

Regulation of Specific Immune Responses by Chemical and Structural Modifications of Allergens

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Key Words

Modified allergens · Recombinant allergens · Antigen conformation · Antigen presentation · IgE regulation · Th1/Th2

Abstract

Specific immunotherapy (SIT) is an efficient treatment of allergic diseases to defined allergens. Despite being used in clinical practice since early in this century, more rational and safer regimens are required, because SIT is faced with the risk of anaphylaxis and standardization problems of allergen-extract-based treatments. A better understanding of the pathogenesis of allergy and of the mechanisms of SIT has led to various approaches to overcome these problems. Knowledge of the influence of IgE-facilitated antigen presentation on allergen-specific Th2 responses increased the efforts to generate non-IgE-binding allergens. The current principal approach to allergen modification is to modify B cell epitopes in order to prevent IgE binding and effector cell cross-linking while preserving T cell epitopes to retain the capacity of inducing tolerance. In this way, the modified allergen will be directed to T cells by a phagocytosis/pinocytosis-mediated antigen uptake mechanism, by-

passing IgE cross-linking and IgE-dependent antigen presentation. Accordingly, a differential regulation of allergen-specific T cell cytokine patterns and IgE:IgG production was demonstrated by modifications of the three-dimensional structure of allergens because of linearity in T cell epitopes and conformation dependence in B cell epitopes. In this context, chemically modified allergen extracts with low IgE-binding capacity have been developed to reduce anaphylactic side effects since the early 1980s. The progress of recombinant techniques for producing allergens and allergen derivatives has led to a dramatic improvement in the ability of developing novel vaccines for the treatment of allergy. This has enabled mutation or deletion of decisive amino acids in B cell epitopes and fractionation or oligomerization of allergens by genetic engineering as fruitful approaches to generate hypoallergenic vaccines. Moreover, non-IgE-binding short T cell epitope peptides and single-amino-acid-altered peptide ligands represent potential candidates for future SIT.

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Introduction

Type I allergy represents an immune disorder which affects almost 30% of the population in some communities [1]. Exposure of sensitized individuals to allergen cross-links specific IgE bound to the surface of mast cells and basophils, triggering a rapid release of mediators which cause the immediate allergic reaction. This early response is characteristically followed by a late response some hours later with cellular infiltrates of activated T cells, eosinophils, macrophages and fewer neutrophils [2, 3]. While immediate as well as late symptoms can be ameliorated by pharmacological treatment, allergen-specific immunotherapy (SIT) represents the only curative approach to type I allergy. SIT is an efficient treatment of allergic diseases to defined allergens and is most effectively used in allergy to insect venom and allergic rhinitis [4–8]. However, three major problems need to be overcome: First, SIT is currently performed with allergen extracts containing hardly standardized allergen mixtures, nonallergic proteins as well as cell-toxic proteins and is not tailored to patients' requirements. Second, systemic administration of allergens can cause severe anaphylactic reactions in the course of treatment. Third, administration of immunologically efficient high doses of allergen required for successful SIT is often not fulfilled because of nonstandardized extracts or side effects. Therefore, a better understanding of the pathogenesis of allergy and particularly the mechanisms of SIT has led to various approaches to overcome these problems.

The mechanism by which SIT achieves clinical improvement was proposed in a number of reports [reviewed in ref. 9–11]. A rise in IgG antibodies, particularly of the IgG4 class, which supposedly block the allergen and IgE-facilitated antigen presentation [12–15], the generation of IgE modulating CD8⁺ T cells [16] and a reduction in the numbers of mast cells and eosinophils, including the release of mediators [17–19], were claimed to be responsible for successful SIT. Moreover, successful SIT of allergic diseases was associated with a decrease in IL-4 and IL-5 production by T cells [20–22]. Also a shift from a Th2 cytokine pattern towards increased IFN- γ production in SIT of allergy to bee venom (BV), wasp venom, grass pollen and house dust mite was observed [20, 21, 23]. It appears, however, that the induction of an anergic state in peripheral T cells represents an essential step in SIT [20, 24–27]. T cell anergy is characterized mainly by suppressed proliferative and cytokine responses against the major allergen(s) and its T cell recognition site [24, 28]. Anergy is initiated by an autocrine action of IL-10,

which is increasingly produced by the antigen-specific T cells [9, 25]. Anergized T cells can be reactivated to produce either distinct Th1 or Th2 cytokine patterns depending on the cytokines present in the tissue microenvironment, and thus determining successful or unsuccessful SIT [24, 29].

Differential Regulation of Specific Immune Responses by Conformational Allergen Variants

Development of specific immune responses requires the encounter of antigens with the cells of the immune system and interaction between T and B lymphocytes and other antigen-presenting cells (APC) [30–32]. The presentation of soluble antigens to T cells by APC involves a complex process including antigen capture, processing of the antigen and presentation of antigen fragments by MHC molecules [30–32]. Antigen presentation by different types of APC can promote the development of CD4⁺ Th subsets with distinct cytokine patterns [33, 34]. Th1 cytokine profiles are characterized by IL-2, IFN- γ and TNF- β and are implicated in cell-mediated defense against intracellular microorganisms and in promotion of memory IgG responses [35–37]. Th2 cells secrete IL-4, IL-5 and IL-13 and predominantly mediate B cell responses and allergic inflammation [35–37]. Besides MHC-TCR interaction, all professional APC are able to deliver other costimulatory signals such as ligands for CD28, CD2 and cytokines that are secreted or expressed on the surface of APC required in T cell activation [38–41]. Monocytes and macrophages present the antigen to T cells in combination with secretion of proinflammatory cytokines such as IL-1, TNF- α , or cytokines and factors like TGF- β , IL-10, IL-12, prostaglandin E₂ that are known to suppress IgE. In contrast, B cells that act as APC produce these cytokines to a low extent only when they are activated [38–42]. Recently, dendritic cell subsets have been identified that are derived from peripheral blood monocytes (DC1) or tonsil lymphocytes (DC2) and that reciprocally induce Th1 and Th2 type cytokines from naive T cells, respectively [41].

BV phospholipase A₂ (PLA) represents the major antigen and allergen of BV [43, 44]. The response to PLA provides a specially suited model to study the cellular and molecular mechanisms of specific immune response regulation in humans [24–26, 43–46, reviewed in ref. 47]. Its three-dimensional structure, which has been resolved by X-ray crystallography, is stabilized by five intrachain cys-

teine disulfide bridges [48]. The protein has been cloned and produced in *Escherichia coli*. Formation of enzymatically active recombinant (r) PLA with full lipid binding affinity requires an additional step of protein refolding in vitro [48, 49]. Generally, anti-PLA antibodies which recognize conformational, discontinuous epitopes are elicited by bee stings [50]. In a normal immune response initially low-affinity IgG1 anti-PLA antibodies are detected, whereas high-affinity IgG4 anti-PLA antibodies are generated after repeated exposure [51, 52] or artificially by prolonged SIT in allergic patients [24, 52]. In contrast, individuals allergic to bee sting develop IgE anti-PLA antibodies [24, 43, 52].

The three-dimensional structure and recognition of the antigen is pivotal in the development of distinct T cell cytokine profiles by preferential usage of different APC. As shown by reduced and alkylated Fel d I allergen [53] or cysteine mutations of Lep d 2 [54], recombinant or chemically modified PLA preparations that do not display the tertiary structure are not recognized by specific antibodies elicited against BV [42, 48, 49]. Thus, APC that utilize phagocytosis or pinocytosis for antigen uptake, such as monocytes, macrophages and dendritic cells, can internalize modified allergen molecules independently of their structural features. Recently, we have demonstrated that PLA preparations lacking the original tertiary structure are presented by monocytes in human peripheral blood mononuclear cells (PBMC). This induced high T cell proliferation, lower IL-4, IL-5 and IL-13 secretion and lower IgE formation in PBMC from BV-allergic individuals. In contrast, the same allergen expressing the original native conformation was presented by B cells in addition to monocytes, and induced more Th2-type cytokines and IgE formation [42]. The modified PLA but not the native PLA induced detectable IL-12 production by monocytes [42]. Specific B cells most efficiently present antigen at low concentrations [32, 45]. In spite of the low frequency of specific B cells in PBMC of allergic individuals, due to its high affinity to lipids (K_a : 10^{-13} M), PLA with a correctly folded structure can directly bind to non-PLA-specific cells, which then are also able to present PLA to T cells with the same potency as PLA-specific B cells [55]. The membrane phospholipid affinity of native PLA enables a model for differential antigen presentation between B cells and monocytes. Similarly, in a study with mice, glutaraldehyde-polymerized ovalbumin has selectively induced cytokine production characteristic of Th1-dominated responses [56]. Thus, these observations point to the pivotal role of physicochemical properties of antigen leading to presentation by different types of APC and

subsequent induction of particular cytokine profiles in T cells, which differentially regulate IgE and IgG responses [42].

Effect of IgE-Mediated Antigen Presentation on Allergen-Specific Immune Responses

The immune response to foreign proteins is strongly dependent on the efficiency and selectivity of antigen uptake by APC. Kehry and Yamashita [57] have shown that IgE bound to CD23 on mouse B cells may be used to focus antigen to T cells. This finding initialized the understanding of focused antigen presentation via IgE receptors. Previously, the high-affinity receptor for IgE has been believed to be present on mast cells and basophils. This is now followed by the recent demonstration of IgE RI on Langerhans cells, eosinophils and epidermal cells [58–60]. Low-affinity IgE receptor (CD23) has a widespread cellular distribution [61]. Both receptors are thought to play a role in IgE-mediated antigen presentation [62–65]. This mechanism operates selectively at low doses of allergens, and the presentation of extremely low doses of allergen to CD4⁺ T lymphocytes is greatly enhanced by IgE-facilitated antigen presentation [15, 65]. Blocking of IgG antibodies in IgG-containing serum fractions induced by SIT of birch pollen allergy inhibits the IgE-facilitated antigen presentation at very low allergen concentrations [15]. Therefore IgE acts as an immunoglobulin specialized in antigen capture or antigen focusing. In atopic patients, especially those with extrinsic type atopic dermatitis, the presence of high levels of specific IgE in serum and CD23 on APC (activated B cells) [66], together with FcεRI expressing APC (Langerhans cells in the skin) [60], may strongly contribute to overstimulation of the allergen-specific immune responses.

Capture of antigens via surface IgE and signal transduction through IgE cytoplasmic chain were shown as crucial events in specific IgE responses [67]. Clinical trials with a neutralizing anti-IgE mAb treatment have demonstrated an inhibition in bronchial late-phase responses and the number of eosinophils in sputum of allergic asthma patients [68]. Correspondingly, the production of IL-4 and IL-5 and lung eosinophilia of mice sensitized to house dust mite is inhibited by anti-CD23 or anti-IgE monoclonal antibody (mAb) treatment [69]. Moreover, studies performed using anti-CD23 mAb and CD23-deficient mice suggest that IgE-mediated antigen presentation plays a major role in Th2 cytokine production and lung eosinophilia [69]. Altogether, previously published data

Table 1. Strategies for bypassing effector cell surface IgE cross-linking and directing allergens to T cells

(a)	Chemical modification of allergens
	Mineral oil precipitation [70]
	Alum precipitation [71]
	Urea denaturation [72]
	PEG conjugation [73, 74]
	Poly <i>D</i> -glutamic acid: <i>D</i> -lysine conjugation [75]
	Allergoids [76, 78–83, 84]
(b)	B cell epitope mutants [85–90]
(c)	Targeting disulfide bonds [42, 53, 54, 88, 91]
(d)	Allergen fragments [92, 93]
(e)	Allergen oligomers [94, 95, 97]
(f)	T cell epitope peptides [26, 101]
(g)	Altered peptide ligands [103]

suggest that inhibition of IgE-facilitated antigen presentation by non-IgE-binding variants of allergens represent a key target for future SIT vaccine development.

Strategies for Bypassing Effector Cell Surface IgE Cross-Linking and Directing Allergens to T Cells (table 1)

It can be expected that due to the hypoallergenic nature of allergens that retain T cell reactivity, much higher allergen doses can be administered to allergic patients. This possibility may permit immunotherapy with fewer injections and will likely favor an allergen-specific tolerance in the Th2 immune response and development of an allergen-specific Th0-Th1 immune response associated with blocking antibodies. Modification strategies for bypassing IgE and directing allergens to T cells include chemical modifications of allergens that are currently available in the clinical practice; T cell epitope peptides on which very limited clinical studies have been performed, and genetic engineering of allergens that are promising for the future of SIT.

Chemically Modified Allergens

To overcome the major problems faced by SIT, earlier studies were mainly based on chemical modification of allergens. Historically, the first attempts were to impede the release of allergens from the injection site. For this purpose, mineral oil [70] and alum precipitation [71] and urea denaturation [72] were used. Conjugation of antigen with polyethylene glycol (PEG) was shown to activate antigen-specific suppressor cells in an animal model [73].

In a double-blind trial with PEG-modified ragweed extract in patients with ragweed hay fever, there was suppression of the seasonal IgE increase with a rise in ragweed-specific IgG [74]. Another trial of SIT in patients with ragweed hay fever was conducted with allergen extract conjugated to poly *D*-glutamic acid:*D*-lysine. The treated patients had significantly lower symptom scores than the controls, although there were increases in both serum specific IgE and IgG levels [75]. An additional approach to modify allergens was to polymerize allergens into high-molecular-weight molecules by using glutaraldehyde as a cross-linking agent. Ragweed AgE with an original molecular weight of 38,000 kD, which has been modified to high-molecular-weight polymers of 200,000–20,000,000 kD, has been shown to be less allergenic and retain immunogenicity [76]. Allergens chemically conjugated to APC in spleen cells have also demonstrated specific IgE-suppressive activity without affecting IgG in a study with mice [77].

Several studies were carried out with allergens modified by means of aldehydes [78–83]. Formaldehyde- and glutaraldehyde-treated allergens termed ‘allergoids’ displayed low IgE binding [78, 79]; they were much better tolerated than the native allergen extract and fewer injections were required to reach higher maintenance doses [78–82]. Several studies have reported monomeric chemically modified allergens obtained by using potassium cyanate at neutral pH to substitute ϵ -amino groups of lysine residues and thus reduce IgE-binding reactivity [83]. Relatively low-molecular-weight allergoids were able to cross biological membranes and were suitable for sublingual administration [83]. Moreover, synthesis of various acid anhydride derivatives, such as succinic anhydride or maleic anhydride derivatives of allergens was described [84]. A chemical approach for altering the conformation of allergens by targeting disulfide bonds has been discussed above [42].

Site-Directed Mutagenesis of B Cell Epitopes

Rapid progress is being made by recombinant DNA techniques replacing the chemical procedures for the preparation of complex allergen extracts. Recombinant allergens represent promising tools for diagnosis and therapy of type I allergy. Primary amino acid sequences of allergens can be identified from cloned genes, and T cell epitopes can be determined by synthetic peptides. Antibody-binding epitopes are identified by means of various approaches. Numerous studies have been performed to modify antibody-binding sites of allergens. Site-directed mutagenesis is one of the most straightforward ap-

proaches of these methods [85–90]. Ferreira et al. [85] have demonstrated the importance of six individual amino acid residues for IgE binding to Bet v 1. Two isoforms of Bet v 1 showed low IgE reactivity when tested with a panel of sera from birch pollen allergic patients, but preserved T cell epitopes. Such an approach may be generally applied to generate allergen variants to be used in safer SIT, as recently demonstrated for house dust mite and peanut allergens [88–90]. Moreover, by genetic engineering, decisive amino acids in the B cell epitopes can be deleted and deletion mutants may show lower capacity of IgE binding, but normal T cell reactivity, as shown in timothy grass pollen allergen, Phl p 5b [87] and house dust mite allergen, Der p 2 [88]. Site-directed mutagenesis of the cystein residues changes the conformation of allergens and represents a fruitful approach to find candidate molecules for improved immunotherapy [54, 88, 91].

Hypoallergenic Allergen Fragments

The presence of conformational B cell epitopes suggested a novel approach to reduce anaphylactic activity by fragmentation of allergens. The three-dimensional structure of the major birch pollen allergen Bet v 1 and cow dander allergen Bos d 2 were disrupted by expression of two parts of the cDNA in *E. coli* [92, 93]. Recombinant fragments of Bet v 1 and Bos d 2 were shown to have almost no allergenicity, but the fragments induced proliferation of specific T cell clones. They did not show any IgE binding in ELISA and western blots. Skin test reactivity and histamine release from peripheral blood granulocytes were greatly reduced by about 100-fold in comparison with the native proteins [92–95]. Moreover, in skin blister chambers, a lower capacity to activate eosinophils and to release proinflammatory mediators and cytokines was demonstrated by rBet v 1 fragments [96]. These results demonstrated that IgE epitopes could be hampered, but T cell reactivity could be preserved by making two fragments of recombinant allergens.

Hypoallergenic Allergen Oligomers

Another approach to obtain hypoallergenic derivatives of recombinant allergens was to obtain oligomers of allergens by genetic engineering. Using the Bet v 1 cDNA as template, Vrtala et al. [97] have constructed a dimer and a trimer of rBet v 1 by Bet-v-1-encoding cDNA subunits containing different restriction sites. This has allowed the construction of expression plasmids containing defined numbers of Bet v 1 cDNAs linked together. Using plasmids containing two or three copies of the Bet v 1 cDNA, stable recombinant Bet v 1 dimers and trimers have been

expressed [97]. The rBet v 1 oligomers bound IgE antibodies of patients allergic to Bet v 1 and inhibited IgE binding to ELISA plate-coupled Bet v 1 monomer; however, their anaphylactic activity was strongly reduced, as determined by basophil histamine release and skin prick testing. The recombinant Bet v 1 oligomers strongly stimulated Bet-v-1-specific T cell clones and PBMC of birch pollen allergic individuals. Together, these data indicate that T cell epitopes are protected but anaphylactogenic activity is reduced in recombinant Bet v 1 oligomers [94–97, Vrtala et al., unpubl. data]. It is remarkable that the rBet v 1 oligomers containing IgE binding epitopes exhibited strongly reduced anaphylactogenic activity. A number of explanations for this surprising and reproducible effect could be microaggregation of oligomers, lower affinity for IgE recognition, and orientation of IgE epitopes on the surface of oligomers in such a way that they prevent efficient effector cell triggering.

T Cell Epitope Peptide Immunotherapy

The epitopes of allergens recognized by T cells and antibodies are not identical. To identify T cell epitopes, antigen-specific T cell lines and T cell clones are generated. Using peripheral blood T cells, T cell lines and clonal T cells, overlapping synthetic peptides spanning the known sequence of the allergen molecule are screened for T cell reactivity. Autologous irradiated APC (PBMC or Epstein-Barr-virus-transformed B cells) are used as APC. In this way, multiple sites of T cell epitopes with an active core region of 9–11 amino acids could be identified from allergens [47, 52]. In a recent study, Haselden et al. [98] have shown that short T cell epitope peptides can directly initiate a T-cell-dependent late asthmatic reaction, without the requirement for an early IgE/mast-cell-dependent response, in sensitized asthmatic subjects. This study demonstrated the important role of T cells that respond to single epitope peptides and initiate allergic reactions and an important rationale for targeting T cells in SIT. In mice, several studies demonstrated that the administration of peptides based on dominant T cell epitopes induced specific T cell tolerance [99, 100]. Clinical trials of allergen peptide immunotherapy (PIT) have been performed in cat and BV allergy. Relatively longer (27 mer) peptides of the major cat allergen Fel d 1 were applied in SIT of cat allergy with variable efficacy. Some patients experienced local reactions a few hours after the injection, and this treatment induced decreased IL-4 production by patients' T cells [101]. PIT of BV allergy was performed with short peptides that represent the T cell epitopes (17, 12, 11 mer) of the BV major allergen PLA. The study

Altered T Cell Epitope Peptides

It was demonstrated that single amino acid alterations in T cell epitope peptides, so-called altered peptide ligands (APL), can modify specific T cell activation and cytokine production by their diversity in binding the TcR or MHC-class-II-presenting molecules [102]. Recently, two dominant T cell epitopes of PLA by alanine substitutions of each amino acid have been screened [103; Faith et al., unpubl. data]. A decrease in proliferation of specific T cells was obtained by alanine substitution of the immunodominant epitope PLA 81-92 [103]. This was associated with a change in the proportions of cytokine release. Only the Phe⁸²-substituted APL inhibited IL-4 but had no effect on IFN- γ production. This effect of a particular amino acid substitution on cytokine secretion appeared to result from changes in affinity to the MHC class II of the peptide. Moreover, this APL was a strong stimulator of IgG production. The defective T cell activation was associated with alterations in transmembrane signaling, specifically with a lack of tyrosine phosphorylation of the ZAP70 tyrosine kinase [103]. These results demonstrate that studies with APL of other major allergens may represent future ways of better controlled PIT.

Conclusion

SIT is a method that involves the administration of gradually increasing doses of allergen over several years. Usually, 3–5 years of regular injections are required [4, 5, 22]. Although it is the only curative and successful approach in type I allergy, it is faced with major problems. However, clinical efficacy, which was demonstrated in numerous studies, varies according to the type of allergen used. There is a risk of systemic anaphylactic reactions. Various attempts were made to increase the success and decrease the risk of side effects of SIT. The current availability of recombinant allergen technology of complex extracts will permit excellent standardization of preparations for clinical use. The aim of allergen modification was to decrease allergenicity while retaining immunogenicity. This could be fulfilled by destroying conformational B cell epitopes while preserving linear T cell epitopes of allergens. Figure 1 demonstrates the difference in antigen presentation and immune responses between native and modified allergens. Native allergens cross-link IgE and induce mast cell and basophil degranulation. They utilize IgE-facilitated antigen presentation leading to higher Th2 cytokine production. High amounts of IL-4 and IL-13 produced by classical allergens induce higher IgE produc-

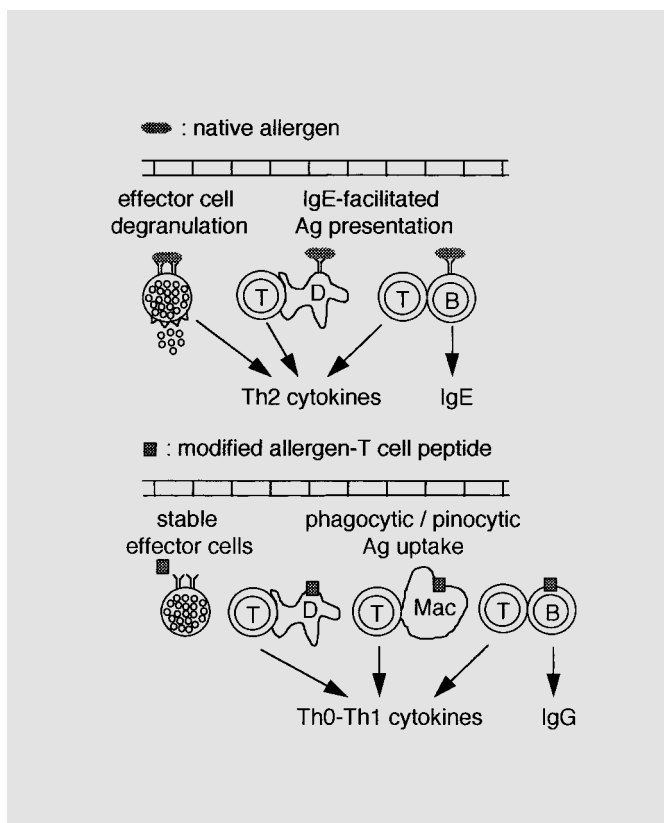


Fig. 1. Difference in antigen presentation and immune response between native and modified allergens. Native allergens cross-link IgE and induce mast cell and basophil degranulation. They utilize an IgE-facilitated antigen presentation leading to higher Th2 cytokines and IgE production. In contrast, modified allergens lack IgE binding or effector cell degranulation and utilize phagocytic or pinocytotic antigen uptake mechanisms. This induces a balanced Th0- or Th1-like cytokine production by T cells, and a lower IgE and a higher IgG production by B cells.

demonstrated an immunomodulation towards the whole allergen with specific T cell anergy and a decrease in specific IgE:IgG4 ratio in successfully treated patients [26]. The question whether intermolecular bystander suppression is possible still remains to be solved in PIT of complex allergens. BV sensitivity was easier to downregulate because this allergy is restricted to a single major allergen. However, the challenge for future PIT will be to design suitable peptide vaccines for more complex allergens. The solution for complex allergens will probably be allergen molecules which have been modified chemically or by means of genetic engineering for bypassing IgE cross-linking but targeting T cells.

tion, whereas high IL-5 production may lead to eosinophil activation and increased eosinophil life span. Because of severe side effects, native allergen extracts may not reach the high doses required for successful SIT and cannot be used in anaphylactogenic food and in latex allergy. In contrast, modified allergens that lack IgE binding or effector cell degranulation do not employ IgE-mediated antigen presentation. They utilize phagocytic or pinocytic antigen uptake mechanisms. This induces a balanced Th0- or Th1-like cytokine production by T cells and lower IgE,

higher IgG production by B cells. Targeting T cells by modified allergens will further enable higher dose administration to induce Th2-type T cell tolerance without the risk of anaphylaxis.

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