REVIEW ARTICLE

Markers of tolerance development to food allergens

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Abstract

IgE-mediated reactions to food allergens are the most common cause of anaphylaxis in childhood. Although allergies to cow's milk, egg, or soy proteins, in contrast to peanut and tree nut allergens, resolve within the first 6 years of life in up to 60% due to natural tolerance development, this process is not well understood. At present, there is no cure or treatment for food allergy that would result in an induction of tolerance to the symptom-eliciting food. Avoidance, providing an emergency plan and education, is the standard of treatment. Oral immunotherapeutic approaches have been proven reasonable efficacy; however, they are associated with high rates of side-effects and low numbers of patients achieving tolerance. Nevertheless, mechanisms that take place during oral immunotherapy may help to understand tolerance development. On the basis of these therapeutic interventions, events like loss of basophil activation and induction of regulatory lymphocyte subsets and of blocking antibodies have been described. Their functional importance at a clinical level, however, remains to be investigated in detail. Consequently, there is eminent need to understand the process of tolerance development to food allergens and define biomarkers to develop and monitor new treatment strategies for food allergy.

IgE-mediated food allergy represents the most important cause of anaphylaxis in childhood. While milk and egg allergy are associated with a high rate of natural tolerance development within the first 6 years of life, this is less clear for other food allergies. Consequently, once it is communicated the diagnosis persists often lifelong severely impacting the quality of life of the patient and the environment despite the recommendation to reconsider clinical tolerance development from time to time.

Currently, avoidance, providing an emergency plan and teaching, is the standard of treatment; however, there is no cure or treatment that has reached the level of recommendation to re-induce tolerance to the symptom-eliciting food. Tolerance assessment occurs either via oral food challenges or due to unintended exposure. Given the cost and time intensiveness of oral food challenges and its potential harmfulness, there is eminent need to understand the process of tolerance development and define markers thereof to develop and monitor new treatment strategies for food allergy. This review aimed to provide an overview on recent advances regarding markers and mechanisms of tolerance development to food allergens.

Oral tolerance in the context of food allergy is considered to occur if the antigen/food can be ingested without problems despite prolonged periods of avoidance, while the status of desensitization is strictly dependent on regular ingestion of the respective food in order to confer protection from allergic reactions. Currently, there is no general consensus on the exact time frame of avoidance and re-exposure that would allow the usage of the terminus tolerance that is considered equivalent to cure in the context of oral immunotherapy. Therefore, the terminus 'sustained unresponsiveness' has been introduced which defines the ability to retain an increased threshold (equivalent to a passed oral food challenge) despite cessation of ingestion of the respective food for weeks to months (usually 4–8 weeks) (1, 2).

Tolerance induction in the prenatal/perinatal period

Food allergens are part of the nutrients that the pregnant mother supplies to the embryo/fetus. The quantity and quality of food determines the transmission through the materno-fetal barrier in the placenta. As allergenic molecules do not differ in terms of structure, polarity, or other characteristics

from nonallergenic molecules, there is no means to assume that allergens would not cross the materno-fetal barrier. In fact, substantial experimental work on *ex vivo* human placenta perfusion models have shown that allergens provided on the maternal placenta side would readily cross the placenta and reach the fetus (3–5). However, transfer of inhalant (birch pollen Bet v1) and nutritive (ovalbumin, beta-lactoglobulin) allergens was limited by a strong binding of allergens in the placenta to syncytiotrophoblast cells (3). By which mechanism allergens interact with the immune system at the feto-maternal interface to trigger either sensitization or tolerance has not been studied directly in the human system (6). In mouse models, allergen exposure to the pregnant animal would induce tolerance upon postnatal re-exposure (7). For postnatal/perinatal tolerance induction, several mechanisms have been described for oral tolerance, including anergy or deletion of antigen-specific T cells, and the development of regulatory T cells (8, 9). It is known that high doses of antigen induce T-cell anergy/deletion, whereas exposure to low doses induces Tregs. Whether these mechanisms also account for prenatal allergen tolerance mechanisms is not completely clarified. However, emerging evidence suggests that exposures during pregnancy and the early postnatal period can modify gene expression and thus disease propensity (10). Thus, it could be shown that cord blood regulatory T cells are depressed in quantity (total numbers) and quality (function) in atopic as compared to nonatopic mothers (11, 12). In addition, maternal smoking or exposure to tobacco smoke during pregnancy was also associated with decreased cord blood Treg numbers, and even more relevantly children with lower Treg numbers at birth had a higher risk to develop atopic dermatitis (adj. OR = 1.55, 95% CI = 1.00–2.41) and sensitization to food allergens (adj. OR = 1.55, 95% CI = 1.06–2.25) during the first year of life. TH17 cells have also been found to be defective in cord blood (mediated by reduced RORC2 mRNA content) as compared to adult TH17 cells (13), but rapid constitutive regeneration is observed at the age of 3 months (14). In the attempt to analyze the role of 'farming' on Treg cell and TH17 cell maturation, Lluis et al. reported a positive correlation of TH17 and Treg cell markers, which were positively influenced by maternal farm exposure. This suggests that prenatal exposure and genetic predisposition may play a role during early TH17 immune maturation and may regulate the development of immune-mediated diseases (15). More strikingly, farm milk exposure in early life led to an increased Treg cell numbers at the age of 5 years and thereby potentially contributes to a protective effect against the development of childhood allergy (16).

Interventional trials with polyunsaturated fatty acids, probiotics, and oligosaccharides suggest also preliminary but not confirmed benefits. In this respect, food allergen exposure in the prenatal/perinatal period has been implicated to rather induce tolerance than to trigger disease-causing reactions, such as allergic sensitization (10). Most of recent human studies have focused on peanut as allergen. Until recently, data have been controversial: In a case–control study of peanut-allergic 18-month-old children, cases were neither more

nor less peanut exposed during pregnancy than nonallergic controls (17). On the other hand, Du Toit et al. (18) could show that high and regular peanut exposure of pregnant mothers in Israel were associated with less peanut allergy in their children as compared to low peanut exposure in UK pregnant women. The LEAP study significantly added to this knowledge. The early introduction of peanut in a high-risk cohort (4- to 11-month-old children with severe atopic dermatitis and/or egg allergy and skin prick test (SPT) <4 mm to peanut) via integration of 6 g peanut three times a week into the diet resulted in an impressive reduction in peanut allergy after 60 months of therapy by 81.7% as compared to the avoidance group (19). Importantly, this effect persisted in the majority of the patients even after a prolonged avoidance period of 1 year (20).

The process of natural tolerance development occurs in the first years of life, but still the sequence of events and the underlying mechanisms are not understood. Cow's milk-allergic children will become tolerant in 60–75% at the age of 5 years (21). Hen's egg allergy will resolve in 56% (22) and peanut allergy in 20% at the age of 3–5 years (23) and 22% at the age of 4 years (Fig. 1A). Understanding the natural process of tolerance development in infancy and early childhood would help to design strategies how to treat food allergic children or predict responses to oral immune therapy.

Novel insights in mucosal immune homeostasis based on mouse models

Mouse models, as the most valid *in vivo* system to investigate immunological mechanisms, mimic the situation in humans. Mouse models for food allergy are diverse as there are a number of different routes to induce the allergic response in the animal. Different immunization protocols including various routes of allergen application and adjuvants are available, which try to trigger a food allergic reaction as physiological and as similar as it is found in humans. However, in mice two different mechanisms have been reported to induce anaphylaxis. The classical IgE -mediated pathway involves, similar to the human organism, the release of mast cell mediators, such as histamine and mast cell protease-1, after the cross-linking of IgE on effector cells (24–26). In contrast, platelet-activating factor (PAF) triggers systemic anaphylaxis in the alternative pathway by activation of macrophages and basophils via IgG binding to the low-affinity receptor FcγRIII (27–29). Irrespective of the limitations, the mouse has clear advantages as study organism, in particular due to the ability to assess mechanistic issues via the generation of knockout mice.

Mucosal tolerance *vs* food allergy

The mucosal immune system is complex involving a functional epithelial barrier against luminal antigens as well as innate and adaptive immune mechanisms (30, 31). Damage of the epithelial barrier can facilitate an allergic response against harmless luminal food antigens if pro-inflammatory
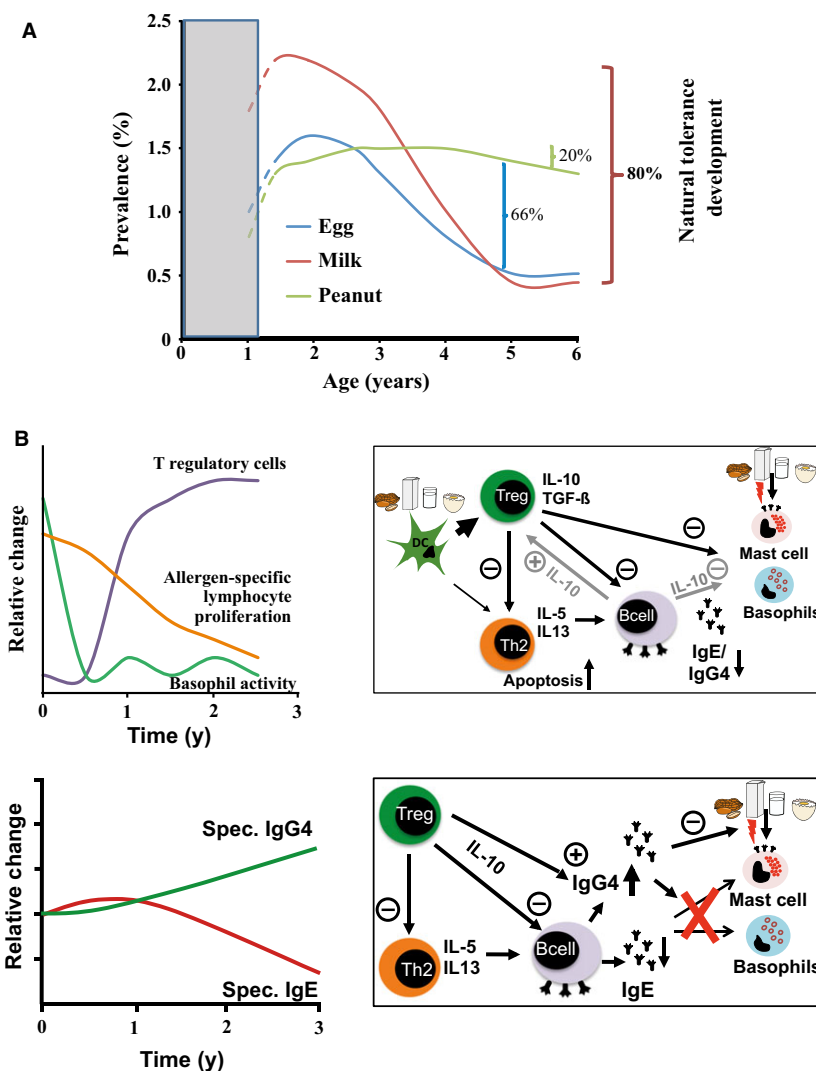
Figure 1 Natural tolerance development in children and immunological changes during oral immunotherapy. While natural tolerance development to milk and egg is a common phenomenon during childhood, peanut allergy often persists to adulthood (A). The underlying mechanisms that take place during that process are not well understood. During oral immunotherapy, similar immunological signals are present. events are considered to take place. Initially, loss/significant reduction in basophil activation, induction of pro-tolerogenic DCs, the generation of allergen-specific regulatory T cells and regulatory B cells take place. Over time, the induction of allergen-specific blocking antibodies mainly of the IgG4 type and a slow reduction in allergen-specific IgE occur (B).

signals are present. Sphingolipid homeostasis has been recently linked to the development of food allergy by influencing epithelial cell integrity. Sphingosine-1 phosphate (S1P), a sphingolipid metabolite, increased the intestinal epithelial uptake of antigens (32). In addition, S1P was proposed to be decisive for the induction of food allergy in mice as the modulation of the S1P homeostasis by the knockout of the sphingosine kinases SphK1 or SphK2 resulted in a reduced production of allergen-specific IgE and mucosal mast cells upon oral sensitization (32).

With regard to cellular and humoral regulatory components, mucosal tolerance depends on the immune exclusion by secretory antibodies, such as dimeric IgA and pentameric IgM, and on local anti-inflammatory cellular mechanisms

(reviewed by Corazza et al. 33). Recent mouse studies focused on the interaction of antigen-presenting cells and the development of regulatory T and B cells, which, in cooperation, suppress the development of allergic responses.

Oral tolerance in the mouse has been shown to involve $CD11c^+CD11b^+CD103^+$ dendritic cells (DCs), which are located in the intestinal lamina propria. $CD11c^+CX_3CR1^+$ macrophages sample soluble antigens from the intestinal lumen by protrusions. A cell–cell antigen transfer between $CD11c^+CD11b^+CD103^+$ DCs and the macrophages is accomplished via CX43-containing gap junctions (34). Once the DC has contact to the antigen, it migrates to the mesenteric lymph nodes, where it can stimulate the induction of $FOXP3^+CD4^+CD25^+$ Treg cells by the production of

retinoic acid (RA) and TGF-β, but only when pro-inflammatory signals are absent (33). In the case of strong pro-inflammatory signals, for example, by the use of adjuvants, such as cholera toxin, in a peanut allergy mouse models, the predominant subset of DCs in intestinal tissue shifts from a $CD11c^+CD103^+$ DC to a TH2-priming $CD11c^+CD11b^+$ cDC subtype (35).

Retinoic acid also affects the mesenteric lymph node DCs. When vitamin A is insufficient either by the knockout or by the use of an RA-antagonizing antibody, mesenterial $B220^-CD8a^-CD11b^+CD103^-$ DCs induced the induction of a TH2-like subset, characterized by higher IL-13 and TNF-α levels and strong IgE and IgG1 responses, which was IL-6 and OX40 ligand dependent. These effects were inhibited by RA, indicating that RA can inhibit allergic responses by preventing the DC-driven induction of TH2 cells (36).

In addition to $CD11c^+CD11b^+CD103^+$ DCs, $CD11c^+B220^+mPDCA^+$ plasmacytoid DCs (pDCs) are associated with the induction of immune tolerance against food allergens. The expansion of DCs triggered by the DC growth factor Flt3L before peanut extract (PE) exposure inhibited PE-allergic manifestation, such as allergen-specific IgE, mast cell degranulation, and the production of the TH2 cytokines IL-4, IL-5, and IL-13. However, when the pDC compartment was depleted, TH2-mediated allergic response took place (35). Interestingly, DC-bound IgE restrains allergic inflammation at mucosal sites (37). In an OVA mouse model of food allergy where humanized FcεRI is expressed on DCs, FcεRI expressing DCs were found in the small intestine. Upon oral allergen challenge of sensitized mice, reduced mucosal inflammation as well as systemic responses, such as lower TH2 cytokines (IL-4 and IL-13), less inflammatory mediators (CCL-2 and IL-6) and lower levels of the mast cell protease MCP-1 were found, indicating an immune regulatory feature of IgE independent of the regulatory T-cell compartment (37).

There is ample evidence for the role of Tregs in tolerance induction at mucosal sites. Small intestinal extrathymic-derived $FOXP3^+$ Tregs develop from conventional T cells in response to microbial or dietary antigens. Interestingly, removal of antigens via feeding of 'antigen-free' elemental diet exhibited significantly decreased small intestinal $CD4+$ T-cell activation and consequently reduced $FOXP3^+$ T cells. These Treg populations could be restored after re-introduction of a normal chow diet. These pTreg cells are continuously generated in the small intestine in response to dietary antigens with an expected half-life of up to 6 weeks. The $CD103^+CD11b^+$ DC subset, which is responsible for pTreg cell development, is reduced to 40%, being normalized after introduction of dietary macromolecules. 'Antigen-free' mice with a lack of pTreg cells were more susceptible to ovalbumin-induced food allergy compared with controls, indicating that dietary antigens from solid food are necessary for pTreg cell induction and thus for the control of mucosal inflammatory and allergic response (38).

In addition to $FOXP3^+CD4^+CD25^+$ Tregs, TR1 cells contribute to mucosal tolerance via the production of IL-10 (39). A novel immune regulatory property involving TR1

cells has been assigned to the complement system. In a mouse model, C3 deficiency was associated with lower IFN-γ and IL-2 and higher IL-4 and IgE production (40, 41). In a C3 knockout model, C3 deficiency shifted the local cytokine balance in the jejunum from a tolerogenic IL-10 based to a pro-inflammatory IFN-γ- and IL-17A-dominated milieu (42). Based on these results and based on the fact that PAF plays an important role in coagulation by the activation of platelets, it might be speculated that the complement system affects anaphylaxis mediated by the alternative pathway. However, recent data indicated that both IgE- and IgG-mediated anaphylaxes were complement independent. Mice pretreated with cobra venom factor to deplete complement before immunization with goat anti-mouse IgD and challenged with goat IgG did not reveal any changes in anaphylaxis (25). On the other hand, the complement regulator Crry, which is expressed on T cells and protects them from complement attack, provides co-stimulatory signals via activation of ERK and JNK (43). Via co-stimulation, binding of C3 to Crry leads to the expansion of isolated natural $CD4^+CD25^+$ Treg cells, increased levels of FOXP3 expression suggesting a contribution of the complement system to immune tolerance (43–45). In addition, γδ T cells, which represent a considerable proportion of intestinal epithelial lymphocytes (IEL) (46), can suppress TH2-dependent IgE responses (47). In a cholera toxin-based food allergy model, sensitization with peanut extract together with the adjuvant caused a reduction of $CD8^+$ γδ T cells in the intestine. Blocking of the γδ T-cell receptor increased IgE-mediated immune response and mast cell degranulation (47). However, the underlying mechanisms of the tolerogenic effects of γδ T cells still need to be identified.

It was reported that not only T regulatory cells but also regulatory B cells contribute to mucosal immune homeostasis. A subpopulation of B cells, $CD5^+CD19^+CD138^-$ tolerogenic B cells (TolBCs), in the mouse intestine carries the integrin alpha v beta 6 (αvβ6) captured from exogenous sources, such as the intestinal epithelial cells. This subset of TolBCs migrates to the source of αvβ6 in a CX3CR1-dependent manner, as intestinal cells express the ligand of CX3CR1, fractalkine. Once αvβ6 is captured, TGF-β production is induced, which leads to the conversion of TH0 cells to Tregs and to the inhibition of food allergic symptoms and local TH2 inflammation (48).

Based on these recent mouse studies, it becomes obvious that a number of different regulatory cells and signals are required to result in mucosal immune homeostasis awaiting confirmation in the human system.

Allergen-specific immunotherapy (AIT) as a model to study tolerance development

Lessons learned from AIT to inhalant allergens

At present, allergen-specific immunotherapy is the only curative treatment for allergic patients. A prolonged status of clinical effectiveness is proven for both subcutaneous and sublingual immunotherapy with inhalant allergens (49–53). A

number of immunological changes accompanying clinical success have been described (54–57).

During the course of allergen-specific immunotherapy to inhalant allergens, a sequence of events takes place, which basically can be subdivided into four. First, a decrease in mast cell degranulation and basophil activity takes place within hours to days and may be accompanied by clinical relevant desensitization, as it is the case for hymenoptera immunotherapy. Secondly, there is the induction of allergen-specific T regulatory and thirdly of allergen-specific B regulatory cells reflected in a suppression of effector T cells. In fourth place, within weeks to months, a dose-dependent increase in allergen-specific IgG4 is observed (58). These allergen-specific IgG4 antibodies are supposed to act as blocking antibodies interacting at the level of IgE-mediated effector cell activation (49, 59–62). In particular, IL-10 produced by allergen-specific T regulatory cells and B regulatory cells has been linked to IgG4 (63). Although data on the function of blocking antibodies is robust, correlation of IgG4 or specific IgE/IgG4 ratio with clinical response depends on the immunotherapy used and the allergen applied. IgG4 itself is largely dependent on allergen exposure but cannot be used as tool to monitor or predict individual clinical response (2, 64–67). Allergen-specific IgE increases within the first weeks to months of specific immunotherapy (2, 68) and decreases in many cases later on. The drop in specific IgE does not correlate with clinical improvement (69) and is not a suitable marker to be used for therapy monitoring or to evaluate SIT efficacy (49). This dissociation of events has been linked to long-living IgE-secreting plasma cells that survive in bone marrow and spleen (70). Another cluster of events is initiated months after initiation of SIT and is defined as the reduction of tissue effector cells such as mast cells and eosinophils and a decrease in skin prick test reactivity.

Oral and sublingual immunotherapy in food allergy

Throughout the last decade, desensitization protocols for oral immunotherapy (OIT) against food allergy have been developed and proven to be effective and safe in the hands of institutions with a high degree of expertise and at level of clinical trials. However, depending on the inclusion criteria, adverse effects ranged from mild to severe and up to 50% patients did not reach the maintenance dose (71–77). Among those individuals who achieved the maintenance dose, depending on the respective allergen (peanut vs milk or egg allergy), the percentage of patients that experience desensitization (clinical effects restraint to the duration of OIT) ranges from 42% to 93% (1, 76–81) and those who experience long-term clinical effects that persist despite OIT discontinuation equivalent to sustained unresponsiveness and possibly tolerance development are ranging from 28% to 50% (1, 2, 75, 76, 82). Consequently, new approaches for peanut, milk, and egg oral immunotherapy with hypoallergenic variants or combined with the anti-IgE antibody omalizumab are under investigation to reduce allergic reactions during dose-up phase and confer a safer oral desensitization (83). Although the body of evidence is increasing and the

results are encouraging, these treatment modalities so far did not reach a level of a clinical recommendation (84). Based on existing literature, the following immunological changes have been reported during OIT.

One of the consistently reported events during OIT is loss or significant reduction in basophil reactivity and sensitivity to the respective allergen. Basophils express the high-affinity receptor for IgE (FcεRI) on their surface and are capable of releasing mediators like histamine, leukotrienes, cytokines, and chemokines that reflect effector cell populations of the immediate-type response. Degranulation results in translocation of lysosomal-associated membrane proteins (LAMPs), like CD63 (LAMP-3), to the cell surface. CD203c, as an ectonucleotide pyrophosphatase/phosphodiesterase, is expressed on basophils and upregulated during basophil activation, however via mechanisms and pharmacokinetics distinct from the LAMPs. The basophil activation test (BAT) has recently also been described as the best method to assess clinical tolerance to peanuts (84). Good sensitivity and specificity have also been described for milk (85, 86) and egg allergy (87). However, its usefulness to delineate whether patients can consume baked egg or milk is less clear (85, 86). In the context of OIT, reduced basophil activation corresponds to desensitization in OIT with peanut (80, 88, 89), egg (1, 90), and milk (91). Despite the lack of sufficient data to define the exact temporal resolution, the loss of basophil activation may be considered one of the earliest readouts to be observed at an immunological level during OIT. Significantly, suppression of CD63 and CD203c expression of basophils was reported during the first 4 months of OIT (80, 89). Interestingly, there was a bystander effect, that is, a suppression of basophil activation to other food allergens and to IgE cross-linking, observed. Although there is no consistency of the reports, a suppression of FcεRI signaling during OIT seems likely (89). Having a closer look at the tolerant population vs the nontolerant individuals under OIT, suppression of basophil activation did not differ between these two groups (88). Consequently, BAT may be rather reflecting desensitization and its ability to estimate tolerance development requires further evaluation.

Changes in the T-cell compartment occur during the course of OIT. Upon allergen-specific stimulation, a reduced allergen-specific IL-5 (77, 80, 92) and IL-13 production (93), (80) and an IL-10 induction were reported (88).

These changes have been attributed to an induction of regulatory T cells. Syed et al. demonstrated that desensitized individuals after peanut OIT displayed increased numbers of allergen-induced Tregs defined as $CFSE^{lo}CD25^{bright}FOXP3^{+}IL10^{+}CD45RO^{+}Helios^{lo/-}LAG3^{+}$ cells after allergen-specific stimulation, whereas natural Tregs remain stable. Allergen-induced Tregs upregulated CCR8, but not CCR4 or CCR7, and had an enhanced chemotactic index toward intestinal cells (88), suggesting an increased number of these induced regulatory cells to the sides of inflammation. Immunotolerant individuals also had a higher FOXP3 expression and FOXP3 hypomethylation. In addition, this study reported the induction of hypomethylation of Teff FOXP3 CpG sides via dendritic cells after OIT but not at

baseline (88). In line with these findings, a significant increase in FOXP3$^+$ Tregs upon stimulation with peanut extract at 6 and 12 months of peanut OIT and a decrease of apoptosis-related genes in unstimulated T cells after 6 months of peanut OIT were described applying genomewide oligonucleotide micorarray analysis (80). During peanut OIT, an increased FOXP3$^{high:}$ FOXP3^{intermediate} ratio within the CD4$^+$CD25$^+$ T-cell compartment was observed (80). In a recent trial, omalizumab pretreatment was applied followed by a rush protocol for milk OIT. The authors provide evidence for an elimination of allergen-specific T-cell responses within a week, without evidence for an induction and expansion of regulatory T cells or a bystander effect. This may be explained either by anergy or by elimination of allergen-specific responses. After discontinuation of the omalizumab treatment and continuation of the OIT, an increase in the IFN-γ/IL-4 and IFN-γ/IL-13 ratio was observed (94). Another study defined a subset of CD4$^+$ T cells that were hypoproliferative upon TCR stimulation and displayed a CD4$^+$CD38$^+$CD45RO$^-$ phenotype in an OIT with hen's egg allergy. This was also reflected in reduced TH1-type (IL-2, TNF-α, and IFN-γ), TH2-type (IL-4, IL-5, IL-9), and TH17-type cytokine levels in serum after oral immunotherapy in egg-allergic children (95). In a randomized, double-blind, placebo-controlled oral Immunotherapy trial with and without omalizumab, anti-IgE treatment significantly improves the safety profile of OIT. However, there was no evidence for a pro-tolerogenic effect (96).

The induction of allergen-specific blocking antibodies of the IgG, in particular IgG4 antibodies, is considered to be one of the central mechanisms of allergen-specific immunotherapy. IgE is normally induced after the onset of OIT and decreases thereafter slowly. The induction of allergen-specific IgG4 starts within the first months of treatment, and levels further increases over time (1, 2, 77). IgG4 induction was more pronounced, and the IgE/IgG4 ratio was lower in peanut OIT as compared to peanut SLIT, whereas significant specific IgE induction was seen only during OIT (92). In a trial with egg OIT, IgG4 production correlated with sustained unresponsiveness (1). In case of milk OIT, neither IgG4 nor IgE levels correlated with tolerance development (91). Discontinuation of peanut OIT led to a reduction of IgG4 (77). Vickery et al. performed an in-depth analysis of epitopes during peanut OIT. Despite considerable interindividual variability, they reported an induction of IgG4 during OIT along with the detection of new IgG4 epitopes. The reduction in peanut-specific IgE, however, went along without affecting the number of recognized IgE epitope diversity. OIT expanded the preexisting small IgG4 epitope repertoire pre-OIT by the magnitude of >10. No change regarding affinity of antibodies was detected. Specific IgG induction was described during egg allergy (76). In another study, the reduction in specific IgE was linked to responders to milk and egg OIT (75).

Skin prick test is included in most of the OIT studies performed. Allergen-specific SPT diameter/size in general decreased during successful OIT (1, 2, 77–80, 88, 91, 93, 94, 97, 98), with rare exceptions (1).

Despite the data suggesting comparable events for AIT to food allergens as compared to inhalant allergens (Table 1), there is eminent need to understand the induction of immunological pro-tolerogenic events at a cellular and humoral level to food allergens to delineate desensitization from tolerance development (77, 88, 99) (Table 1).

Lessons learned from natural tolerance development and asymptomatic sensitization

While IT trials provide considerable insights in changes that go alongside clinical response and some data on the development of sustained unresponsiveness, there is relative little data available on natural tolerance development in allergic individuals. Forty to sixty percent of children with milk or egg allergy will outgrow their food allergy until the age of 6 years (100, 101), whereas this occurs at a lower rate in children with allergy to peanut or tree nuts. These individuals may be considered prototypic for tolerance acquisition after established potentially life-threatening food allergy (100, 102–105). The sequence of events and biomarkers that define individuals with persistent food allergy and those who outgrow their food allergy remain unclear. Sequential rather than conformational epitopes as well as greater diversity, higher affinity, and different sensitization patterns is associated with persistent allergy and different severity of the allergic reaction (106–111), suggesting an important role for B-cell antigen presentation and T-cell help in the maintenance of food sensitization (112). In addition, the subgroup that develops tolerance displays lower levels of allergen-specific IgE and SPT.

IgG4 levels and IgE/IgG4 ratio are being evaluated as possible predictive markers of allergy resolution. Some studies reported that specific IgG4 levels (113, 114) and specific IgE/IgG4 ratio were not predictive of food allergy resolution (115), neither was specific IgE/total IgE ratio (116), while tolerance to milk in atopic children and adults is related to elevated levels of specific IgG4 in combination with low specific IgE (117–119). In egg-allergic children, higher levels of ovomucoid-specific IgE were found in those reacting to baked egg than in those tolerant to baked egg and regular egg. In cow's milk-allergic individuals, high levels of casein-specific IgE antibodies have been identified as a risk factor for persistence of cow's milk allergy (120). Data from a large prospective observational trial on 560 high-risk inner-city children reported an increased production of IgG4 in allergic and sensitized nonallergic individuals. This may also reflect a preallergic state whereas IgG4 increase during specific immunotherapy may represent an active state of tolerance development that may be IL-10 dependent. Recent data support a relevant suppressive effect of IgG4 in patients that acquired tolerance to peanut or keep a status of tolerance despite sensitization. IgG4 from peanut-sensitized nonallergic individuals suppressed allergen-specific activation of mast cells and basophils loaded with IgE from allergic individuals (121), although other studies showed that allergen-specific IgG seems not to be involved in peanut natural clinical tolerance (111).

Table 1 Evidence of different mechanisms of action in OIT/SLIT and natural tolerance

Mechanisms of action	OIT/SLIT	References	Natural tolerance	References
Basophils, mast cells and eosinophils				
Suppression of basophil activation	+++	(1, 80, 88, 91, 133)	+	(84, 85, 134)
Bystander suppression of basophil activation	+	(89)		
T-cell response				
Decreased allergen-induced proliferation	+	(95)	++	(135, 136)
Induction of allergen-specific TR1 cells	+	(88)		
Induction of FOXP3^{pos} Tregs	+++	(80, 88, 93, 95, 137)	+	(123)
Increased secretion of IL-10	+	(80)	+	(118)
Suppression of allergen-specific TH2-type responses	+++	(77, 93, 95)	+	(134, 138)
B-cell response				
Initial increase in specific IgE levels and long-term reduction in specific IgE	+++	(75, 78, 80, 82, 91, 92)	n.a.	
Increased allergen-specific IgG4 production	+++	(1, 76–78, 80, 91–93, 98, 99, 139)	++	(118, 19, 85, 138, 142)
Broadening of allergen-specific IgG4 repertoire	+	(140)		
Increased allergen-specific IgA production (saliva, food SLIT)	+	(141)	+	(142)
Suppressed IgE-mediated facilitated antigen presentation	+	(2)		
IgG4-dependent suppression of mast cell and basophil activation			+	(121)
Dendritic cells				
Induction of pro-tolerogenic dendritic cells	+	(88)		

Specific regulatory T cells were related to the outgrowth of milk allergy. Higher frequencies and maturation were correlated with better prognosis, as seen with CD45RO marker expression in children with milk allergy. CD45RO marker expression was present in 30% of CD4+CD25+ T cells of milk-tolerant children compared with 5% of children with milk allergy (21, 115, 122–124). Children with milk allergy that displayed gastrointestinal symptoms had milk-specific T cells in gastrointestinal mucosa, and these T cells showed a TH2 cytokine secretion profile after specific stimulation (125). At an allergen-specific T-cell level, Turcanu et al. (126) reported a TH1 skewing of T cells from tolerant individuals in response to peanut antigens (IFN-γ^{hi}, TNF-α^{hi}, IL-4^{lo},

IL-5^{lo}, IL-13^{lo}), similarly to nonallergenic. A more recent publication from the same group defined IL-9 as a potential marker differentiating between peanut-sensitized and peanut-allergic individuals at an allergen-specific T-cell level. They demonstrated a significant upregulation of this cytokine among other TH2-type cytokines in peanut-specific activated gut and skin homing memory T-cell populations (127). This finding corresponds with data from peanut-stimulated PBMCs (128). To attribute a role of the TH9 subset in peanut allergy is interesting; however, more data are required.

Children who have developed clinical tolerance to milk had a decrease in *in vitro* peripheral blood mononuclear cell proliferation in response to bovine β-lactoglobulin as a

consequence of an increased number of circulating $CD4^+CD25^+$ T regulatory cells (122). Several studies reported raised T regulatory cell numbers in patients with immune tolerance (80, 129), and antigen-induced T regulatory cells in the gut are associated with natural loss of food allergy (122). Shreffler reported during tolerance development (milk-allergic individuals that tolerate heated milk, which is considered to occur before the development of tolerance to nontreated milk) an increased proliferation of the allergen-specific Treg compartment. Interestingly, no difference regarding this Treg compartment was observed between allergic and nonallergic, nonsensitized individuals (123). In patients who naturally developed tolerance to egg or peanut, $CD25^+CD127^{lo}Foxp3^+$ cell numbers were higher compared with allergic or nonallergic controls. Moreover, the $IL-10^+$ producing $CD25^+CD127^{lo}$ subset as well as the $CD25^+CD127^{lo}Foxp3^+$ subsets expanded *in vitro* compared with those from allergic and nonallergic controls (130). Thus, the Treg compartment is suggested to play a role in tolerance development to milk, egg, and peanut.

Casein-specific IL-4- and IL-13-secreting T-cell numbers appeared to be increased in cow's milk-allergic children and inversely correlated with the tolerated dose (131). Elevated levels of the alarmin uric acid have been found in serum from peanut-allergic children compared with nonallergic healthy controls (104).

One study reported that children allergic to the most prevalent food allergy in childhood, cow's milk protein, have significantly lower levels of mast cell protein-1 (MCP-1) and macrophage inflammatory protein (MIP-1α) as compared to atopic children without allergy. During OIT, both parameters increased significantly, while specific IgE decreased during the course of tolerance development (2 years postintroduction of milk immunotherapy). Consequently, MCP-1 and MIP-1α were considered useful markers to follow-up milk protein desensitization but also a marker for natural tolerance development (132). Further publications in this context are needed.

Recently, the LEAP study provided data for primary and secondary prevention of peanut allergy in a high-risk cohort (19, 20), which will soon be reflected in new guidelines. Nevertheless, no therapies capable of restoring tolerance are available.

Taken together, available markers that are readily available with good correlation of tolerance development are certainly the development of IgE negativity and SPT negativity has a good predictive value and should encourage challenge to the respective food. However, tolerance may occur much earlier leading to prolonged dietary restrictions. The need for repetitive clinical evaluation and reconsideration of challenge procedures in allergic individuals is clearly emphasized in current position statements. Moreover, the lack of usability for therapeutic or secondary prophylactic approaches is apparent.

The basophil activation test may be a valuable test in the future. However, further prospective studies that have the BAT in the center of decision making, possibly in combination with component-resolved diagnosis, are demanded. Moreover, dose titrations over several log scales are likely to be helpful. Currently, BAT is not certified and available in many countries, and allergens and allergen extracts linked to current kits do not allow titrations along 4 log scales to keep the costs within a reasonable range.

In the future, peptide arrays and more sophisticated component-based approaches in combination with functional assays may be applicable. However, their availability outside of clinical trials seems unlikely within the next years. The progression of new treatment options in food allergy may facilitate that. Functional Treg assays will remain restricted to study settings due to their complexity and costs.

Conclusions and outlook

Tolerance development to allergens is the default process. Upon loss of this tolerance, the mechanisms and the sequence of events that have to take place to re-establish tolerance are poorly understood. Biomarkers that predict tolerance development as compared to desensitization are needed. This is decisive to consult patients on treatment duration and risk behavior if OIT is performed or under avoidance regimens. New therapeutic approaches have to prevent allergy development on the first place and learn from natural tolerance development to develop strategies to treat food allergies.

Acknowledgments

Author contributions

Thomas Eiwegger initiated, structured, coordinated, and worked on the manuscript. Marta Ponce, Susanne Diesner, and Zsolt Szepfalusi worked on the manuscript and read the manuscript.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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