

## Molecular mechanisms in allergy and clinical immunology

Series editors: Joshua A. Boyce, MD, Fred Finkelman, MD, William T. Shearer, MD, PhD, and Donata Vercelli, MD

### Mechanisms of allergen-specific immunotherapy

Mübeccel Akdis, MD, PhD, and Cezmi A. Akdis, MD Davos, Switzerland

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Allergen-specific immunotherapy (SIT) has been used for almost a century as a desensitizing therapy for allergic diseases and represents the only curative and specific method of treatment. Administration of appropriate concentrations of allergen extracts has been shown to be reproducibly effective when patients are carefully selected. The mechanisms by which allergen-SIT has its effects include the modulation of T-cell and B-cell responses and related antibody isotypes as well as effector cells of allergic inflammation, such as eosinophils, basophils, and mast cells. The balance between allergen-specific T-regulatory (Treg) and T<sub>H</sub>2 cells appears to be decisive in the development of allergic and healthy immune responses against allergens. Treg cells consistently represent the dominant subset specific for common environmental allergens in sensitized healthy individuals. In contrast, there is a high frequency of allergen-specific T<sub>H</sub>2 cells in patients with allergy. The induction of a tolerant state in peripheral T cells represents an essential step in allergen-SIT. Peripheral T-cell tolerance is characterized mainly by generation of allergen-specific Treg cells leading to suppressed T-cell proliferation and T<sub>H</sub>1 and T<sub>H</sub>2 cytokine responses against the allergen. This is accompanied by a significant increase in allergen-specific IgG<sub>4</sub>, and also IgG<sub>1</sub> and IgA, and a decrease in IgE in the late stage of the disease. In addition, decreased tissue infiltration of mast cells and eosinophils and their mediator release including

circulating basophils takes place. Current understanding of mechanisms of allergen-SIT, particularly the role of Treg cells in peripheral tolerance, may enable novel treatment strategies. (J Allergy Clin Immunol 2007;119:780-9.)

**Key words:** Allergen immunotherapy, T-regulatory cells, tolerance, IgE, IgG, T cells, B cells, mast cells, basophils, eosinophils, IL-10, TGF- $\beta$

The physiopathology of allergic immune responses is complex and has been shown to be influenced by several factors, including genetic susceptibility, route of exposure, allergen dose, and, in some cases, the structural characteristics of the allergen.<sup>1</sup> Allergic immune response requires sensitization and development of specific immune response toward the allergen. During sensitization to allergen, priming of allergen-specific CD4<sup>+</sup> T<sub>H</sub>2 cells results in the production of T<sub>H</sub>2 cytokines (such as IL-4 and IL-13) that are responsible for class switching to the  $\epsilon$  heavy chain for IgE production by B cells, mucus production, and activation of endothelial cells for T<sub>H</sub>2 cell and eosinophil migration to tissues.<sup>2,3</sup> IgE sensitizes mast cells and basophils by binding to the high-affinity receptor for IgE (Fc $\epsilon$ RI) expressed on their surface. On cross-linking of the IgE-Fc $\epsilon$ RI complexes by allergen, mast cells and basophils degranulate, releasing vasoactive amines (principally histamine), lipid mediators (prostaglandins and cysteinyl leukotrienes), cytokines, and chemokines, all of which characterize the immediate phase of the allergic reaction.<sup>2,3</sup> After the sensitization phase, allergic inflammation and reactions to allergen challenge are observed in the target organ, leading to development of allergic rhinoconjunctivitis, eczema, asthma, or systemic anaphylaxis. T cells constitute a large population of cellular infiltrate in allergic inflammation, and a dysregulated immune response appears to be an important pathogenetic factor. Cardinal events during allergic inflammation can be classified as activation, organ-selective homing, survival and reactivation, and effector functions of immune system cells.<sup>4</sup> T cells are activated by aeroallergens, food antigens, autoantigens, and bacterial superantigens in allergic inflammation. They are under the influence of skin, lung, or nose-related chemokine networks, and

From the Swiss Institute of Allergy and Asthma Research.

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Reprint requests: Cezmi A. Akdis, MD, Swiss Institute of Allergy and Asthma Research, Obere Strasse 22, CH7270 Davos, Switzerland. E-mail: [akdisac@siaf.unizh.ch](mailto:akdisac@siaf.unizh.ch).

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*Abbreviations used*

- LPR: Late-phase reaction
- PIT: Peptide immunotherapy
- SIT: Specific immunotherapy
- SLIT: Sublingual immunotherapy
- TLR: Toll-like receptor
- Tr1: Type 1 T-regulatory
- Treg: T-regulatory

they show organ-selective homing. A prolonged survival of the inflammatory cells in the tissues and consequent reactivation is observed in the subepithelial tissues. Finally, T cells display effector functions, which result in the induction of hyper-IgE, eosinophil survival, and mucus hyperproduction; and interact with bronchial epithelial cells, smooth muscle cells, and keratinocytes, causing their activation and apoptosis.<sup>4,5</sup> Peripheral T-cell tolerance to allergens can overcome all of these pathological events in allergic inflammation because they all require T-cell activation.

## SEQUENTIAL EVENTS IN ALLERGEN-SPECIFIC IMMUNOTHERAPY AND THEIR UNDERLYING MECHANISMS

### Very early desensitization effect

The underlying immunological mechanisms of allergen-specific immunotherapy (SIT) are continuously being elucidated (Table I; Fig 1). Very early effects are related to mast cell and basophil desensitization. Intermediate effects are related to changes in allergen-specific T cells, and late effects are related to B cells and IgE as well as mast cells, basophils, and eosinophils. Although definite decrease in IgE antibody levels and IgE-mediated skin sensitivity normally requires years of SIT, most patients are protected against bee stings already at an early stage of venom-SIT. An important observation starting from the first injection is an early decrease in mast cell and basophil activity for degranulation and systemic anaphylaxis (Fig 1). Although it seems similar to rapid desensitization for hypersensitivity reactions to drugs, the mechanism of this desensitization effect is yet unknown. Acute oral desensitization to penicillin V in mice demonstrated that antigen-specific mast cell desensitization is one of the main underlying mechanisms for oral desensitization.<sup>6</sup> It has been shown that mediators of anaphylaxis (histamine and leukotrienes) are released during SIT and sting challenges without inducing a systemic anaphylactic response.<sup>7</sup> Their piecemeal release below the threshold of systemic anaphylaxis may decrease the granule content of mediators and also may affect the threshold of activation of mast cells and basophils, because decreased mediator release in these cells is a well demonstrated feature in *in vitro* analysis a short time after the start of allergen-SIT.<sup>7-9</sup> Although there are fluctuations and a risk for developing systemic anaphylaxis

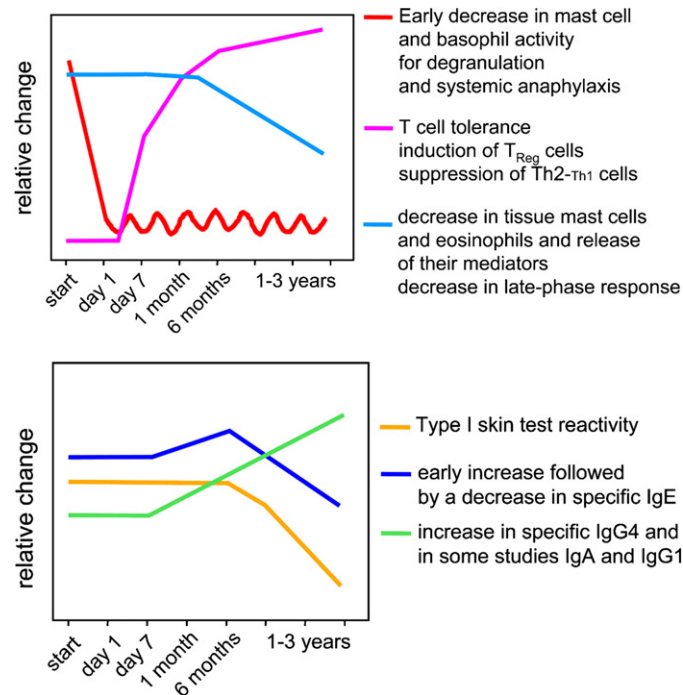
**TABLE I.** Effects of allergen-SIT on clinical and immunologic parameters

|                     |  |
|---------------------|--|
| Clinical parameters | Long-term cure<br>Decreased clinical symptoms and drug use<br>Decreased response to allergen challenge tests<br>Decreased size and cellular influx in skin LPR<br>Decreased skin type I hypersensitivity response<br>Improved quality of life  |
| Mast cells          | Very early desensitization effect<br>Reduction of tissue numbers<br>Decrease in mediator release<br>Decrease in proinflammatory cytokine production  |
| Basophils           | Very early desensitization effect<br>Decrease in mediator release<br>Decrease in proinflammatory cytokine production   |
| Eosinophils         | Reduction of tissue numbers<br>Decrease in mediator release  |
| T cells             | Decreased allergen-induced proliferation<br>Induction of Treg cells<br>Increased secretion of IL-10 and TGF- $\beta$<br>Suppression of T <sub>H</sub> 2 cells and cytokines<br>Decreased T-cell numbers in LPR   |
| B cells             | Early increase and late decrease in serum specific IgE<br>Increased serum specific IgG <sub>4</sub><br>Increased serum specific IgG <sub>1</sub> (relatively low compared with IgG <sub>4</sub> )<br>Increased serum specific IgA<br>Suppressed IgE-facilitated antigen presentation |
| Dendritic cells     | Different subsets can be targeted depending on the type of the adjuvant<br>Suppressed IgE-facilitated antigen presentation   |
| Monocytes           | Increased IL-10 production   |

during the course of allergen-SIT, the suppression of mast cells and basophils continues to be affected by changes in other immune parameters such as the generation of allergen-specific T-regulatory (Treg) cells and decreased specific IgE.

### Generation of Treg cells and peripheral T-cell tolerance

The induction of a tolerant state in peripheral T cells represents an essential step in allergen-SIT (Table I; Fig 1). Peripheral T-cell tolerance is characterized mainly by generation of allergen-specific Treg cells, suppressed proliferative and cytokine responses against the major allergen (Fig 2).<sup>10-12</sup> Subsets of Treg cells with distinct phenotypes and mechanisms of action include the naturally occurring thymic selected CD4<sup>+</sup>CD25<sup>+</sup> Treg cells and the inducible type 1 Treg (Tr1) cells.<sup>13</sup> In allergen-SIT, peripheral T-cell tolerance is initiated by autocrine action of IL-10 and TGF- $\beta$ , which is increasingly produced by the antigen-specific T cells.<sup>11,14,15</sup> The suppression by these cells could be partially blocked by the use of neutralizing antibodies against secreted or membrane-bound IL-10 and TGF- $\beta$ .<sup>12</sup> However, these cells do express CD4 and CD25, raising the question whether these are inducible Tr1 cells, which have upregulated CD25, or naturally occurring CD4<sup>+</sup>CD25<sup>+</sup> Treg cells that

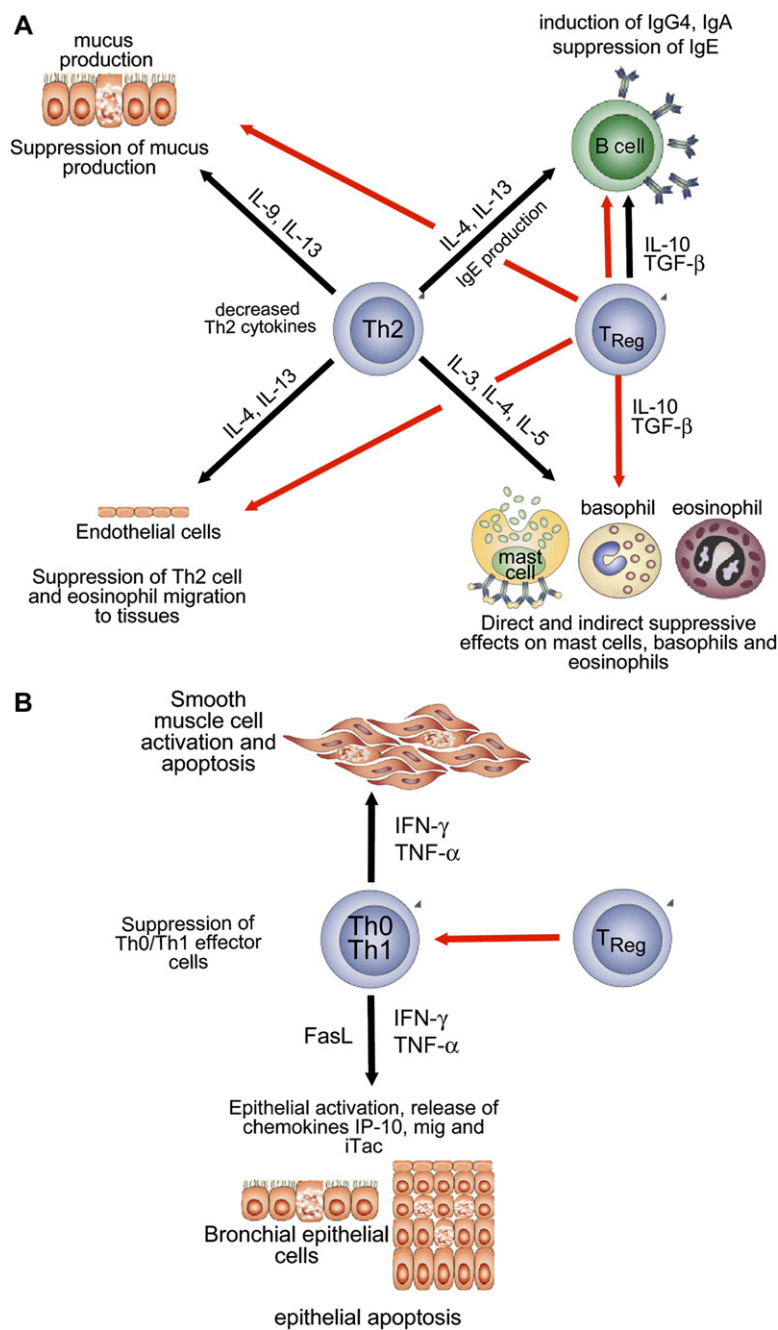


**FIG 1.** Immunologic changes during the course of allergen-SIT. Although there is significant variation between donors and protocols, starting from the first injection, an early decrease in mast cell and basophil activity and degranulation and decreased tendency for systemic anaphylaxis is observed. This is followed by generation of allergen-specific Treg cells and suppression of allergen-specific  $T_H1$  and  $T_H2$  cells. An early increase of specific IgE and late decrease is observed. This is in parallel to an increase of IgG<sub>4</sub> in particular and, in some studies, IgG<sub>1</sub> and IgA. A significant decrease in the allergen-specific IgE/IgG<sub>4</sub> ratio occurs after several months. A significant decrease in type I skin test reactivity is also observed relatively late in the course. Decrease in tissue mast cells and eosinophils and release of their mediators and decrease in LPR is observed after a few months.

produce suppressive cytokines.<sup>13</sup> In addition, it has been shown that  $CD4^+CD25^+$  Treg cells from atopic donors have a reduced capability to suppress the proliferation of  $CD4^+CD25^-$  T cells.<sup>16</sup> Therefore, it has been suggested that upregulation of  $CD4^+CD25^+$  Treg cells plays a role in allergen-SIT. TGF- $\beta$  plays a dual role in allergic disease: it suppresses allergen-specific T cells and plays a role in remodeling of the tissues. It remains to be elucidated in allergic inflammation whether the remodeling and the suppressive roles of TGF- $\beta$  show an imbalance that aggravates the disease instead of controlling the immune response.<sup>17</sup>

Studies on the mechanisms by which immune responses to nonpathogenic environmental antigens lead to either allergy or nonharmful immunity have demonstrated that Treg cells are dominant in healthy individuals.<sup>18,19</sup> If a detectable immune response is mounted, Tr1 cells specific for common environmental allergens consistently represent the dominant subset in healthy individuals. They use multiple suppressive mechanisms, IL-10 and TGF- $\beta$  as secreted cytokines, and cytotoxic T-lymphocyte antigen 4 and programmed death 1 as surface molecules. Healthy and allergic individuals exhibit all 3— $T_H1$ ,  $T_H2$ , and Tr1 allergen-specific subsets—in different proportions.<sup>18</sup> Accordingly, a change in the dominant subset and the balance between  $T_H2$  and Treg cells may

lead to either allergy development or recovery. Another study on healthy immune response to allergens demonstrated that  $CD4^+CD25^+$  Treg cells have been associated with the spontaneous remission of cow's milk allergy. Children who outgrew their allergy (tolerant children) had higher frequencies of circulating  $CD4^+CD25^+$  T cells and decreased *in vitro* proliferative responses to bovine  $\beta$ -lactoglobulin in PBMCs compared with children who maintained clinically active allergy.<sup>20</sup> Several studies have been reported in other diseases in the same line. The *in vitro* proliferative response to nickel of human  $CD4^+$  T cells from healthy, nonallergic individuals was strongly augmented when  $CD4^+CD25^+$  Treg cells were depleted.<sup>21</sup> Furthermore, a human *in vivo* study on immunotherapy of rheumatoid arthritis also showed a marked increase in the number of  $FoxP3^+CD4^+CD25^+$  Treg cells in peripheral blood.<sup>22</sup>  $CD25^+$  Treg cells are characterized by the expression of the transcriptional regulator *Foxp3* (*FOXP3* in human beings), which appears to be the master switch gene for Treg cell development and function. The spontaneous development of allergic airway inflammation, hyper-IgE, and eosinophilia in addition to various autoimmune diseases in *Foxp3* mutant mice provides compelling evidence for its importance in allergic inflammation.<sup>23</sup> Human beings with immune dysregulation polyendocrinopathy enteropathy X-linked syndrome are



**FIG 2. A**, Suppression of  $T_H2$  cell-mediated features of allergic inflammation by Treg cells. Treg cells use multiple suppressor factors to regulate undesired activity of effector  $T_H2$  cells. IL-10 and TGF- $\beta$  suppress IgE production and induce IgG<sub>4</sub> and IgA, respectively. Both cytokines directly suppress allergic inflammation induced by effector cells such as mast cells, basophils, and eosinophils. In addition,  $T_H2$  cells are suppressed by Treg cells and can therefore no longer provide cytokines such as IL-3, IL-4, IL-5, IL-9, and IL-13. These cytokines are required for the differentiation, survival, and activity of mast cells, basophils, eosinophils, and mucus-producing cells, as well as for the tissue homing of  $T_H2$  cells and eosinophils (red line, suppression; black line, stimulation). **B**, Suppression of  $T_H1$  cell-mediated features of allergic inflammation by Treg cells. The  $T_H1$  cytokine, IFN- $\gamma$ , in combination with TNF- $\alpha$  and/or FasL, induces apoptosis of smooth muscle cells, keratinocytes, and bronchial epithelial cells as essential tissue injury events in atopic dermatitis and asthma (red line, suppression; black line, stimulation). FasL, Fas-ligand; IP-10, IFN- $\gamma$ -inducible protein 10; mig, monokine induced by IFN- $\gamma$ ; iTac, IFN- $\gamma$ -inducible chemoattractant.

similarly affected and mostly develop hyper-IgE and eczema because of mutations in the *FOXP3* gene.<sup>23</sup> Supporting these findings, a dysregulation of disease-causing effector T cells is observed in atopic dermatitis lesions in association with an impaired CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T-cell infiltration in the dermis.<sup>24</sup>

The role of Treg cells is not limited to suppression of T<sub>H</sub>2 cells. Peripheral tolerance uses multiple mechanisms to suppress allergic inflammation. Apparently, Treg cells contribute to the control of allergen-specific immune responses in 5 major ways: suppression of antigen-presenting cells that support the generation of effector T<sub>H</sub>2 and T<sub>H</sub>1 cells; suppression of T<sub>H</sub>2 and T<sub>H</sub>1 cells; suppression of allergen-specific IgE and induction of IgG<sub>4</sub> and/or IgA; suppression of mast cells, basophils, and eosinophils; and interaction with resident tissue cells and remodeling.<sup>13</sup>

### Modulation of allergen-specific IgE and IgG subtype responses during allergen-SIT

Specific IgE in serum and on the surface of mast cells and basophils bound to FcεRI in patients with allergy is a hallmark of atopic disease. Although peripheral T-cell tolerance is rapidly induced during SIT, there is no evidence for B-cell tolerance in the early course.<sup>10</sup> Natural exposure to a relevant allergen is often associated with an increase in the IgE synthesis. Similarly, SIT frequently induces a transient increase in serum specific IgE, followed by a gradual decrease over a period of months or years of treatment (Fig 1).<sup>25,26</sup> In pollen-sensitive patients, desensitization prevents elevation of the serum specific IgE during the pollen season.<sup>27</sup> However, the changes in IgE levels cannot explain the diminished responsiveness to specific allergen as a result of SIT, because the decrease in serum IgE is relatively late and does not correlate with clinical improvement after SIT.

Antibody responses induced during allergen-SIT are functionally heterogeneous, which may account for the conflicting data in relation to the protective effects of IgG.<sup>12,25,26</sup> Subclasses of IgG antibodies, especially IgG<sub>4</sub>, are thought to capture the allergen before reaching the effector cell-bound IgE, and thus to prevent the activation of mast cells and basophils. However, the relationship between the efficacy of SIT and the induction of allergen-specific IgG subgroups remains a controversial issue, with serum concentrations of allergen-specific IgG correlating with clinical improvement in some studies but not in others.<sup>28,29</sup> Allergen-specific IgG may be directed against the same epitopes as IgE, resulting in direct competition for allergen binding and a blocking effect. By contrast, induction of IgG specific for other epitopes may result in a failure of the IgG response to compete with IgE, even when IgG is present in molar excess. The concept of blocking antibodies has recently been reevaluated. Analysis of the IgG subtypes induced by SIT has shown specific increases in IgG<sub>1</sub> and particularly IgG<sub>4</sub>, with levels increasing 10-fold to 100-fold.<sup>30,31</sup> There is accumulating evidence that SIT also influences the blocking activity on IgE-mediated responses by IgG<sub>4</sub>, and cellular assays are commonly used to investigate these changes.

Recently, a novel assay that detects allergen-IgE binding by using flow cytometry has been used to detect functional SIT-induced changes in IgG antibody activity. Results suggest that successful SIT is associated with an increase in IgG blocking activity that is not solely dependent on the quantity of IgG antibodies.<sup>32,33</sup> It seems to be relevant to measure the blocking activity of allergen-specific IgG or IgG subsets, particularly IgG<sub>4</sub> and also IgG<sub>1</sub>, instead of the crude levels in sera. In this context, the role of anti-IgE treatment in the induction phase of allergen-SIT on safety and efficacy has been questioned. Anti-IgE mAb pretreatment enhances the safety of SIT for allergic rhinitis and may be an effective strategy to permit more rapid and higher doses of allergen immunotherapy.<sup>34</sup> Its function on long-term efficacy is still under investigation.

The noninflammatory role for IgG<sub>4</sub> may arise because the IgG<sub>4</sub> hinge region has unique structural features that result in a lower affinity for certain Fcγ receptors and the ability to separate and repair, leading to bispecific antibodies that are functionally monomeric.<sup>35</sup> Furthermore, IgG<sub>4</sub> does not fix complement and is capable of inhibiting immune-complex formation by other isotypes, giving this isotype anti-inflammatory characteristics. By using well defined recombinant allergen mixtures, all treated subjects developed very strong allergen-specific IgG<sub>4</sub> and also increased IgG<sub>1</sub> antibody responses. Some patients who were not initially sensitized to Phl p 5 developed strong specific IgG<sub>4</sub>, but not IgE antibody responses to that allergen.<sup>30</sup> This demonstrates that extract-based antibody measurements may provide incorrect information, and studies on mechanisms of allergen-SIT should be performed with single allergens. Nevertheless, IgG<sub>4</sub> antibodies can be viewed as a marker of introduced allergen dose, and they have the ability to modulate the immune response to allergen and thus the potential to influence the clinical response to allergen. In addition, the affinity of newly produced IgG<sub>4</sub> and decreasing IgE to allergens has not been intensely studied and may have a very decisive role. Affinity maturation of specific antibodies in allergen immunotherapy and preseasonal versus postseasonal changes in their affinity remain to be elucidated.<sup>36</sup>

IL-10 that is induced and increasingly secreted by SIT appears to counterregulate antigen-specific IgE and IgG<sub>4</sub> antibody synthesis.<sup>11</sup> IL-10 is a potent suppressor of both total and allergen-specific IgE, and it simultaneously increases IgG<sub>4</sub> production. Thus, IL-10 not only generates tolerance in T cells but also regulates specific isotype formation and skews the specific response from an IgE-dominated to an IgG<sub>4</sub>-dominated phenotype (Fig 2). The healthy immune response to Der p 1 demonstrated increased specific IgA and IgG<sub>4</sub>, small amounts of IgG<sub>1</sub>, and almost undetectable IgE antibodies in serum.<sup>12</sup> House dust mite-SIT did not significantly change specific IgE levels after 70 days of treatment; however, a significant increase in specific IgA, IgG<sub>1</sub>, and IgG<sub>4</sub> was observed.<sup>12</sup> The increase of specific IgA and IgG<sub>4</sub> in serum coincides with increased TGF-β and IL-10, respectively. This may account for the role of IgA and TGF-β as well as IgG<sub>4</sub> and IL-10 in peripheral mucosal immune responses to allergens in

healthy individuals.<sup>11,37</sup> Most probably the decrease in IgE/IgG<sub>4</sub> ratio during allergen-SIT is a feature of skew from allergen-specific T<sub>H</sub>2 to Treg cell predominance. However, although Treg cell generation happens within days, a significant decrease in IgE/IgG<sub>4</sub> ratio occurs after years. The reason for the long period between the change in T-cell subsets but not IgE/IgG<sub>4</sub> levels is not easily explainable by the half-life of antibodies. In this context, the role of bone marrow-residing IgE-producing plasma cells with very long life spans remains to be investigated.<sup>38</sup>

### Suppression of effector cells and inflammatory responses during allergen-SIT

Peripheral T-cell tolerance to allergens, which is characterized by functional inactivation of the cell to antigen encounter, can overcome both acute and chronic events in allergic reactions (Fig 2). Allergen-SIT efficiently modulates the thresholds for mast cell and basophil activation and decreases IgE-mediated histamine release.<sup>39</sup> In addition, IL-10 was shown to reduce proinflammatory cytokine release from mast cells.<sup>40</sup> Furthermore, IL-10 downregulates eosinophil function and activity and suppresses IL-5 production by human resting T<sub>H</sub>0 and T<sub>H</sub>2 cells.<sup>41</sup> Moreover, although demonstrated in a model of myocarditis, IL-10 gene transfer significantly reduces mast cell density, local histamine concentration, and mast cell growth, and prevents mast cell degranulation.<sup>42</sup>

Long-term SIT is associated with reduction of not only the immediate response to allergen provocation but also the late-phase reaction (LPR) in the nasal and bronchial mucosa or in the skin. The mechanism of LPR is different from the mast cell-mediated immediate reaction and involves the recruitment, activation, and persistence of eosinophils and activated T cells at the sites of allergen exposure.<sup>3</sup> Successful SIT results not only in the increase of allergen concentration necessary to induce immediate or LPR in the target tissue but also in the decreased responses to nonspecific stimulation. Bronchial, nasal, and conjunctival hyperreactivity to nonspecific stimuli, which seems to reflect underlying mucosal inflammation, decreases after SIT and correlates with clinical improvement.<sup>43</sup> During birch pollen SIT, reduced plasma levels of eosinophil cationic protein, a marker of eosinophil activation, as well as chemotactic factors for eosinophils and neutrophils correlated with decreased bronchial hyperreactivity and clinical improvement.<sup>44</sup> Inhibition by SIT of the seasonal increase in eosinophil priming has also been demonstrated.<sup>45</sup> In biopsies taken during grass pollen SIT, decreased eosinophil and mast cell infiltration in nasal and bronchial mucosa after SIT correlated with the anti-inflammatory effect.<sup>46</sup>

### MECHANISMS OF SUBLINGUAL IMMUNOTHERAPY

Because of potentially severe, albeit infrequent, side effects associated with injection SIT, mucosal routes of administration are being investigated to conduct allergenic

desensitization.<sup>47</sup> Although its safety and efficacy have now been largely documented, much remains to be investigated on the immunologic mechanisms underlying sublingual immunotherapy (SLIT). A meta-analysis of the double-blind, placebo-controlled trials performed in the past decade has shown that SLIT is clinically efficacious although, at present, the treatment benefit is approximately half that achieved with subcutaneous SIT.<sup>48</sup> The immunologic mechanisms of sublingual SIT seem to be similar to subcutaneous SIT, although the magnitude of the change in most parameters is modest or no change has been observed. It seems likely that the contact of the allergen with the oral mucosa is critical for the success of SLIT.<sup>49</sup> It is postulated that most likely oral Langerhans cells are critically involved in this process.<sup>50</sup> During SLIT, the allergen is captured within the oral mucosa by oral Langerhans cells, and subsequently these cells mature and migrate to proximal draining lymph nodes. Those local lymph nodes may favor the production of blocking IgG antibodies and the induction of T lymphocytes with suppressive function.<sup>51,52</sup> Most studies using SLIT have reported increased levels of serum IgG<sub>4</sub> with a relatively modest increase compared with injection immunotherapy.<sup>53</sup> There is no consistency in T-cell and IgE and effector cell responses, and a significant number of studies has failed to detect systemic immunologic changes.<sup>48,53</sup> This may be related to the different doses of allergen administered in different studies, or to the development of more localized immunologic changes. In a recent study, PBMCs were stimulated with pollen allergen extract after 1 and 2 years of SLIT. The expression of IL-10 mRNA was increased in both high and low doses and showed a positive correlation with TGF- $\beta$  expression. IL-5 was suppressed with a high dose, which negatively correlated with TGF- $\beta$ .<sup>54</sup> In a recently reported birch pollen extract SLIT, patients showed improved nasal provocation scores to birch pollen; however, apple-induced oral allergy syndrome caused by cross-reactivity was not significantly reduced. Bet v 1-specific T-cell tolerance to all epitopes and those cross-reactive with Mal d 1 from apple were shown. However, neither Mal d 1-specific IgE and IgG<sub>4</sub> levels nor Mal d 1-induced T-cell proliferation changed significantly, probably because of noncross-reactive epitopes.<sup>55</sup> These results may explain why pollen-associated food allergy is frequently not ameliorated by pollen immunotherapy. Although SLIT is increasingly being used, several points need further investigation, such as its efficacy in asthma, its mechanisms of action, the optimal dose and time to be administered, its combination with injection immunotherapy, age of onset for its safe use in young children, and its preventive role in the development of allergy.

### UNDERLYING MECHANISMS OF NOVEL AND EMERGING SIT VACCINES

Intensive studies are being performed to improve efficacy and safety of allergen-SIT (Table II). A basic requirement for an allergen vaccine in achieving successful SIT

**TABLE II.** Novel approaches for future of allergen-SIT

| Type of the vaccine/approach                                 | Description and mechanism   |
|--|---|
| Fusion of major allergens <sup>59</sup>                      | Several major allergens (Api m 1, Api m 2) are fused and expressed as a single recombinant protein. IgE binding is attenuated. T-cell reactivity is preserved. Preventive effect on generation of IgE is demonstrated in mice.          |
| Chimeric allergens <sup>60</sup>                             | Fragments of major allergens (Api m 1, Api m 2, Api m 3) are fused and expressed as a single protein. IgE binding is attenuated. T-cell reactivity is preserved. Preventive effect on generation of IgE is demonstrated in mice.        |
| Fragments <sup>61</sup>                                      | Major allergen (Bet v 1) is divided into non-IgE binding fragments. IgE binding is attenuated. T-cell reactivity is preserved. A multicenter clinical trial has been reported.  |
| Peptides of major allergens <sup>58</sup>                    | Non-IgE binding T-cell epitope peptides (Fel d 1, Api m 1) have been used in cat and bee venom allergy.   |
| Polymers of major allergens <sup>61</sup>                    | Major allergen (Bet v 1) is trimerized. Mast cell, basophil degranulation is attenuated. T-cell reactivity is preserved. A multi-center clinical trial has been reported.   |
| Unrefolded native allergens <sup>57</sup>                    | Major recombinant allergens (Api m 1, Bet v 1) are not refolded to the native conformation. This decreases or abolishes IgE binding but preserves T-cell reactivity.  |
| Mixture of several major recombinant allergens <sup>30</sup> | Clinical efficacy of a mixture of 5 recombinant grass pollen allergens (Phl p 1, Phl p 2, Phl p 5a, Phl p 5b, Phl p 6) in reducing symptoms and need for symptomatic medication in patients with grass pollen allergy was demonstrated. |
| GpG oligonucleotide-conjugated allergens <sup>64</sup>       | Major allergen (Amb a 1) is bound to a TLR9-triggering CpG oligonucleotide. In this way, allergen and innate immune response stimulating agent are expressed in 1 molecule.   |
| Allergens coupled to viruslike particles <sup>65</sup>       | Der p 1 peptides coupled to highly repetitive virus capsid-like recombinant particles induced high specific IgG titers.   |
| Combination of conventional SIT with anti-IgE <sup>34</sup>  | Anti-IgE mAb pretreatment enhances the safety of SIT for allergic rhinitis. Its role on long-term efficacy is still under investigation.  |
| Intralymphatic vaccination <sup>62</sup>                     | Allergen-SIT vaccines administered directly into a lymph node are currently being investigated. The aim is to deliver high amounts of allergens into secondary lymphatic organs.  |

without risk of anaphylaxis is to express T-cell epitopes, which induce T-cell tolerance and lack antibody-binding sites that mediate IgE cross-linking.<sup>56</sup> Conformation dependence of B-cell epitopes and linearity of T-cell epitopes may induce a different regulation of allergen-specific T-cell cytokine toward a nonallergic phenotype. Native allergens use IgE-facilitated antigen presentation by dendritic cells and B cells, which activates T cells to produce T<sub>H</sub>2-type cytokines and B cells to produce further IgE in a secondary response. In contrast, B-cell epitope deleted allergens, which do not bind IgE, do not initiate effector cell degranulation. They use phagocytotic or pinocytic antigen uptake mechanisms in dendritic cells, macrophages, and B cells.<sup>57</sup> T cells may be subsequently induced to generate a balanced T<sub>H</sub>0/T<sub>H</sub>1-type cytokine pattern in lower quantities as well as T-cell tolerance, which involves Treg cells. Accordingly, targeting T cells and bypassing IgE by modified allergens will enable the administration of higher doses of allergens, which is required to induce T-cell tolerance without the risk of anaphylaxis.<sup>57</sup>

In line with this concept, immunotherapy using peptides (PIT) is an attractive approach for safe SIT. To date, clinical trials of PIT have been performed in 2 allergies.<sup>58</sup> Induction of T-cell tolerance and increased IL-10

production have been demonstrated both in cat Fel d 1 and bee venom Api m 1 PITs.<sup>58</sup> A potential barrier to PIT of allergy is the apparent complexity of the allergen-specific T-cell response in terms of epitope use and dominant epitopes in human beings and stability of peptides. To overcome these problems, genetically engineered recombinant hybrid molecules that span the whole T-cell repertoire but do not bind IgE have been developed. In cysteine-containing proteins, folding is complicated by the formation of intramolecular and intermolecular disulphide bond formation, whereas any formed disulphide bond can fix the conformation and limits the freedom of further folding processes. With an increasing number of cysteines, the probability of a correct or natively like folding rapidly decreases because of the increasing probability of incorrect disulfide-bound formation. A fusion protein consisting of the 2 major allergens of bee venom, Api m 1 and Api m 2, has been generated to investigate this concept. Destroyed conformational B-cell epitopes but intact T-cell epitopes of the 2 allergens characterize this protein. By providing decreased allergenicity with preserved T-cell tolerance-inducing capacity, the Api m [1/2] fusion protein represents a novel vaccine prototype for allergen-SIT.<sup>59</sup>

Another interesting approach was to cut the major allergens to fragments and fuse them in a different order without missing any T-cell epitopes in 1 reassembled mosaic allergen.<sup>60</sup> In this study, 2 fragments of Api m 1, 3 fragments of Api m 2, and Api m 3 are reassembled in a different order with overlapping residues, so no T-cell epitopes are missed. Single injection of both vaccines, which only target T cells, demonstrated a preventive effect on IgE generation in mice. The advantage of these 2 approaches is that only 1 molecule has to be produced and purified instead of several recombinant allergens. T-cell epitopes are preserved, and B-cell epitopes can be deleted or preserved depending on the type of the fusion molecule. Another interesting approach was to use fragments and a trimer of major birch pollen allergen Bet v 1 to treat birch pollen allergy. A double-blind, placebo-controlled study has been completed in 3 centers, which demonstrated an increase in IgG<sub>1</sub>, IgG<sub>2</sub>, IgG<sub>4</sub>, and IgA and suppression of seasonal increases of IgE.<sup>61</sup> In a different approach, the effectiveness of a mixture of 5 recombinant grass pollen allergens in reducing symptoms and a need for symptomatic medication in patients with grass pollen allergy were demonstrated. All treated subjects developed strong allergen-specific IgG<sub>1</sub> and IgG<sub>4</sub> antibody responses.<sup>30</sup> Allergen-SIT vaccines are generally administered subcutaneously, intradermally, or sublingually, from where they must reach secondary lymphatic organs to induce an immune response. In a mouse study, an MHC class I-binding peptide from lymphocytic choriomeningitis virus enhanced immunogenicity by as much as 10<sup>6</sup> times compared with subcutaneous and intradermal vaccination. Intralymphatic administration induced CD8 T-cell responses with strong cytotoxic activity and IFN- $\gamma$  production that conferred long-term protection against viral infections and tumors.<sup>62</sup> The efficacy of allergen-SIT vaccines administered directly into inguinal lymph nodes of human beings is currently being investigated.

Allergen-SIT uses aluminium hydroxide as an adjuvant. These preparations have generally proven to be efficacious and have a good safety profile, but might be improved in efficacy. A new class of adjuvants—so-called *immune response modifiers*—act on antigen-presenting cells through the Toll-like receptors (TLRs), which recognize pathogen-associated molecular patterns on microorganisms. Depending on the type of TLR, different types of antigen-presenting cells can be targeted. TLR-triggering compounds that can control the overexpression of T<sub>H</sub>2 cytokines or skew the T<sub>H</sub>1:T<sub>H</sub>2 balance toward a T<sub>H</sub>1 profile have been effective in murine models of allergy.<sup>56</sup> Oligodeoxynucleotides containing immunostimulatory CpG motifs that trigger TLR9, linked to the allergen in ragweed allergy in human beings, have been used. Amb a 1-immunostimulatory DNA sequence conjugate SIT led to a prolonged shift from T<sub>H</sub>2 immunity toward T<sub>H</sub>1 immunity and appeared to be safe.<sup>63</sup> In a recent study, the same Amb a 1 CpG conjugate was shown to be effective for the treatment of allergic rhinitis in 2 consecutive seasons. Although an early increase in Amb a 1-specific IgE was observed during the injection phase, a

seasonal increase in Amb a 1-specific IgE did not occur, and a reduction in the number of IL-4-positive basophils was reported.<sup>64</sup> In another study, vaccination with a peptide antigen covalently coupled to highly repetitive virus-like particles induced high IgG antibody titers in human beings, suggesting the possibility of using allergens coupled to viruslike particles in allergen-SIT.<sup>65</sup> As a different immunologic approach, the fusion of allergens with human Fc $\gamma$  has been reported to inhibit allergen-induced basophil and mast cell degranulation by cross-linking Fc $\gamma$  and Fc $\epsilon$ RI receptors.<sup>66</sup>

## CONCLUSION

There is increasing evidence to support IL-10 and/or TGF- $\beta$  secreting Treg cells and immunosuppressive cytokines as key players in mediating successful allergen-SIT and a healthy immune response to allergens. In addition to allergy, these mechanisms may have implications in autoimmunity, graft-versus-host disease, tumor cell growth, parasite survival, chronic infections, and the development of AIDS. In sensitized individuals, peripheral T-cell tolerance represents the key mechanism in healthy immune responses to allergens. Peripheral tolerance to allergens induced by allergen-SIT involves control of the allergen-specific immune response in multiple phases including specific T-cell suppression, generation of noninflammatory antibody isotypes such as IgG<sub>4</sub> and IgA, suppression of IgE and type 1 hypersensitivity responses, and suppression of LPRs and mast cells, eosinophils, and basophils. Taking the recent advances in knowledge of peripheral tolerance mechanisms into account, developments of safer approaches and more efficient methods of allergen-SIT await.

New approaches to allergen-SIT, such as the use of recombinant proteins, peptides, fragments, and hybrid allergens, are promising, but are only in an early stage of human clinical trials. There are several essential requirements underlying novel strategies for the development of safe and more efficient allergen-SIT vaccines: (1) the vaccine should be constituted of perfectly well standardized native proteins or recombinant allergens; (2) the vaccine should induce tolerance in allergen-specific effector T cells, suppress IgE production, and promote IgG<sub>4</sub> or IgA isotype blocking antibody production; (3) the vaccine should not induce severe side effects and should be well tolerated; (4) the vaccine should be easily administered; (5) the treatment should achieve clinical success and long-term protection in a short time with few doses; and (6) early biological/immunologic markers to assess clinical success, before the onset or early in the course of the treatment, should be identified.

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