Mechanisms of allergen immunotherapy for inhaled allergens and predictive biomarkers

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Allergen immunotherapy is effective in patients with IgE-dependent allergic rhinitis and asthma. When immunotherapy is given continuously for 3 years, there is persistent clinical benefit for several years after its discontinuation. This disease-modifying effect is both antigen-specific and antigen-driven. Clinical improvement is accompanied by decreases in numbers of effector cells in target organs, including mast cells, basophils, eosinophils, and type 2 innate lymphoid cells. Immunotherapy results in the production of blocking IgG/IgG4 antibodies that can inhibit IgE-dependent activation mediated through both high-affinity IgE receptors (FceRI) on mast cells and basophils and low-affinity IgE receptors (FceRII) on B cells. Suppression of Th2 immunity can occur as a consequence of either deletion or anergy of antigen-specific T cells; induction of antigen-specific regulatory T cells; or immune deviation in favor of Th1 responses. It is not clear whether the altered long-term memory resides within the T-cell or the B-cell compartment. Recent data highlight the role of IL-10–producing regulatory B cells and “protective” antibodies that likely contribute to long-term tolerance. Understanding mechanisms underlying induction and persistence of tolerance should identify predictive biomarkers of clinical response and discover novel and more effective strategies for immunotherapy. (J Allergy Clin Immunol 2017;140:1485-98.)

Key words: Immunotherapy, mechanisms, allergic rhinitis, allergic asthma, long-term tolerance, T cells, B cells, type 2 innate lymphoid cells, IgE-FAB, biomarkers

Abbreviations used

- Breg: Regulatory B
- C1Q: Component 1Q
- CRTH2: Chemoattractant receptor–homologous molecule expressed on Th2 lymphocytes
- DAO: Diamine oxidase
- DC: Dendritic cell
- DCreg: Regulatory dendritic cell
- ELIFAB: Enzyme-linked immunosorbent–facilitated antigen-binding assay
- FOXP3: Forkhead box P3
- ILC: Innate lymphoid cell
- ILC2: Group 2 innate lymphoid cell
- iTreg: Inducible regulatory T
- LT: Leukotriene
- nTreg: Natural regulatory T
- RIPK4: Receptor-interacting serine/threonine-protein kinase 4
- sIgE: Allergen-specific IgE
- sIgG: Allergen-specific IgG
- ThF: Follicular helper T
- Treg: Regulatory T
- VLA4: Very late antigen 4, integrin α4β1

Allergen immunotherapy is effective in selected patients with allergic rhinitis, including those with mild/moderate asthma. There is heterogeneity in the populations studied, the different allergen products and protocols used, and the clinical outcomes used to document efficacy and safety. Nonetheless, recent guidelines confirm that immunotherapy is particularly effective in patients with seasonal rhinitis, and recent data strongly support its use in perennial allergy caused by house dust mites.

Subcutaneous immunotherapy involves weekly updosing injections, followed by monthly maintenance injections for at least 3 years. In view of occasional systemic allergic side effects, subcutaneous immunotherapy requires administration in a specialist allergy clinic with access to resuscitative measures. Sublingual immunotherapy involves daily drops or tablets placed under the tongue. Sublingual immunotherapy is effective and safer than subcutaneous immunotherapy, such that it is self-administered by the patient at home. Sublingual and subcutaneous immunotherapy are effective generally within 2 to 4 months of initiating treatment and can be given preseasonally/coseasonally for short-term benefit. Indirect
comparisons have suggested that immunotherapy might be more effective than antiallergic drugs. In contrast to antiallergic drugs and currently available mAb therapies, when allergen immunotherapy is given continuously for 3 years, both routes have been shown to be disease-modifying, manifest as long-term remission of symptoms for at least 2 to 3 years after discontinuation.9,10

In this review we explore historical and recent data on the mechanisms of immunotherapy for inhalant allergens. Our expectation is that a greater understanding of the underlying mechanisms of tolerance will identify potential biomarkers that could predict and/or monitor the response to treatment. Such knowledge could inform new potential treatment strategies.

OVERVIEW OF MECHANISMS OF ALLERGIC RHINITIS AND ASTHMA

IgE and mast cells

The cardinal features of allergic rhinitis include increased allergen-specific IgE concentrations to clinically relevant allergens, IgE-dependent activation of mast cells, and local eosinophilia in target organs. In addition to systemic and regional lymphatic sources of IgE, specific IgE can be synthesized and produced locally by B cells within the respiratory mucosa,11 thereby accounting for the occasional phenomenon of “local allergic rhinitis” with symptoms on allergen exposure in the absence of detectable serum specific IgE or positive immediate skin test results to relevant allergens.12

IgE-dependent activation is detectable during the immediate (0- to 60-minute) response after nasal allergen provocation. Allergen cross-linking of adjacent surface IgE molecules on mast cells and basophils triggers within seconds or minutes the release of preformed mediators, such as histamine,13 and tryptase,14 contained within intracytoplasmic granules. Newly formed mediators derived from arachidonic acid within the membrane lipid include sulphidopeptide leukotrienes (LTs; LTC4, LTD4, and the terminal metabolite LTE4),12 platelet-activating factor, and prostaglandin D2. The biological properties of these mediators are consistent with the local vasodilatation, edema formation, local neurogenic stimulation, and mucus hypersecretion that characterize typical nasal allergen-induced immediate type I hypersensitivity. In the lower airways bronchial smooth muscle contraction, as well as edema and mucus hypersecretion, contribute to acute bronchoconstriction.

A proportion of subjects have a late response at 2 to 10 hours after challenge. The late response is characterized by tissue eosinophilia, nasal congestion, and mucosal hyperreactivity to both allergic and nonallergic triggers that can last for days or even weeks after a single nasal allergen challenge. In contrast

GLOSSARY

Bet v 1: A potent allergen from trees within the order Fagales, which is the main cause of type I allergies observed in early spring and characterized by hay fever, dermatitis, and asthma.

METHYLATED CpG SITES: CpG sites are regions of DNA in which a cytosine nucleotide occurs next to a guanine nucleotide separated by only 1 phosphate. Methylation of the cytosine within a gene can turn the gene off.

c-kit (CD117): A cytokine receptor most notably expressed on the surfaces of hematopoietic stem cells and other cell types, including mast cells. CD117 is a receptor tyrosine kinase type III protein that binds to stem cell factor and forms a dimer that activates its intrinsic tyrosine kinase, resulting in phosphorylation and activation of signal transduction molecules that produce cell signaling.

CYTOTOXIC T LYMPHOCYTE–ASSOCIATED PROTEIN 4 (CTLA-4): A receptor that functions as an inhibitory signal and downregulates immune responses when bound to CD80 and CD86. CTLA-4 is constitutively expressed in regulatory T cells but only upregulated in conventional T cells after activation.

IFN-γ: A type II interferon, IFN-γ is a cytokine required for innate and adaptive immunity against viral, bacterial, and protozoal infections. IFN-γ has been shown to be an important activator of macrophages and inducer of class II MHC molecule expression. IFN-γ is produced predominantly by natural killer (NK) and NKT cells as part of the innate immune response and by CD4 T\(_{\gamma,1}\) and CD8 cytotoxic T lymphocyte effector T cells once antigen-specific immune development.

ImmuCAP (A REGISTERED TRADEMARK OF PHARMACIA DIAGNOSTICS AB): An in vitro quantitative assay that measures allergen-specific IgE levels in human serum. ImmunoCAP assays can be performed on hundreds of allergens by using cellulose polymer, which provides high binding capacity of clinically relevant allergen proteins, including those present at very low levels.

IL-3: A growth-promoting cytokine capable of supporting the proliferation and activation of a broad range of hematopoietic cell types, including basophils.

IL-6: A cytokine also known as IFN-β2 and implicated in a wide variety of inflammation-associated disease states. IL-6 has been associated with B-cell maturation and has been shown to act as an endogenous pyrogen capable of inducing fever in patients with autoimmune diseases or infections.

IL-12: A cytokine produced by dendritic cells, macrophages, neutrophils, and human B-lymphoblastoid cells (NC-3T) in response to antigenic stimulation and has been shown to be required for differentiation of naïve T cells into Th1 cells.

IL-21: A cytokine expressed in human CD4\(^+\) T cells and found to be upregulated in Th1, Th17 subsets and follicular T cells, which induces cell division/proliferation of various cells of the immune system, including natural killer cells and cytotoxic T cells.

IL-25: A proinflammatory cytokine that shares sequence similarity with IL-17 and has been shown to favor the Th2-type immune response. IL-25 can induce nuclear factor \(k\)B activation and stimulate IL-8 production.

IL-33: A member of the IL-1 family of cytokines expressed on Th2 cells, mast cells, and group 2 innate lymphocytes that potentially drives production of Th2-associated cytokines.

IL-35: An IL-12 family cytokine produced by regulatory, but not effector, T and B cells that plays a role in immune suppression.

PROGRAMMED CELL DEATH 1 (PD-1): A cell-surface receptor that plays an important role in downregulating the immune system and suppressing inflammatory T-cell activation. PD-1 is an immune checkpoint that serves a dual role of promoting apoptosis in antigen-specific T cells while simultaneously reducing apoptosis in regulatory T cells.

THYMIC STROMAL LYMPHOPOIETIN (TSLP): A cytokine that stimulates T-cell maturation through activation of antigen-presenting cells, such as dendritic cells and macrophages.

TGF-β: A cytokine secreted by many cell types, including macrophages, that controls proliferation, cellular differentiation, and inflammatory processes in a variety of cells. It also plays a role in T-cell regulation and differentiation.

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to findings in patients with allergic asthma and nasal polyposis, morphologic and immunohistochemical features of airway remodeling are not a consistent feature of even moderate/severe allergic rhinitis.15

TH2 lymphocytes and group 2 innate lymphoid cells

The above pathophysiologic events are under the regulation of a distinct subset of TH2 cells. TH2 cells produce IL-4, the key cytokine responsible for TH2 cell differentiation.16-18 IL-4 and IL-13 induce B lymphocytes to produce ε-germline gene transcripts,19 the first step in heavy chain gene rearrangement in favor of IgE production. IL-4 and IL-13 upregulate vascular cell adhesion protein 1 expression on the vascular endothelium, promoting adhesion of very late antigen 4, integrin α4β1–expressing eosinophils. Both stimulate mucus production from glands in the upper and lower airways. IL-5 is responsible for terminal differentiation and release of eosinophils from the bone marrow and prolongs eosinophil survival by inhibiting eosinophil apoptosis in tissues.20 Along with stem cell factor, IL-9 is a key cytokine for the differentiation and maturation of mast cells.21 Release of TH2 cytokines and tissue eosinophilia are apparent during the late-phase response that occurs at 4 to 12 hours after allergen challenge.22

Group 2 innate lymphoid cells (ILC2s) represent an alternative source of TH2 cytokines in the nasal mucosa. Innate lymphoid cells (ILCs) are morphologically similar to lymphocytes, although they are distinct in not expressing surface antigen receptors or other cell lineage markers and act in an antigen-independent manner.23,24 ILCs consist of 3 different groups referred to as group 1 ILCs, ILC2s, and group 3 ILCs. Group 1 ILCs constitutively express RAR-related orphan receptor α and GATA-3; produce TH2 cytokines, particularly IL-5 and IL-13; and provide immunity to helminths, as well as stimulating allergic responses. Group 3 ILCs are characterized by the transcription factor RAR-related orphan receptor γt, express IL-17a and/or IL-22, afford protection against extracellular bacteria, and are involved in tissue repair processes.

The role of ILC2s in allergic rhinitis was first identified in patients with cat allergy, who showed increases in peripheral blood ILC2 numbers at 4 hours after a cat allergen nasal challenge.25 Subsequently, increases in circulating ILC2 numbers have been identified in both patients with grass allergy and rhinitis26 and asthmatic patients27 during the grass pollen season. ILC2s represent an abundant alternative source of TH2 cytokines and likely serve to amplify and maintain local TH2-driven allergic inflammation. In view of recently identified plasticity within ILC2s in tissues of patients with chronic obstructive pulmonary disease and chronic rhinosinusitis,28 this concept must be revisited in the context of allergic rhinitis.18

The respiratory epithelium and dendritic cells

Although IgE-dependent mast cell activation and tissue eosinophilia are driven by TH2 lymphocytes, TH2 cell differentiation is dependent on the local cytokine milieu provided by interactions between the respiratory epithelium, local dendritic cells (DCs), and regional lymph nodes.

In an atopic subject allergens pass through the inflamed nasal epithelium, and activated epithelial cells release CCL2 and CCL20, which recruit immature DCs (Fig 1, A). Activated DCs migrate to regional draining lymph nodes and polarize naive T cells into TH2 cells. DC migration is primed by IL-13 produced by ILC2s and also by IL-4 produced principally by basophils. Within the germinal center of the lymph node, a subset of TH2 cells constitutively express RAR-related orphan receptor α and GATA-3; produce TH2 cytokines, particularly IL-5 and IL-13; and provide immunity to helminths, as well as stimulating allergic responses.
differentiates into follicular helper T (T<sub>FH</sub>) cells. T<sub>FH</sub> cells produce both IL-4 and IL-21, which, along with TH2 cell–derived IL-4, promote immunoglobulin heavy chain class-switching to IgE in B cells.

The respiratory epithelium of atopic allergic subjects expresses cytokines that include IL-25, IL-33, and thymic stromal lymphopoietin. These epithelial cytokines favor development of a proallergic DC phenotype that provides help for TH2 cell differentiation. Additionally, these epithelially derived cytokines are major growth factors for ILC2s that amplify and maintain local TH2-driven allergic inflammation. During subsequent allergen exposure, IgE-facilitated allergen recognition through FcεRI on DCs and FcεRII on B cells amplifies the development of TH2 responses to inhaled allergens (Fig 1, B).

DCs, depending on their maturation phase, their location, and the associated local cytokine milieu, can either initiate and maintain allergic inflammation (proallergic DC2s) or alternatively promote a state of immune tolerance (tolerogenic regulatory dendritic cells [DCregs]) to sensitizing allergens. DC2s express the markers CD141, GATA-3, OX40 ligand, and receptor-interacting serine/threonine-protein kinase 4 (RIPK4). When DC2s were exposed to allergen and cocultured subsequently with T cells, they promoted preferential T<sub>H2</sub> T-lymphocyte responses.

MECHANISMS OF ALLERGEN IMMUNOTHERAPY

Further details on mechanisms of allergen immunotherapy can be found in Fig 2.

IgE, IgG, and IgA responses

Both sublingual and subcutaneous immunotherapy have been associated with transient early increases in serum allergen-specific IgE antibody levels that are followed by blunting of the usual seasonal increases in IgE levels during natural allergen exposure. These early increases are not accompanied by untoward side effects, and it has been suggested that early TH2 priming by high allergen exposure might be important for successful immunotherapy. Prolonged subcutaneous immunotherapy over several years can result in a decrease in allergen-specific IgE (sIgE) concentrations, an event that might contribute to long-term tolerance.

In 1935, Robert Cooke and colleagues demonstrated the passive transfer of suppressive activity for immediate ragweed IgE sensitivity in the skin by use of serum obtained from patients who had undergone ragweed subcutaneous immunotherapy. Serum and nasal inhibitory activity for IgE after subcutaneous immunotherapy was subsequently shown to reside within serum IgG, IgG4, and IgA fractions. Studies have shown 10- to 100-fold increases in serum concentrations of IgG,
particularly IgG4. Sublingual immunotherapy has also been shown to induce allergen-specific IgG4, IgG4, and IgA antibodies. These increases in levels of immunoreactive antibodies have been observed after immunotherapy to both seasonal pollens and perennial allergens, such as house dust mite. Serum specific IgG4 levels have been shown to increase in a time- and dose-dependent manner during grass pollen immunotherapy.

Several studies have highlighted the inhibitory capacity of IgG4 for IgE-dependent events. IgG4 antibodies are bispecific and have the capacity to exchange F(ab) arms by swapping heavy-light chain pairs between IgG4 molecules with diverse specificities. IgG4 can compete with IgE for allergen, thereby blocking allergen-IgE complex formation. This prevents cross-linking of high-affinity IgE receptors (FcεRI) on basophils and mast cells, inhibiting histamine release. Competition of IgG/IgG4 for IgE can also block binding of allergen-IgE complexes to low-affinity receptors (FcγRIIb) on B cells, thereby inhibiting IgE-facilitated antigen presentation to T cells, a major driver of allergen-specific Th2 responses.

Paradoxically, although immunoreactive IgG/IgG4 levels decreased by 80% to 90% within 1 year of stopping allergen immunotherapy, IgG-associated serum IgE-inhibitory activity persisted for several years and accompanied long-term clinical efficacy. This suggests that despite lower levels, IgG antibodies that persist after discontinuation of immunotherapy can have either higher avidity or higher affinity. These data raise the possibility that long-lived memory B cells induced by immunotherapy can persist as a result of low-level environmental allergen stimulation, thereby contributing to long-term tolerance.

IgG antibodies have been detected locally in both nasal fluid and serum after immunotherapy. Both specific IgG4 levels and associated inhibitory activity for IgE-facilitated antigen binding (IgE-FAB) were increased in the nasal fluid of patients undergoing sublingual immunotherapy compared with untreated participants. The IgG4 dependency of IgE-inhibitory activity has been shown in depletions experiments using IgG4 affinity chromatography. The magnitude of IgE suppression was greater with nasal fluid than with serum, thereby highlighting the potency of local IgG inhibitory antibodies.

Allergen immunotherapy and effector cells

The influence of allergen immunotherapy on effector cells has been studied after nasal allergen provocation and during natural seasonal pollen exposure. Both subcutaneous and sublingual Immunotherapy inhibit early- and late-phase responses after allergen challenge. Suppression is accompanied by improvements in seasonal symptoms and a decrease in requirements for rescue medication. A direct correlation was noted between reductions in IL-5 levels and nasal mucosal eosinophil numbers and also between eosinophil numbers and the severity of seasonal symptoms. In house dust mite–sensitive patients sublingual immunotherapy with mite extract inhibited local mucosal vascular intercellular adhesion molecule 1 expression and also decreased local eosinophilia. These data illustrate that both sublingual and subcutaneous immunotherapy result in decreases in recruitment, activation, or both of effector cells at allergic tissue sites.

Allergen immunotherapy and T-lymphocyte responses

Decreases in Th2 cell numbers. Suppression of allergen-induced late nasal responses during subcutaneous grass pollen immunotherapy has been associated with decreases in numbers of CD4+ T cells and local IL4 mRNA–positive T cells in the nasal mucosa. These findings are supported by the observation of decreases in Th2 cytokine levels in nasal lining fluid after nasal challenge. Recent techniques that include ex vivo tetramer analysis have enabled the phenotyping of peripheral circulating allergen-specific T cells. This has permitted identification of key T-cell surface markers, such as CD27, chemoattractant receptor–homologous molecule expressed on TH2 (CRTH2), CD161, and chemokine receptor 4 (CCR4), associated with type 2 proallergic responses. In patients with grass pollen allergy, tetramer-specific T cells that did not express CD27 mostly expressed the surface markers CRTH2 and CCR4. This was in contrast to nonallergic subjects, whose T cells expressed low levels of CRTH2 and CCR4 and high levels of CD27. Patients with higher pollen allergy expressed a high frequency of CD27− Th2 cells that decreased after subcutaneous immunotherapy. Similarly, the Gauging Responses in Allergic Rhinitis to SCIT versus SLIT Trial (GRASS), both subcutaneous and sublingual immunotherapy resulted in clinical improvement during 2 years that was paralleled by a decrease in peripheral tetramer-positive CRTH2−CCR4−CD27−CD4+ Th2 cell numbers. These changes were paralleled by a decrease in levels of local nasal Th2 cytokines, including IL-4, IL-5, and IL-13, in nasal fluid after allergen provocation. Both numbers of circulating tetramer-positive Th2 cells and levels of local nasal Th2 cytokines rebounded during year 3, along with a deterioration in seasonal symptoms 1 year after discontinuation of immunotherapy. The failure of 2 years of immunotherapy (in contrast to 3 years of continuous treatment) to induce durable tolerance might have been related to this re-emergence of antigen-specific Th2 immunity.

Increases in numbers of regulatory T cells. Immune tolerance during immunotherapy has been shown to be associated with induction of allergen-specific regulatory T (Treg) cells.

Treg cells can be grouped into 2 subsets: natural regulatory T (nTreg) cells, which express the transcription factor forkhead box P3 (FOXP3), and inducible regulatory T (iTreg) cells, which produce regulatory cytokines, such as IL-10, IL-35, and TGF-β. nTreg cells. nTreg cells were first described by Sakaguchi et al. In addition to the transcription factor FOXP3, nTreg cells have increased expression of the IL-2 receptor (CD25) and low
expression of the IL-7 receptor (CD127). nTreg cells exert their suppressive capacity in a direct cell-cell contact–dependent manner.81,82 Functional roles have been proposed for membrane cytotoxic T lymphocyte–associated protein 4 (CTLA-4), surface-bound TGF-β, the glucocorticoid-induced TNF receptor, and programmed cell death 1 (PD-1). nTreg cells have also been shown to modulate allergen-specific T-cell responses in healthy nonatopic subjects.89 Subcutaneous immunotherapy was associated with local increases in FOXP3+CD25+ T-cell numbers in the nasal mucosa compared with those in untreated control subjects. After sublingual grass pollen immunotherapy, immunofluorescence studies on sublingual biopsy specimens identified increases in FOXP3+CD3+ cell numbers in the sublingual mucosa.97 Human in vitro studies of biopsy specimens of human buccal mucosa and associated lingual tonsils and adenoids identified the oropharyngeal mucosa as an environment rich in protolerogenic DCs and Treg cells.90,91 Altered nTreg cell function has been associated with epigenetic modification at the FOXP3 promoter region. In a randomized controlled study of dual sublingual immunotherapy in participants allergic to both house dust mite and grass pollen, methylated CpG sites within the FOXP3 locus of enriched peripheral memory Treg cells were decreased after 12 months of treatment.92

TFH/TFR Cells. TFH cells are characterized by surface CXCR5, the transcription factor B-cell lymphoma 6 protein, and increased expression of IL-4, IL-21, and IL-6. TFH cells reside in the marginal zones of germinal follicles within regional lymph nodes, where they provide essential help for B-cell maturation and class-switching. In 2004, a distinct population of CXCR5-expressing FoxP3+ Treg cells was identified, which possessed the ability to migrate into germinal centers and suppress T- and B-cell responses.93,94 However, it was not until 2011 that this population of cells was recognized as a distinct subset of CD4+ T cells with regulatory capacity, namely follicular regulatory T (TFR) cells. One study has shown that memory TFH cells were significantly reduced after immunotherapy.95 Moreover, TFR cells from immunotherapy-treated patients were shown to have higher capacity to produce IL-10 compared with TFH cells. When CXCR5+ TFH cells were enriched from immunotherapy-treated donors and cultured in the presence of T-cell receptor stimulation and IL-2 for 5 days, flow cytometric analysis revealed an increase in TFR cell numbers. These findings highlight the plasticity of TFR cells and their likely role in suppressing Th2 responses and IgE antibody production during allergen immunotherapy.95

iTreg cells. iTreg cells produce either IL-10 (T_{R1}) or TGF-β (T_{H3}) and have been shown to modulate allergen-driven T-cell proliferative responses and T_{H2} cytokine release.93 Studies of nasal biopsy specimens obtained before and at 2 years after grass pollen immunotherapy identified a shift in favor of local iTreg cell responses in the nasal mucosa. There was an increase in numbers of IL-10–expressing T cells during the pollen season that was associated with an increase in serum IgG4 levels.96 Seasonal increases in numbers of TGF-β+ T cells in the nasal mucosa correlated with increases in peripheral circulating IgA concentrations.97

Induction of peripheral IL-10+ Treg cells was reported after grass and birch pollen sublingual immunotherapy.56,85
A time-course study during subcutaneous grass pollen immunotherapy demonstrated that PBMCs obtained as early as 2 to 4 weeks during early updosage, when coculturated with grass pollen allergen for 6 days, produced high levels of IL-10 in supernatants. This early IL-10 signal was paralleled closely by suppression of the allergen-induced late-phase response (Fig 3). Increases in IL-10 production and suppression of the late response were followed sequentially by increases in serum IgG4 levels at 6 to 8 weeks that peaked at 16 weeks, along with parallel suppression of immediate skin responses. Postimmunotherapy serum was shown to have IgG-associated IgE-blocking activity for both basophil activation (increased allergen stimulated basophil CD63) and IgE-FAB inhibition that paralleled increases in IgG4 levels. The in vivo time course of PBMC IL-10 production and associated changes in serum blocking antibodies, allergen-induced skin responses and hypothetical changes in Th2 lymphocytes during updosping, and maintenance of grass pollen immunotherapy for 3 years and during immunotherapy withdrawal are shown in Fig 3.

Regulatory B cells. Regulatory B (Breg) cells are a subset of B cells that produce IL-10 and have the capacity to inhibit T cell-- and DC-mediated inflammatory responses and maintain natural immunologic tolerance. Purified populations of IL-10–producing Breg cells in bee venom–tolerant subjects exhibited high surface expression of CD25 and CD71 and low expression of CD73. These cells had the capacity to suppress bee venom–specific T-cell proliferation. Moreover, the provenance of allergen-specific IgG4 antibodies after bee venom immunotherapy was shown to be from phospholipase A2–specific IL-10+ Breg cells. In addition to IL-10, Breg cells have been shown to exert their suppressive capacity through production of TGF-β and IL-35. It is likely that similar Breg cell responses can be elicited during immunotherapy with grass pollen or house dust mite allergens. Whether the same phenotype is expressed by B cells after immunotherapy with inhalant allergens remains to be determined.

TH1 immune deviation. Suppression of TH2 immunity during both subcutaneous and sublingual immunotherapy has also been associated with immune deviation and induction of TH1 cells. In situ hybridization studies of the nasal mucosa after successful subcutaneous immunotherapy demonstrated increases in IFNG mRNA+ T cells after allergen challenge that correlated with decreases in nasal symptoms during the pollen season. Pollen immunotherapy was associated with decreases in the ratio of IL5/IFNG mRNA+ cells in the mucosa and increases in nasal IFN-γ protein levels in nasal fluid during natural seasonal allergen exposure. Similarly, subcutaneous grass pollen immunotherapy resulted in increases in IL12 mRNA+ macrophages in the skin that accompanied suppression of late cutaneous responses and correlated positively with local IFN-γ+ T cells and inversely with IL-4–expressing T cells. Evidence for/against TH1 deviation in peripheral blood studies has been more controversial. One study suggested that the shift from TH2 to TH1 responses might have been related to activation-induced cell death of allergen-responder TH2 cells. During birch pollen subcutaneous immunotherapy, a transient increase in Bet v 1–specific IL-10–secreting cells at 3 months was followed at 12 months by a reduction in the ratio of Bet v 1–specific IL-5/IFNG-γ-secreting T cells. Moreover, survival of TH1 cells has been reported after deletion of TH2 cells.

Allergen immunotherapy and ILCs

The influence of immunotherapy on ILC2 cells has been studied in peripheral blood but not in target organs, partly because of difficulties in identifying these cells that do not express cell lineage markers accessible to immunohistochemical localization in tissues. After grass pollen subcutaneous immunotherapy, there was a marked inhibition of seasonal increases in lineage-negative CRTH2+CD127+ ILC2s that correlated with the severity of self-reported symptoms during the pollen season. These results were highly significant when compared with seasonal increases in ILC2 numbers observed in matched untreated control subjects with seasonal allergic rhinitis. These data were supported by inhibition of seasonal increases in numbers of CD117+ (c-kit+) ILC2s and in the proportion of IL-13+ ILC2s, as determined by means of intracellular cytokine staining. In a study of immunotherapy in participants with seasonal asthma, there was no change in the number of ILC2s, although this is likely explained by the measurements having been performed out of season when the participants were asymptomatic. To our knowledge, there have been no reports of the influence of immunotherapy on innate epithelially derived cytokines, which are known to be closely involved in the regulation of both local TH1-mediated events and ILCs.

Allergen immunotherapy and DCs

The buccal mucosa is exposed constantly to foreign proteins in foods and represents a distinct protolerogenic environment. Ex vivo studies of buccal mucosal biopsy specimens from patients with grass pollen allergy have shown that oral mucosal Langerhans cells bind the major grass pollen allergen Phl p 5 in a dose- and time-dependent manner that plateaus at 5 minutes and leads to a decelerated maturation of oral Langerhans cells in parallel with an enhanced migratory capacity and increased production of tolerogenic cytokines that include IL-10 and TGF-β.

In a randomized controlled trial of sublingual immunotherapy, despite local increases in numbers of FOXP3+ Treg cells in sublingual mucosal biopsy specimens, there was no change in local monocyte-derived DCs, although CD1a+ Langerhans cells were not examined specifically. However, the influence of allergen immunotherapy on subtypes of DCs in the blood circulation has been studied. PCR studies of peripheral blood samples taken before and after 4 months of sublingual immunotherapy was used to characterize changes in DC phenotype. A significant increase in the numbers of DCs with a DCreg phenotype was observed. The DCreg signature was reflected by an increase in mRNA expression for stabilin-1 and complement component 1Q (C1Q), as predicted from in vitro studies. Interestingly, this DCreg signature was observed only in those “responders” to immunotherapy, as reflected by a significant decrease in rhinoconjunctivitis symptoms. In support of these findings, a 1-year treatment with sublingual immunotherapy in children with mite allergy resulted in peripheral DCs that showed an immature phenotype and an increased capacity to produce IL-10 and decreased IL-12 levels.

BIOMARKERS OF RESPONSE TO ALLERGEN IMMUNOTHERAPY

International guidelines highlight the need for quantitative and validated measurements for potential biomarkers. A European
Academy of Allergy and Clinical Immunology Task Force reported a consensus statement on potential biomarkers of allergen immunotherapy. These were classified into 7 domains (Table I): (1) IgE (total IgE, sIgE, and sIgE/total IgE ratio), (2) IgG subclasses (allergen-specific IgG [IgG1] and sIgG4, including the sIgE/IgG4 ratio), (3) serum inhibitory activity for IgE (IgE-FAB), (4) basophil activation, (5) cytokines and chemokines, (6) cellular markers (Treg cells, Breg cells, and DCs), and (7) in vivo biomarkers, which include provocation tests.

**IgE (total IgE, sIgE, and sIgE/total IgE ratio)**

Inclusion criteria for initiation of immunotherapy rely on a history of symptoms on exposure to allergen and increased serum sIgE levels to the clinically relevant allergen, as measured by using the ImmunoCAP system. Patient selection has been refined by the availability of recombinant allergen technology to identify specific IgE to the major allergen determinants and to recognize irrelevant cross-reacting allergens. For example, with a patient high IgE levels to Phl p 1 and Phl p 5 might be a good candidate for grass pollen immunotherapy. In contrast, IgE sensitivity to the grass pollen profilin Phl p 12 might result in high increased IgE levels and false-positive skin test responses to whole birch pollen extract because of cross-reactivity with the birch profilin Bet v 2.

An initial early increase in sIgE levels during both subcutaneous and sublingual pollen immunotherapy has been shown to be followed by blunting of seasonal increases in sIgE levels. In long-term studies of subcutaneous immunotherapy, a gradual decrease in sIgE levels over several years was observed, although there was no clear association between changes in sIgE levels and the magnitude of the clinical response. The ratio of specific IgE/total serum IgE at baseline was reported to correlate with clinical response to immunotherapy, although others have not replicated these findings.

**IgG subclasses (sIgG1, sIgG4, and sIgE/IgG4 ratio)**

Immune-reactive IgG1 and IgG4 antibodies can be measured by using ImmunoCAP (Fig 4, A) and by using an allergen microarray (eg, ImmunoSolid Allergen Chip Assay [ISAC]). Allergen-specific IgG subtypes, including IgG1 and particularly IgG4 have been shown to be increased in the range of 10- to 100-fold compared with baseline values during immunotherapy, although with no consistent correlation with clinical response to treatment. ISAC can be performed by using very small volumes of serum or nasal fluid. A large component of the observed increase in serum allergen-specific IgG or IgG4 levels after allergen immunotherapy is likely to reflect high allergen exposure and could be used potentially to monitor patients’ adherence to immunotherapy regimens. A decrease in the sIgE/IgG4 ratio has been reported after subcutaneous immunotherapy and was associated with a reduction in late cutaneous skin reactions. However, this finding has not been reproduced in other studies.

**Serum IgE inhibitory activity (IgE-FAB and enzyme-linked immunosorbent–facilitated antigen-binding assay)**

IgG-associated IgE-inhibitory activity can be assessed by using a flow cytometry–based assay (IgE-FAB) that has been validated according to the International Conference on Harmonisation guidelines. This assay measures the ability of IgG-containing serum obtained after allergen immunotherapy to inhibit the FceRII-dependent binding of allergen-IgE complexes to B cells, a surrogate for IgE-facilitated antigen presentation to T cells (Fig 4, B). An alternative approach is the enzyme-linked immunosorbent–facilitated antigen-binding assay (ELIFAB). The IgE-FAB assay is reproducible but technically more complex and is currently confined to specialized laboratories. Limited data available suggest a modest correlation between IgE-FAB and
ELIFAB results and the clinical response to immunotherapy over and above that observed when simply measuring immunoreactive IgG levels.43,59 This is likely related to IgE-FAB and ELIFAB, providing a further functional measure of affinity and/or avidity of antibody binding.

Basophil activation
In flow cytometry–based assays using whole blood, basophil activation can be studied by monitoring expression of surface markers, such as CD63 and CD203c. Although CD63 expression measures basophil degranulation,111 CD203c is a specific basophil marker that also measures IL-3–dependent activation of basophils. A novel functional assay that detects intracellular staining of phycoerythrin-conjugated diamine oxidase (DAO) has also been validated. DAO binds tightly to its substrate histamine, such that allergen stimulation results in a reduction in basophil intracellular DAO levels proportional to the amount of intracellular histamine released. This reduction has been detected during both subcutaneous and sublingual immunotherapy (Fig 4, C and D).112,113

Cytokines and chemokines
Recent advances in miniaturized multiplex cytokine analysis with the Meso Scale Discovery and Luminex platforms have enabled the measurement of cytokines and chemokines in nasal fluid that increase in response to allergen provocation and are modified by immunotherapy.69,70

Cellular and molecular markers
Cellular markers of potential use for assessing or predicting response to immunotherapy include phenotypic markers for T cells (Th2, Treg, Th17/TFR, and Th1 cells) and subpopulations of Breg cells, all of which have been shown to be modified during immunotherapy, principally by using flow cytometry. Although in clinical trials these markers have been able to distinguish between treatment groups and correlate overall with clinical outcomes of
efficacy, they have been unable to distinguish responders from nonresponders or predict response in individual subjects. Furthermore, there is a need for optimal cell processing and transfer and storage of samples such that complex flow cytometry for multiple T and B cell–associated markers is beyond the scope of routine clinical laboratories.

DCs express distinct molecular markers according to their T cell–differentiating capacity. DCregs preferentially express C1Q and FcγRIII, which favor preferential Treg cell development, whereas DC2s express CD141, GATA-3, OX40 ligand, and RIPK4, which favor polarizing naïve T cells into T\(\text{T}_{\text{H}2}\) cells. Expression of these markers in PBMCs was evaluated before and at 2 and 4 months after sublingual grass pollen immunotherapy. This quantitative RT-PCR–based method correlated with clinical outcomes. Remarkably, an optimal combination of 5 molecular markers that included 3 DC2 markers (CD141, GATA-3, and RIPK4) and 2 DCreg markers (C1QA and FcγRIIA) was able to distinguish clinical responders from nonresponders with a sensitivity of 90.48% and a specificity of 61.9%. These interesting results demand further evaluation in clinical trials and ultimately in clinical practice.

**In vivo biomarkers**

In vivo biomarkers refer to the use of allergen provocation tests to evaluate patients’ allergen-specific reactivity before and after treatment. Provocation tests include skin prick tests, intradermal tests, and nasal, conjunctival, and bronchial provocation tests. For example, the magnitudes of early and late responses after grass pollen nasal challenge and after intradermal testing were inhibited by both subcutaneous and sublingual immunotherapy. A modest correlation was observed between recorded total nasal symptom scores after challenge, the late skin response, and participants’ subjective assessment of hay fever severity during the pollen season. The European Medicines Agency has elaborated on the use of provocation testing for proof of concept for novel approaches, allergen dose finding, and use as supportive secondary efficacy end points during clinical trials of allergen immunotherapy.

**Summary**

In the context of a clinical history of symptoms on exposure to relevant inhaled allergens, the serum sIgE level is the single most relevant biomarker for selection of patients for allergen immunotherapy, and this has been refined by the availability of recombinant allergen technology. However, at present, the level of sIgE is unable to reliably predict or monitor the clinical response to immunotherapy. At present, the ratio of IgE/to total IgE at baseline remains under evaluation as a possible predictor of response. Functional assays of IgG-associated inhibitory activity for IgE (IgE-FAB and ELIFAB) have better correlation with clinical response in clinical trials than immunoreactive IgG/IgG\(_4\) levels but do not predict efficacy in individual subjects. Serum-based assays have the advantage of ease of sample handling and storage, and it seems likely that IgG/IgG\(_4\) levels might be more effective as a surrogate for compliance with treatment, which could be of particular value for monitoring patients receiving sublingual immunotherapy.

The various cellular assays reported are restricted to specialist centers. Basophil responsiveness assays and T/B-cell phenotypic assays require flow cytometry and involve, respectively, either processing of fresh blood or complex cell separation and storage protocols. They are informative for proof of concept in clinical trials but are not feasible for routine clinical practice.

Studies of DC phenotype by use of RT-PCR on whole blood or PBMCs have been shown to separate clinical responders and nonresponders to grass pollen sublingual immunotherapy, and further studies are needed to replicate these findings and assess their value in individual patients.

**NOVEL APPROACHES FOR IMMUNOTHERAPY**

A better understanding of mechanisms should translate ideally into novel immunotherapy approaches. The aim has been to improve efficacy over standard allergen extract–based extracts while permitting shorter, safer, and more convenient strategies for patients. Alternative routes, such as epicutaneous and intralymphatic approaches, have proved safer than conventional subcutaneous immunotherapy, although there are no head-to-head trials to assess comparative efficacy. The intradermal route was ineffective for grass pollen allergen and might have exacerbated seasonal symptoms. Targeting immune deviation using the Toll-like receptor 4 agonist monophosphoryl lipid A in combination with subcutaneous grass pollen allergoid immunotherapy was effective with 4 preseasonal injections without an increase in side effects. A trial of bacterial DNA oligonucleotides rich in CpG sequences covalently linked to the major ragweed allergen Amb a 1, was effective in a phase II trial, possibly by inducing Treg cells and/or immune deviation, although this approach failed at phase III. Targeting IgE or type 2 cytokines (IL-4 and IL-5) has been successful in reducing exacerbations in asthmatic patients, although without durable effects after discontinuation. Anti-IgE in combination with allergen immunotherapy was highly effective in reducing the risk of systemic allergic reactions. The combination of anti–IL-4 with subcutaneous allergen immunotherapy was effective in suppressing circulating T\(\text{T}_{\text{H}2}\) cells and allergen-induced late responses but showed no advantage over allergen extract alone. Current novel approaches to reduce systemic adverse events include the use of engineered recombinant hypoallergenic molecules and allergen peptide–based approaches that specifically target either T-cell epitopes or B-cell epitope–based strategies that selectively promote allergen-specific IgG responses.

For the future, targeting the innate immune response using antibodies directed against the epithelial cytokines IL-33, IL-25, or thymic stromal lymphopoietin in combination with allergen immunotherapy would be an attractive combination strategy to likely reduce inflammation, suppress ILC2s, and promote a more tolerogenic DC phenotype.

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