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Immunological mechanisms for desensitization and tolerance in food allergy

Rima Rachid, MD[1] and **Dale T. Umetsu, MD, PhD**[1,2]

[1]Division of Immunology and Allergy, Boston Children's Hospital, Harvard Medical School Karp Laboratories, Rm 10127, One Blackfan Circle, Boston MA 02115

Abstract

Food allergy is a major public health concern in westernized countries, estimated to affect 5% of children and 3-4 % of adults. Allergen specific immunotherapy for food allergy is currently being actively evaluated, but is still experimental. The optimal protocol, in terms of the route of administration of the food, target maintenance dose, duration of maintenance therapy and the optimal patient for these procedures are still being worked out. The mechanisms underlying successful food desensitization are also unclear, in part because there is no standard immunotherapy protocol. The mechanisms involved however, may include mast cell and basophil suppression, development of food-specific IgG4 antibodies, reduction in the food specific IgE/IgG4 ratio, up-regulation and expansion of natural or inducible regulatory T cells, a skewing from a Th2 to a Th1 profile and the development of anergy and/or deletion in antigen specific cells. Additional studies are required to elucidate and understand these mechanisms by which desensitization and tolerance are achieved, and which may reveal valuable biomarkers for evaluating and following food allergic patients on immunotherapy.

Keywords

Food allergy; Immunotherapy; Desensitization; Tolerance

Introduction

Food allergy is a major public health concern in westernized countries, estimated to affect 5% of children and 3-4 % of adults. (1) Over the past decade, the prevalence of food allergies has increased markedly. (2) Approximately ninety percent of allergic reactions to food are secondary to the “big 8” allergenic foods, namely milk, egg, peanut, soy, wheat, tree-nuts, fish and shellfish (3). Peanut allergy alone likely exceeds 1% of school-aged children in the United Kingdom and the United States. (4, 5) Many food allergies are spontaneously outgrown with time; most children outgrow their milk, and egg allergy without treatment by the end of the first or second decade of life. (6, 7) On the other hand, it is estimated that less than 20% of patients with peanut allergy will become tolerant to peanut with time. (8) This is of major concern, given that peanut and tree-nut allergy account for the majority of near fatal and fatal anaphylactic reactions to food. (9, 10)

The currently accepted standard of care for patients with food allergies consists of strict avoidance of the food, nutritional counseling and constant preparedness for treatment in the event of accidental ingestion with antihistamines and/or injectable epinephrine.

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[2]Corresponding Author: dale.umetsu@childrens.harvard.edu, Phone: 617 919 2439, Fax: 617 730 0384.

Unfortunately, this approach negatively affects the quality of life for many patients and their family, as it leads to heightened anxiety from the fear of accidental ingestion, and limits participation in social events and school activities.(11, 12) In addition, up to 40-75% of patients have accidental food ingestions over a 1-10 year period, with exposures commonly occurring in schools, day care, restaurants or other food establishments.(11, 13-16) There is an obvious need for new therapeutic modalities that would either cure food allergy, or at least allow patients to tolerate a defined quantity of food, thereby eliminating the possibility, or at least reducing the severity, of reactions upon accidental ingestion.

Food immunotherapy as an approach for food allergy is currently being actively investigated. As with inhaled allergen-specific immunotherapy (allergy shots), food immunotherapy is performed by the administration of increasing doses of an allergen extract, followed by maintenance dosing for a currently undefined duration of time. Allergen-specific immunotherapy has been shown to be effective for many allergic problems (e.g., allergic rhinitis, bee venom allergy and drug allergy). The mechanisms by which allergen-specific immunotherapy, which has been used for a century for allergic rhinitis, reduces allergy has been studied for decades. Allergen-specific immunotherapy for allergic rhinitis and bee venom allergy is thought to induce peripheral T cell tolerance, modulate the thresholds for mast cell and basophil activation and decrease IgE-mediated histamine release.(17) By contrast, the mechanisms by which immunotherapy for food allergy might work are much less studied and are very poorly understood. (18)

Routes of immunotherapy

Multiple protocols have evaluated the role of food immunotherapy, using different routes of administration. Initially, subcutaneous immunotherapy to food was used, but was associated with a very high rate of systemic reactions (39%) (19), which halted enthusiasm for this approach. Over the past 10 years, oral immunotherapy (OIT) and more recently sublingual immunotherapy (SLIT)(20-27), or both together(28) have been evaluated. In SLIT, a food extract is placed under the tongue and then either swallowed or spit out, while in OIT, the food is ingested. Both therapies rely on administering a small dose first, followed by increasing amounts during the build-up phase to reach a maintenance dose that is usually administered daily. While many of these studies require several months before to reaching the maintenance phase (29-32), rush desensitization has been evaluated in others. (25, 33-38) More recently, epicutaneous immunotherapy trials, which consist of applying the food allergen as a patch on the skin, have been launched.(39) Food desensitization has been studied to different foods, including cow's milk (28, 31, 34, 40), hen's egg (36, 41), peanut (26, 29, 30, 32, 38), hazelnut (25, 27), kiwi(23, 24) and peach (20-22). In addition, one study examined the role of anti-IgE mAb (omalizumab) as an adjunct therapy to milk OIT, which reduced allergic reactions that occurred during the desensitization process (33) (discussed below). Based on the promising findings of this study with omalizumab, three additional trials are being conducted to evaluate the effect of anti-IgE mAb as a adjunct therapy to food OIT, one with milk OIT (Mt Sinai) and 2 others with peanut OIT (Duke University and Boston Children's Hospital-Harvard Medical School) (clinicaltrials.gov).

Tolerance versus desensitization

The ultimate goal of food allergy immunotherapy is cure, resulting in permanent tolerance, as defined by the absence of symptoms after ingestion of the food even after prolonged and/or erratic periods of avoidance. Usually however, only desensitization is achieved, i.e., the patient is able to ingest the food without reactions, but only while remaining on maintenance dosing. True tolerance to foods after desensitization has been evaluated in only a few studies, with approaches varying from stopping the food for 2 weeks to a few months,

followed by an oral food challenge (OFC). (23, 27, 28, 38, 42, 43) As we will discuss in this review, the outcome of these different studies suggest that, while desensitization in the setting of food allergy can be achieved in most cases, some patients regain sensitization after interruption of food intake. We will review the different immuno-modulatory changes that have been described with successful food desensitization.

Evaluation of tolerance

The details of multiple studies of food immunotherapy using different protocols have been reviewed elsewhere.(44-46) Very few trials have evaluated the development of tolerance, which may be affected by different factors, including the route of immunotherapy, the maintenance dose, the length of the maintenance phase, the food-specific IgE level and intercurrent illness.

In one open label study, 21 children aged 5-10 years received cow's milk oral immunotherapy (OIT) over a mean period of 201 days.(31) Fifteen children (71.4%) achieved a daily intake of 200 ml of milk during a 6-month period, while; three of 21 children (14.3%) tolerated 40–80 ml/day, and the other three (14.3%) failed the desensitization. At the final visit, the children who tolerated milk were advised to take cow's milk and/or dairy products *ad libitum*. Four year later, in a follow up study (47) nine of the remaining 14 patients who were desensitized totally to milk were consuming it daily without interruption, taking about 250 mL of cow's milk (corresponding to ~ 8 grams of milk protein) and its derivatives freely, and four were either eating other dairy products, or ingesting milk occasionally. Interestingly, one patient stopped taking milk for a month secondary to a viral illness that occurred 6 months after the desensitization period. When milk consumption was resumed, he developed urticarial and asthma symptoms, indicating that even after successful desensitization, food allergy can return when the food is stopped for a few weeks, even after 6 months of maintenance food ingestion.

In an open label pilot study of egg OIT, Buchanan et al enrolled 7 children (median age 48 months) with a history of non-anaphylactic allergy to egg. After a modified rush desensitization followed by a build-up phase to 300 mg of egg protein, patients remained on a two year maintenance phase. (36) Four patients passed a DBPCFC with 8 grams of egg protein, and all patients tolerated significantly more egg protein during DBPCFC than at study onset. Interestingly, only 2 of 4 patients who passed the DBPCFC were able to tolerate the full amount of egg 3-4 months off OIT. One patient reacted to as low as 24 mg and the others to 2 grams of egg protein. This indicated that even after 2 years of OIT, desensitization may be lost after few months off of the food, suggesting that maintenance with higher daily doses of food may be required in order to achieve a more durable desensitized state. In addition, although a state of tolerance may have been achieved in 2 patients, natural tolerance may have developed spontaneously given that the study was over 24 months and that egg allergic patients are known to become tolerant to egg with time.(7) In a follow up study, the effects of higher maintenance dosing was investigated. (48) The mean egg white-specific IgE in enrolled patients was 18.8 kU/L. After desensitization and daily intake of a maintenance dose of 300 mg egg protein for 4 months, patients with serum egg-specific IgE <2 kU/L underwent an oral food challenge to egg and the dose was increased according to the highest tolerated dose. Cycles of maintenance for 4 months followed by food challenges were continued until a maximum dose of 3.6 g/day was achieved. Patients were then followed every for 4 months on this dose, and whenever egg-specific IgE<2kU/L, OIT was stopped and DBPCFC was performed with up to 10 grams of egg protein. All patients passed the challenge and were taken off OIT for 4 weeks, then successfully passed a DBPCFC. This study suggested that with higher maintenance doses, a state of desensitization can be maintained when the food ingestion is interrupted for 4

weeks. However, it is not clear whether these subjects have reached a permanent state of tolerance. In addition, the study also suggested that low levels of food-specific IgE level may indicate tolerance.

Blumchen et al enrolled 23 peanut-allergic pediatric patients (median age 5.6 years) who underwent a first confirmatory DBPCFC at baseline (threshold dose for reaction<25 mg peanut protein).(49) The median peanut-specific IgE was 95.6 kU/L (range 3-2071 kU/L). Subjects underwent in-hospital rush desensitization for up to 1 week, then a dose escalation over a median of 7 months to reach a maximal target maintenance dose of 500 mg. Maintenance phase lasted for a median of 8 weeks, and was followed by 2 weeks of peanut avoidance. At the final DBPCFC, patients tolerated a median of 1 gram compared to 0.19 gram peanut at baseline prior to OIT. Therefore, after food interruption for 2 weeks, peanut desensitization was still maintained. As in other studies, the effect of longer period of food interruption remains unclear.

Overall, **sublingual immunotherapy** (SLIT) for food allergy has been evaluated in far fewer studies compared to that for OIT. The limitations of SLIT is that the maximal dose of the food is dependent on the small volume that can be administered sublingually, therefore limiting the maximum dose that can be achieved, which may limit consistency in the beneficial outcome. The first case report of food SLIT described a 29-year-old woman with severe anaphylaxis on exposure to small amounts of kiwi fruit.(24) During SLIT, the dose of kiwi extract was increased over a 5 week period to a maintenance of dose of 1 mL, during which time she experienced significant allergic reactions. A maintenance dose of a $1cm^3$ piece of kiwi was continued daily for about five years, at which time it was discontinued for 4 months because of severe tonsillitis.(23). She was then challenged with $1cm^3$ cube of fresh Kiwi, which she tolerated. This study demonstrated that after 5 years of maintenance therapy, the desensitization effect persisted even after a 4 month interruption of food exposure, suggesting that a state of tolerance could be maintained off of the food for at least 4 months.

Most recently, Keet et al explored the efficacy of milk SLIT versus SLIT followed by OIT. Thirty children age 6-17 years received either SLIT alone to 7 mg maintenance dosing, SLIT to 3.7 mg, followed by OIT to 1 gm maintenance dosing, or SLIT to 3.7 mg followed by OIT to 2 gm maintenance dosing.(50) After 1.4 years of maintenance therapy, 10% in the first group, 60% in the 2nd group and 80% in the 3rd group passed an 8 gm oral challenge, indicating that SLIT/OIT is significantly more efficient than SLIT alone in desensitizing patients. However, allergic reactions were much more common in the OIT versus SLIT alone groups. Moreover, 10% of patients in the OIT groups withdrew due to adverse effects. To evaluate for tolerance, the 15 patients who passed the OFC were taken off of maintenance dosing for 6 wks. 50% of the patients in the 2nd group and 38% in the 3rd group regained milk reactivity, indicating that the desensitization status may be lost quickly after stopping oral dosing, despite a long maintenance period. This study is consistent with the findings of Vickery et al and egg allergy, (48) suggesting that higher maintenance dosing may lead to a more durable desensitization state.

It remains unclear why food immunotherapy is more successful in some patients, why others are resistant to desensitization, and why allergic reactions develop during desensitization. Contributing factors may include sub-optimally controlled asthma leading to more severe reactions, physical exertion after dosing and menstruation (51) and higher level of food-specific IgE, perhaps indicative of greater polarization of the immune response. In addition, the role of psychosocial stress in exacerbating or potentiating “allergic” reactions to the food is unclear, and possibly underestimated. There may be a subgroup of patients who are more resistant to desensitization and may require modified protocols including a much slower

build-up phase, and later a longer maintenance period. Potentially, some of these patients may benefit from adjunct therapy to food immunotherapy, such as monoclonal anti-IgE antibodies (mAb). Furthermore, whether a state of permanent tolerance can be achieved in the majority of patients is not known, particularly because few studies examining this issue have been performed, and in these studies, only short periods of food avoidance have been employed. Whether longer maintenance periods and higher maintenance doses are required for real tolerance to develop are also not yet clear. The plethora of unknowns makes the study of the mechanisms of food immunotherapy difficult, but important for improving these therapies.

Mechanisms of food allergen immunotherapy

The mechanisms by which food allergen immunotherapy effectively reduces allergic symptoms are poorly understood, in part because they have been inadequately studied. Presumably many of the features involved in immunotherapy for food allergy are similar to those observed in inhalant allergen immunotherapy, although the precise mechanisms of allergen SCIT or SLIT are still controversial and remain to be fully elucidated. However, there are significant differences between oral and subcutaneous immunotherapy, for example in the doses used (in OIT the doses are many orders of magnitude greater than that used in SCIT). In addition, the immunological mechanisms involved in oral versus sublingual immunotherapy may differ substantially. Furthermore, the mechanisms involved with different food desensitization protocols may be quite distinct, due to differences in the rapidity of dose escalation and in maintenance doses achieved.

Mechanisms in development of allergic diseases

The mechanisms of food desensitization and tolerance must be understood in the context of the underlying mechanisms of the disease, the understanding of which has evolved considerably over the past several years. For example, early cutaneous exposure to food protein through a disrupted skin barrier has been suggested to lead to allergic sensitization, while early oral exposure to food allergen may induce tolerance (52). This hypothesis is currently being tested in 2 randomized controlled trials (learning about peanut allergy (LEAP) study, and the enquiring about tolerance (EAT) study). Classically, the development of food allergy has been viewed as an inappropriate Th2 response to foods, associated with increased allergen-specific IgE production, and an increase in the number of allergen-specific Th2 cells. (18) Clinically, patients often develop reactions with 2 phases, one early and one late. The early phase response is due to mast cell degranulation mediated by allergen-specific IgE leading to the release of preformed and newly synthesized mediators including histamine and leukotrienes, which trigger symptoms of immediate type hypersensitivity. The late phase reaction develops 4-8 hours after the immediate phase response, and involves the development of complex effector functions related to tissue inflammation and injury, with the infiltration of allergen-specific effectors cells, such as Th2 cells and Th9 cells, as well as eosinophils and basophils. (53) In the skin, the late phase response is characterized by significant swelling, pruritis, erythema and warmth; in the lungs the late phase response is associated with a significant reduction in pulmonary function that is difficult to reverse, and that persist for hours. The late phase response is also associated with the development of airway hyperreactivity, a prominent feature of chronic asthma. The specific symptoms of the intestinal late phase response are not well described, but may involve increased intestinal permeability, vomiting and diarrhea.

Th2 and Th9 cells, like Th1, Th17, Th22 and inducible T_{Reg} cells, comprise subsets of, and derive from naive, $CD4^+$ T cells. These subsets differentiate from naïve $CD4^+$ T cell depending on conditions present when antigen is initially introduced. Th2 cells produce

IL-4, IL-5, IL-9, IL13, (53-55) which orchestrate the allergic inflammatory response. Th9 cells secreting IL-9 appear to be a discrete T helper cell subset, and can be induced to develop by the presence of TGF-β and IL-4 in both mice and humans, and this is enhanced by the presence of IL-25. IL-9 enhances the growth of mast cells, and can lead to inflammation in the lung and intestines, including intestinal anaphylaxis. IL-9, in combination with TGF-β, can induce the development of Th17 cells.

Th17 cells represent another recently described cell type, frequently found at epithelial cell surfaces. Th17 cells, induced by the presence of TGF-β, IL-6 and IL-1β, produce IL-17 and IL-22, and protect against bacterial infection by enhancing the recruitment of neutrophils. Absence of IL-17 function in humans is also associated with severe fungal infections in the skin, whereas overproduction of IL-17 is associated with autoimmune diseases such as inflammatory bowel disease and psoriasis. IL-17 is also found in the lungs of patients with severe asthma, in association with neutrophils, and in the skin of patients with chronic atopic dermatitis. Th17 cells produce IL-22, which is also produced by Th22 cells. Th22 cells only produce IL-22, which is an IL-10 cytokine family member that plays an important role in the lungs, skin and intestines by inducing the production of anti-microbial peptides, and by promoting the survival of epithelial cells in the lungs, gut and liver. However, it may also play a pathological role in psoriasis, possibly by potentiating the role of IL-17. IL-22 levels are also increased in the skin of patients with atopic dermatitis.(56)

T_{Reg} cells are thought to down-regulate all of the above-mentioned T effector cell subsets, by producing anti-inflammatory cytokines such as IL-10 and TGF-β. (57, 58) There are 2 main types of T_{Regs} cells: the first one is called natural T_{Reg} (nT_{Regs}) cells, and are selected in the thymus as $Foxp3^+CD4^+CD25^+$ T_{Regs} cells. These nT_{Regs} cells constitute approximately 10-15% of peripheral blood lymphocytes, and prevent the development of autoimmune disease. The other T_{Reg} cell type is referred to as antigen-specific or inducible T_{Regs} (iT_{Regs}) cells, and are generated in the periphery following antigen exposure.(59) iT_{Regs} can be further subdivided into $Foxp3^+$ iT_{Regs} cells, and $IL\text{-}10^+$ Tr1 cells. (59, 60) iT_{Regs} cells can not only suppress Th2 cells, but can also limit the function of mast cells, basophils, eosinophils and dendritic cells. iT_{Regs} are able to inhibit mast cell degranulation by an OX4-OX-40 Ligand interaction. By producing IL-10 and TGF-β, they may also induce IgG4 and IgA production and regulate allergen-specific-IgE. (61-64)

The important role of $Foxp3^+$ T_{Reg} cells in allergy is highlighted by the fact that Foxp3 mutant mice develop an intense multi-organ inflammatory response associated with allergic airway inflammation, significant hyper IgE, eosinophilia, and dysregulated Th1 and Th2 cytokine production.(65) Severe allergic inflammation is a cardinal manifestation of loss-of-function Foxp3 mutations in humans, where affected subjects develop multiple food allergy, atopic dermatitis, asthma, increased IgE levels, and eosinophilia. (66). The specific role of iT_{Reg} cells in allergy is highlighted by the observation that mice deficient in iT_{Reg} cells, but not nT_{Reg} cells, spontaneously develop pronounced Th2 type pathologies in the gastrointestinal tract and lungs. (67) iT_{Regs} cells inhibited allergic eosinophilia and Th2 cytokine expression in murine lung, indicating that iT_{Regs} cells play an important inhibitory role in airway inflammation. (68) Murine studies also suggest that oral tolerance does not require $CD4^+Foxp3^+$ nT_{Reg} cells; on the other hand the establishment of oral tolerance correlates with the *de novo* induction of antigen-specific $CD4^+Foxp3^+$ iT_{Regs}.(69) Finally, the establishment of iT_{Reg} cells, may require specific intestinal microflora (70), as is discussed in another chapter in this series.

In humans, the number of local $Foxp3^+CD25^+CD3^+$ cells in the nasal mucosal increases after allergen immunotherapy and their up-regulation is associated with clinical efficacy and suppression of seasonal allergic inflammation. (71, 72) IL-10 down-regulates T cells by

blocking CD2, CD28, and inducible co-stimulator (ICOS) co-stimulatory signaling(73). IL-10 was also shown to reduce pro-inflammatory cytokine release from mast cells. In addition, IL-10 down-regulates eosinophils, and suppresses IL-5 production by resting Th0 and Th2 cells. (74, 75) TGF-β inhibits the function of both Th1 and Th2 cells, and induces the conversion of naive $CD4^+CD25^-$ T cells into $CD4^+CD25^+$ T cells by inducing the expression of Foxp3.(76)

Innate immunity in allergy

While allergen-specific $CD4^+$ T cells play a critical role in regulating allergy in the gastrointestinal tract, newly described innate immune mechanisms also contribute to food allergy. Three recently described innate cytokines, produced by intestinal epithelial cells, greatly enhance Th2 responses. The first, called **Thymic Stromal Lymphopoietin (TSLP)**, has been shown to be highly increased in the skin and blood of patients with atopic dermatitis, (77, 78) and in patients with eosinophilic esophagitis and asthma. TSLP, an IL-7-like cytokine, alters dendritic cells, causing them to selectively induce allergen-specific Th2 cells. Moreover, TSLP appears to directly enhance basophil hematopoiesis in a pathway that is distinct from that induced with IL-3.(79) Selective expression of IL-13 in the skin of mice caused an atopic dermatitis phenotype and the condition was associated with enhanced production of TSLP. (80) Elimination of TSLP signaling significantly diminished the allergic asthma responses, immune cell production of Th2 cytokines (IL-4, IL-13), and serum IgE. In mouse models of food allergy, the presence of TSLP is required to amplify Th2 responses. In humans, TSLP polymorphisms are highly associated with eosinophilic esophagitis, and with food allergy.

IL-25, an IL-17-like cytokine (also called IL-17E), is another innate cytokine produced by intestinal epithelial cells. It is found in the lungs of patients with asthma, and is associated with allergen sensitization in humans. IL-25 also enhances the growth and differentiation of basophils and mast cells. In addition, increased IL-25 production by mothers was associated with food sensitization in the child. **IL-33** is the third recently described innate cytokine important in allergic diseases. IL-33 is also produced by intestinal epithelial cells, lung epithelial cells and by alternatively activated macrophages. It is a member of the IL-1 cytokine family and is found in the blood of patients undergoing anaphylaxis, (81) in the skin of patients with atopic dermatitis, and in the lungs of patients with severe asthma. The genes for *IL33* and its receptor *ST2* are highly associated with asthma, and both are highly expressed in the intestines during helminth infections in mice, suggesting they may play an important role in food allergy.

The importance of TSLP, IL-25 and IL-33 in allergic disease became clear with the discovery two years ago of a novel innate lymphoid cell type called **nuocytes**, or **natural helper cells**, or **innate lymphoid type 2 cells**. (82) Nuocytes are non-T, non-B cells that do not express mature hematopoietic lineage markers, but produce large quantities of IL-5 and Il-13. Importantly, TSLP, IL-25 and IL-33 greatly enhance the growth and activation of nuocytes. They have been implicated in immune responses in the gut against helminth infections. (83) In addition, nuocytes have been found in the lungs of mice and in humans. (84, 85) Although their role in food allergy has not yet been determined, it is likely that they may amplify Th2 responses, as they do in the lungs.

In summary, the mechanism leading to allergic diseases is complex and involves multiple pathways, some of which have only been discovered recently. Although it is clear that food immunotherapy has a beneficial clinical effect, the immuno-modulatory changes induced by this modality have not been thoroughly studied in terms of all of these pathways.

Immunomodulation in food immunotherapy

The known immuno-modulatory changes induced by food immunotherapy include suppression of mast cells and basophils, the generation of allergen-specific IgG-4 “blocking antibodies”, the down regulation of effector cells (antigen-specific IgE producing cells and allergen-specific Th2 cells), and the induction of T cell tolerance and its associated changes in cytokine secretion. We will discuss all of these mechanisms.

Early effects: Mast cells and basophils suppression

During the initial phases of food allergen-immunotherapy, allergen-specific IgE levels and IgE-mediated skin sensitivity wheal size often increase, although these levels decrease after 1-2 years of immunotherapy. (86, 87) However, the beneficial clinical effects are seen much sooner, well before IgE levels drop, as treated individuals tolerate increasing amounts of the pertinent food. Similar finding have been observed in subcutaneous immunotherapy with bee venom, or with acute desensitization with drugs, where a decrease in mast cell and basophil degranulation activity is observed early during the desensitization process. (17) This effect on mast cells and a clinical protection against bee stings, and its discrepancy with skin test reactivity is not yet understood, but may be related to the dose of antigen seen by the mast cell, and to “piecemeal release” of small amounts of mediators, which somehow increases the threshold of activation of mast cells and basophils.(88-90) An inhibitory histamine receptor (HR2) may also be involved in rapidly inhibiting the function of mast cells and basophils. Thus, mast cell activation (skin test reactivity) performed in OIT studies after maintenance dosing has been achieved show a decrease in food-specific skin prick test (SPT) wheal size compared to baseline.(26, 28, 30, 49, 91) In another study, 12 months after completing a peanut SLIT protocol, SPT was significantly reduced in the treatment group compared to that done at baseline, with a median wheal size of 4 mm in the former and 11 mm in the latter, indicative of a decrease in mast cell reactivity after peanut SLIT.

Studies of mast cell/skin tests during food immunotherapy generally correlate with analyses of peripheral blood basophils. For example, Jones and co-worker found that basophil activation decreased significantly 6 months into peanut OIT, as did skin test reactivity. (30) Similarly, basophil reactivity diminished with peanut OIT, as demonstrated by the number of activated $CD63^+$ basophils appearing after *in vitro* stimulation with peanut extract. (26) In patients who underwent milk OIT combined with omalizumab as an adjunct therapy, milk-induced basophil activation was reduced, as demonstrated by examination of basophil expression of CD203c and CD63, histamine release and Syk expression after *in vitro* allergen challenge. (92) Initially, basophil inactivation to all allergens was due to omalizumab treatment and the clearance of antigen-specific IgE from the basophil surface. However, after omalizumab was discontinued (after week 16), only milk-specific (but not egg or cashew-specific) basophil unresponsiveness persisted, suggesting that milk desensitization exclusively reduced milk-specific basophil degranulation, presumably related to a reduction in food-specific IgE or IgG. The reduction in milk-specific basophil degranulation after discontinuation of omalizumab treatment correlated with a reduction in the milk-specific skin test reactivity, consistent with a reduction in milk-specific IgE production.

On the other hand, in a study comparing SLIT with SLIT/OIT, Keet et al found that while cow's milk SPT reactivity was reduced over time, as did milk-specific IgE in the OIT group, allergen- or anti-IgE-induced basophil histamine release did not fall at any point in the study. (28) This suggests that skin testing may have greater sensitivity compared to basophil histamine release. Interestingly, constitutive expression by basophils of CD203c (a basophil activation marker) was reduced after immunotherapy, and, subjects who had an increased

constitutive basophil expression of CD203c early in therapy had a poorer outcome later in the study.

These studies together suggest that food specific immunotherapy can reduce allergen-specific mast cell and basophil degranulation and skin test reactivity at late time points, in part due to late reductions in allergen-specific IgE production. Larger studies are needed to clarify the early effects of food immunotherapy on mast cells and basophil activation and their role in the development of clinical desensitization and possibly tolerance.

Oral and intestinal mucosa

The mechanism of food desensitization may be partly dependent on the route of dose administration, as observed with SLIT versus OIT /SCIT. The oral route may be inherently associated with tolerance, presumably because oral intake is primarily associated with nutrient absorption, even in the face of high exposure to bacteria and potential infection. (93) In SLIT, Langerhans cells within the oral mucosa take up antigen, which is initially recognized by allergen-specific IgE bound to surface FcεR1 (the high-affinity IgE receptor) (94, 95). These cells also express high levels of MCH class I and II as well as co-stimulatory and co-inhibitory molecules. Scadding et al speculated that Langerhans cells and possibly other mucosal dentritic cells migrate to regional lymph nodes where the antigen is presented to T cells, leading to the development of anti-inflammatory allergen-specific Th1 or T_{Reg} cells.(93) These cell types then have the capacity to inhibit Th2 mediated inflammation, particularly in non-allergic individuals. Production of IL-10 and TGF-β by oral Langerhans cells have been shown to enhance the development of IL-10, TGF-β producing T_{Regs} cells after grass SLIT. (72). In addition, ligation of TLR4 may enhance IL-10 production of Langerhans cells (94), and contribute to the induction of T_{Reg} cells.

Other factors that contribute to the natural capacity of the gastrointestinal track to induce tolerance include the capacity of intestinal epithelial cells and monocytes to produce IL-10 and TGF-β (96, 97), which enhance T_{Reg} cell generation (98), the production of antigen-specific IgA and secretory IgA (S-IgA) in the intestines, which can reduce absorption of undigested antigens at mucosal surfaces through a mechanism known as immune exclusion, thereby preventing inflammatory responses (99). Thus, Scadding et al found that sublingual grass pollen immunotherapy was associated not only with increases in sublingual $Foxp3^+$ Treg cells but also elevated allergen-specific IgA and IgG4. (100) Similarly, another recent study on peanut SLIT showed that salivary levels of peanut-specific IgA increased significantly for most subjects receiving SLIT but not for subjects receiving placebo. (101)

B cell tolerance and modulation of specific IgE and IgG4

In most studies of OIT, regardless of the variation between protocols, a significant rise in food specific IgG4 levels have been observed. (25, 30, 36, 102) In addition, in the majority of the trials, food-specific IgE initially increased for several months after initiation of food OIT or SLIT, but then decreased either to baseline level or lower by the end of the study.(26, 30, 48) After peanut OIT, IgE complex formation was inhibited by serum factors in an IgE-facilitated allergen binding assay.(30) On the other hand, Shripak and colleagues did not find any significant change in milk-specific IgE levels after 3-4 months of milk OIT maintenance therapy though it did increase in some patients. However, milk IgG and particularly IgG4 levels increased significantly in patients in the active treatment group compared to baseline (by a median 764% for IgG4), while there was no change in the placebo group.(40) Consistent with the findings of others, Blumchem et al found that there was a significant reduction in peanut SPT wheal size and an increase in peanut-specific IgG4 after peanut OIT. (49) However, 2 weeks after discontinuing maintenance peanut therapy, peanut skin test wheal size increased. In addition, there was a small but significant

drop in peanut-specific IgG4 levels. Interestingly however, many (but not all) patients passed the DBPCFC after 2 weeks of peanut ingestion interruption. It is not known whether the desensitization state would have been maintained if the food interruption were continued for a longer period of time. The drop in peanut-specific IgG4 levels suggests that there may be a role for food-specific IgG4 in maintaining desensitization and possibly tolerance, and that a longer maintenance phase (months and likely years) may be required to maintain important immunological changes that sustain immunological tolerance.

The rise in allergen specific IgG subtypes has been studied in many clinical trials of food and inhaled allergens, but its role in immune modulation remains controversial. In many studies of allergen SCIT, IgG1 and particularly IgG4 levels increase 10-100 folds (103, 104), but concentration of allergen-specific IgG does not always correlate with clinical improvement..(105, 106) IgG4 is a non-inflammatory, non-complement binding isotype that is thought to capture the allergen before it reaches the effector cell IgE, therefore preventing the activation of mast cells and basophils. This could lead to a competition for allergen biding, or “blocking” effect. (107) However, recent studies suggest that the beneficial effects of allergen-specific IgG is not solely dependent on the level of IgG, but rather on their blocking activity and affinity or the ability to bind to the inhibitor FcγRIIB on antigen-presenting cells. (108, 109) Interestingly, while one IgG molecule against a single epitope on the Feld1 was able to inhibit the degranulation of mast cells and basophils in patients with cat allergy, a combination of 2 or 3 different IgGs had a greater inhibitory effect. The increased inhibitory activity of several antibodies might be the result from stronger crosslinking of FcgRIIB by higher-order allergen-antibody complexes. (110) Alternatively, the effectiveness of allergen-specific IgG may be dependent on the IgE/IgG4 ratio, since allergen-specific IgG4/IgE ratios were found to be about thousand times higher in non-allergic beekeepers compared with bee venom allergic individuals. (111) The decrease in IgE/IgG4 ratio during immunotherapy may be secondary to IL-10 up-regulation production, which decreases IL-4 induced IgE switching and increases IL-4 induced IgG4 production. Further studies are required to evaluate the role of IgG, IgG4 levels in food immunotherapy desensitization and induction of food tolerance.

Immune deviation, T_{Reg} cells and T cell tolerance

Peripheral T cell tolerance is characterized by the one or more of the following: the deletion of allergen-specific T effector cells, anergy in allergen-specific effector T cells, or the generation of allergen-specific T_{Reg} cells. These events together or alone result in limiting the function of food-specific Th2 cells, mast cells and other allergic effector cells, and are thought to be essential steps for successful allergen specific immunotherapy. The few studies that evaluated T cell changes during and after food immunotherapy have not provided consistent results, and therefore the role of immune deviation and T cell tolerance remains controversial.

Allergen specific iT_{Reg} cells have been described to develop in the context of SCIT for aeroallergens and bee venom, using doses that are generally <50 μg. In the context of food allergy, iT_{Reg} cells have been observed, for example in patients who are tolerating products containing heated milk. In such patients, a significantly higher percentage of proliferating casein-specific $CD25^+CD27^+$ T_{Reg} cells were present than in subjects with milk allergy. (112) The casein-specific T_{Reg} cells were found to be $FoxP3^+CD25^{hi}CD27^+$, cytotoxic T lymphocyte-associated antigen 4^+, $CD45RO^+CD127^-$ and were derived from circulating $CD25^{hi}$ T cells. Depletion of the $CD25^{hi}$ cells before *in vitro* culture significantly enhanced casein-specific effector T-cell expansion. Their depletion causes the enhanced proliferation of casein-specific, effector T-cell proliferation, demonstrating that they are functionally suppressive. Finally, these cells were found to be capable of suppressing the proliferation of

$CD4^+$ T cells in a mixed lymphocyte response. These results are consistent with the possibility that iT_{Reg} cells develop when exposure to the particular food is relatively low.

In OIT, where the doses of the food range from very low to very high, Jones and coworkers found that the secretion of IL-10, IL-5, IFN-γ, and TNF-α increased over a period of 6 to 12 months after peanut OIT. (30). The number of $FoxP3^+$ T cells observed in cultures of peripheral blood cells stimulated with peanut increased until 12 months and decreased thereafter. An increase in peanut-induced inflammatory cytokines/chemokines IL-5, IL-1β, TNF-α and MIP-1β and growth factors G-CSF and GM-CSF was observed. In addition, microarray analysis of resting T cells showed down-regulation of genes in apoptotic pathways. In another study, Varshney *et al* found that peanut OIT subjects had an increase from baseline of the ratio of $FoxP3^{hi}/FoxP3^{intemediate}$ $CD4^+CD25^+T_{Reg}$ cells in cultures stimulated for 7 days with peanut antigen, from cells taken at 12 months after initiation of peanut OIT (at the time of the DBPCFC). This increase in the number of T_{Reg} cells, some of which may be peanut specific, was not observed in placebo-treated patients. (29) IL-5 and IL-13 decreased significantly from baseline, and there was a transient increase in TGF-β at 9 months that decreased to baseline at DBPCFC. Although there was no change in IL-10 or INF-γ levels, these studies suggest that 12 months of peanut OIT changes the function of peanut specific T cells, and may increase the number of peanut specific T_{Reg} cells, at least transiently.

On the other hand, Blumchen and co-workers found that 9 months after the initiation of peanut OIT, there was no evidence for an upregulation of Th1 or T_{Regs} cells. They however observed a downregulation of IL-2, IL-4 and IL-5 cytokine production from peanut-stimulated PBMCs compared to baseline suggesting a skewing away from a Th2 response. (49) These findings were peanut-specific, as no such changes were found with the milk-specific response. However, there was no difference in INF-γ, TNF-α and IL-10 production. These findings suggested that clinical improvement might be secondary to clonal anergy or clonal deletion. (113, 114) After discontinuing peanut for 2 weeks, while these changes were still noted in the majority of the patients, 4 subjects showed an increase in IL-2 production almost returning to baseline value. However, these changes did not correlate with the clinical outcome of the final DBPCFC, or the period of time necessary to achieve the maintenance OIT dose.

Enrique at al found that after hazelnut SLIT, in which the doses are relatively low, there was a significant increase of IL-10 levels.(27) On the other hand, twelve months after peanut SLIT, IL-5 level was significantly lower in the treatment group compared to placebo, and no significant differences between the 2 groups were found in IL-10, INF-γ or IL-13 levels. (26) In addition, while an increased T_{Reg} cell number was seen in the active treatment group compared to the placebo group, this result did not reach statistical significance. These findings were in contrast with what has been observed in grass SLIT immunotherapy, where an increased production of IL-10 and TGF-β by oral Langerhans cells and an increased number of IL-10 and TGF-β producing T_{Regs} cells were reported (72). The authors postulated that is possible that peanut SLIT may preferentially induce $nFoxp3^+$ T_{Regs} cells, which mediate their effect more via cell to cell contact than cytokine secretion, unlike type iT_{Regs} that are hypothesized to be IL-10 or TGF-β producing cells. (72)

After egg OIT, Vickery and co-workers did not find statistically significant changes in IL-10, TGFβ, IL-13/INF-γ ratio nor in egg-specific $CD4^+CD25^{hi}$ cell expression.(48) On the other hand, Itoh and co-workers found a significant change in Th1/Th2 ratio 6 months after rush hen's egg desensitization, but the changes were not significant at 12 months.(102) Paradoxically, serum IL-10 level decreased and plasma TGF-β1 level increased at 6 months and 12 months compared to baseline. There were no changes in INF-γ and IL-4 levels.

OIT in combination with omalizumab

When milk OIT was performed in combination with omalizumab, patients with very significant milk allergy (the mean milk-specific IgE was 98 kU/L) could be desensitized rapidly with relatively few allergic reactions (115). At the end of the study, long after omalizumab was discontinued, 82% of patients tolerated a dose of 8 grams of milk protein, compared to 36% and 42% in other studies of severe milk allergy.(34, 40) However, within a week of the initiation of milk desensitization, $CD4^+$ T cell proliferation was strikingly decreased compared to baseline. The dose of milk, which reached 1,000 mg within one week of desensitization, and increased to 2,000 mg/day over the next 7-10 wks, (92) rapidly reduced the milk-specific T cell but not the tetanus toxoid T cell proliferative response. The reduction in milk-specific proliferation was not associated with an increase in $CD4^+Foxp3^+$ T_{Reg} cells, nor was it associated with increased IL-10 production, as it was not inhibited by the presence of anti-IL-10 monoclonal antibodies (mAb) or anti-TGF-β mAb, indicating that IL-10 and TGF-β were not responsible for the decrease in the milk response. These results suggest that milk-specific T cell anergy or deletion may develop when the allergen dose is rapidly increased during oral desensitization.

In this study, omalizumab was used only for 16 wks, but during this time omalizumab may have reduced FcεRI on mast cells and basophils (116), which decreases the responsiveness of mast cells and basophils to antigen challenge (117), and reduces mast cell and basophil survival. (118). In addition, omalizumab can decrease the interaction of IgE with FcεRII and FcεRI present on antigen-presenting cells and dendritic cells (119), which can reduce antigen presentation, interfere with the total IgE production by B cells, and decrease the activation of Th2 cells producing IgE-stimulating cytokines, such as IL-4, IL-5 and IL-13. (120, 121). However in the study, several months after omalizumab treatment was discontinued, and after maintenance dosing was achieved, the milk-specific $CD4^+$ T cell proliferation returned. This increase in T cell proliferation was associated with an increase in INF-γ production, associated with the return of the vigorous milk-specific response, suggesting the development of immune deviation towards a Th1-skewed response. A similar Th1-like allergen-specific response was found in peanut-allergic patients who had naturally outgrown their sensitivity (122) and in milk-specific Peyer's patch T cells from non-allergic individuals. (123)

The different and sometimes contradictory findings between studies (e.g., with regard to T_{reg} cells) may be due in part to multiple factors, including differences in build-up phase protocol, target maintenance dose, duration of maintenance phase and sometimes a variable period of food interruption. In murine studies, iT_{Reg} cells are more prominent with low doses of oral antigen are used, and less prominent with high doses are used, where deletion and anergy become evident. Thus, food specific iT_{Reg} cells might be more likely to be observed in protocols with slow dose escalation. In addition, the seemingly contradictory findings may be secondary to large variations between studies in terms of the timing of immunological evaluation during food immunotherapy, as well as the laboratory techniques that were followed. Larger studies are required to elucidate the role of T_{Regs} cells and the different cytokines in inducing desensitization after food immunotherapy, and explore the possibility of development of anergy and clonal deletion. It should also be noted that identifying allergen-specific T_{Reg} cells is difficult because their frequency is extremely low, and special techniques, which have only been used in a few studies, may be required. Additional techniques, such as use of MHC class II-antigen peptide tetramers may be useful in this regard. Finally, it is also possible that several different mechanisms maybe involved in different situations, some involving antigen-specific iT_{Reg} cells, antigen-specific T_R1 cells, or immune deviation, in which the cytokine profiles of antigen-specific T cells evolve with time.

T cell anergy

Anergy in T cells is defined as lack of responsiveness to the antigen that can be restored with IL-2 and associated with the absence of T_{Reg} immunosuppressive activity (124). In our study using omalizumab as an adjunct therapy for milk-OIT, milk-specific T cells acutely lost the capacity to proliferate in response to milk antigen at the time of rush desensitization. The loss in ability to proliferate was not associated with the presence of T_{Reg} cells, and was partially reversed in the presence of IL-2, suggesting that the high-dose milk administration used for rush desensitization over 6 hours resulted in the development of milk-specific T cell anergy and possibly partial deletion (92). Rapid reduction in allergen-specific $CD4^+$ T-cell responses have been observed in beekeepers exposed to multiple bee stings (average cumulative antigen dose <1-4 mg over 7 months), although it was not clear if the reductions were due to anergy/deletion or the development of venom-specific T_{Regs}.(125). In grass and bee venom SCIT in which doses ranged from 4 to 100 mcg per dose, allergen-specific tolerance was associated with the induction of allergen-specific T_{Regs} producing IL-10. (58, 126) It is possible that high dose immunotherapy (using gram amounts of antigen) used in oral immunotherapy leads to anergy and/or deletion of antigen-specific $CD4^+$ cells, while low dose can lead to the development of T_{Regs}. It is possible however that the T_{Regs} cells could develop later in the course of food immunotherapy. Additional studies will be required to evaluate the role of anergy in reaching a state of desensitization and later tolerance in food IT.

Conclusions

There have been many recent clinical studies evaluating the effects of food immunotherapy. The studies are heterogeneous in terms of routes and rates of administration of the food antigen, different target maintenance dose and duration of maintenance therapy. The definition of success also varies between investigators, as some aim for achieving a tolerated threshold dose during final OFC large enough to limit allergic reactions with accidental ingestions, while others aimed for much higher doses that would allow ingestions of more substantial quantities of food. In addition, there may be a subset of patients who are resistant to food desensitization, possibly secondary to uncontrolled asthma, recurrent illnesses, psychosocial stressors, or due to a “higher sensitivity” to food. Most studies did not include patients with a history of severe anaphylaxis, therefore excluding patients with more severe disease, who may require modified protocols with slower build-up and longer maintenance phase. These patients may benefit from adjunct therapy to food immunotherapy, such as anti-IgE mAb. Although some studies attempted evaluation for tolerance, most have relied on food interruption for only few weeks. Even with maintenance doses as long as 60 weeks, many patients redeveloped sensitization after food interruption for these few weeks. It is likely from the outcome of these observations that maintenance doses may be needed for years before permanent tolerance and therefore cure is achieved. It is also possible that the maintenance dose and duration may depend partly on the severity of the allergy, and possibly on food-specific IgE level. The mechanisms underlying a successful food desensitization are still very unclear, and may involve similar mechanisms observed in allergen SCIT and SLIT, including mast cell and basophil suppression, food-specific IgG4 antibodies formation or a changes in food specific IgE/IgG4 ratios, up-regulation of natural or inducible T_{Regs} cells, a skewing from aTh2 to a Th1 profile and the development of anergy or deletion of allergen-specific cells. The role of Th9, Th17, Th22 cells and innate cytokines such as TSLP, IL22, IL-25 and IL-33, as well innate immunity pathways such as Myd88 in intestinal immunity have only recently been described and have not been investigated in depth in food allergy. Additional studies will be required to more fully elucidate the mechanisms by which desensitization and tolerance is achieved, as this may

also reveal biomarkers that could measured serially to evaluate and monitor for tolerance or possibly cure.

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Abbreviations

DBPCFC	Double blind placebo controlled food challenge
iT_{Regs}	inducible regulatory T cells
IT	immunotherapy
mAb	monoclonal antibody
nT_{Regs}	natural regulatory T cells
OFC	oral food challenge
OIT	oral immunotherapy
SIgA	secretory IgA
SLIT	sublingual immunotherapy
SPT	skin prick test
Syk	spleen tyrosine kinase
T_{Regs}	Regulatory T cells