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/IgG$_4$ antibodies that can inhibit IgE-dependent activation mediated through both high-affinity IgE receptors (FcεRI) on mast cells and basophils and low-affinity IgE receptors (FcεRII) on B cells. Suppression of T$_H$2 immunity can occur as a consequence of either deletion or anergy of antigen-specific T cells; induction of antigen-specific regulatory T cells; or immune deviation in favor of T$_H$1 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 T$_H$2 lymphocytes
DAO:	Diamine oxidase
DC:	Dendritic cell
DCreg:	Regulatory dendritic cell
ELIFAB:	Enzyme-linked immunosorbent–facilitated antigen-binding assay
FOXP3:	Forkhead box P3
ILC:	Innate lymphoid cell
ILC2:	Group 2 innate lymphoid cell
iTreg:	Inducible regulatory T
LT:	Leukotriene
nTreg:	Natural regulatory T
RIPK4:	Receptor-interacting serine/threonine-protein kinase 4
sIgE:	Allergen-specific IgE
sIgG:	Allergen-specific IgG
T$_{FH}$:	Follicular helper T
T$_{FR}$:	Follicular regulatory T
Treg:	Regulatory T
VLA4:	Very late antigen 4, integrin α4β1

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Allergen immunotherapy is effective in selected patients with allergic rhinitis, including those with mild/moderate asthma.[1,2] There is heterogeneity in the populations studied, the different allergen products and protocols used, and the clinical outcomes used to document efficacy and safety.[3] Nonetheless, recent guidelines[4] 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.[5]

Subcutaneous immunotherapy involves weekly updosing injections, followed by monthly maintenance injections for at least 3 years.[1,6,7] 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.[1,8] Sublingual and subcutaneous immunotherapy are effective generally within 2 to 4 months of initiating treatment and can be given presesonally/coseasonally for short-term benefit. Indirect

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Terms in boldface and italics are defined in the glossary on page 1486.

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; LTC_4, LTD_4, and the terminal metabolite LTE_4),[12] platelet-activating factor, and prostaglandin D_2. The biological properties of these mediators are consistent with the local vasodilatation, edema formation, local neurogenic stimulation, and mucus secretion 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_H1 and CD8 cytotoxic T lymphocyte effector T cells once antigen-specific immunity develops.

ImmunoCAP (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-37) in response to antigenic stimulation and has been shown to be required for differentiation of naive T cells into T_H1 cells.

IL-21: A cytokine expressed in human $CD4^+$ T cells and found to be upregulated in T_H2 and T_H17 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 T_H2-type immune response. IL-25 can induce nuclear factor κB activation and stimulate IL-8 production.

IL-33: A member of the IL-1 family of cytokines expressed on T_H2 cells, mast cells, and group 2 innate lymphocytes that potently drives production of T_H2-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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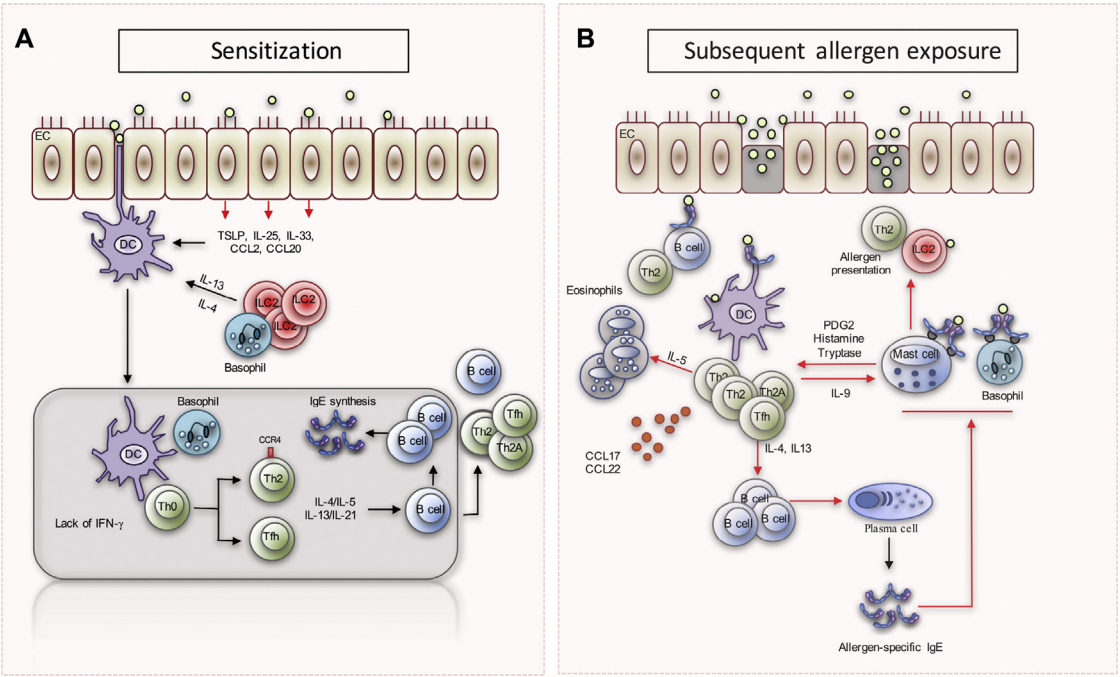

FIG 1. **A** and **B,** Mechanisms of allergic inflammation: summary of immunologic response to initial triggers of allergic sensitization and allergic inflammation after re-exposure to inhalant allergens (see text). *EC,* Epithelial cells.

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]

T_H2 lymphocytes and group 2 innate lymphoid cells

The above pathophysiologic events are under the regulation of a distinct subset of T_H2 cells. T_H2 cells produce IL-4, the key cytokine responsible for T_H2 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 T_H2 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 T_H2 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 T-box transcription factor and produce the T_H1 cytokines ***IFN-γ*** and TNF and provide protection against intracellular bacteria and parasites. ILC2s constitutively express RAR-related orphan receptor α and GATA-3; produce T_H2 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 rhinitis[26] and asthmatic patients[27] during the grass pollen season. ILC2s represent an abundant alternative source of T_H2 cytokines and likely serve to amplify and maintain local T_H2-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 T_H2 lymphocytes, T_H2 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 aeroallergens 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 T_H2 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 T_H cells

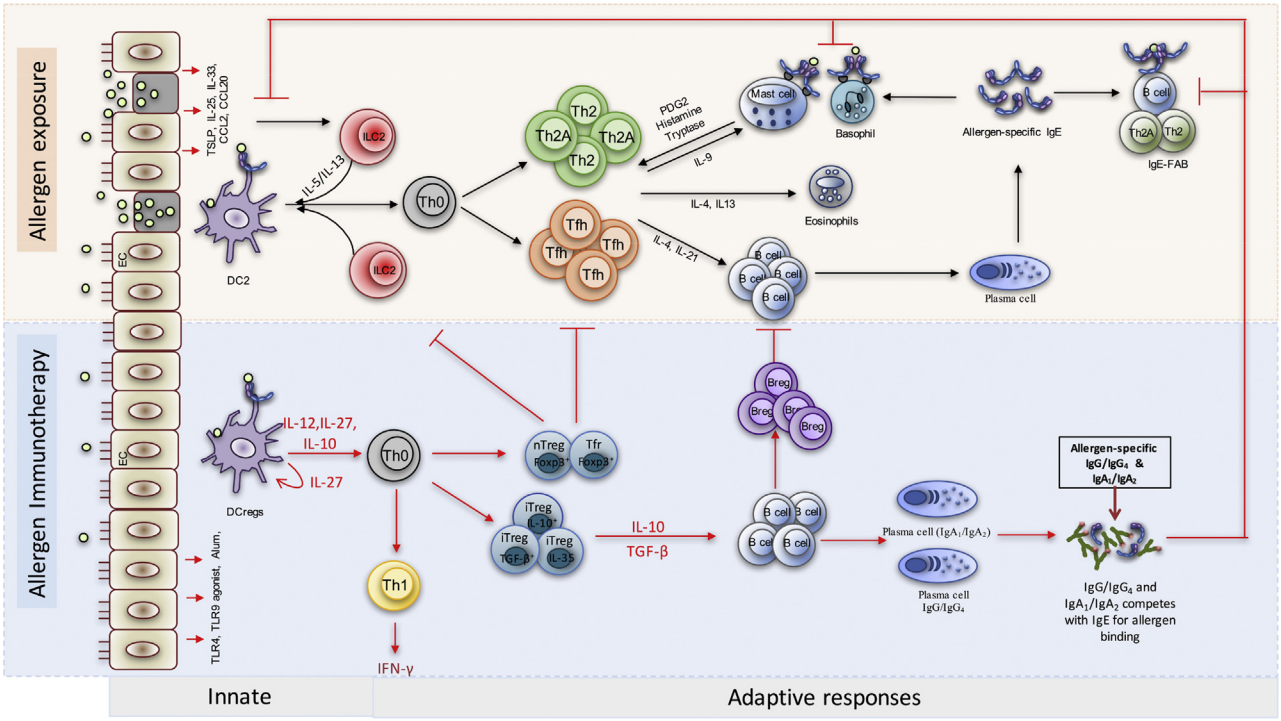

FIG 2. Mechanisms of AIT. During the initial sensitization phase in patients with allergic rhinitis, low allergen exposure at the nasal mucosal surface results in activation of epithelial cells, which then activate DCs. DCs uptake and present antigens to naive T cells to induce allergic T_H2 *(Th2A)* responses and IgE-facilitated antigen presentation. Subsequent allergen re-exposure leads to mast cell and basophil degranulation, causing classic early-phase reactions. Subsequent infiltration of other leukocytes leads to late-phase allergic inflammation. High-dose allergen exposure by immunotherapy restores DC function, which produces IL-12, IL-27, and IL-10 and promotes immune deviation from a T_H2 to T_H1 response and induction of Treg and Breg cells (including other B-cell subsets) that produce IgA, IgG, and IgG_4 blocking antibodies. Suppressive activities of Treg cells, Breg cells, and IgG-blocking activity is indicated by *red arrows*. *EC*, Epithelial cells; *TLR*, Toll-like receptor.

differentiates into follicular helper T (T_{FH}) cells. T_{FH} cells produce both IL-4 and ***IL-21***, which, along with T_H2 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***,[29] ***IL-33***,[30] and ***thymic stromal lymphopoietin*** protein.[31] These epithelial cytokines favor development of a proallergic DC phenotype[32,33] that provides help for T_H2 cell differentiation. Additionally, these epithelially derived cytokines are major growth factors for ILC2s that amplify and maintain local T_H2-driven allergic inflammation.[34-36] During subsequent allergen exposure, IgE-facilitated allergen recognition through FcεRI on DCs and FcεRII on B cells amplifies the development of T_H2 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)[32,33,37,38] or alternatively promote a state of immune tolerance (tolerogenic regulatory dendritic cells [DCregs])[32,33,39-42] to sensitizing allergens. DC2s express the markers CD141, GATA-3, OX40 ligand, and receptor-interacting serine/threonine-protein kinase 4 (RIPK4).[33] When DC2s were exposed to allergen and cocultured subsequently with T cells, they promoted preferential T_H2 T-lymphocyte responses.[35-39]

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.[43] These early increases are not accompanied by untoward side effects, and it has been suggested that early T_H2 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,[44,45] an event that might contribute to long-term tolerance.

In 1935, Robert Cooke and colleagues[46] 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, IgG_4, and IgA fractions.[47-51] Studies have shown 10- to 100-fold increases in serum concentrations of IgG,

particularly IgG_4.[7,52-55] Sublingual immunotherapy has also been shown to induce allergen-specific IgG_1,[56] IgG_4, and IgA antibodies.[10,49,57-59] These increases in levels of immunoreactive antibodies have been observed after immunotherapy to both seasonal pollens and perennial allergens, such as house dust mite.[60,61] Serum specific IgG_4 levels have been shown to increase in a time- and dose-dependent manner during grass pollen immunotherapy.[59]

Several studies have highlighted the inhibitory capacity of IgG_4 for IgE-dependent events. IgG_4 antibodies are bispecific and have the capacity to exchange F(ab) arms by swapping heavy-light chain pairs between IgG_4 molecules with diverse specificities.[62] IgG can compete with IgE for allergen,[63] 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/IgG_4 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 T_H2 responses.[16,64-66]

Paradoxically, although immunoreactive IgG/IgG_4 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.[49] 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.[53] Both specific IgG_4 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.[43] The IgG_4 dependency of IgE-inhibitory activity has been shown in depletion experiments using IgG_4 affinity chromatography. The magnitude of IgE suppression was greater with nasal fluid than with serum, thereby highlighting the potency of local IgG inhibitory antibodies.[52]

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.[57,67] Suppression is accompanied by a reduction in early increases in local nasal histamine, tosyl L-arginine methyl ester–esterase, and tryptase concentrations in nasal fluid. Inhibition of late responses is associated with a decrease in eosinophil numbers[68] and levels of T_H2 cytokines, including IL-4, IL-5, IL-9, and IL-13.[69,70] A reduction after immunotherapy is also noted in nasal fluid levels of the CC chemokine eotaxin, which contributes to eosinophil recruitment. A double-blind trial of subcutaneous grass pollen immunotherapy resulted in decreases in numbers of effector cells, including $CD117^+$ (***c-kit***+) mast cells,[71] basophils,[72] and eosinophils,[73] in the nasal mucosa compared with pretreatment numbers that were significant compared with placebo-treated participants.[72,74] These local changes detected in nasal biopsy specimens were 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.[72] 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 T_H2 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.[74] These findings are supported by the observation of decreases in T_H2 cytokine levels in nasal lining fluid after nasal challenge.[69] Recent techniques that include *ex vivo* tetramer analysis[75-78] have enabled the phenotyping of peripheral circulating allergen-specific T cells.[79-82] This has permitted identification of key T-cell surface markers, such as CD27, chemoattractant receptor–homologous molecule expressed on T_H2 lymphocytes (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.[75-78] This was in contrast to nonallergic subjects, whose T cells expressed low levels of CRTH2 and CCR4 and high levels of CD27. Patients with alder pollen allergy expressed a high frequency of $CD27^-$ T_H2 cells that decreased after subcutaneous immunotherapy. Similarly, in the Gauging Responses in Allergic Rhinitis to SCIT versus SLIT Trial (GRASS),[45,70] both subcutaneous and sublingual immunotherapy resulted in clinical improvement during 2 years that was paralleled by a decrease in peripheral tetramer-positive $CRTH2^+CCR4^+CD27^-CD4^+$ T_H2 cell numbers. These changes were paralleled by a decrease in levels of local nasal T_H2 cytokines, including IL-4, IL-5, and IL-13, in nasal fluid after nasal allergen provocation. Both numbers of circulating tetramer-positive T_H2 cells and levels of local nasal T_H2 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)[9,10] to induce durable tolerance might have been related to this re-emergence of antigen-specific T_H2 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.[83-88] 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***.[59,79,83]

nTreg cells. nTreg cells were first described by Sakaguchi et al.[80] In addition to the transcription factor FOXP3, nTreg cells have increased expression of the IL-2 receptor (CD25) and low

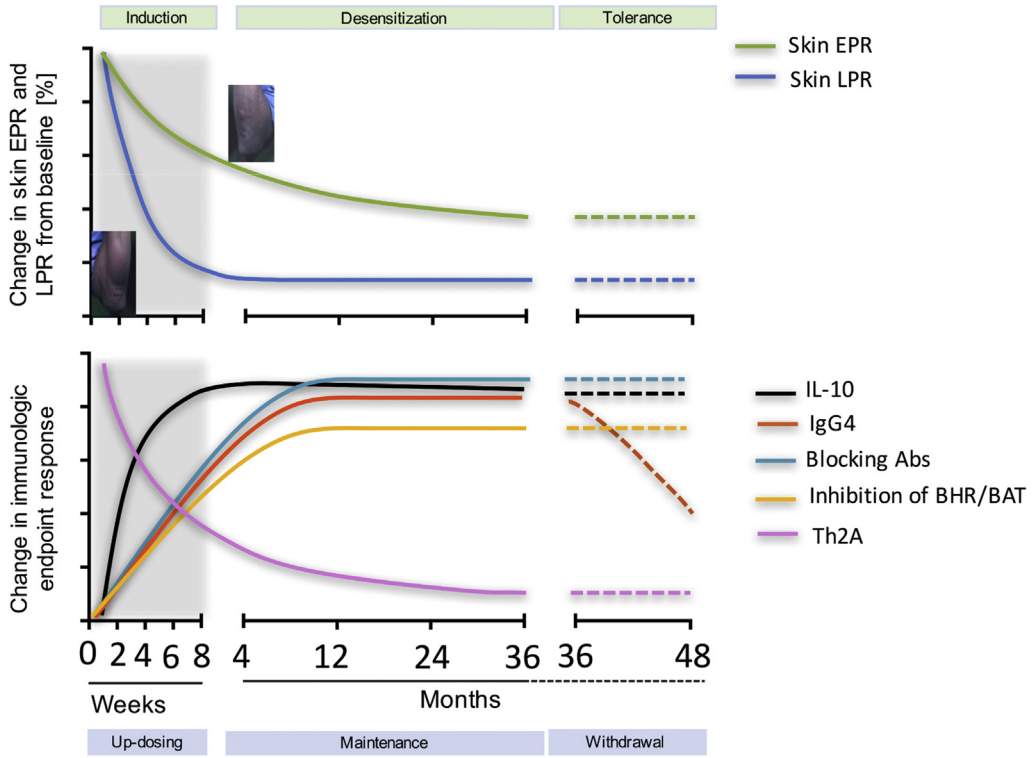

FIG 3. Time course of the effect of immunotherapy on surrogate clinical markers (early and late cutaneous responses) and associated immunologic events during the induction and maintenance phases of immunotherapy (desensitization), as well as their persistence after withdrawal of treatment (tolerance phase). *Abs*, Antibodies; *BAT*, Basophil Activation Test; *BHR*, basophil histamine release response; *EPR*, early-phase response; *LPR*, late-phase response.

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***, surface-bound TGF-β, the glucocorticoid-induced TNF receptor, and ***programmed cell death 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[84] 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.[87] 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]

T_{FH}/T_{FR} cells. T_{FH} 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***. T_{FH} 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 (T_{FR}) cells. One study has shown that memory T_{FH} cells were significantly reduced after immunotherapy.[95] Moreover, T_{FR} cells from immunotherapy-treated patients were shown to have higher capacity to produce IL-10 compared with T_{FH} cells. When $CXCR5^+$ T_{FH} 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 T_{FR} cell numbers. These findings highlight the plasticity of T_{FR} cells and their likely role in suppressing T_H2 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.[83] 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 IgG_4 levels.[96] Seasonal increases in numbers of $TGF\text{-}\beta^+$ T cells in the nasal mucosa correlated with increases in peripheral circulating IgA concentrations.[51]

Induction of peripheral $IL\text{-}10^+$ 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 updosing, when cocultured with grass pollen allergen for 6 days, produced high levels of IL-10 in supernatants.[57] 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 IgG_4 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 IgG_4 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 updosing,[57] and maintenance of grass pollen immunotherapy for 3 years and during immunotherapy withdrawal[49] 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.[97] 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.[98] Moreover, the provenance of allergen-specific IgG_4 antibodies after bee venom immunotherapy was shown to be from phospholipase A_2–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.[97] 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.

T_H1 immune deviation. Suppression of T_H2 immunity during both subcutaneous and sublingual immunotherapy has also been associated with immune deviation and induction of T_H1 cells.[9,99] *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.[9] 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.[52] 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.[100] Evidence for/against T_H1 deviation in peripheral blood studies has been more controversial.[101,102] One study suggested that the shift from T_H2 to T_H1 responses might have been related to activation-induced cell death of allergen-responder T_H2 cells.[103] 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/IFN-γ–secreting T cells.[104,105] Moreover, survival of T_H1 cells has been reported after deletion of T_H2 cells.[106]

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.[26] 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,[27] 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 T_H2-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.[107]

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.[87] 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 grass pollen immunotherapy was used to characterize changes in DC phenotype. A significant increase in the numbers of DCs with a DCreg phenotype was observed.[32] 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.[32] Interestingly, this DCreg signature was observed only in those "responders" to immunotherapy, as reflected by a significant decrease in rhinoconjunctivitis symptoms.[32] 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.[102]

BIOMARKERS OF RESPONSE TO ALLERGEN IMMUNOTHERAPY

International guidelines highlight the need for quantitative and validated measurements for potential biomarkers.[108] A European

TABLE I. Six domains of biomarkers: (1) antibodies, (2) serum inhibitory activity for IgE, (3) basophil activation, (4) cytokines and chemokines, (5) cellular biomarkers, and (6) in vivo biomarkers

Domains	Biomarkers	References
Antibodies	IgE (sIgE, total IgE, sIgE/total IgE)	44, 51, 59, 96, 109, 110
	$sIgG_4$	83, 95, 106
	IgA	51, 87
Serum Inhibitory activity for IgE	IgE-FAB	59
	ELIFAB	43
Basophil activation	CD63	111, 115-117
	CD203c	112, 118
	DAO	16, 112, 113
	Basophil histamine release	16, 112, 113
Cytokines and chemokines	T_H2: IL-4, IL-13, IL-9, IL-17, eotaxin, TNF-α	99, 119-122
	T_H1: IFN-γ, IL-12	99
	Regulatory: IL-10, TGF-β	83
Cellular biomarkers	Treg cells	83-88
	Breg cells	123, 124
	DCs	32, 33, 102, 114
In vivo biomarkers	Allergen provocation tests (SPT, ID, NPT, and EEC)	9, 72, 125-127
	Chamber studies	128, 129

EEC, Environmental exposure chamber; *ID,* intradermal test; *IgE-BF,* IgE blocking factor; *NPT,* nasal provocation test; *SPT,* skin prick test.

Academy of Allergy and Clinical Immunology Task Force reported a consensus statement on potential biomarkers of allergen immunotherapy.[101] These were classified into 7 domains (Table I)*: (1) IgE (total IgE, sIgE, and sIgE/total IgE ratio), (2) IgG subclasses (allergen-specific IgG $[IgG]_1$ and $sIgG_4$, including the $sIgE/IgG_4$ 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.[108]

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[1,130,131] 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.[132] For example, a patient with 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[59] and sublingual[109] 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[44] was observed, although there was no clear association between changes in sIgE levels and the magnitude of the clinical response.[51,96] The ratio of specific IgE/total serum IgE at baseline was reported to correlate with clinical response to immunotherapy,[110,133] although others[53,60,134] have not replicated these findings.

IgG subclasses ($sIgG_1$, $sIgG_4$, and $sIgE/IgG_4$ ratio)

Immune-reactive IgG_1 and IgG_4 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 IgG_1 and particularly IgG_4 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.[83,95,106] 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 IgG_4 levels after allergen immunotherapy is likely to reflect high allergen exposure and could be used potentially to monitor patients' adherence to immunotherapy regimens.[108] A decrease in the $sIgE/IgG_4$ ratio has been reported after subcutaneous immunotherapy and was associated with a reduction in late cutaneous skin reactions.[101] However, this finding has not been reproduced in other studies.[101]

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.[135] This assay measures the ability of IgG-containing serum obtained after allergen immunotherapy to inhibit the FcεRII-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).[43] The IgE-FAB assay is reproducible but technically more complex and is currently confined to specialized laboratories.[59] Limited data available suggest a modest correlation between IgE-FAB and

*References 9, 16, 32, 33, 43, 44, 51, 58, 72, 83-88, 95, 96, 99, 102, 106, and 109-129.

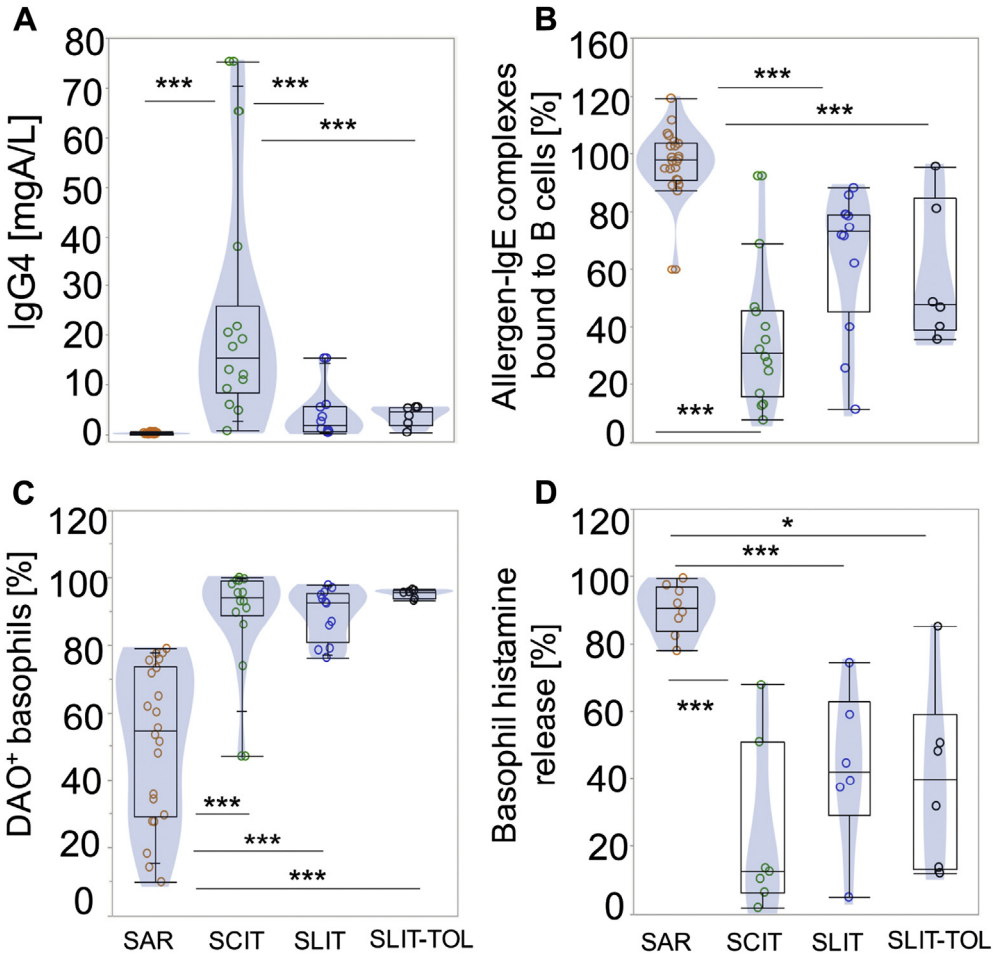

FIG 4. Induction of IgG_4 antibodies and associated IgE-inhibitory activity during immunotherapy. **A,** Specific IgG_4 levels. **B,** IgE-facilitated allergen binding to B cells. **C,** Histamine release at the single-cell level using labeled DAO (increased intracellular DAO demonstrates inhibition of histamine release). **D,** Histamine ELISA. Measurements were from untreated patients with grass allergy *(SAR)*, subcutaneous immunotherapy *(SCIT)*– and sublingual immunotherapy *(SLIT)*–treated patients, and those who had completed 3 years of SLIT, followed by discontinuation for up to 3 years *(SLIT-TOL)*. Data are expressed as individual data (quintile box plots with contour). $*P < .05$ and $***P < .001$, Mann-Whitney *U* test.[16]

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 (T_H2, Treg, T_{FH}/T_{FR}, and T_H1 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,[69,83,88,136,137] 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,[32] whereas DC2s express CD141, GATA-3, OX40 ligand, and RIPK4, which favor polarizing naive T cells into T_H2 cells.[33] 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.[33,114] Remarkably, an optimal combination of 5 molecular markers that included 3 DC2 markers (CD141, GATA-3, and RIPK4) and 2 DCreg markers (C1QA and FcγRIIIA) 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.[101] 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.[69,70] 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.[138]

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.[132] 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/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.[1,139] 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[140] and intralymphatic[141,142] 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.[143] 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.[144] 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,[145] although this approach failed at phase III.[94] 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.[146] Anti-IgE in combination with allergen immunotherapy was highly effective in reducing the risk of systemic allergic reactions.[147] The combination of anti–IL-4 with subcutaneous allergen immunotherapy was effective in suppressing circulating T_H2 cells and allergen-induced late responses but showed no advantage over allergen extract alone.[148] Current novel approaches to reduce systemic adverse events include the use of engineered recombinant hypoallergenic molecules[132] and allergen peptide–based approaches that specifically target either T-cell epitopes[149,150] or B-cell epitope–based strategies that selectively promote allergen-specific IgG responses.[151,152] 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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