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The Use of Biomarkers to Predict Aero-Allergen and Food Immunotherapy Responses

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Abstract

The incidence of allergic conditions has continued to rise over the past several decades, with a growing body of research dedicated toward the treatment of such conditions. By driving a complex range of changes in the underlying immune response, immunotherapy is the only therapy that modulates the immune system with long-term effects and is presently utilized for the treatment of several atopic conditions. Recent efforts have focused on identifying biomarkers associated with these changes that may be of use in predicting patients with the highest likelihood of positive clinical outcomes during allergen immunotherapy (AIT), providing guidance regarding AIT discontinuation, and predicting symptomatic relapse and the need for booster AIT after therapy. The identification of such biomarkers in food allergy has the additional benefit of replacing oral food challenges, which are presently the gold standard for diagnosing food allergies. While several markers have shown early promise, research has yet to identify a marker that can invariably predict clinical response to AIT. Skin prick testing (SPT) and specific IgE have commonly been used as inclusion criteria for the initiation of AIT and prediction of reactions during subsequent allergen challenge; however, existing data suggests that changes in these markers are not always associated with clinical improvement and can be widely variable, reducing their utility in predicting clinical response. Similar findings have been described for the use of allergen-specific functional IgG4 antibodies, basophil activation and histamine release, and type 2 innate lymphoid cells. There appears to be a promising association between changes in the expression of dendritic cell-associated markers, as well as the use of DNA promoter region methylation patterns in the prediction of allergy status following therapy. The cellular and molecular changes brought about by immunotherapy are still under investigation, but major strides in our understanding are being made.

Keywords Biomarkers · Immunotherapy · Prognostic · Allergen · Food allergy

Introduction

As defined by the National Institute of Allergy and Infectious diseases, allergic diseases comprise symptomatic conditions such as asthma, atopic dermatitis, and food allergy (FA). The incidence of these allergic conditions has continued to rise over the past few decades, with an increasing number of individuals having more than one of these allergic conditions. For patients whose symptoms are not abated by conventional pharmacotherapies such as nasal glucocorticoids and antihistamines, allergen immunotherapy (AIT), in the form of subcutaneous immunotherapy (SCIT) or sublingual immunotherapy (SLIT), is the only safe and effective option [1, 2] that reduces symptoms and the need for rescue medications [3–5], improves quality of life, [6] and could provide long-term clinical benefits after cessation of treatment [7–9]. AIT is the only FDA-approved therapy that modifies the underlying immune response in IgE-mediated diseases, such as allergic rhinitis, allergic asthma, stinging insect hypersensitivity, and atopic dermatitis [1, 10–13]. Presently, immunotherapy is under research for its application in IgE-mediated FA, and although not FDA-approved, it is gaining popularity in off-label use.

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The Immune Response in Allergic Disease

Allergic diseases are due to a dysregulated immune system [14–17] that is associated with an increase in inflammation and the formation of specific IgE antibodies against otherwise harmless environmental and food antigens [18, 19]. The inflammatory response is Type 2 T helper (Th2) cell mediated and involves both the innate and the adaptive immune arms [19]. The production of several cytokines is associated with this response, including a cluster of cytokines encoded on chromosome 5q31–33: interleukin (IL)-3, IL-4, IL-5, IL-9, and IL-13 [20]. IL-17 is produced by Type 17 T helper (Th17) cells [21–23], and IL-25, IL-31, IL-33, and thymic stromal lymphopoietin (TSLP) are produced by tissue cells [20].

There are two phases to the immune response in allergic diseases, including the sensitization phase and the effector phase [24–26]. During the sensitization phase (Fig. 1), specific mucosal-resident dendritic cell (DC) subsets capture allergens in the skin, airways, or gut and subsequently internalize and transport the allergens to draining lymph nodes [18]. Within the lymph nodes, the antigens are processed and presented to naïve $CD4^+$ T cells by MHC class II molecules [18] leading to their differentiation into allergen-specific $CD4^+$ Th2 cells which produce high levels of IL-4 and IL-13. Activation of $CD4^+$ Th2 cells occurs through phosphorylation of the *trans*-acting T cell–specific transcription factor GATA-3 [14, 18], driving the production of IgE isotypes by B cells [18]. Natural killer T (NKT) cells and basophils contribute to the process of sensitization by producing IL-4 early in the process [18, 27]. As the IgE memory B cells mature, they differentiate into plasma cells and begin to produce large amounts of allergen-specific IgE antibodies (sIgE). These sIgE antibodies bind to the high-affinity FcεRI receptors on the surface of basophils and mast cells. Following the sensitization phase, subsequent exposure to allergen results in crosslinking of the surface bound sIgE to the allergen and activation of mast cells and basophils, leading to clinical symptoms [18].

Resident tissue cells also contribute to allergen sensitization. While epithelial cells normally form a barrier, providing the first line of defense against environmental allergens, this barrier function is compromised in allergic individuals, allowing allergens to enter the submucosa. This entry results in chronic inflammation and contributes to further sensitization [26, 28, 29]. Additionally, epithelial cells are able to promote sensitization through the production of various proinflammatory cytokines, including IL-25, IL-31 or TSLP, and IL-33, that act on DCs and type 2 innate lymphoid cells (ILC2s) to further promote a Th2-skewed response [30, 31].

Following the sensitization phase, the effector phase (Fig. 2) is initiated when an allergen is presented and causes cross-linking of the sIgE bound to the FcεR1 receptor on sensitized mast cells and basophils [18]. Crosslinking of FcεRI receptors leads to the subsequent release of preformed and de novo synthesized proinflammatory mediators, such as

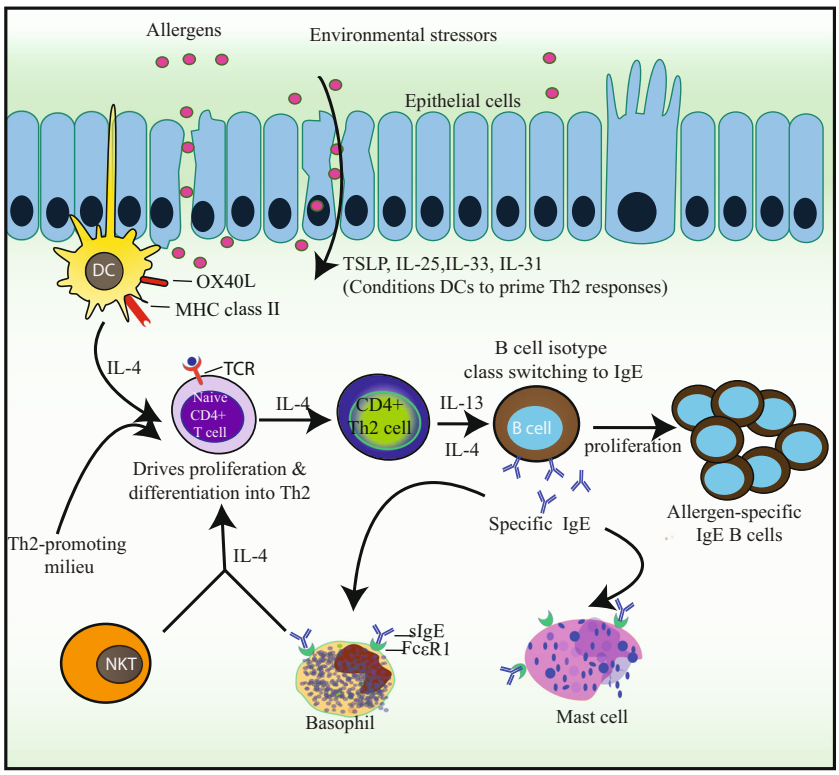

Fig. 1 Mechanism of sensitization phase in allergic disease. During the allergic sensitization phase, mucosal dendritic cells (DC) capture allergen, which are transported to neighboring lymph nodes after processing. Within the lymph nodes, allergen-specific Th2 cells proliferate in the presence of IL-4 and endothelial cell-derived Th2 cytokines. Natural killer T (NKT) cells and basophils may provide an early source of IL-4. Activated allergen-specific Th2 cells further produce IL-4 and IL-13 which favors B cell isotype class switching to specific IgE cells, which bind to the surface of effector cells such as mast cells and basophils through the high-affinity IgE receptor FcεR1 leading to sensitization. During this phase, a memory pool of allergen-specific B cells and allergen-specific Th2 cells are generated

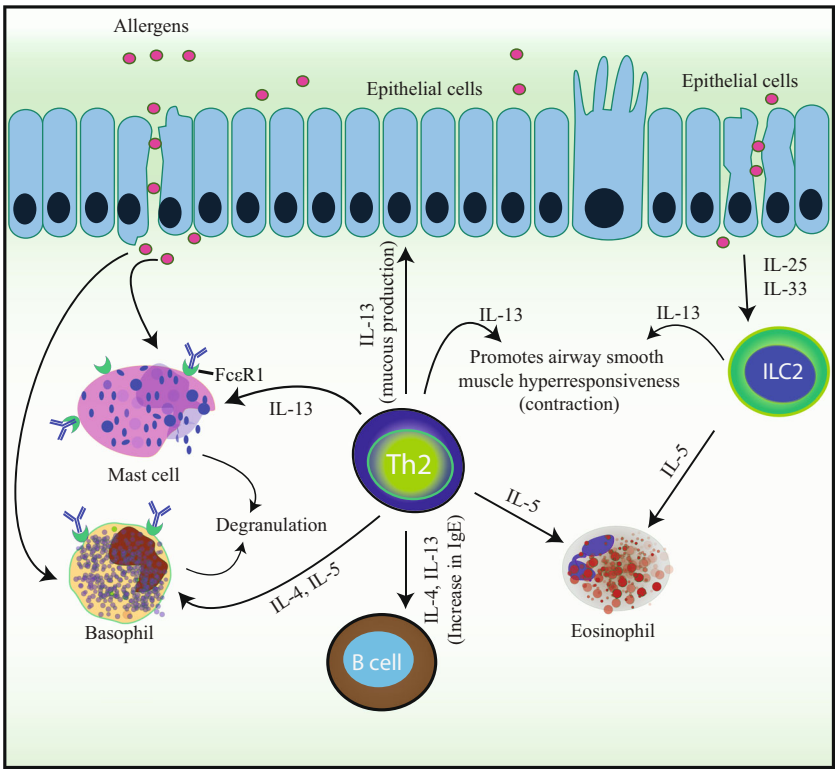

Fig. 2 Mechanism of effector phase in allergic disease. During the effector phase, subsequent encounters to a previously sensitized allergen leads to IgE cross-linking on and activation of basophils and mast cells. Basophil and mast cell degranulation leads to the release of anaphylactogenic mediators such as histamine, proteases, prostaglandins, leukotrienes, and cytokines which are the cause of the immediate symptoms and acute inflammation. As these cytokines accumulate, allergen-specific Th2 cells are activated and produce IL-4, IL-5, and IL-13 among other cytokines, which maintain allergen-specific IgE levels, eosinophilia, mucus production, and recruitment of inflammatory cells to inflamed tissues leading to tissue damage

histamine, heparin, and proteases, as well as prostaglandins, leukotrienes, and cytokines which are all responsible for the immediate phase reaction and acute inflammation [18]. The late-phase reaction is facilitated by the accumulation of the inflammatory mediators produced by the effector cells and the activation of memory allergen-specific Th2 cells. These Th2 cells produce IL-4, IL-5, IL-9, and IL-13, which promote eosinophilia, help maintain allergen-specific IgE levels, and recruit additional inflammatory cells to the tissues, worsening the inflammation and tissue damage [18].

Several additional effector cells participate in driving the ongoing allergic reactions. ILCs, especially ILC2, which are present in the sputum, nasal polyps, esophagus, and peripheral blood [32–34], control the mucosal environment [31]. Additionally, ILCs interact with surrounding tissue cells, such as ECs, and, through the induction and production of cytokines, promote and contribute to a pro-inflammatory milieu [35]. Type 1 helper (Th1) and NKT cells produce interferon-gamma (IFN-γ), which can either worsen the effector phase by inducing epithelial cell damage [36, 37], or it can counteract the allergic inflammation by opposing the Th2 response [38]. NKT cells, in conjunction with IL-17-producing Th17 cells, also enhance IL-8-mediated neutrophil recruitment [24], thus adding to the inflammation in allergic disease. Additionally, through the production of IL-9 and IL-10, Th9 cells further promote tissue inflammation [39]. Finally, Th22 cells have been found to contribute to allergic inflammation in atopic dermatitis by promoting epidermal hyperplasia [24, 40–42].

Two additional cell types, T regulatory cells (Tregs) and B regulatory cells (Bregs), play an essential role in maintaining tolerance in a healthy individual's immune system, and they are also key players in the restoration of tolerance after successful AIT [43, 44]. Information on Tregs and Bregs has recently been reviewed [45]. Briefly, tolerance to harmless food and environmental antigens are essential to prevent allergic diseases. In non-allergic individuals, the immune system maintains a fine balance between the proinflammatory state required to clear pathogens, and the immunosuppressive responses required to prevent chronic inflammation leading to tissue damage. The generation of tolerance to allergens occurs in several tissues including tonsils, GI mucosa, respiratory tract, skin and oral mucosa and leads to the generation of allergen-specific Tregs and Bregs [18] via different immune pathways.

Allergen Immunotherapy

AIT, either environmental or food, increases the threshold of reactivity or desensitizes individuals to allergens. Although maintenance of the state of desensitization appears to require regular allergen exposure in the majority of individuals with food allergy, in some individuals desensitization is maintained ever after a period of cessation of immunotherapy and allergen exposure. However, whether this indicates true tolerance,

defined as a permanent immunological change (a cure), as seen to occur naturally or whether the mechanisms underlying desensitization and natural tolerance differ is still unknown. As biomarkers to determine tolerance are currently unavailable, studies on food allergy are now evaluating the ability to successfully pass a food challenge after a defined period of allergen avoidance after cessation of active therapy (sustained unresponsiveness) [46]. As previously mentioned, environmental AIT is FDA approved for AR, asthma, and AD. Generally, the subcutaneous route of immunization has been the accepted technique; however, SLIT has also been approved as a non-invasive approach to treat allergic rhinitis [47]. New routes of immunization such as epicutaneous, intradermal, and intralymphatic modalities are currently under study [48, 49]. Regardless of the route of immunotherapy, although there are some differences, the same general mechanism of action is elicited [47] (Fig. 3).

The mechanisms behind the actions of AIT have mostly been explored in allergic rhinitis and insect hypersensitivity [50]; however, the same principles most likely apply behind food immunotherapy. For subjects with FA, food immunotherapy is currently under research with studies focusing on the degree of sustained unresponsiveness [51–53], as permanent tolerance has not yet been demonstrated. Over the past decade, research in food immunotherapy has surged [54], and oral immunotherapy (OIT) appears to be efficacious; however, the high rate of adverse events during therapy continues to drive research toward adjunct therapies and safer therapeutic alternatives to minimize reactions. SLIT, although well established in allergic rhinitis, is currently being explored for its efficacy in food immunotherapy, and several clinical trials have shown promising results since the first reported use in 2003 [55]. Epicutaneous immunotherapy (EPIT), a process in which the allergen is contained within a skin patch in soluble form and absorbed into the stratum corneum, is another method that is under exploration for the treatment of FA [51].

In addition to the route of immunotherapy, several potential adjunctive therapies are presently being explored, including the use of omalizumab, an anti-IgE monoclonal antibody. First studied as an adjunct in SCIT, omalizumab demonstrated significant reductions in adverse reactions [56, 57]. The potential role of omalizumab with OIT was first evaluated in a pilot study of 11 subjects with cow's milk allergy [58], and several subsequent studies have been published since [59, 60]. The addition of omalizumab accelerates OIT desensitization in high-risk patients and improves the safety profile [61].

Regardless of the indicated allergic disease or method by which AIT is employed, its efficacy is driven by a complex range of changes in the underlying immune response [14–17, 27]. Improving our characterization of these changes and the associated biomarkers would be of significant benefit in distinguishing patients who have the highest likelihood of responding to AIT, providing guidance regarding when to discontinue AIT, and predicting symptomatic relapse and administering booster AIT [10].

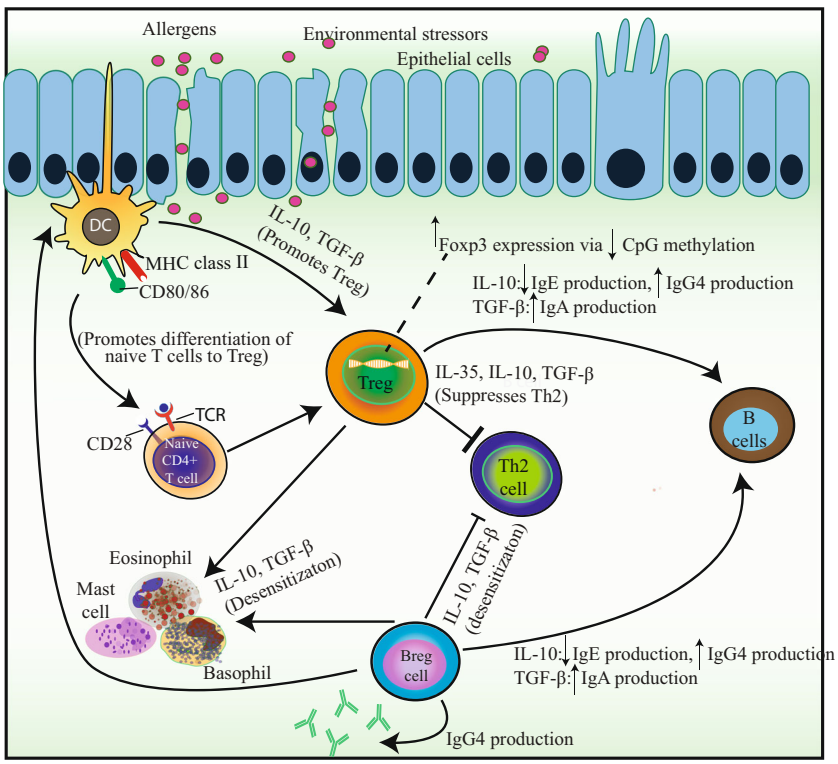

Fig. 3 Mechanism of immune changes after treatment with allergen immunotherapy. During the course of AIT, several cellular and molecular events take place. Within hours, there is a noted decrease in mast cells and basophil activity and degranulation leading to a decrease in type 1 skin test reactivity. Treg cells and their cytokines suppress Th2-type immune responses and help control the allergic diseases. During AIT, Breg cells are activated and suppress T cells and contribute to IgG4 synthesis, whereas Tregs suppress the allergic process by preventing and inhibiting inflammation in many different cell types

The identification of biomarkers associated with response to OIT is of particular interest in the setting of food allergies. Presently, the standard of assessing efficacy is through pre-treatment and post-treatment double-blind, placebo-controlled food challenges (DBPCFCs); however, this procedure can be resource intensive, time consuming, and carries the risk of moderate-to-severe allergic reactions [62]. Reliable biomarkers could help eliminate the need for food challenges and provide a safer and more convenient alternative to these challenges. In this review, we provide an overview of the potential biomarkers to assess response to both aeroallergen and food immunotherapy.

Biomarkers

Mast Cells

As previously mentioned, DBPCFCs and direct allergen exposure represent the current standard for the diagnosis and evaluation of food allergies; however, they can be resource intensive, time consuming, and carry the risk of moderate-to-severe allergic reactions [62]. In an effort to avoid these issues, one of the most widely used diagnostic tests to help confirm or evaluate an allergy is the skin prick test (SPT) due to its relative safety, simplicity, and prompt results. By measuring the wheal size resulting from exposure to minute amounts of an offending allergen introduced into the superficial layers of the skin, the SPT provides a way to measure and monitor the release of histamine and other inflammatory mediators by mast cells in response to the binding of the allergen to surface-bound IgE [63, 64]. While food challenges allow for the direct measurement of an individual's sensitivity to a given allergen, the SPT may allow for the indirect monitoring of changes in mast cell activity during immunotherapy and prediction of reaction during food challenge. Despite the practical advantages SPT provides over food challenges, there has been debate over its utility as a predictor of response to immunotherapy and the outcomes of such food challenges, as positive SPTs do occur in desensitized individuals [65].

While a wheal diameter of ≥ 3 mm has generally been regarded as a positive reaction [66], a prospective study of 467 children with suspected cow's milk, egg, and peanut allergy found this cut-off to be a poor predictor of clinically relevant FA. In order to invariably predict the individuals who reacted at oral food challenge (OFC) before or after immunotherapy, wheal diameter cut-offs of ≥ 8 mm, ≥ 7 mm, and ≥ 8 mm to cow's milk, egg, and peanut, respectively, were needed. Interestingly, when the analysis was limited to children aged 2 years or younger, the same ability to correctly predict reaction at food challenge was achieved at lower values, specifically ≥ 6 mm for cow's milk, ≥ 5 mm for egg, and ≥ 4 mm for peanut. While patients with SPT values falling below these cut-offs would still need to undergo food challenge to confirm their sensitization status due to the presence of false negatives, these results suggest that the use of higher cut-off values may allow for the accurate indication of FA or failure to desensitize during immunotherapy in patients with values exceeding the cut-offs, thus eliminating the need for further food challenge in those with the highest SPT values [65].

In addition to potential variations across age, observed SPT outcomes have also been shown to vary with the extracts and allergens used, the methods and techniques by which the tests are performed and measured, and each individual's intrinsic skin reactivity [67, 68]. Commonly, wheal size is characterized by the mean diameter, defined as the mean value of the longest and the midpoint orthogonal diameter of the wheal; however, several studies have evaluated the use of skin indexes, in which the mean diameter or scanned area of the allergen-induced wheal is divided by the mean diameter or scanned area of the positive histamine-induced control wheal, in an effort to theoretically control for individual skin reactivity and inter-observer variability [69, 70]. As hypothesized, significant differences were identified in the mean wheal diameter and skin index ($P = 0.03$ and $P < 0.001$, respectively) of children with suspected FA when comparing patients with a positive food challenge to those with a negative challenge [69]. While this finding would suggest that the skin index may better predict the outcome of food challenge, an additional study in 172 children with cashew nut sensitization found no significant difference in the ability of mean wheal diameter, scanned wheal area, skin index using mean wheal diameters, or skin index using scanned wheal areas to predict the outcome of food challenge to cashew when comparing areas under the curve in an ROC plot [70].

Given the existing data, SPTs may be of potential use in eliminating the need for food challenges in patients with the highest SPT values following immunotherapy; however, further research is needed in the establishment of cut-off values stratified by age and allergen, as well as standardization in the allergen extracts, techniques, and methods of wheal size measurement.

Basophils

Basophils represent less than < 1% human leukocytes in peripheral blood [10] and, similar to mast cells, contain proteoglycans and histamine within cytoplasmic secretory granules [71]. In sensitized individuals, incoming allergens can cross-link FcεRI-bound sIgE antibodies on the surface of the basophils, leading to degranulation and the release of histamine, leukotrienes, and other proinflammatory mediators that drive the allergic inflammatory response [72, 73]. As a result of AIT, basophil activation is inhibited via allergen-specific

IgG antibodies which compete with sIgE for allergen binding and prevent allergen-IgE receptor cross-linking on the basophils. Additionally, the allergen-IgG complexes bind to FcγRIIB, inhibitory IgG receptors which inhibit downstream IgE receptor activation [74–77]. Decreased basophil responsiveness has been demonstrated in both aero-allergen as well as food IT [78–81].

The quantification of basophil responsiveness has been considered as a potential biomarker for the assessment of immunotherapy efficacy. There are two key surface markers on basophils that aid in the monitoring of basophil activation: CD63 and CD203c. CD63 (granule-associated tetraspan) is detected on allergen-stimulated and activated basophils, while CD203c (ectonucleotide pyrophosphatase/phosphodiesterase 3, a type II transmembrane ectoenzyme) is highly selective for basophils in peripheral blood and is induced rapidly on the external surface of the plasma membrane after activation [74, 82, 83]. A number of other membrane-associated proteins have been identified, including CD13, CD107a, and CD164, each of which is expressed upon activation [10]. CD13 and CD164 follow patterns of expression similar to CD203c, whereas CD107a more closely aligns with that of CD63 [84].

More recently, intracellular expression of fluorochrome-labeled diamine oxidase (DAO) in basophils has been identified as a potential novel biomarker to assess AIT efficacy and the acquisition of tolerance [8]. In a cross-sectional study of AIT [8], grass pollen-induced basophil activation in patients with seasonal allergic rhinitis was found to be significantly diminished in patients who were treated with SCIT or SLIT for grass pollen. The quantification of the reduction in basophil activation and histamine release was performed by measuring DAO using flow cytometry, with reductions being associated with reduced combined symptoms and rescue medication scores [8]; however, studies to date show conflicting results when assessing basophil activation. While a number of studies suggest associations between reductions in basophil activation [85–88], particularly in the reduced expression of CD63, CD203c, and DAO with increases in CD107a [8, 89–92], others do not report a suppression of basophil activation in otherwise successful trials of AIT [93–95].

In addition to SCIT and SLIT, changes in basophils have also been noted in the setting of OIT with adjunct omalizumab use [96]. The IgE-mediated response of basophils is facilitated through the expression of tyrosine kinase syk [97], with increased syk expression increasing basophil sensitivity sIgE cross-linking. Basophil activity during treatment with omalizumab is currently thought to be due to two opposing effects, the net effect determining overall response to therapy. In a study involving participants with peanut allergy, the effect of syk on basophil function affected the subsequent efficacy of omalizumab to suppress reactions during the oral challenge following OIT [96, 98]. Research has suggested that omalizumab may paradoxically increase the sensitivity of basophils via an increase syk expression [97]; however, omalizumab has also been shown to lower FcεRI and sIgE densities on the basophil surface, leading to a desensitized basophil requiring higher concentrations of allergen for activation [97, 99]. This condition has been found to be similar in both food and aeroallergen conditions [96]. Given the current data, the measurement of individual baseline syk expression or IgE-mediated histamine response may have value in predicting the subset of patients with the highest potential for successful treatment with omalizumab in the setting of OIT for FA [98].

Innate Lymphoid Cells

Innate lymphoid cells (ILCs), including ILC1, ILC2, and ILC3 subsets, are similar to lymphocytes, but lack the rearranging antigen receptors [100, 101]. Similar to patterns of cytokine production characteristic of polarized $CD4^+$ T cells, ILCs produce large amounts of proinflammatory cytokines, such as IFN-γ, IL-5, IL-13, IL-17, and IL-22 [47], and play a role in the initiation and maintenance of allergic inflammation [47]. Of the three previously mentioned ILC subsets, ILC2s are most closely tied to type 2 allergic inflammation and tissue repair [100]. Their effector function is driven by IL-33 [102], IL-25 [103], TSLP [104], and leukotriene D4, with interactions leading to the production of Th2 cytokines including IL-4, IL-5, IL-9, and IL-13 [100]. The importance of ILC2s in allergic rhinitis was first explored by Doherty et al. in 2014, wherein the team demonstrated an increased presence of peripheral blood ILC2s in subjects exposed to intranasal cat allergen provocation within 4 h of the exposure, thus demonstrating how acutely ILC2s can be induced in allergic rhinitis [105].

Similar to the study by Doherty et al., a second study involving grass pollen allergic individuals demonstrated increases peripheral blood $CD117^+$ILC2s and IL-13^+ILC2s after natural grass pollen exposure during pollen season [106]. Grass pollen SCIT blunted the seasonal increases of $CD117^+$ILC2s, as well as IL-13^+ILC2s [106]. Outside of the pollen season, the frequency of the ILC2s were not different among grass pollen allergic and non-allergic controls [106]. In contrast, studies utilizing SLIT have failed to show a reduction in ILC2s; however, this may be due to enumeration of ILC2s in active and placebo groups having been conducted outside of pollen season [107], or that SLIT may not effectively be targeting the ILC2s. Specific studies targeting ILC2 response during immunotherapy for food allergy is presently lacking, with additional studies needed to evaluate its use in predicting clinical outcome in such a setting.

Specific and Total IgE

In addition to clinical symptoms, inclusion criteria for initiating AIT nearly ubiquitously includes elevated serum-specific IgE levels [108–110]. Several studies have shown that, during the first few months of AIT, there is an initial, transient boost in allergen-specific IgE serum levels with no significant clinical change in the individual. Additionally, this increase is only noted peripherally and not in the airways [111]. After 6–12 months, however, a progressive decline in the sIgEs, consistent with a decrease in the allergen-specific plasma cells which reside in the bone marrow, has been well documented in long-term AIT studies [112, 113]. This initial increase in allergen-specific IgE levels followed by a gradual decrease has been demonstrated during immunotherapy for food allergy in several studies [55, 81, 114–116]; however, the decrease is not consistently associated with clinical improvement [79, 117]. Similar to sIgE, a transient initial increase with subsequent decrease has been reported for total IgE (tIgE); however, there is a wide variation [50, 74].

The ratio of sIgE to total IgE (sIgE/tIgE) has also demonstrated conflicting efficacy when used as a marker for the prediction of clinical outcome resulting from AIT. While the ratio has shown promise in the setting of grass pollen and house dust mite SCIT and SLIT [118], a subsequent randomized, controlled, open-label study could not replicate the results. Although others have suggested similar correlations to clinical outcome as those described in the setting of SCIT and SLIT [5, 95, 118–120], given the conflicting data, further efforts must be made in the establishment and evaluation of quantitative ratio values that can be reliably associated with clinical reactivity. With additional research and refinement, the use of component resolved testing, especially the sIgE/tIgE ratio, may be important as a potential marker for response to AIT for food allergies in the future [121].

IgG4

In contrast to AIT-induced changes in IgE, both allergen and food immunotherapy have been associated with rises in serum allergen-specific functional IgG subtype 4 antibodies (sIgG4) in the first few months of treatment [78–81, 122]. In addition to inhibiting the binding of IgE-allergen complexes to B cells and decreasing the activation of B cells, mast cells, and basophils by engaging FcγRIIB (CD32) inhibitor receptors [123, 124], sIgG4 antibodies act as a blocking antibody by competing with allergen binding of sIgE, preventing the subsequent activation and degranulation of effector cells, as well as allergen presentation to T cells [75, 120, 123]. Increases in this antagonistic activity during AIT can be quantified using an immune-solid-phase allergy chip (ISAC) [125, 126]. Additionally, a validated flow cytometry-based assay (IgE-FAB) is available for the assessment of IgE-facilitated antigen presentation and activation of T cells during AIT [127].

Several studies have reported a 10–100-fold increase in the concentrations of allergen specific IgG1 and IgG4 during AIT [128, 129], with others exploring whether a reliable correlation exists between the levels of allergen-specific IgG4 and clinical outcome [93, 130–132]. One of the early immunologic changes noted in OIT includes an increase in food-specific IgG4 levels, supporting its potential use as a predictor of clinical outcome during therapy for food allergy [78–81]; however, in a time- and dose-dependent analysis of allergen-IgE binding to B cells and sIgG4 levels [133] during AIT, it was found that the serum inhibitory activity for IgE-FAB, not serum IgG4, best correlated with clinical outcome [132, 133], with increases in activity corresponding with positive clinical outcomes. Additional studies have shown that, although sIgG4 remains increased in active treatment groups, levels revert back to near-pretreatment levels after treatment cessation [74]. Conversely, the serum inhibitory activity for IgE-FAB has persistently been shown to remain elevated post-treatment [133, 134]. While sIgG4 responses appear to be relevant at the cohort level, these findings suggest that sIgG4 alone cannot reliably be used as a biomarker to predict clinical outcomes or reactivity after both aero-allergen and food AIT at the individual patient level [48].

IgE-Blocking Activity

Following AIT, there is a serum IgE inhibitory effect that is largely driven by IgA and IgG antibodies [74, 135, 136]. As previously described, this inhibitory effect prevents allergen binding to IgE, IgE–allergen complex binding to B cells, and the inhibition of basophils [74], thus preventing many aspects of a pro-allergic responsive [120, 137, 138]. Although several studies have suggested increases in IgE blocking factor (IgE-BF) following AIT are positively correlated with clinical outcome, the instrument used for the IgE-BF assay is no longer being produced. As such, the validation and use of IgE-BF as a predictive biomarker is limited [74].

On the other hand, as mentioned previously, the use of IgE-FAB to detect the binding of allergen–IgE complexes to B cells expressing FcεRII (CD23) is a highly reproducible flow cytometry-based bioassay. Recent studies have suggested an inverse correlation between symptom scores, rescue medication scores, and IgE-FAB [87, 133]. Additionally, decreases in IgE-FAB after AIT do appear to correlate with clinical response to grass and birch AIT with an inverse correlation between IgE-FAB and symptom scores and rescue medications [75, 126], with increased serum inhibitor activity for IgE-FAB persisting for 2 years [137]; however, despite these findings, no relationship has been discovered between the

serum inhibitor activity for IgE-FAB in responders to AIT compared to non-responders [74].

Mucosal IgA has also been implicated in IgE-blocking activity. In mouse models of asthma, the efficacy of SLIT was strongly correlated with the induction of IgA in the respiratory mucosa [47, 139]. During AIT, allergen-specific IgAs are produced and are present in the mucosal surfaces as a blocking antibody [113]. Serum IgA has also been found to correlate with response to OIT in the setting of food allergy. In a study of egg OIT, children with egg allergy had lower serum egg white specific IgA compared to healthy controls. In most participants who became tolerant to egg, there was a significant increase of over 28% of egg white-specific IgA over time, suggesting a role of allergen-specific IgA in food tolerance [140]. Due to the presence of multiple IgE-blocking mechanisms, allergen-specific IgE reactivity has the potential to be used as a proxy for AIT efficacy [141] through the quantification of IgE binding in the presence of blocking IgGs and IgAs [141].

Dendritic Cells

There has been interest in monitoring changes in innate immune cells, including peripheral blood monocytes (PBMCs) and DCs, as early predictors of response to immunotherapy due to their role in driving the down-stream orientation of adaptive T-cell responses. As DCs mature under the influence of multiple extracellular signals, they may polarize as proallergic DCs (DC2s) producing inflammatory cytokines and promoting the differentiation of naïve CD41 T cells into IL-5 and 13 secreting Th2 cells. Conversely, others may polarize as tolerogenic DCs (DCregs) driving the differentiation of CD41 T cells into Tregs [142, 143]. These DC-driven changes in the adaptive T-cell response, including the promotion of Tregs, Th1 cells, and IgG4 production over allergen-specific Th2 cells, basophils, and specific IgEs, have been documented in those undergoing aero-allergen IT; however, the data linking changes in these markers to clinical response has been limited [144].

In an effort to identify stronger predictors of clinical efficacy, recent efforts have evaluated changes in the expression of markers associated with DCs themselves to highlight potential correlations between changes in peripheral blood DC-associated markers and clinical response to immunotherapy. In a study of PBMCs from patients undergoing SLIT for grass pollen allergy, the expression of DCreg-associated complement component 1 (C1Q) and stabilin-1 (STAB1) were found to be increased in clinical responders compared to non-responders or those in the placebo group [143]. Follow-up study of PBMC-derived DC2 and DCreg-associated markers for additional associations indicated that 4 months of SLIT promoted a clear down-regulation of the DC2-associated markers CD141, GATA2, OX40 ligand, and receptor-interacting serine/threonine-protein kinase 4 (RIPK4) in the PBMCs of responders, defined as those with percentage of symptom score improvement above the median at month 4, compared to non-responders. This down-regulation was accompanied by a significant increase in the expression of the DCreg-associated complement C1Q subcomponent subunit A (C1QA), FcgRIIIA, ferritin light chain (FTL), and solute carrier organic anion transporter family member 2B1 (SLCO2B1) in responders. While changes in these DC2 and DCreg-associated markers were associated with clinical efficacy after 4 months of AIT, changes in FcgRIIIA were also found to correlate with the onset of clinical efficacy as early as 2 months of AIT. Further ROC analysis yielded an algorithm using an optimal combination of five of these markers, CD141, GATA3, RIPK4, C1QA, and FcgIIIA, that was able to identify responders from non-responders with a sensitivity and specificity of 90.48 and 61.9%, respectively, when using threshold values that varied at the 2- and 4-month time points [144].

To note, these observed changes in DC-associated markers were associated with clinical improvement regardless of whether or not alterations in known markers of adaptive, allergen-specific responses, including Th2 lymphocytes, type 2 innate lymphoid cells, basophils, IgA, IgE, IgG, and IgG4, were identified in the sera, suggesting changes in DC2 and DCreg-associated markers may better correlate with clinical improvement induced by SLIT when compared to previously mentioned markers. While these markers of efficacy still need to be validated in larger cohorts of patients undergoing AIT for other allergens and routes of immunotherapy, they show promise in the early prediction of adaptive immune responses and immunotherapy efficacy on an individual basis [144]. Our understanding of the role of DCs in food allergy in human subjects is limited and further characterization of differences in DC responses in food-allergic individuals undergoing IT is necessary to expand our knowledge of human DC function in food allergy [145].

DNA Methylation

Desensitization during immunotherapy is associated with increases in IgG and reductions in specific IgE antibodies, shifts from Th2 responses toward Th1 with increased IFN-γ production, reduced recruitment or deletion of T effector cells (Teffs), and induction of Tregs. While the previously described diagnostic tests have potential as excellent markers of sensitization, they are generally still poor predictors of clinical reactivity and outcomes of AIT, thus making OFCs and direct allergen exposure a necessity in the differentiation of sensitization and clinical allergy. Recently, additional associations between alterations in the methylation patterns of

specific DNA promoter regions and response to immunotherapy have come into focus. Studies have suggested that DNA methylation patterns of the fifth carbon in CpG dinucleotides of specific gene promoter regions, especially those associated with Forkhead box protein 3 (FoxP3), a protein associated with the tolerogenic and suppressive function of Tregs, are altered during immunotherapy and may be associated with clinical reactivity, thus making these patterns of methylation promising candidates for the monitoring of response to immunotherapy and prediction of food challenge outcomes. Methylation of these promoter sites influences gene expression, with increased methylation being associated with gene silencing [146]. Alterations in methylation have previously been associated with the shift from Th1 to Th2 responses in those with FA, with methylation studies suggesting that specific immunotherapy may promote the methylation of Th2-related gene promoter regions while decreasing rates of methylation in Th1-associated regions [146, 147]. As such, efforts have been made to link these methylation patterns with response to immunotherapy and clinical reactivity.

In a study by Canani et al., peripheral blood mononuclear cell-derived CpG methylation patterns in the promoting regions of specific genes associated with Th2 (IL-4 and IL-5) and Th1 (IL-10 and TNF-γ) cytokines within PBMCs were able to clearly distinguish those with active IgE-mediated cow's milk allergy from those who outgrew the allergy. IL-4 and -5 are critical to the development of IgE-mediated allergic inflammation in the event that they are not adequately counter-regulated by Th1 cytokines, such as IL-10 and IFN-γ. Those with active allergy exhibited significantly lower methylation of IL-4- and IL-5-associated regions and significantly higher rates in those associated with IL-10 and IFN-γ when compared to healthy controls, the greatest predictor of active allergy being the methylation rates of the IL-5-associated region. Additionally, methylation patterns in the regions of those with recent acquisition of tolerance to cow's milk were similar, but not identical, to those of healthy controls [148].

Similar findings have been demonstrated in the assessment of sustained unresponsiveness following successful desensitization of peanut allergy after 24 months of peanut OIT. Previously desensitized patients were reassessed for tolerance after 3 and 6 months of peanut avoidance. Those reacting to food challenge were categorized as non-tolerant, and those without reaction were deemed immune tolerant. Between these groups, there was no significant variation in specific IgE or basophil activation at any time point; however, those who were immune tolerant had significantly increased numbers of antigen-induced Tregs (ai-Tregs) and expression of FoxP3 during OIT and at the 3- and 6-month post-therapy OFCs compared to the non-tolerant and control groups, with significantly lower rates of FoxP3-associated CpG site methylation within ai-Tregs of the immune tolerant group compared to those in the non-tolerant or control groups. Furthermore, the loss of tolerance between 3 and 6 months post-therapy was associated with an increase in methylation at the same CpG sites [94]. It should be noted that these associations between immunotherapy and changes in DNA methylation do not appear to be limited to OIT alone. In a study by Swamy et al., subjects successfully treated with dual SLIT to timothy grass and dust mite produced increased levels of allergen-specific suppressive memory Tregs characterized by reduced CpG methylation within their FoxP3 promoter regions [149].

As a whole, these findings suggest that the evaluation of DNA methylation in ai-Tregs could be of value in predicting individual response to immunotherapy and the presence of sustained unresponsiveness without the need for repeat OFCs or direct allergen exposure. In a recent study, Martino et al. analyzed DNA methylation across a wider range of allergy-associated DNA promoter regions. By profiling genome-wide DNA methylation patterns in PBMCs and comparing CpG sites at which methylation levels vary according to FA status, the team determined a DNA methylation signature of 96 CpG sites able to predict clinical outcomes of food challenges with significantly greater accuracy compared to allergen-specific IgE and SPT. These 96 allergy-associated sites, located on genes stimulated by DCs and expressed by plasma and memory B cells, effector cells, Tregs, and T cells, had significantly higher levels of methylation in those with positive SPT and OFC compared, classified as food allergic, to those with positive SPT but no reaction at food challenge, classified as food sensitized. Through receiver operating characteristic (ROC) analysis, an optimal methylation cut-off value was determined, accurately predicting OFC outcomes with 96.55 and 89.66% specificity and sensitivity, respectively. Application of this cut-off in validation samples produced posterior probabilities greater than 0.9 in each case with an area under the curve of 0.9774 in the comparison of food-allergic patients to food-sensitized, with outcome of food challenge correctly predicted in each patient with no misclassification. Predictive performance was strongest when comparing food-allergic patients to sensitized; however, methylation scores remained significantly different when comparing both food-allergic and sensitized patients to healthy controls [150]. Application of the cut-off value in an additional replication cohort in which methylation scores were derived from total $CD4^+$ T cells, rather than PBMCs, provided correct prediction of food challenge reaction with a reduced accuracy of 79.2%. While differences in food-allergic and sensitized methylation scores were preserved despite methylation data being derived from a different cell type, the decrease in specificity and sensitivity strongly suggests that optimal diagnostic cut-off methylation values are cell specific and would need to be determined for each cell type used as a source of methylation data. Regardless of cell type and cut-off values used, the use of

methylation data appears to be most useful in differentiating food-allergic patients from those who are sensitized, after patients have been classified as sensitized using SPT and/or specific IgE data, potentially avoiding the need for OFCs in patient who would be difficult to classify based on SPT and IgE data alone [150].

While more studies are needed to develop and refine optimal gene panels, cell sources, and methylation cut-off values, these methylation biomarkers are readily detectable in the blood and appear to be highly promising as predictors of reaction during OFC or direct allergen exposure, especially when used in combination with IgE and SPT data [150].

Summary

We have reviewed some of the major biomarkers found to be important in the process of allergy and immunotherapy. Immunotherapy protocols are being tested for their safety and efficacy in desensitizing individuals to specific allergens; however, recurrence of allergic sensitization is common after discontinuation of therapy. Interestingly, in a subset of individuals, immunotherapy is protective against food allergens even after discontinuation of immunotherapy. Whether this protection is permanent is currently unknown because of inadequate long-term follow-up data. Research on understanding the underlying mechanisms may assist in modifying protocols to improve outcome and enable sustained unresponsiveness, rather than a temporary relief against food allergies. The cellular and molecular changes brought about by immunotherapy are still under investigation, but major strides in our understanding are being made.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

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