

REVIEW

Recent developments and highlights in immune monitoring of allergen immunotherapy

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Abstract

Allergic diseases are the most common chronic immune-mediated disorders and can manifest with an enormous diversity in clinical severity and symptoms. Underlying mechanisms for the adverse immune response to allergens and its downregulation by treatment are still being revealed. As a result, there have been, and still are, major challenges in diagnosis, prediction of disease progression/evolution and treatment. Currently, the only corrective treatment available is allergen immunotherapy (AIT). AIT modifies the immune response through long-term repeated exposure to defined doses of allergen. However, as the treatment usually needs to be continued for several years to be effective, and can be accompanied by adverse reactions, many patients face difficulties completing their schedule. Long-term therapy also potentially incurs high costs. Therefore, there is a great need for objective markers to predict or to monitor individual patient's beneficial changes in immune response during therapy so that efficacy can be identified as early as possible. In this review, we specifically address recent technical developments that have generated new insights into allergic disease pathogenesis, and how these could potentially be translated into routine laboratory assays for disease monitoring during AIT that are relatively inexpensive, robust and scalable.

KEYWORDS

allergen immunotherapy, allergy, biomarker, immune monitoring

1 | INTRODUCTION

1.1 | Allergen immunotherapy: a prototype of precision medicine

As a corrective treatment for allergic diseases, allergen immunotherapy (AIT) has been explored and utilized for over a century.¹ Treatment involves repeated exposure to defined doses of allergen for a prolonged period of time. It can lead to clinical tolerance,²⁻⁵ that is a sustained reduction of allergen-induced symptoms following

treatment⁶ and prevent progression from milder to more severe disease in children.⁷

Conventionally, AIT for aeroallergens is given by subcutaneous injections subcutaneous immunotherapy (SCIT)⁸ or sublingually sublingual immunotherapy (SLIT).^{9,10} These approaches are deemed safe, but in a minority of patients, a strong reaction can already be observed at the (very low) starting dose, postponing or stopping further therapy. Furthermore, it has become evident that at least 3 years of treatment is needed to confer long-term clinical tolerance.^{11,12} To overcome these limitations, several newer approaches are under investigation with altered administration or allergen preparation.¹³

Intralymphatic immunotherapy (ILIT) is being trialled for aeroallergens with the aim to shorten the treatment period to 2 months using three ultrasound-guided injections of low-dose allergen applied with 1-month intervals. Based on six clinical trials reported to date, the procedure is deemed safe and promising, although long-term follow-up is needed with standardized and validated scoring methods.¹⁴

Food allergen-specific immunotherapy via the oral route as well as the sublingual route has attracted a great deal of clinical research interest in recent years although both treatment modalities remain experimental at this stage.^{5,15-17} Epicutaneous immunotherapy (EPIT) for peanut allergy has successfully been trialled in Phase 1 and 2B studies with the aim of increasing the threshold of reactivity of patients to peanut extract.^{18,19} In addition, immunotherapy with allergen-derived T-cell epitope-based peptides is being trialled in intradermal and subcutaneous formulations.^{20,21} These peptides do not invoke IgE responses and mitigate the risk of anaphylaxis, but target the specific, disease-driving T cells. Similarly, the use of hypoallergenic but T-cell-reactive allergen peptides derived by proteolytic hydrolysis for grass pollen allergy treatment also shows promise.¹⁷ Other types of allergen formulation are being explored, including the use of recombinant allergens designed to minimize adverse events.^{22,23} The inclusion of different adjuvants^{24,25} including nanoparticle encapsulation²⁶ is also being investigated to more actively drive the immune response away from the dominant type 2 response.

Hence despite proven efficacy, AIT is still very much in development, and changes that could improve safety and adherence are an ongoing need. Currently, the gold standard evaluation of AIT success is scoring of clinical symptoms following allergen challenge. With AIT duration being 3-5 years, this poses two major issues: (a) the long period of uncertainty to find out whether treatment is effective and (b) the potential risks of adverse events the patient is exposed to upon allergen challenge when assessing efficacy. Thus, given these risks and the economic cost of ineffective treatments, there is a need for markers that can predict therapy success and monitor therapy effects early on. With such markers, the right treatment can be given to the right patient at the right time, that is precision medicine.

1.2 | Current status of biomarkers for immune monitoring of AIT

As specified by the Biomarkers Definitions Working Group of the National Institutes of Health in 2001, "A biological marker or biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention".²⁷ In the context of AIT for allergic disease, the aim is to alter the established immune response, with the expected changes in immunological parameters that could be utilized as biomarkers for immune monitoring.

Many immune cells and soluble factors are involved in the development of an adverse, IgE-dominant sensitization to allergen, and these show unique and distinct characteristics from the majority of protective immune responses. Importantly, a skewed type 2 immune

Highlights

1. Precise and robust biomarkers for AIT monitoring are emerging with technological advances in high-resolution analysis of rare cells in local tissues.
2. Genome-wide epigenetic changes as well as immunoglobulin and T-cell receptor transcript repertoires are a source of new biomarkers for AIT monitoring.
3. AIT can be monitored through quantification and immunophenotyping of rare, antigen-specific immune cells using sophisticated multi-parameter flow and mass cytometry.

response is initiated with the involvement of cytokines IL-25, IL-33, TSLP and type 2 innate lymphoid cells (ILC2). These skew helper-T-cell maturation towards the Th2 fate,^{28,29} and direct B cells to induce Ig class switching to IgE, processes mediated by type 2 cytokines IL-4, IL-5 and IL-13 (Figure 1). This type 2 skewing persists in an individual, even after the initial response has passed and involuted. Long-lived memory Th2 and memory B cells specific for the allergen remain,^{30,31} and these will trigger a hypersensitivity response when the individual is challenged by renewed encounter with the same allergen.

Repeated allergen exposure through AIT has been shown to alter the allergic immune response. Typically, increased proportions of Th1 and regulatory T (Treg) cells are seen,^{32,33} and cytokines released from these cells are thought to downregulate the Th2 response,³⁴ as well as driving generation of allergen-specific IgG4 (Figure 2A).^{35,36} Together, the modified Th cell response and inhibitory activity of IgG4 antibodies are believed to form the basis of immune tolerance.⁶

Several types of assays have been developed in the past years for laboratory testing of immune cells and molecules driving immune tolerance. Those that are in use to monitor efficacy in clinical trials for AIT have recently been reviewed and classified into six domains based on the immune parameter being assessed (Figure 2B).³⁷ The seventh domain concerns *in vivo* clinical biomarkers including exhaled breath analysis and allergen provocation studies that are beyond the scope of this review but reviewed elsewhere.³⁸⁻⁴² Assays in all six *ex vivo* biomarker domains show promise on the basis of association with clinical improvement, although there are limitations in their predictive nature, specificity and sensitivity.

2 | TECHNOLOGICAL ADVANCES FOR IDENTIFICATION OF BIOMARKERS

The need for better biomarkers to monitor AIT success has been consistently addressed in the past decades and has more recently taken flight with the development of technologies that allow large

data set acquisition in a hypothesis-free manner, referred to as the "Omics revolution." Starting with genomics, multiple different technologies have been categorized, with eight showing great promise for biomarker discovery of AIT success (Table 1).⁴³

Genomics can be applied for discovery of genetic variants, including rare ones in patients with inborn errors of immunity (IEI) where these are causative for the phenotype^{44,45} and more common variants that predispose to atopy^{44,46,47} or affect asthma severity.⁴⁸ If genetic variants that affect the response to AIT are identified, these could potentially be used at baseline to stratify patients. In addition, environmental exposures (exposome), for example dietary food intake^{49,50} and microbiota,⁵¹ can affect immune responses and allergic sensitization. A potential mechanism is through epigenetic changes to DNA or histones that do not change the DNA sequence. The main challenge in epigenetics is to select the right tissue. Although blood cells are readily accessible for these studies,^{52,53} they exhibit heterogeneous expression profiles and function.⁵⁴ The analysis of locally affected tissue would potentially yield more clinically relevant results, and initial studies show promise.^{55,56} Ideally, epigenetic profiles would be linked to changes in gene expression (transcriptomics) to provide

first insights towards functional implications.⁵⁷ New developments in single-cell transcriptomics will allow high-resolution analysis of rare cells otherwise hidden in tissue-based analyses. Furthermore, targeted sequencing of T-cell receptor or Ig genes can reveal T-cell and B-cell signatures associated with disease or immune tolerance.⁵⁸⁻⁶⁰ Proteomic approaches allow for the large-scale study of key proteins in solution, as well as those derived from cell fractions. The latter can also be examined in the context of single cells through advances in multi-parameter flow cytometry and mass cytometry.^{31,61,62} Finally, metabolomic and lipidomic approaches have been developed to evaluate chemical processes in the body through analysis of endogenous (amino acids, fatty acids, vitamins) or exogenous (toxins, drugs) molecules in serum and faecal samples.^{63,64}

Advances in Omic technologies are driving the field with the accumulation of large data sets for interrogation and opportunities to identify immune biomarkers for patient stratification, prognostication and therapy monitoring. Importantly, upon identification of such markers, simplified approaches will have to be developed to measure these, allowing implementation in clinical trials and routine diagnostics (Box 1).

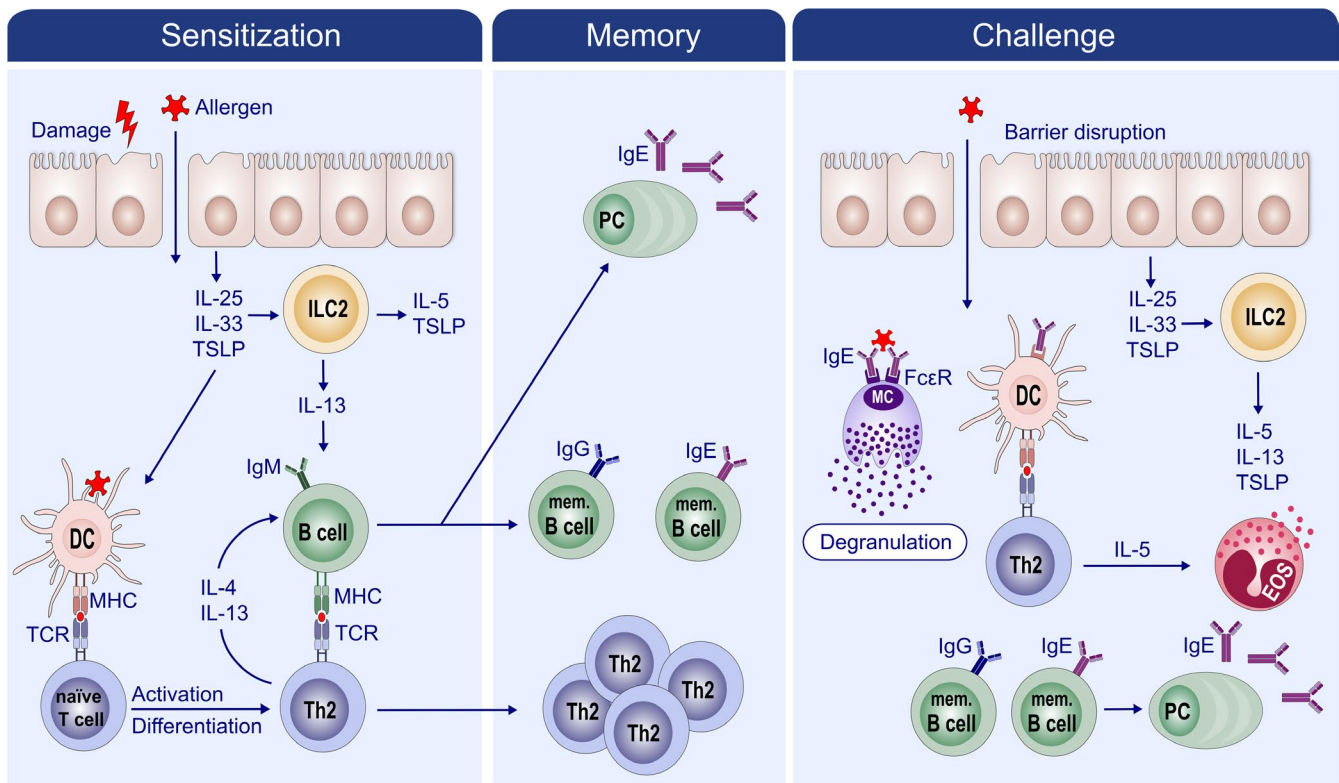
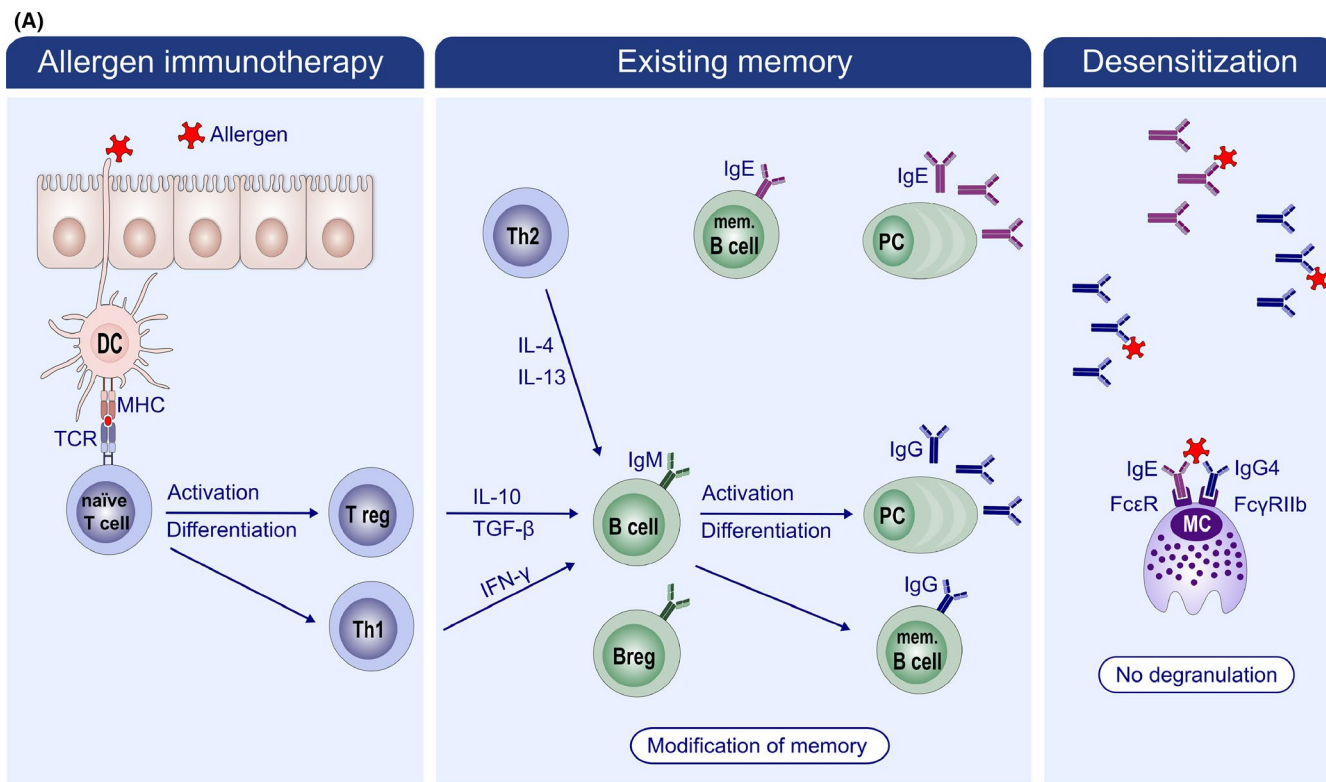


FIGURE 1 Immunological features of allergic sensitization, memory and allergen challenge. *Sensitization* to allergens is thought to be mediated by epithelial leakiness and activation due to potential inherent and external causes, which result in a proinflammatory cytokine and chemokine milieu with specific factors driving a Th2 response (TSLP, IL-25, IL-33). As a result, inflammatory cells are recruited, and of these innate lymphoid cells (ILC2) are early providers of type 2 cytokines IL-5, IL-13 and TSLP and play a role in T- and B-cell activation. This environment drives T cells towards a Th2 phenotype after recognition of cognate antigen by dendritic cells (DC), and these Th2 cells drive B-cell memory and plasma cell differentiation with IgE class-switched antibodies. Following clearance of allergen, the inflammation subsides, but allergen-specific *memory* resides in local and circulating Th2, memory B and plasma cells, as well as effector cell-bound and soluble IgE. Upon *challenge* with the same allergen, IgE-loaded target cells degranulate and drive the immediate hypersensitivity response, while memory T and B cells enhance local Th2 response together with ILC2 and eosinophils

**(B)**

Domain	Type of target	Assays	Strengths	Limitations
I: IgE	Serum protein	Total IgE Allergen-specific IgE	Serum markers; sIgE gold standard patient selection for AIT	No correlation of levels with therapy success
II: IgG subclasses	Serum protein	Allergen-specific IgG1 Allergen-specific IgG4	Serum markers; sIgG4 elevated by AIT	Failure in sIgG4 induction might reflect inadequate treatment compliance
III: Serum inhibitory activity for IgE	Serum protein	IgE-FAB IgE-BF	Serum-based assay; functional output	Laborious and requires specialized reagents/equipment
IV: Basophil activation	Cell activation	Flow cytometry of allergen-activated basophils (CD63, CD203c, BHR, DAO)	Requires small amount of blood	<i>In vitro</i> assay, which is difficult to optimize and standardize
V: Cytokines and chemokines	Serum protein	ELISA or Luminex for: IL-2, IL-2R, IL-4/5/6, IL-8/9/10, IL-13/18, IFN-γ, ECP, Eotaxin, MCP-1, TARC, transthyretin	Serum-based assay; high-throughput	Low sensitivity for many cytokines in serum
VI: Cellular biomarkers	Cell phenotyping / activation	Immunophenotyping of <i>ex vivo</i> or <i>in vitro</i> activated cells: CD4+ T cells, Treg, Breg, DC	Multiple cell subsets can be measured in single analysis from small blood sample	Lack of standardization in definitions and evaluation of most cell types. Antigen-specific cells can currently only be measured after <i>in vitro</i> culture (laborious)

FIGURE 2 Current view on mechanism and biomarkers in use to monitor AIT. A, Scheme of immune modulation by AIT, where low-dose, repeated exposure to allergen is thought to occur with limited to no inflammation. As a result, Th skewing is balanced towards Th1 and Treg, which subsequently modify the B-cell response. Especially, the production of IL-10 is thought to drive IgG4 class switching. Thus, local and systemic memory is rebalanced, both in the T-cell and the B-cell compartment, and there is a strong increase in allergen-specific IgG4 antibodies. Upon allergen challenge, IgG4 and potentially other soluble factors are thought to inhibit IgE-mediated degranulation of target cells, that is desensitization. Together with the loss of Th2 skewing, this underlies the observed clinical tolerance. B, Laboratory biomarkers utilized in diagnostics and clinical trials for AIT (adapted from ³⁷). Abbreviations: IgE-FAB, IgE-facilitated allergen binding; IgE-BF, IgE-blocking factor; BHR, basophil histamine release; DAO, diamine oxidase; Treg, regulatory T cell; Breg, regulatory B cell; DC, dendritic cell

TABLE 1 Omic technologies driving the identification of new biomarkers

Technology*	Platform	New biomarkers	Challenge	References
Genomics	Whole genome sequencing	<i>D2HGDH, IL1RL1, RERE, RUNX3</i>	Identification of functional variant and potential multigenic interactions	85,86
Epigenetics	DNA methylation Histone modification Chromatin remodelling ATAC-sequencing RNA interference	DNA methylation of Th cytokine genes, and Th and Treg transcription factors	Translation into specific and reproducible assays	87,88,90,91
Transcriptomics	Next-generation sequencing Microarray Single-cell RNAseq	Th and Treg cytokine transcripts; Ig and TCR repertoires	Single-cell transcript detection by cytometry; identification of Ig and TCR variable regions encoding allergen-specific receptors	33,58-60
Proteomics	Multiplex immunoassays MALDI-TOF Nano-LC-MS/MS Proximity extension assay (PEA)	Th and Treg cytokine and chemokines	Lowering threshold for sensitivity, and optimization for local tissue analysis	138
Immunophenotyping	Flow cytometry Mass cytometry/CyTOF	Th and Treg cells; IgG subclass and IgE-expressing B cells; Breg	Inclusion of allergen specificity in the analysis	11,32-34,42,92,113,123,128,129
Lipidomics	Liquid chromatography tandem-mass spectrometry (LC-MS)	Hydroxyeicosatetraenoic acids (HETEs), lipoxin A4	Observation to be confirmed	76-79
Metabolomics	Nuclear magnetic resonance (NMR) Mass spectrometry (MS) Extracellular flux analysis of glycolysis and oxidative phosphorylation	Carbohydrates and pyruvate	Observation to be confirmed	76
Microbiomics	16S sequencing Metagenomic sequencing (MGS)	-	Signature bacteria, virus, fungus and archaea species to be identified	51

Abbreviation: ATAC, Assay for Transposase-Accessible Chromatin.

*Adapted from⁴³

Box 1

Ideal features of an immune system biomarker

1. Noninvasive, easily measured, inexpensive and produces rapid results
2. Obtained from readily available sources, such as blood, sputum, urine or faeces
3. High sensitivity, allowing early detection, and no overlap in values between diseased patients and healthy controls
4. High specificity, being greatly upregulated (or downregulated) specifically in the diseased samples and unaffected by comorbid conditions
5. Levels should vary rapidly in response to treatment
6. Levels should aid in risk stratification and possess prognostic value in terms of disease severity and/or treatment outcome
7. Biologically plausible and provide insight into the underlying disease mechanism

Adapted from⁶⁵

3 | CANDIDATE BIOMARKERS FOR IMMUNE MONITORING IN ALLERGEN IMMUNOTHERAPY

3.1 | Soluble markers for immune monitoring

3.1.1 | Immunoglobulins

Traditionally, serum proteins are measured for diagnosis and therapy monitoring of allergic disease. The gold standard is the presence of allergen-specific IgE for diagnosis and the induction of specific IgG4 during AIT. The former typically shows a transient increase early in treatment but then a decline over time, and although the latter is associated with beneficial outcome, it appears more to reflect therapy adherence.³⁷ Other soluble markers of therapy success are needed, and very likely to be present. Serum IgG4 levels are typically very low, but following AIT, they can rise dramatically resulting in an increased IgG4/IgE ratio.⁹ Other Ig isotypes are also found to

increase, possibly contributing to the serum inhibitory activity on allergen-IgE binding.^{11,21,40} Indeed, the reduction in IgE-facilitated allergen binding (IgE-FAB) following grass pollen SLIT was found to be associated with increased specific IgG1 and IgA as well as IgG4 in serum.⁶⁶ More recently, specific IgG2 serum levels were also found to be increased following SLIT for ryegrass pollen allergy with typically higher concentrations than IgG4.³³ Moreover, IgG2 transcripts are highly mutated and potentially bind antigens with high affinity,⁶⁷ contributing further to an inhibitory effect on allergen-IgE binding. However, it should be noted that the relative contribution of IgG4 to allergen binding compared with other Ig isotypes may be underestimated in many immunoassays.⁶⁸ It remains to be determined whether the quantitative changes in serum immunoglobulins during and following AIT are high enough (sensitivity), and whether the effects are specific for a positive therapy outcome (ie clinical tolerance). Finally, it would be of interest to explore allergen-specific IgD levels pre- and post-AIT. The function of IgD remains the most elusive of all Ig isotypes. IgD is co-expressed with IgM on the surface of mature B cells. Even though IgD protein levels exceed that of IgM, the function of this isotype as a surface-expressed receptor remains unclear, and surface IgD appears dispensable for B-cell development and responses.⁶⁹ Secreted IgD levels are typically low in serum, but might be higher in the upper respiratory tract mucosa, where a particular population of plasma cells resides that has undergone IgM to IgD class switching.⁷⁰ Recently, it has been shown that IgD binds to CD44 on basophils via galectin-9.⁷¹ Although IgD enhances the Th2-type humoral response, it can actively inhibit IgE-mediated degranulation of basophils.⁷¹ Potentially, AIT could lead to increased production of allergen-specific IgD thereby contributing to the amelioration of disease symptoms.

3.1.2 | Cytokines, chemokines and other soluble molecules

Cytokine changes from a Th2 predominant phenotype in allergic disease towards a Th1 and Treg phenotype following AIT may provide biomarkers of successful therapy. However, measurements in serum have been challenging due to the frequently low cytokine levels that limit assay detection sensitivity and the fact that the active processes take place in tissue. As a result, downregulation of Th2 cytokines (IL-4, IL-13, IL-9) and upregulation of Th1 (IFN γ , IL-12) and regulatory cytokines (TGF β and IL-10) have not been consistently found.³⁷ Thus, analysis of cellular cytokine production (see below) and detection of cytokines in local tissue might be more promising avenues for AIT monitoring. Data on local cytokine levels are limited. In a cross-sectional analysis, immunotherapy-treated patients with grass pollen allergy had reduced nasal fluid concentrations of IL-4, IL-9 and eotaxin compared with untreated allergies.⁷² These results were not reproduced in a paired analysis of seasonal allergic rhinitis (SAR) patients sensitized to *Olea europaea*, who did not show significant changes in nasal lavage fluid IL-4, IFN- γ , IL-10 and TGF- β after 1 year of treatment.⁷³ Hence, more longitudinal studies are required to determine whether

cytokine levels in local tissue could be used for immune monitoring. There might be a potential for other soluble factors as markers of Th2 responses. Of specific interest is epithelium-derived IL-25, which in contrast to IL-33 and TSLP was found significantly increased in nasal polyps of chronic rhinitis patients.²⁸

Besides cytokines and chemokines, the neurotransmitter acetylcholine is found to be upregulated in the skin of patients with atopic dermatitis.⁷⁴ Considering the capacity of acetylcholine to polarize dendritic cells (DC) towards a Th2-promoting profile,⁷⁵ it might be involved in other local tissues of allergic patients as well. Lipid metabolites are a new target for monitoring of AIT.^{76,77} Specifically, release by immune cells of 15-hydroxyeicosatetraenoic acid (15-HETE) and lipoxin A4 is upregulated following AIT, and potentially, these products can be measured in serum.^{78,79}

3.2 | Molecular markers for immune monitoring

3.2.1 | Genetic variants

There are several established gene variants that have been shown to directly result in allergic disease in hyper-IgE syndrome (*STAT3*, *DOCK8*, *PGM3*, *ERBIN*, *IL6ST* and *CARD11*)⁸⁰ or in Netherton syndrome (*SPINK5*),⁸¹ and others that have been shown to predispose to disease (*STAT6*, *FGN*).^{46,82} In addition, for more than a decade, genome-wide association studies have revealed many genes in which variants are highly associated with allergic disease.^{83,84} With the data sets getting larger, the resolution of this approach has improved, resulting in a limited number of genes that are now reproducibly found and shared between allergic diseases.^{85,86} As these associations have been identified through known polymorphisms that might not be directly causative for the effect, it will now be of great interest to sequence these entire gene regions to identify the functional variants. Ultimately, the combined analysis of these variants will allow for the identification of potential multi-gene variant effects. Specifically, variants of interest are those that associate with good vs poor outcome of AIT, as these could then be utilized going forward as markers to stratify patients prior to treatment start.

3.2.2 | Cytokine transcripts

Analogous to cytokine protein levels, cytokine transcripts are potential markers to examine Th2 skewing and the effects of AIT on this. Again, blood is the most easily obtained tissue, but not the site of inflammation. Moreover, in both blood and local tissue, the cellular composition is heterogeneous, and this can lower sensitivity of measurements. Nevertheless, the Th2 locus could be a suitable target with the advantage being that it contains the *IL4*, *IL5* and *IL13* genes,^{87,88} and combined analysis of these transcripts could overcome sensitivity issues. Conversely, high levels of *IL10* transcripts in the blood of house dust mite allergic patients before AIT were found predictive of therapy effect.⁸⁹ The cytokine responses of Th cells are regulated by epigenetic changes,^{87,88,90} and it would be of interest to

learn whether changes in DNA methylation,⁹¹ for example, could be measured in addition to cytokine transcripts to predict or monitor the effects of AIT.

3.2.3 | Immunoglobulin transcripts

The Ig heavy chain (*IGH*) and Ig light chain (*IGL*) genes coding for antibody molecules are modified following an immune response and gain somatic hypermutations (SHM) in their variable regions.⁶⁷ In the case of *IGH*, there can be a switch from the IgM isotype to IgG, IgA or IgE.⁹² Furthermore, the variable domain of each rearranged Ig gene consists of a unique combination of variable (V), diversity (D; only *IGH*) and joining (J) gene segments with a uniquely processed junctional region.⁹³ Thus, a pool of sequences of *IGH* transcripts contains information on the repertoire diversity, affinity maturation (SHM) and skewing of the effector response (Ig isotype) (Figure 3).

Large-scale sequence analysis of *IGH* transcripts has recently been undertaken for this purpose, and importantly, in blood, allergen-specific *IGH* transcripts can be detected.⁵⁸ In addition, the repertoire of *IGH* transcripts has been shown to undergo changes following AIT with more oligoclonality in *IGHA* and *IGHG* transcripts.⁵⁹ Furthermore, paired analysis of IgG transcripts in ryegrass pollen-allergic patients before and after 3 years of SLIT showed that usage of *IGHG2* and *IGHG4* was increased within the total pool of unique *IGHG* transcripts.³³ Together, these studies demonstrate the potential of measuring changes in the *IGH* transcript repertoire that correlate with allergic disease and effects of treatment, and might be utilized as biomarkers. Similar approaches have been initiated for

T-cell receptor beta (*TRB*) transcripts, where an increase in repertoire diversity was found in allergic individuals as compared to nonallergic controls.⁶⁰ Importantly, rearranged *TRB* genes do not undergo SHM or class switch recombination, and therefore, the origin from naïve or memory T cells cannot be distinguished. Hence, those transcripts from naïve T cells cannot be excluded from the analysis and reduce the sensitivity of the assay. Still, with the identification of allergen-specific *TRB* sequences and advances in large-scale sequencing and bioinformatics analysis, this transcriptomics approach shows promise for identification of molecular biomarkers for AIT success.

3.3 | Cellular markers for immune monitoring

3.3.1 | Myeloid cells

Allergen immunotherapy-induced changes reported in myeloid cell subsets in blood are limited. This is probably because the myeloid cells involved in allergic responses are typically effector cells, such as eosinophils, mast cells and basophils. As a result, eosinophils are typically expanded during active inflammation, such as in grass pollen-allergic individuals during pollen season.¹¹ Still, it appears that in out-of-season measurements, eosinophil frequencies in blood are reduced after grass pollen SLIT.¹¹ Activated basophils are important biomarkers of allergic responses, reflecting specific IgE functional activity. In vitro basophil activation tests (BAT) may be useful in monitoring patients on AIT, by ex vivo measurements of CD63 or CD203c expression, basophil histamine release (BHR) or diamine oxidase (DAO), avoiding the risk of severe allergic reactions with in

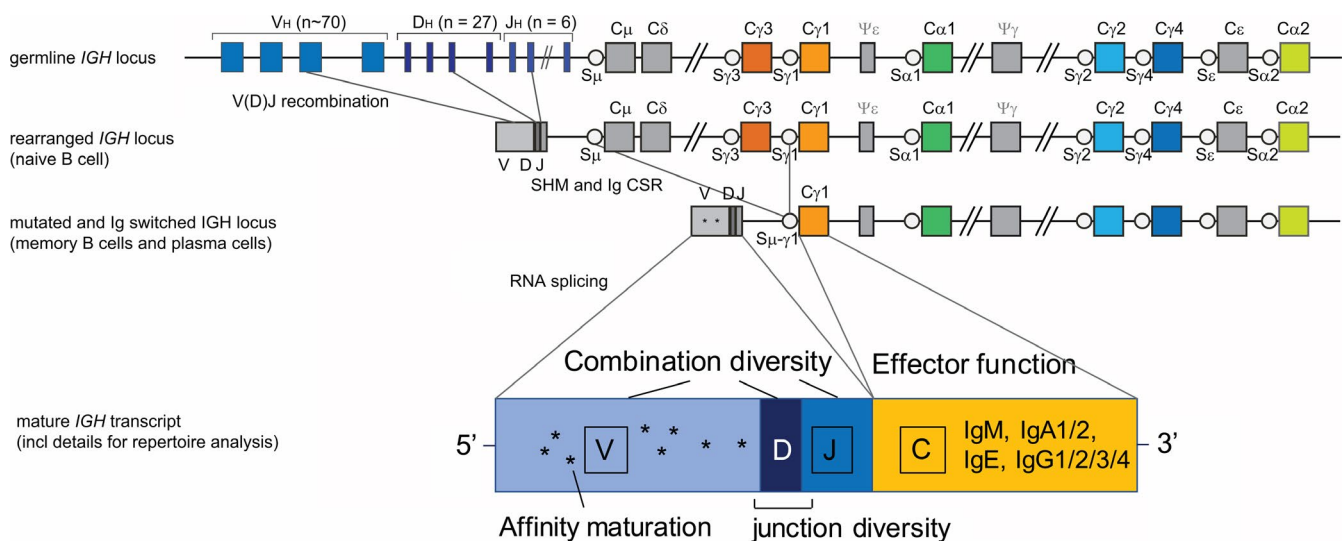


FIGURE 3 Schematic representation of genetic changes to the *IGH* locus and details of a mature *IGH* transcript. The mature *IGH* transcript is composed of a VDJ exon encoding the Ig variable domain responsible for antigen binding and constant exons encoding the Ig constant regions mediating the effector functions. The VDJ exon is formed during genomic V(D)J recombination of one of multiple variable (V), diversity (D) and joining (J) genes during progenitor B-cell differentiation. This VDJ combination is further specific by imprecise joining resulting in a unique junction. Upon antigen encounter, mature B cells proliferate, and each daughter cell introduces somatic hypermutations (SHM) in the VDJ exon. Selection of daughter cells with high-affinity surface Ig will result in affinity maturation. Finally, Ig class switching occurs, which replaces the Ig constant domain from IgM-encoding exons to one of multiple IgA, IgG or IgE, thereby changing the antibody effector function. The mature *IGH* transcript carries all information reflecting the molecular processes that have occurred in the locus. Information that can be obtained from sequence analysis is highlighted

vivo allergen challenge.⁹⁴⁻⁹⁷ However, advances in practical aspects of the test are required for standardization and adaptation for use in routine laboratories. Due to their sequestration into tissues, mast cells remain difficult to investigate in the context of AIT and immune monitoring. Improvement in noninvasive assessment of mast cell quantities and qualities would be of great importance to our understanding of AIT. Recent studies have also examined whether changes in circulating DCs are predictive of AIT success.^{98,99} In vitro, monocyte-derived DCs can be skewed towards phenotypes promoting Th1 (DC1), Th2 (DC2), Th17 (DC17) and Treg (DCreg) differentiation. Several transcripts associated with a DCreg phenotype were found upregulated in PBMC after AIT.⁹⁸ For translation into clinical applications, it would be important to establish whether these changes can be measured with more straightforward and sensitive techniques, such as flow cytometry.

3.3.2 | Innate lymphoid cells (ILC)

Due to their recently identified role in allergic responses, ILC2 have been examined both in local tissue and peripheral blood.^{29,100,101} This minor lymphocyte population in blood is defined by the absence of lineage markers CD3, CD14, CD16, CD19, CD56 and FcεRIα (lin-) to exclude T cells, B cells, NK cells, monocytes and basophils, and positivity for CRTh2 and CD127. In patients with allergic asthma and during the pollen season in patients with SAR, these cells are increased in blood.¹⁰²⁻¹⁰⁴ Importantly, SAR patients receiving SLIT do not show this expansion of ILC2 in blood,¹⁰⁴ nor do asthma patients in whom disease is well-controlled by medication.¹⁰³ However, the utility of ILC2 as a biomarker is limited by two factors: (a) the expansion of ILC2 in blood disappears when allergen is avoided, limiting treatment evaluation for food allergies and for SAR out-of-season^{104,105}; and (b) ILC2 numbers are extremely low in blood, ranging from 0.01% to 0.1% of lymphocytes.¹⁰²⁻¹⁰⁵

3.3.3 | T cells

Effective immunotherapy has been shown to reverse the Th2 dominance with increased IFNγ production and reduction in Th2 cytokines, and to result in induction of Treg^{11,106-108} that produce TGF-β and IL-10, which are pivotal for the successful immune deviation in AIT.^{109,110} These changes in CD4 T-cell cytokine production can be assessed through in vitro stimulation of PBMC from patients with allergen extracts. Typically, after 1-7 days of culture, cytokine production in culture supernatant can be assessed to determine Th1/Th2 shifts and Treg involvement.^{33,111} The advantage of in vitro stimulation is that allergen-specific cells can be examined subsequently based on the upregulation of activation markers (eg CD154 and CD137) or proliferation as assessed by dilution of labelling dyes.^{33,112} Flow cytometry of activation markers can be combined with intracellular staining for cytokines and intranuclear staining for the Treg transcription factor FoxP3. The latter approach allows for single-cell measurements and multi-parameter analysis, which have been applied to demonstrate reduction in Th2 cells following SLIT

for HDM³⁴ and grass pollen,¹¹ as well as plasticity of Th2 cells developing a Treg phenotype following oral AIT for peanut allergy.¹¹³ Furthermore, allergen-specific Tregs have been shown to expand following AIT for HDM and grass pollen allergies thereby increasing the ratio of allergen-specific Tregs over effector T cells.^{32,33,114,115}

To facilitate translation towards routine measurements of T-cell biomarkers, immunophenotyping approaches have been developed that only use membrane markers and allow for direct ex vivo T-cell evaluation without in vitro cell culture. The CD4+CD25+CD127- phenotype specifically distinguishes human Treg,^{116,117} and these cells can be quantified in allergic patients³¹ with increases following SLIT for grass pollen¹¹ and AIT in HDM-allergic individuals.³² Furthermore, chemokine receptor expression patterns correlate strongly with Th cell function¹¹⁸⁻¹²⁰ and can be used to identify Th1 (CCR6-CXCR3-CCR4-), Th2 (CCR6-CXCR3-CCR4+) and Th17 (CCR6-CXCR3-CCR4+) subsets within CD4 memory cell populations (CD4+CD45RA-).^{30,121} Grass pollen immunotherapy was shown to reduce the percentage of CCR3+CD4+ cells in fresh PBMC of allergic rhinitis patients to levels similar to those in normal individuals.¹²² Analogously, a regulatory CD8 T-cell population has been identified recently, and using the membrane phenotype CD25+CD137+ only or the CD8+CD25+Foxp3+ phenotype, this subset has been shown to expand following AIT.^{42,123}

Many abnormalities in Treg and Th cell number and frequency have been observed within the total pool of circulating T cells, independent of antigen specificity.^{30,31,42,121} With the use of MHC-peptide reagents to detect antigen specificity,¹¹⁵ the sensitivity of these changes can be significantly enhanced to detect early effects of AIT.^{32,113} As the application of MHC tetramers, especially for MHC class II, is limited by the diversity of alleles in the human population, there is a need for alternative markers for allergen-specific cells. In early immunophenotyping studies of allergen-specific cells, the CD27-CRTh2+ phenotype was shown to correlate well with the Th2-skewed (CCR4+) subset,¹²⁴ and CD4 T cells with this phenotype (CCR4+CD27-CRTh2+) were specifically reduced in patients with birch pollen-related apple allergy following SLIT with recombinant Mal d 1.²³

3.3.4 | B cells

In contrast to the long-term attention on soluble IgE and IgG4, immunophenotyping of the B-cell compartment to monitor the effects of AIT has only recently gained traction. This is for the most part due to the technical difficulties in detecting IgE-expressing and IgG4-expressing B cells, which are both very infrequent. With the advances in multi-parameter flow cytometry and the availability of many fluorochromes with high staining intensity, it has become possible to detect IgE-expressing B cells following exclusion of naïve B cells that have bound IgE through surface CD23 as well as memory B cells expressing other Ig isotypes.⁹² This approach is technically demanding, and high cell numbers and an optimized flow cytometry panel are required for reliable detection.^{30,31,92,125} Similar to total and specific IgE in serum, blood IgE-expressing B cells are increased in adults with atopic dermatitis,⁹² and in children with asthma, food

allergy and atopic dermatitis.³⁰ However, as for serum IgE, it is unlikely that IgE-expressing B cells will be useful biomarkers for AIT. In a recent study, during 3 years of SLIT for grass pollen, the frequency of IgE-expressing B cells within the total B-cell fraction was found to not change significantly, but using IgG subclass detecting reagents, the frequencies of IgG2-expressing B cells and IgG4-expressing B cells were found to be specifically increased following SLIT.³³ It appears that changes in the B-cell compartment are quite restricted, as despite early reports of more global changes involving transitional and naive mature B cells in atopic dermatitis,¹²⁶ these could not be replicated,¹²⁷ and do not appear to be affected by AIT.³³ The exception to this would be an increase in IL-10-expressing B cells, or Breg, which is associated with tolerance to bee venom and SLIT for grass pollen and HDM allergies.^{36,128,129}

4 | LESSONS FROM ANIMAL MODELS

Many clinical studies of new therapeutic strategies for enhancing the immunoregulatory effects of AIT are underpinned by data from animal models, usually in mice.^{24,130-132} Elucidation of potential new biomarkers for clinical efficacy of AIT may also follow from such studies. For example, a mouse model of peanut allergy was recently used to demonstrate the association of clinically effective AIT with sustained hypermethylation of *Gata3* in Th2 cells and hypomethylation of *Foxp3* in CD62L⁺ Tregs.⁹⁰ A particularly interesting novel strategy reported recently for identifying candidate biomarkers for AIT efficacy is the use of a bioinformatic approach, applying data mining tools to search for hidden trends within large data sets derived from experimental murine models of AIT.¹³³

While murine studies enable testing on large groups of genetically identical animals and examination of more relevant target tissue cells, valuable lessons for human allergic disease may also be obtained from studies on larger animals. AIT is an accepted treatment for allergic diseases in veterinary practice. Atopic dermatitis is the most common allergic disease in companion animals such as dogs, cats and horses, and SCIT with mites, pollen or moulds has been shown to be effective.¹³⁴ Similar immunological changes in allergy are observed in animals to those in humans, and AIT has been shown to be accompanied by similar increases in Treg and specific IgG4 with decreased specific IgE.¹³⁴ Even more so, reliable biomarkers for AIT efficacy would be valuable in veterinary medicine but to date none are recognized. Although experimental models of allergic disease, whether in small or large animals, may not entirely mimic clinical human disease, they can provide valuable insight into disease mechanisms and are useful tools for defining appropriate immune markers of immunoregulation in allergy.

5 | FUTURE PERSPECTIVES

Over the past decade, many technical advances and immunological insights have been applied to gain further understanding of the allergic

response, as well as the development of new treatments. Interestingly, despite being the oldest form of treatment, AIT remains the only modality that alters the skewed Th2 response and can result in long-term clinical benefit. The gold standard of treatment evaluation is a controlled allergen challenge, which can result in adverse effects and is typically only informative after 3-5 years of treatment. Currently, several objective laboratory assays are used to evaluate treatment effects, but serum IgG4, BAT and IgE-FAB measurements have their limitations. Advanced technical approaches have yielded potential new biomarkers, but each has their challenges for straightforward implementation. Molecular biomarkers such as epigenetic and transcriptional changes take place in specific cell types and will require targeted evaluation to improve sensitivity and more inexpensive assays for detection. Potentially, simultaneous multi-parameter detection of proteins and RNA in single cells can be applied to implement these insights.¹³⁵ Reproducible information on changes in soluble cytokine and chemokine levels in serum remains a challenge despite more sensitive assays being available. Most likely, the best alternative would be to measure these either in local tissue where the pathological process takes place or to measure production of these factors in the cells of interest. These cellular measurements can now be performed to much greater detail with advances in multi-parameter cytometry, and especially, the adaptive immune cells that carry the memory of the allergic response appear to be stably affected in allergic patients. The sensitivity of detecting changes separately in, for example Th2, Treg, IgG4+ and IgG2+ B cells, might not be high enough, but there is the potential to combine these and develop a score for treatment success based on per-patient changes as compared to pretreatment.¹³⁶ Ultimately, the detection of allergen-specific T cells and B cells^{32,137} could provide the most specific evaluation of treatment success, and with the advances in high-throughput antigen-receptor transcript sequencing, this is becoming more feasible.

6 | CONCLUSIONS

New technical developments and insights into immunological processes are driving the identification of pathological processes in allergic responses. With the use of diverse Omic approaches, multiple molecular, serological and cellular factors have been shown to be dysregulated in disease and modified by AIT. The main objective now is to identify which of these correlates with AIT with sufficiently high sensitivity and specificity, and to develop robust, routine assays to measure these through noninvasive diagnostic tests.

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CONFLICTS OF INTEREST

All authors declare no conflict of interest.

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