Mechanisms of immunotherapy to aeroallergens
M. H. Shamji and S. R. Durham

Allergy and Clinical Immunology Section, Medical Research Council and Asthma UK Centre for Allergic Mechanisms of Asthma, Faculty of Medicine, National Heart and Lung Institute, Imperial College London, London, UK

Correspondence:
Dr Mohamed Shamji, Allergy and Clinical Immunology Section, Medical Research Council and Asthma UK Centre for Allergic Mechanisms of Asthma, National Heart and Lung Institute, Faculty of Medicine, Imperial College London, Room 365, Third Floor, Sir Alexander Fleming Building, South Kensington Campus, London SW7 2AZ, UK.
E-mail: m.shamji99@imperial.ac.uk

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Summary
Allergen immunotherapy is allergen-specific, allergen dose- and time-dependent and is associated with long-term clinical and immunological tolerance that persists for years after discontinuation. Successful immunotherapy is accompanied by the suppression of numbers of T-helper 2 (Th2) effector cells, eosinophils, basophils, c-kit+ mast cells and neutrophils infiltration in target organs, induction of IL-10 and/or TGF-β+ Treg cells and increases in ‘protective’ non-inflammatory blocking antibodies, particularly IgG4 and IgA2 subclasses with inhibitory activity. These events are accompanied by a reduction and/or a redirection of underlying antigen-specific Th2-type T cell-driven hypersensitivity to the allergen(s) used for therapy. This suppression occurs within weeks or months as a consequence of the appearance of a population of regulatory T cells that exert their effects by mechanisms involving cell–cell contact, but also by the release of cytokines such as IL-10 (increases IgG4) and TGF-β (increases specific IgA). The more delayed-in-time appearance of antigen-specific T-helper 1 responses and alternative mechanisms such as Th2 cell anergy and/or apoptosis may also be involved. The mechanisms of sublingual immunotherapy are similar to those following a subcutaneous administration of allergen, whereas it is likely that additional events following antigen presentation in the sublingual mucosa and regional lymph nodes are involved. These insights have resulted in novel approaches and portend future biomarkers that may be surrogate or predictive of the clinical response to treatment.

Introduction
Conventional allergen-specific immunotherapy (SIT) involves the repeated administration of sensitizing allergen subcutaneously. Incremental allergen doses are given for a period of 8–16 weeks, followed by monthly maintenance injections for a period of 3–5 years [1]. Recently, sublingual allergen-specific immunotherapy (SLIT) has emerged as an effective and safer alternative route of immunotherapy for IgE-mediated hypersensitivity against aeroallergens. To date, SIT is the only immunomodulatory intervention that induces allergen-specific immunological tolerance [1, 2]. Injection allergen–SIT for seasonal pollinosis is associated with the amelioration of seasonal symptoms, bronchial hyperresponsiveness (BHR) and a reduced requirement for rescue medication [3–5]. Clinical responsiveness to SIT has been shown to exceed the duration of SIT treatment by several years, a clear advantage over the use of anti-IgE or anti-allergic drugs [6, 7]. SIT reduces the onset of new sensitizations in children [8] and there is preliminary evidence that SIT may prevent the progression of allergic rhinitis to physician-diagnosed asthma [9, 10]. In this article, we present a review of the immunological mechanisms of subcutaneous (SCIT) and SLIT. Furthermore, the mechanisms of long-term clinical benefits and potential biomarkers of clinical efficacy are evaluated.

Effects of injection immunotherapy on early and late allergic responses
The suppression of the late-phase allergic responses in the skin [11], nose [12] and the lung [13] has been reported following allergen–SIT. Several studies have reported inhibition of the early and the late response following an intradermal allergen challenge [6, 12, 14–16]. Reduced numbers of T lymphocytes and suppression of eosinophil, basophil and neutrophil infiltration in skin biopsies have been reported following allergen–SIT [14, 15]. Furthermore, suppression of the influx of effector cells in the skin
after allergen challenge may explain the observed decrease in the size of the late cutaneous response. Despite a significant reduction of mast cell numbers in the skin, the early allergic response was only partially suppressed following SIT [6]. The time-course of the early and late allergic inflammatory response in the skin has been studied in detail following timothy grass pollen SIT [17]. Interestingly, suppression of the late allergic response to an intradermal allergen challenge was significant within 2 weeks and evident even at low allergen doses. In contrast, a modest but significant reduction in the early allergic response occurred later and only after achieving maintenance doses following 8–12 weeks of up-dosing therapy. Similarly, allergen-induced early and late nasal responses are suppressed following immunotherapy for grass pollen [18] and cat allergens [19], whereas late asthmatic responses are inhibited following mite [13] and birch pollen SIT [20].

Cellular responses following allergen-specific immunotherapy

A schema for the underlying mechanisms of allergen-SIT is presented in Fig. 1. SCIT is associated with changes in cellular and humoral responses with a significant reduction of tissue infiltrating inflammatory effector cells. The induction of long-term tolerance after immunotherapy is associated with a shift in the ratio of T-helper 2 (Th2) and T-helper 1 (Th1) cytokines. However, not all studies have confirmed these findings. Although increases in Th1 cytokines in peripheral blood cells have been reported following allergen-SIT [21, 22], other studies failed to report any changes in Th1 cytokine production by these cells [23, 24]. However, the change in the ratio of Th1 and Th2 cytokines has been observed in local target organs [23, 25] and may control allergen–induced allergic responses by inhibiting the pro-inflammatory effects of dendritic cells (DCs) [26, 27].

The influence of allergen-SIT on peripheral DCs has been demonstrated recently [27]. Allergen-SIT increased DC TLR9-mediated innate immune function, which was previously impaired at baseline in allergic subjects. In this study, a robust innate immune response from isolated pDCs was re-established among house dust mite (HDM)-allergic subjects undergoing allergen-SIT, resulting in a three- to fivefold increase in IFN-γ production in response to CpG stimulation [27]. Furthermore, the proportions of peripheral blood pDCs, but not mDCs, are significantly reduced after the initiation of Hymenoptera venom-SIT. This was associated with changes in the expression of function-associated surface molecules

Fig. 1. Immunological mechanisms of immunotherapy to aeroallergens. Low-dose and repeated allergen exposure at mucosal surfaces in atopic individuals drives type I IgE-mediated allergic responses. High-allergen dose through a subcutaneous or a sublingual route results in the shift of T cell polarization from a Th helper 2 (Th2) to a Th helper 1 (Th1) response. This is accompanied by an increase in the ratio of Th1 cytokines (IFN-γ, IL-12) to Th2 cytokines (IL-4, IL-5 and IL-13). The induction of T regulatory cells (inducible Treg cells (iTreg) and natural Treg cells (nTreg)) and cytokines such as IL-10 and TGF-β following immunotherapy play an important role in suppressing Th1 and Th2 responses and contributes towards the induction of allergen-specific IgA1, IgA2 and in particular IgG4 antibodies with inhibitory activity. IgG4 antibodies are able to suppress FceRI- and CD23-mediated IgE-facilitated allergen presentation and basophil histamine release.
such as CD32, CD40 and TLR2 on DCs during allergen-SIT. Altogether, numeric and phenotypic changes of blood DCs may contribute to a suppression of allergic inflammatory response during SIT [28].

Peripheral T cell tolerance following SIT is characterized by the induction of allergen-specific regulatory T cells (Treg cell; IL-10-producing T cells and CD4+ CD25+ T cells). The regulatory function of IL-10/TGF-β and CD4+CD25+ T cells has been reported to overlap. Induction of cytokines such as IL-10 and TGF-β by CD4+CD25+ following SIT has been reported in several studies [21, 24, 29, 30]. These cells have been shown to suppress antigen-driven proliferative T cell responses and Th2 cytokine release in an IL-10-dependent manner [21]. Several novel biomarkers that include increased expression of the intracellular transcription factor FoxP3 together with increased cell surface expression of the receptor CD25 and reduced CD127 have been shown to characterize regulatory T cells. Phenotypic regulatory T cells (CD4+CD25+CD127lo) remained unchanged following a comprehensive, longitudinal study of birch pollen SIT [31]. Furthermore, the proportion of CD25+ and CD25hi populations within peripheral CD4 T cells did not change in patients treated with birch pollen-SIT compared with untreated birch-allergic controls [32]. In contrast, increased functional Treg cell activity has been identified following an ex vivo allergen stimulation of T cell populations in peripheral blood. Thus, elevated levels of IL-10 production in peripheral blood mononuclear cell (PBMC) cultures have been demonstrated in patients treated with SIT compared with placebo-treated controls [17]. The time course of increases in IL-10 occurred early, within 2 weeks, and paralleled the suppression of the associated, T cell-driven, cutaneous late response. Furthermore, increases in the frequency of antigen-specific IL-10+ cells have been demonstrated by the EliSpot assay [31]. In the same study, enriched peripheral IL-10+CD4+ T cells using IL-10 secretion assay suppressed proliferative responses in an IL-10-dependent manner.

Moreover, TGF-β has been shown to have a potent regulatory property. It is proven essential for the maintenance of immunological self-tolerance in the CD4 T cell compartment. TGF-β inhibits the differentiation of Th1 and Th2 cells by inhibiting the expression of transcription factors T-BET and GATA-3 [33, 34]. The lack of Th1 and Th2 polarization during T cell differentiation results in the generation of Treg cells [35]. TGF-β induces the expression of FOXP3, which promotes the induction of T cells with a regulatory phenotype [36]. It also induces CTLA-4 expression in Treg cells [37, 38]. TGF-β modulates IgE, FcεRI expression on Langerhans cells and is a class switch factor for non-inflammatory IgA antibodies [39]. These findings suggest its role in inducing peripheral tolerance.

Furthermore, recent studies have demonstrated elevated numbers of IL-10+ and TGF-β+ T cells and FoxP3+CD4+ and FoxP3+CD25+ phenotypic regulatory T cells within the nasal mucosa [40, 41]. These local increases in phenotypic regulatory T cells within the target organ paralleled clinical improvement and reduced inflammatory allergic responses (a reduction in mast cells [39], basophils [42] and eosinophils [43] in the nasal mucosa).

More recently, HDM-SIT has been associated with the induction of CD8+CD25+Foxp3+ Treg cells [44]. PBMCs isolated from healthy control subjects and from HDM-sensitive asthmatic patients before treatment, 6 and 12 months showed a significant increase in CD8+Foxp3+ Treg cells expressing intracellular IL-10 and granzyme B. Co-stimulation of PBMCs with Pam3CSK4 (a synthetic TLR2 ligand) and *Dermatophagoides pteronyssinus* II expanded the CD8+CD25+Foxp3+ Treg population and inhibited *D. pteronyssinus* 2-induced IL-4 production [44].

**Immunoglobulin responses following allergen-specific immunotherapy**

In atopic individuals, exposure to relevant allergen results in an increased concentrations in allergen-specific IgE in the serum and in the local target organs. van Ree and colleagues demonstrated a transient increase in rye grass pollen (Lol p 1, 2, 3 and 5)-specific IgE in patients during the season, followed by a gradual decrease over time [45]. SIT in patients with hayfever is associated with transient early increases in allergen-specific IgE, followed by blunting of seasonal increases in IgE [1, 46, 47]. In a proportion of individuals who received birch pollen-SIT, low concentrations of IgE antibodies to previously unrecognized proteins were identified [48]. The clinical relevance of these new sensitizations is doubtful as they paralleled clinical improvement with no apparent adverse events.

Quantitative measurements of allergen-specific IgG subclasses in SIT-treated patients have revealed increases in allergen-specific IgG1 and IgG4 antibody concentrations in the serum and in the local target organs [21, 49, 50]. Changes in IgG2 and IgG3 levels were not significant [51]. Despite these increases in the levels of allergen-specific antibodies, many studies have failed to show a correlation between allergen-specific IgG1 and IgG4 antibodies and clinical efficacy.

The possible functional relevance of these allergen-specific inhibitory IgG1 and IgG4 antibodies has been illustrated in several studies. Bet v1-specific IgG1 and IgG4 antibodies from SIT-treated patients inhibited basophil histamine release in an antigen-specific fashion [52–54]. These monoclonal Bet v1-specific IgG1 and IgG4 could compete with Bet v1-specific IgE and prevent its interaction with Bet v1 allergen. In a murine model of allergy, the inhibitory activities of IgG were shown to be mediated via the FcγRIIB receptor. Incubation of IgE, allergen and IgG immune complexes with mouse mast cells resulted in the juxtaposition or the co-aggregation of
the FcγRIIB and FcεRI resulting in the suppression of mast cell degranulation [55]. Additionally, inhibition of basophil histamine release was demonstrated using the recombinant chimeric Fcγ–Fcε construct that potentiates FcγRIIB and FcεRI co-aggregation on human basophils in vitro [56]. This inhibitory effect was dependent on immunoreceptor tyrosine-based inhibitory motif phosphorylation, resulting in the activation of intracellular phosphatases and counterbalance of the influence of immunoreceptor-based activation motifs present within the intracellular tail of the FcεRIγ expressed on human basophils [57]. In contrast, in a human model [58], blockade of downstream signalling of FcγRIIB with a monoclonal antibody directed against CD32 did not block IgG-mediated inhibitory activity following birch pollen-SIT, which implies direct competition with IgE for an allergen rather than a mechanism involving downstream inhibition of the IgE receptor signalling pathway as observed in the murine model.

van Neerven and colleagues demonstrated that the serum obtained from subjects following birch pollen-SIT was able to inhibit IgE-facilitated allergen presentation by B cells to an allergen-specific T cell clone, resulting in decreased T cell proliferation and reduced cytokine production by birch pollen-specific T cells [59]. Wachholz and colleagues showed that the serum obtained from subjects who participated in a randomized double-blind placebo-controlled trial of grass pollen-SIT could inhibit IgE-facilitated allergen presentation to a grass-specific T cell clone [60]. Moreover, using a simplified assay where allergen-IgE complexes bound to FcεRI on the surface of B cells were detected by flow cytometry (IgE-FAB), it was shown that the vigour of proliferative responses by T cell clones reflects the binding of these complexes [60]. This was confirmed in patients following allergen-SIT for birch allergy [61]. Nouri-Aria and colleagues further confirmed increases in allergen-specific IgG4 antibodies in patients treated with grass pollen-SIT. In the same study, post-allergen-SIT serum was able to inhibit IgE-facilitated allergen binding. This serum inhibitory activity was shown to co-purify with IgG4-containing fractions following affinity chromatography [62]. These findings suggest that IgG antibodies are involved in the underlying mechanisms of successful SIT. Hence, the measurement of inhibition of IgE-facilitated allergen binding has been utilized as a potential surrogate marker of successful allergen-SIT in several clinical trials [17, 61, 63–65].

Immunoglobulin isotype class switching, in particular for IgG4 and IgE, is dependent on Th2 cytokines (IL-4 and IL-13) and cognate interaction of CD40 on Th cells and the CD40 ligand on B cells [66–68]. Furthermore, the induction of IL-10 immunoregulatory cytokine following SIT has a profound effect on isotype class switching. In the presence of IL-4, additional IL-10 induces a preferential class switch in favour of IgG4 and has been shown to suppress both total and allergen-specific IgE responses [69, 70]. A detailed time-course of IL-10 induction in patients treated with grass pollen-SIT has revealed a significant increase in IL-10 production in parallel with the suppression of late responses and preceding IgG4 induction and clinical responsiveness [17]. Therefore, immunoreactive IgG4 may be a surrogate biomarker of IL-10 induction [17, 71]. Elevated levels of allergen-specific IgA2 antibodies and polymeric IgA2 have been reported following grass pollen-specific injection immunotherapy. Passive sensitization of monocytes in vitro using purified polymeric IgA2 from IgA-containing serum obtained following allergen–SIT, followed by cross-linking in vitro of IgA on monocytes by antigen or anti-IgA resulted in IL-10 production [39]. This indirect production of IL-10 from accessory cells may in turn favour isotype class switching in favour of IgG4 antibody production. These findings implicate a possible role for IgA antibodies in the induction of tolerance following SIT.

Sublingual allergen-specific immunotherapy

The clinical efficacy of the sublingual route as an alternative to subcutaneous immunotherapy has been confirmed in Cochrane Systematic Reviews and meta-analyses [72, 73]. Optimal, high doses for grass pollen-SLT have been established in two large independent randomized double-blind placebo-controlled studies that used grass allergen tablets [74, 75]. A 4- to 6-month pre-seasonal treatment, followed by a continued seasonal treatment with allergen-SLT resulted in increased clinical efficacy compared with a 2-month pre-seasonal treatment [76]. A recent trial using a rapidly dissolving grass allergen tablet showed a consistent decrease in symptom and rescue medication scores compared with placebo treatment [77] that persisted for 3 years of continued treatment and persisted for at least 1 year following a double-blind cessation of treatment [78].

Immunological mechanisms of sublingual allergen-specific immunotherapy

The oral mucosa is considered as a site of natural immune tolerance [72]. There are resident professional antigen-presenting cells, namely oral Langerhans cells (oLC), that express high levels of MHC class I and II molecules. Co-stimulatory molecules such as CD40, CD80, CD86 and FcεRI/II are also expressed. oLC constitutively expressed high levels of FcεRI [79] and have been shown to produce IL-10 in a TLR4-dependent manner [80]. Reduced T cell proliferative responses and the induction of T cells with a regulatory phenotype have been demonstrated in coculture experiments. Cross-linking of FcεRI on monocytes isolated from the oral mucosa induced IL-10 [81] and indoleamine 2,3-dioxygenase [82]. Indoleamine
2,3-dioxygenase is associated with reduced tryptophan levels and impaired T cell effector function. Furthermore, attenuated maturation and a gradual up-regulation of CD83 and CCR7 on oral DCs following allergen uptake during migration to the lymphoid tissue have been demonstrated. During this migration, oral DCs may prime T cells in the oral lymphoid foci before the classical contact with T cells in the lymphoid tissue. DCs residing in the oral mucosa are capable of priming Treg cell, including FoxP3+ Treg cells, which is increased in the oral mucosa during SLIT. IL-10, IL-18 and signalling lymphocytic activation molecule expression and the amount of FoxP3-expressing T cells are increased in the peripheral blood during allergen-SLIT [83, 84]. Moreover, during the pollen season, increased expression of programmed cell death ligand (PD-L1) on B cells and monocytes was associated with reduced IL-4 levels in PBMCs from SLIT-treated patients [85]. Thus, these immunological changes following allergen-SLIT may be associated with the induction of allergen-specific tolerance and the clinical effect of SLIT. These findings suggest a network of Langerhans cells, epithelial cells, monocytes and oral DCs capable of producing IL-10, TGF-β and activins and priming Treg cells [26, 86–88] and may play a potential role in the induction of allergen-specific tolerance and the clinical effect of SLIT. These possibilities require testing pre- and post-allergen-specific sublingual immunotherapy. Additionally, there are limited numbers of mast cells in the oral mucosa, which may possibly account for the established safer profile of SLIT [80] compared with SIT.

The effects of SLIT on allergic Th2 effector cells have been demonstrated in patients treated with *Parietaria*-specific sublingual immunotherapy. A significant reduction in the number of eosinophils, neutrophils and ICAM-1 expression in the nasal mucosa was observed [89]. A reduction of eosinophil cationic protein [89, 90] and numbers of eosinophils have been reported in several but not all studies [91]. The suppression of T cell proliferative response following birch and grass pollen-specific SLIT has been observed [92, 93]. Elevated levels of IFN-γ and/or reduced in Th2 cytokines [90, 94–97] have been reported in some but not all studies [93, 98, 99]. A recent study assessed immunological changes in patients who received pre-seasonal, seasonal or prolonged ragweed sublingual therapy. A reduced expression of CD80 and CD86 expression on CD14+ monocytes, increased IL-10 production and a reduced IL-4 synthesis were observed. Elevated levels of PD-L1 expression and PD-L1-associated IL-10 synthesis were also observed [100]. These findings are in agreement with the previously reported induction of IL-10 at protein and mRNA levels, IgG4 and the modulation of PD-L1, IL-10 and IgG4 [85, 93, 101].

The role of regulatory T cells was elegantly demonstrated following birch pollen SLIT [92]. Higher numbers of circulating CD4+CD25+ T cells were detected after 4 weeks. Elevated levels of FoxP3 and IL-10 were observed in actively treated patients when compared with pre-treatment. IL-4 production and IFN-γ mRNA expression in PBMC cultures were reduced. The loss of suppressor activity was observed when CD25+ cells were depleted from PBMC and was reversed on adding anti-IL-10, which implied the involvement of regulatory T cells. In a blinded trial of SLIT in HDM-sensitive adults, an increased proportion of non-dividing CD4+CD25+FoxP3+ suppressor cells was observed only in mite-treated patients and at 6 and 12 months in 7-day HDM stimulated-PBMC cultures. These phenotypic CD25-associated Treg cells had functional activity in that they were able to suppress mite-antigen-induced CD4+ T cell proliferation and this suppression could be inhibited at 6 months, but not later, by the addition of soluble TGF receptors, implying a transient role for TGF-β in the mechanism of suppression after mite-SLIT. Interestingly, the proportion of these phenotypic Treg cells decreased to baseline levels after 24 months in the actively treated group despite persistent clinical improvement [102]. Serum TGF-β levels have also been shown to correlate with visual analogue scores and rescue medication scores following sublingual pollen-SIT [103]. Taken together, these findings suggest that Treg cells may play a role in reducing Th2-mediated allergic inflammation that is manifest at 3–6 months and IL-10 [92] and/or TGF-β [102] dependent. However, the persistence of antigen-induced suppression and clinical efficacy at later time-points during allergen-SLIT may be mediated by other immunological mechanisms such as immune deviation in favour of antigen-specific Th1 responses [18, 92, 94].

**Immunoglobulin responses following sublingual allergen-specific immunotherapy**

During pollen-SLIT, paradoxical increased increases in allergen-specific IgE may occur within weeks, although they do not appear to be associated with adverse events. These early increases are followed by blunting of seasonal increases in serum IgE. There follows an increase in allergen-specific IgG and IgG4. These elevations are both time- and allergen-dose dependent [75] and progressive for at least 2 years [77], although of a lower magnitude than that observed during allergen-SIT [4].

Some studies have shown increases in specific IgG4 in the absence of demonstrable efficacy [93], whereas others have shown no difference in the IgG levels (Table 1), likely related to the lower allergen doses used [98], particularly in relation to HDM-specific sublingual immunotherapy [8, 91, 99, 104]. These findings raise the issue of causality vs. bystander effects of IgG. In functional assays, sera obtained after grass pollen-specific sublingual immunotherapy were able to inhibit IgE-allergen binding *in vitro* [77]. The heterogeneity of immunological responses in particular in relation to allergen-specific IgE and IgG4

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A particular feature of allergen-SIT is the induction of long-term antigen-specific tolerance, namely persisting natural pollen exposure following allergen-SIT [64]. Long-term clinical benefit for several years after treatment is well documented [6]. These findings, after 3 years of grass pollen-SIT have now been confirmed in a separate study after 2 years of blinded treatment and withdrawal [6]. Long-term clinical tolerance was associated with the persistence of allergen-specific T cells induced following sublingual allergen-SIT. Although there are similarities following sublingual allergen-SIT, the cells expressing CD25+FoxP3 cells within the sublingual mucosa after SLIT and their increase in numbers during natural pollen exposure following allergen-SLT [64].

In a recent study, we showed that sublingual grass-pollen-SLT induced significant increases in serum-specific IgG4 antibodies following sublingual grass pollen-specific immunotherapy (p < 0.05, vs. placebo)

Table 1. Summary of IgE and IgG4 following sublingual grass pollen-specific immunotherapy ([↑], increase; [↓], decrease)

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<td>RDBPCT</td>
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<td>Subjects</td>
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<td>Ragweed pollen</td>
<td>HDM</td>
<td>Timothy grass</td>
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<td>Primary clinical end points were not significantly different when compared with placebo</td>
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<tr>
<td>Allergen outcome</td>
<td>Primary clinical end points were not significantly different when compared with placebo</td>
<td>Effective in subjects treated with a high dose only</td>
<td>No change in daily symptoms</td>
<td>Effective in subjects treated with a high dose</td>
<td>No change in symptom scores in subjects treated with a high dose compared with a low dose</td>
<td>No change in symptom scores in both treated groups when compared with placebo</td>
<td>Primary clinical end points were not significantly different when compared with placebo</td>
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Specific IgE

| Specific IgE | ↑ vs. placebo | ↓ in specific IgE in treated groups | Dose-dependent increases vs. placebo | Not measured | ↑ vs. placebo |

Specific IgG4

| Specific IgG4 | ↑ vs. placebo | No change | Dose-dependent increases vs. placebo |↑ in monotherapy-treated group only | IgG4, IgG1, IgA, IgA2, vs. placebo |

Inhibition of CD23-dependent IgE-FAB

| Inhibition of CD23-dependent IgE-FAB | Not measured | No change | Not measured | Not measured | Not measured (although, Sustained ↑ IgE blocking factor 1 year after withdrawal) |↑ vs. placebo |

Down-regulation of IgE-facilitated binding is a surrogate for the suppression of allergen-SLT-induced IgG4 antibodies following allergen-specific immunotherapy. In this study, there were no antibody levels following SLIT in contrast to the findings observed in the SLIT-induced IgG4 antibodies. These data suggest a likely mechanism of action of SLIT in the suppression of IgE-facilitated IgG4 production and associated symptoms on allergen exposure and associated symptoms on allergen exposure and associated symptoms on allergen exposure. These data suggest a likely mechanism of action of SLIT in the suppression of IgE-facilitated IgG4 production and associated symptoms on allergen exposure and associated symptoms on allergen exposure.

Natural pollen exposure following allergen-SLT induced significant increases in serum-specific IgG4 antibodies following sublingual grass pollen-specific immunotherapy (p < 0.05, vs. placebo).
of IgG-associated inhibitory activity against binding of IgE-allergen complexes to B cells, a surrogate of IgE-facilitated antigen presentation and activation of antigen-specific T cells [65]. In this study, IgG-associated inhibitory bioactivity rather than absolute levels of the IgG4 antibody correlated with clinical tolerance as reflected in maintained suppressed combined symptom and rescue medication scores 2 years after immunotherapy withdrawal. This effect has been demonstrated following grass pollen immunotherapy withdrawal, whereas we could not confirm the maintained suppression of IgE-FAB following the withdrawal of bee venom immunotherapy [110], implying possible different underlying mechanisms for maintained tolerance after the discontinuation of venom immunotherapy.

More recently, a 3-year large randomized double-blind controlled trial of sublingual grass tablet immunotherapy has been shown to provide clinically relevant, sustained disease-modifying effects after a 1-year withdrawal [78]. Throughout the 3-year treatment period, a progressive increase in IgG4 and the IgE-blocking factor (the IgE-blocking factor is the result of a functional assay that measures the amount of IgE actually hindered from binding to allergen to elicit symptoms) in the actively treated group was observed with no change in the placebo. One year after treatment, the differences in change from the baseline values between the groups were still significantly different for both immunoreactive IgG4 and the IgE-blocking factor. These findings strengthen the role of functional inhibitory IgG, in particular IgG1 and IgG4 antibodies, as surrogate biomarkers of persistence of clinical tolerance to grass pollen immunotherapy.

Potential biomarkers for monitoring allergen-specific immunotherapy

In a trial of grass pollen immunotherapy, the possible predictive value of the ratio of serum specific-IgE/total IgE was evaluated [111]. Patients who received active treatment showed a transient increase, followed by seasonal blunting in specific IgE. The ratio of serum specific-IgE/total IgE correlated with the clinical response to immunotherapy, whereas this was not observed in another study in relation to the clinical response to HDM immunotherapy [112]. Thus, the usefulness of this ratio requires further evaluation. Although there is evidence that SIT is associated with early increases in the concentration of allergen-specific IgG4 blocking antibodies and modest reductions in allergen-specific IgE as determined by ELISAs, these antibody levels did not correlate with clinical efficacy [46, 113, 114]. Despite this lack of correlation, previous reports have demonstrated that fractionated IgG4 antibodies in serum from patients who had received grass pollen immunotherapy were responsible for the inhibition of IgE-FAB to B cells [60, 62]. This suggests a functional role of IgG4 in the inhibition of IgE-FAB, which may be due to changes in IgG antibody specificity or affinity. Another role of allergen-specific IgG4 antibodies induced by immunotherapy is their ability to block allergen-induced IgE-dependent histamine release by basophils, confirming the functional blocking activity of these antibodies [115].

Clinical surrogate markers such as end-point skin prick test (SPT) titration to allergens, airway-specific and non-specific hyperresponsiveness and nasal or conjunctival challenges have been assessed for their potential role in monitoring immunotherapy [116, 117]. In an open non-randomized study in mite-sensitive children, the suppression of immediate skin test reactivity at 3 years protected against relapse and the need for re-treatment in the following 3 years after the discontinuation of immunotherapy [118]. In blinded trials, Walker and colleagues demonstrated complete inhibition of seasonal increases in methacholine-induced BHR, whereas Roberts and colleagues demonstrated significant reductions in allergen-induced cutaneous and conjunctival responses, both following grass pollen immunotherapy. Although there are associations between clinical responses to grass pollen immunotherapy and clinical surrogates such as SPT titration to allergens, allergen-specific bronchial responsiveness and nasal or conjunctival challenges, true correlations with the magnitude of the clinical response in terms of reduced symptoms or improved quality of life have yet to be convincingly demonstrated. This partially reflects the complexity and interdependency of the underlying mechanisms, but also the multiple additional factors involved such as neurogenic influences, target organ responsiveness and, not the least, psychosocial influences of altered environmental factors.

Mechanisms of peptide-specific immunotherapy

Peripheral T cell tolerance has also been demonstrated following the administration of a synthetic Fel d 1 and Api m 1 peptide-based vaccine. These short peptides consist of a native sequence or a sequence that has undergone amino acid substitution and has reduced IgE cross-linking activity. A mixture of large number of short peptides resulted in methacholine-induced BHR, whereas Roberts and colleagues demonstrated complete inhibition of seasonal increases in methacholine-induced BHR, whereas Roberts and colleagues demonstrated significant reductions in allergen-induced cutaneous and conjunctival responses, both following grass pollen immunotherapy. Although there are associations between clinical responses to grass pollen immunotherapy and clinical surrogates such as SPT titration to allergens, allergen-specific bronchial responsiveness and nasal or conjunctival challenges, true correlations with the magnitude of the clinical response in terms of reduced symptoms or improved quality of life have yet to be convincingly demonstrated. This partially reflects the complexity and interdependency of the underlying mechanisms, but also the multiple additional factors involved such as neurogenic influences, target organ responsiveness and, not the least, psychosocial influences of altered environmental factors.
a therapeutic vaccine, PBMC proliferative responses were assessed for each of the 16 Fel d 1 peptides (12 treatment peptides and four Fel d 1 peptides that were not included in the vaccine). Responses to all 12 treatment peptides were significantly reduced in the 16 cat-allergic patients receiving active treatment, but not in the eight subjects receiving placebo [120].

**Concluding comments**

Allergen-SIT is effective, antigen-specific and induces long-lasting tolerance. Successful immunotherapy is accompanied by the suppression of allergic inflammation and the induction Treg cells and 'protective and non-inflammatory' antibodies such as IgA2, IgG and particularly IgG4 that inhibit facilitated antigen presentation and basophil histamine release and augment monococyte-derived IL-10 production. The underlying mechanisms involve immune deviation in favour of Th1 responses, induction of FOXP3 and IL-10/TGF-β+Treg in the peripheral blood and in the nasal/oral mucosa. A greater understanding of mechanisms has informed novel immunotherapy approaches [121], whereas the identification of predictive biomarkers for the success of immunotherapy remains elusive.

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