Corresponding author: M. Cecilia Berin, PhD, Pediatrics, Box 1198, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029. E-mail: cecilia.berin@mssm.edu. Or: Lloyd Mayer, MD, Immunology Institute, Mount Sinai School of Medicine, 1425 Madison Ave, Box 1089, New York, NY 10029. E-mail: lloyd.mayer@mssm.edu.

Terms in boldface and italics are defined in the glossary on page 15.

mature lymphocytes outside the thymus or bone marrow, and this process is known as peripheral tolerance. Exposure of naive T cells to antigens presented in the absence of costimulatory signals results in inactivation or anergy of the responder cell. In the absence of activation of the innate immune system by microbial signals (pathogen-associated molecular patterns) or damage signals (damage-associated molecular patterns), presentation of self-antigens or environmental antigens does not generate an effector T-cell response but rather deletion or anergy.

The site of antigen presentation also plays a significant role in determining the nature of the T-cell response. We know that antigen presentation in the gastrointestinal tract under homeostatic conditions results in the generation of an active regulatory response, which is termed oral tolerance, that is mediated by the generation of antigen-specific Treg cells. The preferential induction of T cells with regulatory activity is provided by tissue-specific factors, suggesting that the route of antigen exposure might be a critical factor in the development of immune tolerance.

TOLEROGENIC CAPACITY OF THE GASTROINTESTINAL MUCOSA

The phenomenon of oral tolerance was first described by Wells and Osborne in 1911.[5,6] They used guinea pigs to show that inclusion of egg white, purified egg allergens, or oats in the diet rendered the animals hyporesponsive to sensitization and anaphylaxis to those proteins. Six decades later, a number of research groups showed that antigen feeding led to the development of suppressor T cells first in the gastrointestinal lymphoid tissue (Peyer patches and mesenteric lymph nodes) and at later time points in the spleen.[7-9] These suppressor cells, when transferred to naive animals, could inhibit IgE responses or delayed-type hypersensitivity responses in the recipient mice. IgE production is highly sensitive to oral tolerance, and feeding of antigen has been shown to prevent symptoms in experimental models of asthma[10,11] and food allergy or anaphylaxis.[12-15]

Weiner and colleagues initially showed that oral tolerance to myelin basic protein could be mediated by either CD4 or CD8 T cells,[16,17] and subsequent work from the group focused on a subset of Treg cells that they termed T_H3 cells.[18] T_H3 cells produce **TGF-β** and variable levels of IL-4 and **IL-10** and mediate their suppression in a TGF-β–dependent manner.[19] These cells are induced in both human subjects[18] and mice[20] after antigen feeding, and in mice they suppress the clinical severity of experimental autoimmune encephalitis (a model of multiple sclerosis). Regulatory cells other than T_H3 cells have been shown to be involved in oral tolerance. Similar to the early findings that CD8 T cells could transfer tolerance, feeding of mice with an MHC class I epitope of ovalbumin induced oral tolerance to ovalbumin in mice in a CD8-dependent manner.[21] Interestingly, these CD8$^+$ Treg cells could suppress T_H1 and **T_H17** responses but not T_H2 responses. Thymus-derived nTreg cells have been shown to be dispensable for oral tolerance induction,[10] but in contrast, iTreg cells (CD4$^+$**CD25**$^+$FoxP3$^+$ cells) are required for tolerance induction. This was shown by ablation of FoxP3$^+$ cells by using a transgenic mouse expressing the diphtheria toxin receptor under the control of the FoxP3 promoter (the DEREG mouse).[13,22] Injection of diphtheria toxin into the mice abolishes all FoxP3$^+$ Treg cells, including those induced after antigen feeding. After allowing the global Treg cell population to rebound, mice were immunized.

GLOSSARY

CD11c: Also known as p150, CD11c is an integrin expressed on DCs (much less on macrophages) and is involved in leukocyte adhesion through ligands, such as intercellular adhesion molecule 1.

CD25: CD25 is the α chain of the IL-2 receptor and is expressed on activated T cells and Treg cells. Daclizumab, a humanized anti-CD25 antibody, has been used in the treatment of allograft rejection and adult T-cell leukemia.

CD103: Also known as integrin αE, CD103 binds to β7 to form αEβ7 on intraepithelial T cells that are retained in the intestinal mucosa (by binding to E cadherin).

CD154: CD154 is also known as CD40 ligand (CD40L), is expressed on activated T cells, and is required for isotype switching. Mutations in CD40L can cause X-linked hyper-IgM syndrome.

CYTOTOXIC T LYMPHOCYTE–ASSOCIATED ANTIGEN 4 (CTLA-4): Also known as CD152, CTLA-4 is upregulated by activation of T cells and is constitutively expressed by Treg cells. CTLA-4 is a member of the immunoglobulin superfamily and contains an immunoreceptor tyrosine-based inhibitory motif (ITIM). CTLA-4 binds to CD80 and CD86 on the antigen-presenting cell and counteracts activation delivered by the T cell receptor and CD28.

CX$_3$CR1: Part of the chemokine receptor family, all chemokine receptors are 7-transmembrane G protein–coupled receptors. CX$_3$CR1 binds fractalkine (CX$_3$CL1), a membrane-bound chemokine.

FORKHEAD BOX PROTEIN 3 (FoxP3): FoxP3 is expressed in some Treg cells. Congenital absence of Foxp3 Treg cells causes immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome, an immunodeficiency associated with polyorgan autoimmunity.

IL-10: IL-10 is associated with dampening immune responses by working through DCs and macrophages (decreased class II expression, costimulatory molecule expression, and costimulatory cytokine levels) and is produced by Treg cells (T$_R$1 cells).

OX40 LIGAND (OX40L): OX40L is a second signal molecule that is involved in multiple aspects of T_H2 inflammation, including eosinophilic inflammation.

REGULATORY T (Treg) CELLS: Some Treg cells can be CD4$^+$CD25$^+$FoxP3$^+$ and function to dampen the immune response to both allergenic and autoimmune antigens.

RETINOIC ACID: In the intestinal tract retinoic acid production promotes the development of FoxP3$^+$ Treg cells by inducing CD103.

SIGNR1: SIGNR1 is a C-type lectin that is expressed on DCs (DC-SIGN homologue), binds intercellular adhesion molecules 2 and 3, is a receptor for nonendosomal/nonlysosomal-mediated uptake, and is involved in T cell–mediated primary immune responses.

TETRAMERS: MHC peptide tetramers are used to stain antigen-specific T cells for flow cytometric analysis. Tetramers are multimers of peptide–MHC II molecules that can bind to the antigen-specific T-cell receptor.

TGF-β: TGF-β is a pleiotropic growth factor produced by epithelial cells and inflammatory cells, including eosinophils, mast cells, and T cells. TGF-β1 can have profibrotic effects, be a switch factor for IgA, and be a very immunosuppressant cytokine. TGF-β1 can also be produced by Treg cells.

T$_H17: T_H17 cells are CD4$^+$ T cells that are defined by the production of IL-17A, IL-17F, IL-21, and IL-22. T_H17 cells are involved in autoimmunity and defense against bacteria, stimulated to produce IL-17 by IL-23, and maintained by the transcription factor retinoic acid–related orphan receptor γt.

The Editors wish to acknowledge Seema Aceves, MD, PhD, for preparing this glossary.

Transient ablation of the Treg cell population resulted in a loss of oral tolerance.[13] T_H3 and iTreg cells might not be mutually exclusive in their function because T_H3 cells can promote the development of FoxP3+ Treg cells.[23] A number of investigators have shown that TGF-β is necessary for the induction of tolerance through the oral mucosa.[10,19,20,24] In contrast, there are conflicting data about the role of IL-10 in oral tolerance.[10,14,22,24] In addition to effects on other T cells mediated by secretion of cytokines, Treg cells induced by antigen feeding can affect other T cells indirectly by acting through a common antigen-presenting cell.[25]

In addition to tolerance mediated by the generation of regulatory cells, antigen feeding can also result in deletion of antigen-specific effector T cells.[26-28] This phenomenon of deletion was initially described by using mice transgenic for a T-cell receptor against a peptide from ovalbumin, and they described that a high dose of antigen administered orally could induce deletion of these antigen-specific CD4+ T cells, whereas low doses led to expansion of cells with a regulatory phenotype.[26] However, there are several reports of the induction of regulatory CD4+ T cells in response to high doses of antigen administered orally,[13,29] suggesting that this paradigm of deletion at a high antigen dose and regulatory induction at a low antigen dose might not always hold true. Feeding of hapten before induction of hapten-induced contact hypersensitivity has shown that deletion of cells (CD8+ T effector cells) and induction of CD4+ Treg cells can be coexisting mechanisms promoting the development of immune tolerance.[27,28] Mice that have a defect in the gene related to anergy in lymphocytes *(GRAIL)* in their T cells cannot be orally tolerized.[30] Anergy is defined as nonresponsiveness of the T cells without having suppressive or tolerogenic activity, and the lack of tolerance to fed antigens in *GRAIL*-deficient mice suggests an additional role for anergic T cells in peripheral tolerance. It is likely that all 3 mechanisms of deletion, anergy, and active regulation play a role in maintaining immune tolerance to fed antigens.

The selective induction of Treg cells in response to antigen delivered to the gastrointestinal mucosa is mediated by a specialized subset of gastrointestinal dendritic cells (DCs). There are 2 developmentally distinct lineages of mononuclear phagocytes expressing **CD11c** within the intestinal lamina propria: those that express the surface marker **CD103** and those that express the chemokine receptor **CX₃CR1**.[31,32] CX₃CR1 mononuclear phagocytes can extend dendrites between epithelial cells and sample antigen directly from the lumen.[33,34] However, they are thought to be nonmigratory and are not able to transmit these antigens to the mesenteric lymph nodes for the induction of an adaptive immune response.[32] Recent evidence suggests that these CD103− mononuclear phagocytes are transcriptionally closer to macrophages than DCs.[35] CD103+ DCs are migratory and constitutively traffic to the mesenteric lymph nodes.[32] CD103+ DCs were recently found to acquire antigen through intestinal goblet cells that functioned as a conduit for delivery of antigens from the intestinal lumen.[36] Under homeostatic conditions, these CD103+ DCs selectively induce the development of iTreg cells through mechanisms dependent on TGF-β, *retinoic acid*, the enzyme indoleamine 2,3-deoxygenase, and the cosignaling molecule 4-1BB.[37-40] In addition to imprinting regulatory function on naive T cells, these CD103+ DCs also imprint gut homing in a retinoic acid–dependent manner[41] and promote the generation of gut-homing, IgA-secreting B cells.[42] Surgical ablation of the mesenteric lymph nodes abolishes oral tolerance,[43] whereas Peyer patches have been shown to be dispensable for tolerance.[44,45]

Is the gastrointestinal mucosa uniquely tolerogenic? Although the gastrointestinal tract has specialized mechanisms to suppress immune responsiveness to the gut flora, such as high constitutive levels of IL-10 from intestinal macrophages[46] and high levels of retinoic acid generated by stromal cells,[47] Treg cells can be initiated at other sites. Tolerance to antigen through the respiratory tract is well established,[48-50] and, as in the gut, regulatory responses to antigen in the lung are mediated by distinct airway DC subsets.[51] Immune tolerance has also been described in response to antigen applied through the mouth mucosa[52,53] and the skin.[54,55] As in the gut, a population of skin-draining DCs was found to express high levels of the enzyme retinaldehyde dehydrogenase, which is necessary for retinoic acid production, and facilitate the development of FoxP3+ Treg cells.[56] Therefore it is likely that immune tolerance can be induced at multiple sites in the body.

IS FOOD ALLERGY ASSOCIATED WITH A DEFECTIVE Treg CELL RESPONSE?

The adaptive immune response to food antigens in patients with food allergy is characterized by food-specific IgE production from B cells and a T_H2-skewed T-cell response that drives the IgE class-switching. By definition, this is a failure of immune tolerance, but the Treg cell response to foods has been difficult to study in human subjects. Food antigen–specific T-cell lines grown from PBMCs of subjects with food allergy were found to be primarily of a T_H2 phenotype, secreting IL-4 and IL-13 but little IFN-γ.[57-59] There has been mixed success in growing food-specific T-cell lines from control subjects,[58,60,61] but studies that have grown T-cell lines from control subjects have reported that they have a T_H1 or T_H0 profile in comparison with allergic subjects, who have a T_H2 profile.[61] Short-term stimulation of PBMCs and analysis of the T-cell cytokine phenotype in proliferating cells by using flow cytometry has also indicated that healthy control subjects have detectable peanut-specific T cells that are primarily of a T_H1 phenotype compared with peanut-reactive T cells from subjects with peanut allergy who have a T_H2 profile.[62] The use of more specific detection methods of allergen-specific T cells, such as **tetramers**, or detection of **CD154+** T cells after short-term stimulation with peanut antigen (6 hours) has demonstrated that there is a considerably lower frequency of peanut-specific T cells in healthy control subjects compared with that seen in subjects with peanut allergy,[63,64] and although levels of IFN-γ are similar between groups, healthy control subjects have a noted lack of T_H2 cytokine production.[63] The Treg cell response in food allergy has been addressed by using 2 approaches: depletion of CD25+ T cells (including Treg cells) before *in vitro* restimulation with food allergen and detection of dividing Treg cells (CD4+CD25^high) in PBMCs cultured with food allergen for 7 days in the presence of IL-2. The latter approach was used to show that subjects with milk allergy who were tolerant to heated milk had higher levels of milk-responsive Treg cells than subjects who were reactive to heated milk or those who were tolerant to all forms of milk.[65] These subjects who are tolerant to heated milk are thought to be in the process of outgrowing their milk allergy, and therefore it was hypothesized that this Treg cell expansion was involved in the development of tolerance. However, there was no difference observed in the frequency of milk-specific Treg cells when comparing subjects with milk allergy with control subjects, suggesting that a Treg cell defect might not underlie the development of food allergy. Using the approach of

Treg cell depletion to look at the effect on effector T-cell proliferation in milk-restimulated cultures, it has been found that there was detectable Treg cell activity in children who have outgrown their milk allergy.[66,67] There is little evidence in these latter studies for a significant milk-specific Treg cell population in healthy control subjects, although this might be difficult to observe if there are few milk-specific effector T cells present to proliferate after Treg cells have been depleted. Studies are needed that directly address the frequency of food allergen–specific Treg cells in healthy subjects and subjects with food allergy to determine whether baseline clinical tolerance to foods is associated with an active food-specific Treg cell response.

In animal models the default response to an antigen delivered through the oral route is one of active immune tolerance, and therefore adjuvants must be used to elicit allergic sensitization. Commonly used adjuvants include cholera toxin (CT) and staphylococcal enterotoxin B. Oral administration of CT drives an increase in the migration of the normally tolerogenic CD103$^+$ DCs from the lamina propria to the draining lymph nodes and induces a T_H2 response from naive T cells through the costimulatory molecule *OX40L*.[68] It is not known whether the antigen-specific Treg cell responses are also suppressed by CT. Mice sensitized with staphylococcal enterotoxin B as an adjuvant were found to have reduced levels of TGF-β and FoxP3 expression in antigen-restimulated splenocytes,[69] suggesting a suppressive effect of this adjuvant on regulatory pathways. Van Wijk et al[70] inhibited the regulatory molecule cytotoxic T lymphocyte–associated antigen 4 *(CTLA-4)* during exposure to peanut extract. They observed that when CTLA-4 was blocked during exposure to peanut in the presence of CT, there was an enhancement of sensitization and symptoms on allergen challenge. When CTLA-4 was blocked during feeding of peanut without adjuvant, there was no induction of IgE and there were no symptoms induced on allergen challenge.[70]

CTLA-4 is only 1 potential immunomodulatory mechanism used by Treg cells. It was recently described that mice deficient in iTreg cells (but with normal levels of thymic-derived nTreg cells) have spontaneous T_H2-skewed inflammation in the gastrointestinal tract and antibodies against both gastrointestinal autoantigens and antigens derived from the mouse chow.[71] The latter data suggest that iTreg cells have a constitutive role in the suppression of allergic sensitization to dietary antigens. Fig 1 summarizes *de novo* mechanisms of tolerance and sensitization in the gastrointestinal tract.

Why is it important to know whether food allergy is associated with a defective Treg cell response? This might have important implications for the response to immunotherapy. A defective allergen-specific Treg cell response might indicate that providing allergen alone as immunotherapy might not be sufficient to induce a robust Treg cell response in some subjects and that providing a protolerogenic adjuvant might be required for the induction of immune tolerance mediated by Treg cells. Genetic factors play an important role in the susceptibility to atopic disease, including food allergy. Studies in patients with inflammatory bowel disease and their first-degree relatives suggest that genetic factors can also contribute to defects in the generation of oral tolerance to fed antigens.[72,73]

CAN TOLERANCE BE INDUCED THERAPEUTICALLY IN SUBJECTS WITH FOOD ALLERGY?

Immune tolerance is defined as the absence of an antigen-specific adaptive immune response or, alternatively, as the presence of an active Treg cell response. When we refer to the induction of tolerance in food allergy, we define this as a sustained clinical nonresponsiveness to food allergen after discontinuation of therapy. This is distinct from desensitization, which is clinical nonresponsiveness while antigen-specific immunotherapy is maintained. Desensitization to food allergens through oral immunotherapy (OIT) remains experimental, and the literature to date does not support the routine use of OIT for desensitization.[74,75]

The focus of this review will specifically be on the establishment of tolerance. Tolerance that is generated in a sensitized subject might or might not be mediated by immune mechanisms similar to those involved in experimental oral tolerance. We know from studies on the natural history of food allergy that clinical tolerance can develop spontaneously after allergic sensitization has occurred, and in fact, this occurs in the majority of young children who are allergic to milk or egg. For children sensitized to allergens including peanut, tree nuts, fish, and shellfish, the occurrence of clinical tolerance is much lower but not rare (approximately 20% of patients with peanut allergy and 10% of patients with tree nut allergy were found to outgrow their allergy[76,77]). The immune mechanisms responsible for this development of clinical tolerance are not well understood but, as mentioned above, might involve a transient expansion of Treg cells[65,66] in addition to waning allergen-specific IgE levels. The loss of sensitization in early childhood might represent a maturation of the mucosal immune system and the development of a regulatory tone, potentially through changes in microbial colonization.

For those with persistent food allergy, the question remains whether tolerance can be induced by therapeutic interventions. In the case of anaphylaxis induced by insect stings, subcutaneous immunotherapy (SCIT) leads to complete protection from sting-induced anaphylaxis in the majority (>75%) of subjects.[78] A prolonged duration of SCIT (4-5 years) is associated with sustained clinical protection and a continued waning of allergen-specific IgE levels off therapy.[79] Venom allergy and food allergy are comparable in their clinical manifestations (anaphylaxis) and infrequent allergen exposure. Despite these similarities, SCIT for peanut allergy was attempted but abandoned as a therapeutic approach because of the unacceptable rate of reactions to the therapy.[80,81] The differences in response to immunotherapy of these 2 allergic disorders can tell us something about the unique pathways involved in sensitization and tolerance.

There are reports of successful desensitization to food allergens through the oral route throughout the last century.[82] Renewed interest has led to a number of trials of OIT, demonstrating that the majority of subjects undergoing OIT with peanut, egg, or milk tolerate the immunotherapy and become desensitized, such that they can tolerate a food challenge while receiving daily allergen immunotherapy.[83-87] The question remains whether this approach is disease modifying and whether true tolerance (sustained nonresponsiveness after a period of time off therapy) develops. This question has been addressed in a small number of trials to date.

Children receiving OIT for egg or milk allergy for a median period of 21 months had a tolerance rate of 36% during a double-blind, placebo-controlled food challenge (DBPCFC) performed 2 months after OIT discontinuation.[88] However, the tolerance rate in the control untreated group was surprisingly high at 35%, indicating a lack of efficacy of OIT in the development of immune
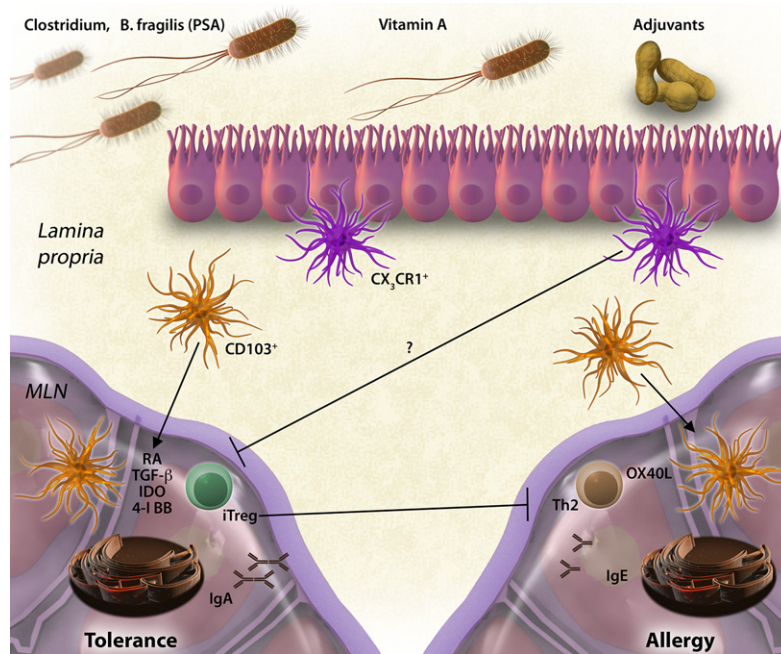
FIG 1. Tolerance and sensitization in the gastrointestinal tract. Under homeostatic conditions, antigens are acquired in the lamina propria and presented in the mesenteric lymph node *(MLN)* by CD103$^+$ DCs. Through mechanisms involving retinoic acid *(RA)*, TGF-β, indoleamine 2,3-dioxygenase *(IDO)*, and 4-1BB, DCs induce the production of gut-homing iTreg cells and IgA-producing plasma cells. Dietary factors (vitamin A) and microbial factors (*Clostridium* species and *Bacteroides fragilis* polysaccharide A *[PSA]*) promote the generation of Treg cells. Under sensitizing conditions that are induced in mice with adjuvants, T$_H$2 cells are generated through mechanisms that involve OX40 ligand *(OX40L)*, and IgE production is induced. Treg cells actively suppress allergic sensitization to foods. These mechanisms have been described in the naive state; it remains to be determined how antigen delivered as OIT in a sensitized subject would be presented by gastrointestinal DCs and how that would modify the adaptive immune response.

tolerance. Buchanan et al[86] performed a 2-year uncontrolled OIT trial for egg allergy, in which 4 of 7 patients passed a DBPCFC at 24 months, and 2 of these 4 patients passed a second DBPCFC 3 months after discontinuation of OIT. This promising but relatively low success rate of tolerance induction was improved in a follow-up trial by Vickery et al[89] that used an OIT dosing regimen in which the maintenance dose was increased stepwise until the egg-specific IgE levels decreased to less than 2 kU/L. At that point, patients underwent a DBPCFC and a second DBPCFC 1 month after OIT discontinuation to determine tolerance development. Six of 6 patients who passed the first DBPCFC also passed the second DBPCFC. Keet et al[90] initially treated patients with milk sublingual immunotherapy (SLIT) before randomization to continue receiving SLIT or receiving OIT at one of 2 maintenance doses for a total of 80 weeks. When a tolerance challenge was performed 6 weeks after completion of immunotherapy, 1 of 10 patients receiving SLIT (7-mg daily maintenance dose) were tolerant, 3 of 10 patients receiving 1000 mg of milk as a maintenance OIT dose were tolerant, and 5 of 10 patients receiving 2000 mg of milk as a maintenance OIT dose were tolerant. The numbers of patients in these trials are small, and the trials are not placebo controlled but provide preliminary data supporting the hypothesis that higher doses and longer duration of immunotherapy can promote sustained nonresponsiveness or tolerance. These data suggest that tolerance might be dose dependent, but this needs to be systematically tested with placebo-controlled trials powered to test significant differences. A recent placebo-controlled trial of OIT for egg allergy with 40 children in the OIT group and 15 in the placebo group demonstrated a desensitization rate of 75% after 22 months of OIT and a tolerance rate of 28% at 24 months, as determined by using a DBPCFC performed 2 months after discontinuation of OIT.[91] No placebo-treated children passed the desensitization challenge at 10 months, but they were not rechallenged at 22 or 24 months except in the case of one subject with an IgE level of less than 2 kU/L (who did not pass the challenge). Children in the OIT group who passed the tolerance challenge added egg to their diet *ad libitum* and did not report any adverse reactions at 30 or 36 months' follow-up. This result suggests that approximately one quarter of children with egg allergy can achieve tolerance after a 2-year period of OIT, although the lack of challenge data in the placebo group at 22 to 24 months is a concern in this interpretation, particularly given the high rate of spontaneous tolerance observed in the placebo group of the trial discussed earlier.[88]

With the caveats discussed above, the data from these studies show that a subset of treated patients achieve sustained nonresponsiveness to foods. Unfortunately, this occurs for only a minority of subjects undergoing this prolonged immunotherapy. The challenge ahead of us is to study these patients to understand how tolerance does occur from an immunologic perspective, so that we can design more rational therapies to facilitate those immune changes in subjects with persistent food allergy. As reviewed in earlier sections and summarized in Fig 1, primary immune tolerance in mice is dependent on the induction of allergen-specific Treg cells that block the generation of allergen-specific IgE. There are very limited data to determine whether the same
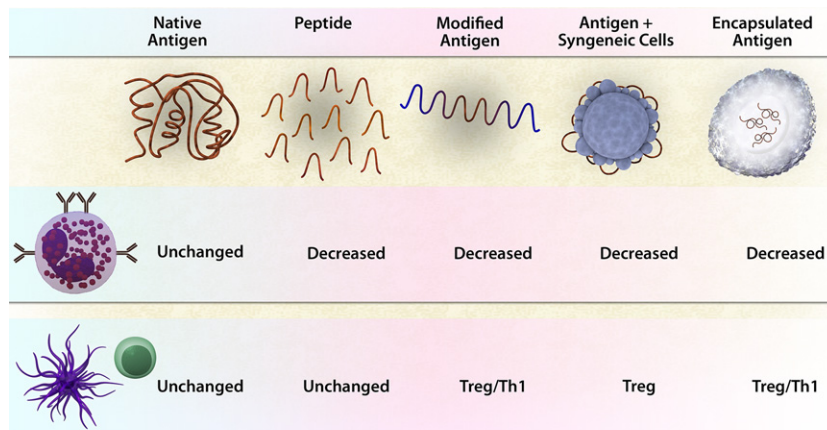
FIG 2. Approaches to maximize the safety and efficacy of immunotherapy with food allergens. Immunotherapy with native antigen results in side effects caused by activation of allergic effector cells and a modification of the adaptive T-cell response to enhance Treg cells and suppress T_H2 cells. Approaches to increase safety by reducing the activation of allergic effector cells includes using peptides that cannot cross-link IgE, modifying the allergens (by heating or mannosylation), binding to particles like syngeneic leukocytes, and encapsulating in nanoparticles or within microbial carriers. Approaches to boost the immunomodulatory effects on the adaptive immune response include modifying the antigen to provide adjuvants that act on antigen-presenting cells (mannosylation and Toll-like receptor ligands), binding to syngeneic leukocytes (providing tolerogenic cues), or encapsulating the antigen together with microbial adjuvants. Studies are needed to test the effect of adding novel immunomodulatory agents (tolerogenic adjuvants or neutralizing antibodies that target antigen presenting cells) to immunotherapy protocols.

Treg cell mechanisms are at play in tolerance induced in patients with food allergy after immunotherapy. OIT has been reported to be associated with changes in various immune parameters, including a boosting of levels of IgG_4 and IgA, which function as blocking antibodies; reduction in basophil and mast cell reactivity; and changes in Treg cell or T effector cell numbers (measured based on T_H2 cytokine release from antigen-stimulated PBMCs).[83,85,86,92,93] There is a rationale for all of these mechanisms to play a role in desensitization, and if these changes are maintained after discontinuation of therapy, they might play a role in tolerance. With the exception of the increase in IgG_4 levels, not all findings are consistent between different trials. Changes in the T-cell response (either induction of Treg cells or anergy or deletion of T_H2 effector cells) have not yet been addressed in tolerance compared with desensitization. In the recent placebo-controlled egg OIT trial, immune markers that were significantly different between those who were tolerized versus desensitized to egg were egg-specific IgG_4 levels after 10 months of treatment (but this difference was no longer apparent after 22 months of treatment) and wheal size after skin prick testing after 22 months of treatment.[91] The relationship between antigen-specific Treg cells and clinical tolerance needs to be carefully explored. The induction of Treg cells can prevent the generation of an IgE response by preventing the T_H2 or T follicular helper response needed for IgE class-switching. When IgE has already been generated in a patient with food allergy, it is not clear how much the generation of an antigen-specific Treg cell response modifies the effector arm of the allergic response, although direct suppression of mast cell activation by Treg cells has been described.[94,95] It remains to be understood whether clinical tolerance is primarily due to an induction of a Treg cell response or a waning of allergic sensitization.

Further studies are needed to determine the relationship between immune markers, such as basophil reactivity, antigen-specific antibody levels or affinity, antigen-specific Treg cells or T effector cells, and clinical reactivity. Performing these immune studies at the time of desensitization and tolerance challenges might be particularly informative for determining the relationship between these immune parameters and clinical tolerance and will help us to understand whether these are biomarkers or potential mechanisms of tolerance. In addition, experimental approaches that are not limited by our current hypotheses of tolerance are needed, such as functional genomic profiling to identify pathways selectively activated in those subjects achieving tolerance. Such an approach is also warranted for the study of immune mechanisms responsible for the natural outgrowth of food allergy, which might be significantly different from the outgrowth induced by immunotherapy. Alternatively, immunotherapy may hasten the outgrowth of food allergy in a subset of patients already predisposed to the development of tolerance. We can begin to address these important questions through advanced immune profiling of subjects who achieve tolerance compared with those with persistent food allergy refractory to immunotherapy.

FUTURE DIRECTIONS OF IMMUNOTHERAPY

Relatively few preclinical studies have addressed immunotherapy from a therapeutic rather than a preventative approach. Feeding of antigen to naive mice efficiently shuts down food-induced allergic responses through the induction of Treg cells that prevent IgE production.[13] In contrast, mice that were orally sensitized to egg white proteins and then subsequently received a course of conventional egg OIT had desensitization but not immune tolerance,[96] which is similar to the response reported for the majority of human subjects. In contrast to these findings, immunotherapy administered through the intraperitoneal route to tree nut–sensitized mice led to an abrogation of anaphylactic symptoms when mice were challenged several weeks after immunotherapy discontinuation.[97] As mentioned previously, immunotherapy through the subcutaneous route was abandoned as a

therapeutic approach because of safety concerns. However, modification of allergens to abrogate their IgE binding yet retain their capacity for presentation to T cells can allow for systemic or subcutaneous delivery of doses high enough to promote tolerance. Supporting this concept, pepsin digestion of cashew allergen decreased the allergenicity of cashew extract *in vivo*, yet when administered systemically as immunotherapy to cashew-sensitized mice, the digested extract was able to abrogate anaphylactic responses to a similar degree as the native protein.[98] Modification of allergen by means of mannosylation[14] or delivery within mannosylated liposomes[99] can effectively prevent the development of food-induced allergic symptoms through a mechanism involving specific ICAM3 grabbing nonintegrin-related 1 (**SIGNR1**) on the DC, and induction of IL-10–producing Treg cells. This approach has been shown to be effective, even when administered after sensitization.[99]

In addition to altering or encapsulating the antigen, other approaches to improve safety include providing antigen by alternative routes. Topical delivery of peanut extract as immunotherapy (epicutaneous immunotherapy) has been shown in preclinical studies to modify clinical reactivity to peanut in mice; this is seen as a reduction in peanut-induced allergic airway inflammation[100] or peanut-induced eosinophilic inflammation of the gastrointestinal tract.[101] Epicutaneous immunotherapy has not yet been tested in food-induced anaphylaxis models to determine its efficacy, but human trials are underway with this novel approach.

In addition to modifying the antigen or route of delivery to have a safer method of immunotherapy, another approach has been to provide adjuvants to promote tolerance or skew the adaptive immune response from a T_H2-dominated response. A vaccine was constructed from *Escherichia coli* bearing modified peanut allergens Ara h 1, Ara h 2, and Ara h 3 that had the IgE-binding epitopes modified. The *E coli* was heat killed and administered through the intrarectal route to peanut-sensitized mice. A sustained reversal of clinical reactivity to peanut was observed in mice treated with this vaccine,[102] leading to the initiation of a current phase I clinical trial in human subjects. Another recent approach used intravenous administration of peanut antigen coupled to syngeneic spleen cells.[103] This approach could provide appropriate self-antigens from the apoptotic cells that can function as a tolerogenic adjuvant. This strategy was safe in that it did not induce anaphylactic responses in peanut-sensitized mice, was a very effective prophylactic approach when given to naive mice, and had modest effects as a therapeutic approach to suppress peanut-induced anaphylaxis in peanut-sensitized mice. The administration of antigen-coupled syngeneic cells is effective in preclinical models of autoimmunity[104] and is currently being tested in human trials for multiple sclerosis.

Provision of antigen with a defined microbial ligand has also been used in preclinical studies for the treatment of food allergy. A fusion protein of flagellin from *Listeria monocytogenes* and ovalbumin was prepared and administered as an intraperitoneal injection before or after sensitization and oral challenge of mice with ovalbumin. Treatment with flagellin-OVA but not flagellin or OVA alone resulted in a significant reduction (but not abrogation) in gastrointestinal symptoms when administered as either a preventative or therapeutic approach.[105] Other potential protolerogenic adjuvants include polysaccharide A from *Bacteroides fragilis*, which promotes the development of IL-10–producing $CD4^+$ Treg cells in a Toll-like receptor 2–dependent manner.[106,107] Colonization with strains of *Clostridium* species also markedly induces IL-10–producing Treg cells in the intestine and prevents the generation of an IgE response after systemic immunization of mice.[108] Manipulation of the gut flora has not been tested in conjunction with immunotherapy, but theoretically, factors that promote the development of Treg cells can be useful adjuvants for the induction of immune tolerance. Fig 2 shows some of the novel approaches to allergen-specific immunotherapy that have been tested in preclinical studies.

CONCLUSIONS

The natural history of food allergy indicates that such allergy can be outgrown and therefore shows that it is possible to acquire tolerance after sensitization has occurred. Unfortunately, natural tolerance is infrequent for antigens such as peanut, tree nuts, fish, or shellfish. Two placebo-controlled trials have been performed that directly address tolerance in response to OIT, one showing no beneficial effect of OIT on tolerance[88] and the other showing tolerance induction in a minority of subjects with egg allergy.[91] Although the latter study shows promise, these findings need to be verified through repetition and expanded to other food allergens. The data do not yet support the use of OIT as a therapy to induce immune tolerance. However, by carefully profiling immune tolerance when it has been successfully established either through natural outgrowth or experimental intervention, we expect to identify means of establishing tolerance in the remaining majority of patients with food allergy.

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