NOVEL ADVANCES IN ALLERGY DIAGNOSIS AND TREATMENT

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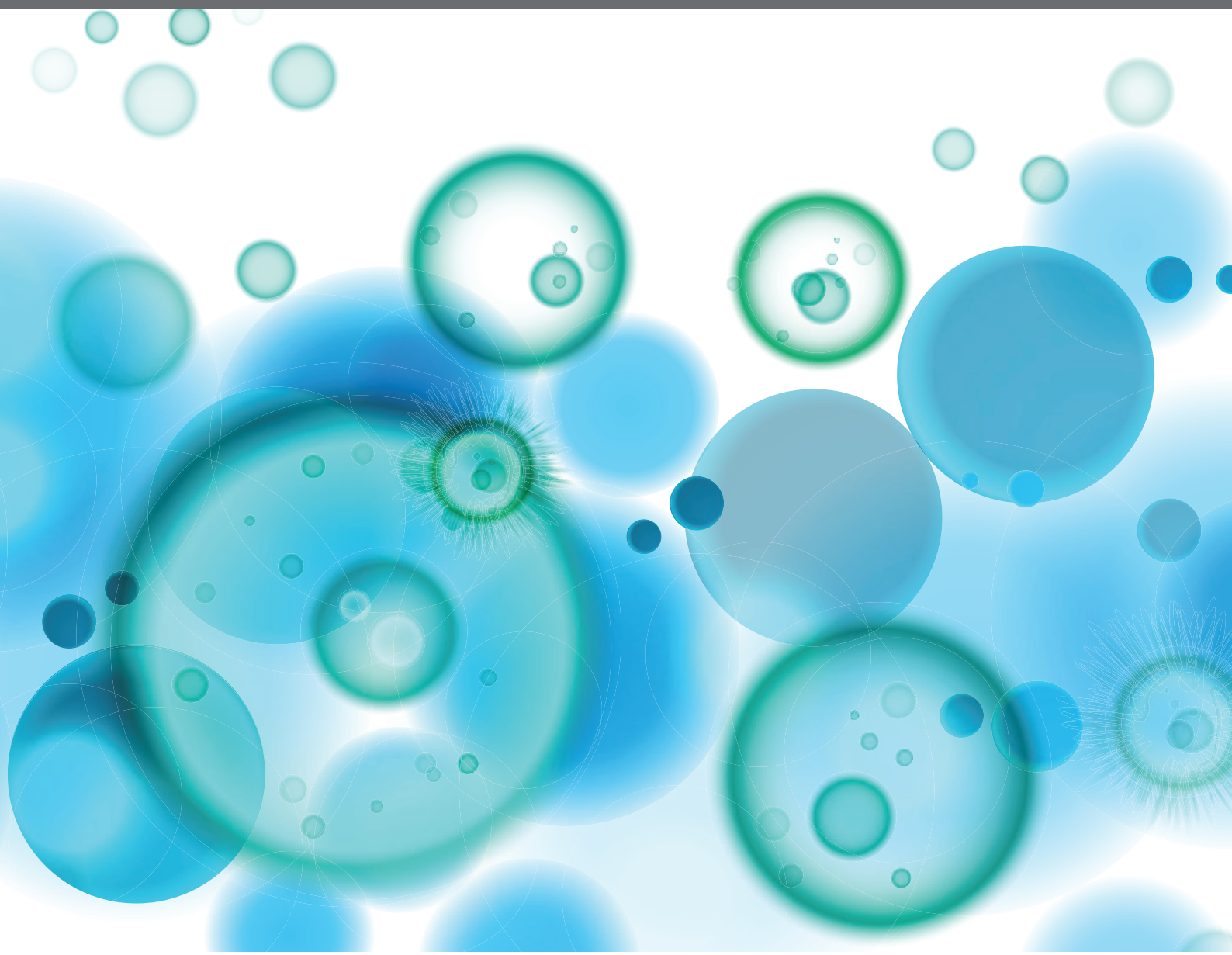

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NOVEL ADVANCES IN ALLERGY DIAGNOSIS AND TREATMENT

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EDITORIAL
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Editorial: Novel Advances in Allergy Diagnosis and Treatment

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Keywords: allergen, allergen-specific immunotherapy (AIT), allergy, allergy diagnosis, atopic diseases

Editorial on the Research Topic

Novel Advances in Allergy Diagnosis and Treatment

The term "allergy" was coined by Clemens von Pirquet in 1906 to describe a general change of the organisms' reactivity in quality, quantity and time, including hyper- and hyposensitivity reactions to exogenous substances (allergens) which are also depending on endogenous factors (1). Today, the word "allergy" is associated with an abnormal, adaptive immunologic hypersensitivity reaction to non-infectious environmental substances. The most common manifestations of allergic diseases are IgE-mediated hypersensitivity reactions which in the last decades have become a major health problem as already more than one quarter of the population in industrialized countries is affected and prevalence is further rising (2). Allergen sources include a wide variety of environmental substances such as pollen, house dust mites, animal dander, foods, drugs or insect venoms and the disease can manifest itself e.g. as rhinitis, conjunctivitis, chronic asthma, urticaria or even life-threatening anaphylaxis (2, 3). Long before the availability of anti-allergic drugs, Leonard Noon demonstrated in 1911 that prophylactic inoculation with grass pollen extract was efficient in suppressing symptoms of hay fewer (4). Since that time, allergen-specific immunotherapy (AIT) remains the only available curative treatment for allergic patients. Nevertheless, in recent times, several novel approaches aiming at enhancing therapeutic efficacy and diagnostic accuracy have been developed. Moreover, the ongoing elucidation of immunological mechanisms of allergic sensitization, disease progression and tolerance induction to allergens will facilitate the development of new preventive and therapeutic strategies against allergy (5).

Although AIT is a well-established disease-modulating treatment for IgE-mediated allergic diseases, the induction of immune tolerance is an evolving area that is still not sufficiently understood. Zissler and Schmidt-Weber give a comprehensive overview on immunological changes during AIT and their usefulness as biomarker for monitoring and predicting therapeutic success. They discuss that clinical allergen tolerance depends on multiple mechanisms across different immune cell and tissue compartments. Hence, it is likely that only combinations or ratios of gene expression levels are promising to achieve predictive value and definition of helpful biomarkers. Outstanding effective tolerance induction can be achieved by AIT of Hymenoptera venom-allergic patients. Blank et al. describe how the classification of venom-allergic patients into different disease endotypes and phenotypes applying available biomarkers and diagnostic tolls can provide therapeutic guidance and strengthen personalized treatment strategies and precision medicine. Along the same lines, Czolk et al. provide an overview on the immune basis for phenotype variations in peanut-allergic individuals. They discuss that deep immune phenotyping and multi-omics technologies can build a reliable basis for novel insights into disease pathophysiology and identification of biomarkers or biomarker signatures

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predictive for reaction phenotypes. This knowledge shall advance the stratification of individuals prior to selection for oral immunotherapy or early food introduction for prevention.

Efficacy and safety of AIT relies on the type of allergy and the disease status of the patient. Several approaches aiming at enhancing therapeutic efficacy and reducing side-effects have been developed or are in the scientific pipeline. For instance, the use of adjuvants may optimize the immunological response to AIT in the most appropriate way for a specific disease manifestation. The current status of the application of the adjuvant Microcrystalline Tyrosine (MCT) and adjuvant systems comprising MCT and Monophosphoryl Lipid A (MPL) in AIT is reviewed by Heath et al. Montamat et al. describe the potential of CpG oligonucleotides as adjuvant for AIT with a focus on dose- and concentration-dependent effects that are crucial for the induction of inflammatory or tolerogenic responses. A broader overview on existing and promising new candidates for formulations of AIT of human and veterinary patients, including adjuvants, immunomodulators, physical packaging, conjugates and combinations thereof to modify allergenic proteins, making them safer, and more efficacious in AIT, is given by Pali-Schöll et al.

There are some disadvantages associated with AIT when using complete protein extracts from allergenic sources. These include among others, problems with standardization and allergen stability, as well as the fact that the patients will be treated with a whole cocktail of allergens and non-allergenic proteins, while having a specific IgE-profile (6). AIT based on recombinant hypoallergenic allergens has raised attention, especially in allergic diseases which are triggered by one dominant allergen as e.g. birch pollen allergy. Aglas et al. demonstrate a robust IgG immune response against a hypoallergenic variant of the major birch pollen allergen Bet v 1 in rats that efficiently blocks human IgE-binding to the wild-type allergen, thereby demonstrating its potential therapeutic value in AIT. Flicker et al. discuss the potential benefit of nanobodies, single domain antibodies with several superior properties compared to conventional antibodies, for passive treatment of IgE-mediated allergy. The study by Krause et al. presents the development of a high throughput analytical platform for unbiased IgE target epitope detection. Such epitopes may represent interesting candidates for diagnosis as well as therapy. Pomés et al. give a comprehensive overview of state-of-the-art approaches to analyze the interaction between IgE-antibodies and corresponding allergen epitopes. This information on antigenic determinants will facilitate the design of hypoallergens for AIT and further elucidate fundamental mechanisms of the IgE immune response.

Evolutionary old responses against helminth parasites closely resemble the patho-mechanisms that drive allergic diseases. This implies that studying the helminth-host-interaction may contribute to novel strategies for fighting against the rise of allergic diseases. Bohnacker et al. review the protective role of helminths in asthma and allergy. The immunomodulatory properties of helminth molecules make them promising candidates to become the next generation of biotherapeutics for the treatment of type 2 inflammatory disorders. Also commensal microbes have a tremendous impact on human health and recent evidence indicates that the susceptibility to food allergy is critically linked to microbial dysbiosis. Kreft et al. give an overview on this important research area and explore future directions for a potential microbial therapy of food allergy. In most cases, proteins are recognized as allergens, although the relevance of carbohydrate-specific antibodies as mediators of IgE-mediated allergy was described decades ago. Hils et al. review the historical development of carbohydrate-allergen-research with a particular focus on clinical and immunological features of the alpha-gal syndrome, the underlying feature of red meat allergy.

Many immune mechanisms of allergic diseases were uncovered by applying animal models of allergy which also often build the basis for the development of anti-allergic treatments. Alessandrini et al. give an overview of currently used type 2 and non-type 2 rodent asthma models and discuss the limits of extrapolation from mice to humans. Currently available biological therapies applying monoclonal antibodies to treat asthma and their possible effects on airway remodeling are reviewed by Kardas et al. There is growing evidence that allergic diseases may develop over lifetime from atopic dermatitis and food allergy in infancy to gradual development into allergic rhinitis and allergic asthma in childhood. Yang et al. give an overview on this so called atopic march and discuss new perspectives for prevention and treatment of atopic diseases that are provided by this concept.

Importantly, the success of AIT depends on an accurate allergy diagnosis that aims at identifying the primary allergen source and risk factors for disease severity. Based on recombinant allergens, molecular or component-resolved allergy diagnosis was introduced into clinical practice and allowed dissecting the molecular sensitization profiles of allergic patients. Huang et al. demonstrate that detection of IgE- and IgG-reactivity to a panel of respiratory allergens micro-arrayed onto silicon elements is more sensitive than glass-based chips. Furthermore, they discuss the advantages of silicon-based allergen microarrays and how this technology will allow addressing hitherto unmet needs in micro-array-based molecular allergy diagnosis. Another powerful tool and sensitive marker that can be used to detect clinically relevant allergy is the basophil activation test (BAT). Eberlein gives an overview on this diagnostic approach and on how the BAT provides information on the severity of an allergic reaction and its use to monitor immunotherapy and desensitization. Barbaud et al. report a multi-center study demonstrating that standardization of intradermal tests with drugs reduces variability and enables a more reliable comparison of results between individuals and centers. Nowadays, it becomes evident that also clinical decision support systems (CDSS) reinforce health care professionals in taking informed decisions during their clinical routine. Dramburg et al. give an overview on existing tools, new developments and novel concepts and discuss the potential of digital CDSS in improving prevention, diagnosis and monitoring of allergic diseases.

In conclusion, state-of-the-art and novel promising diagnostic, therapeutic and basic concepts that will help to fight the global burden of allergic diseases have been introduced and discussed in this Research Topic. Recent advances in diagnostic strategies and biomarker development have greatly improved diagnostic sensitivity and specificity and demonstrate rising potential for detecting clinically relevant allergy and risk factors for severe reactions as well as to predict and monitor therapeutic success

in the future. Furthermore, the development of new therapeutic formulations and the uncovering of basic mechanisms of immune tolerance increasingly will contribute to more efficient AIT regimens, even in difficult to treat patients, and presumably also to completely new therapy concepts for allergy.

AUTHOR CONTRIBUTIONS

SB and CH wrote the manuscript and contributed equally to the editorial process for this collection. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Intradermal Tests With Drugs: An Approach to Standardization

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Background: Intradermal tests (IDTs) are performed and interpreted differently in drug allergy centers making valid comparison of results difficult.

Objective: To reduce method-related and intercenter variability of IDTs by the introduction of a standardized method.

Materials and methods: In 11 centers of the European Network for Drug Allergy, IDTs were prospectively performed with saline and with amoxicillin (20 mg/ml) using (1) the local method and (2) the standardized European Network in Drug Allergy (ENDA) method (0.02 ml). The diameters of the initial injection wheal (Wi) for the different volumes and sites injected obtained from each center were analyzed.

Results: The most reproducible method was to fill a syringe with test solution, then expel the excess fluid to obtain exactly 0.02 ml. The median Wi diameter with 0.02 ml injection using the standardized method was 5 mm [range 2–10 mm; interquartile range (IQR) 5–5 mm; $n = 1{,}096$] for saline and 5 mm (range 2–9 mm; IQR = 4.5–5 mm; $n = 240$) for amoxicillin. IDT injection sites did not affect the Wi diameter. Training improved precision and reduced the variability of Wi diameters.

Conclusion: Using the standardized IDT method described in this multicenter study helped to reduce variability, enabling more reliable comparison of results between individuals and centers.

Keywords: drug allergy, intradermal test, amoxicillin, standardization, specificity of drug skin tests

INTRODUCTION

Allergy skin testing is essential for the correct diagnosis of immediate and delayed drug hypersensitivity (DH). It is also used to identify alternative drugs for patients with positive skin or provocation tests with suspected drugs. The intradermal test (IDT) is the most sensitive skin test and may be used when soluble forms of the drugs are available. A questionnaire survey in 2004 (1) within the European Network in Drug Allergy (ENDA), the Drug Allergy Interest Group of the European Academy of Allergy and Clinical Immunology (EAACI), showed differences in performing drug allergy investigations. Guidelines such as those by the European Society of Contact Dermatitis (ESCD) (2), the EAACI (3), anesthesiology societies (4, 5), and the United States of America (6) differ in their recommendations (**Table 1**), making valid comparison of the results between centers virtually impossible. A position paper providing guidelines on drug concentrations for skin testing was published in 2013 (7), but at the present time, there is no consensus on the methodology and interpretation of drug IDT. The drug concentration, the method used, and the criteria for positive skin tests all influence the sensitivity and specificity of IDT; consequently, thresholds for specific results may vary between different centers (8, 9). Barbaud et al. (8), using the ESCD guideline, showed that the highest specific concentration before causing an irritant reaction for cefotiam was 10 mg/ml and, for cefotaxime, 25 mg/ml, but Torres et al. (9) in the EAACI Interest Group on DH position paper recommended using 1–2 mg/ml for cephalosporin IDT.

A questionnaire survey on the skin test methods used by different centers in the ENDA group was carried in 2008/2009. It showed a wide variation in the method used and in the interpretation of the results. This led to the setting up of a multicenter study comparing local IDT methods with the proposed new standardized IDT method based on the ENDA consensus and to determine if the standardized method will minimize the intercenter variability in performing IDT. As the initial results and analysis had showed large variation in the diameter of initial wheal just after injection (Wi) readings obtained in the different centers, a practical session was organized during an ENDA meeting in one participating hospital. We noted the different ways of filling the syringe and differences in the injection needle gauge, injection, and test measurement methods. A standardized IDT (the Guideline) was written and validated by all coauthors.

MATERIALS AND METHODS

All tests performed in this prospective descriptive study were part of routine investigations in patients who had been referred for investigation of DH, and no additional tests were carried out. Information on the methodology used to carry out the drug IDT was collected from databases of 11 departments in Europe with special interest in DH. The following data were recorded: age and gender of the patients, the method for filling the syringe, the injected volume of saline and amoxicillin (AX), the injection site, and the diameter of Wi and 20 min post-injection. For AX (20 mg/ml), the parenteral AX powder (the manufacturer may vary depending on the center and dispensed by local hospital pharmacy) was dissolved in sterile isotonic saline and used within 2 h of the IDT as recommended (9).

TABLE 1 | Comparison of international guidelines published for performing drug intradermal tests.

	ESCD (2)	EAACI (3)	BSACI (4)	SFAR (5)	Macy et al. (6)
Volume injected	0.04 ml sequential dilutions in saline or phenolated saline	0.02–0.05 ml	0.03 ml	0.02–0.05 ml	0.02 ml with a 27-gauge tuberculin
Measurement of the Wi	Yes Raising a wheal of 4–6 mm	Yes Raising a bleb of 3 mm	Yes Raising a bleb of 4–6 mm	Yes Wheal ≤4 mm	No
Time interval to immediate skin test reading (minutes)	30	15–20	20–30	20	15
Criteria for immediate positivity	Wheal ≥10 mm	W20 ≥ Wi+ 3 mm with surrounding flare	A wheal that is ≥3 mm larger than the initial bleb with surrounding flare	W20 ≥ Wi × 2	Wheal ≥5 mm with a surrounding erythema
Criteria for delayed positivity	Papule at 24 h	24–72 h infiltrated erythema	Not defined	Not defined	Not defined
Site	Extensor surface of the arm	Volar aspect of the forearm (or other regions)	Not defined	Back, arm or forearm	Not specified
Negative control with saline	Yes	Yes	Yes	Yes	Yes, with Tris-buffered saline

ESCD, European Society of Contact Dermatitis; EAACI, European Academy of Allergy and Clinical Immunology; BSACI, British Society for Allergy and Clinical Immunology, Societe Francaise d'Anesthesie et Reanimation.

Wi, diameter of initial wheal just after injection; W20, diameter of the wheal 20-min post-injection.

Questionnaires on the Methods for Doing IDT

A questionnaire was sent by email to all the members of the ENDA group. The main questions aimed at highlighting differences in the local practice between centers, and the returns are summarized in **Table 1**.

Evaluating Injection Volume Obtained by Using Different Syringe Sizes and Syringe-Filling Methods

Two methods, used in participating centers, to fill a syringe with 0.02 ml normal saline (NS) were evaluated. In Nancy, three nurses specialized in drug allergy workup drew up exactly 0.02 ml into a 1-ml syringe using a 25-G needle, which was then emptied into a small vial (Method 1). Another nurse filled a syringe with 0.05–0.07 ml saline, then expelled the excess fluid and air bubbles to obtain exactly 0.02 ml, which was similarly emptied into a vial (Method 2). The two procedures were repeated 10 consecutive times. The weight of the NS collected by both methods was determined using a precision scale (KERN EW/EG version 2.4 11/2006) and then converted into milliliters. Method 2 was also carried out in Copenhagen using a 1-ml syringe as in Nancy, and a 0.5-ml syringe and a 27-G needle in both instances.

Comparing Non-standardized Methods With Injection of a Standardized Volume

The first part of the study was done in order to determine if adhering to previously published international guidelines minimizes intercenter variability in performing drug IDT; each participating center performed IDT using its local IDT protocol with NS and AX (20 mg/ml) test solutions. In the second part of the study, the ENDA method, injecting a fixed volume (0.02 ml) of AX or NS, was carried out on additional new patients. We also analyzed if the injection site, the syringe size, and the needle gauge influenced the size of Wi.

The injection sites used for saline or AX IDT were the lateral aspect of the upper arm (UA) and/or the flexor aspect of the forearm (FA), and, in a limited number of patients, the back (B). All centers used 25- to 27-G needles except one, which used

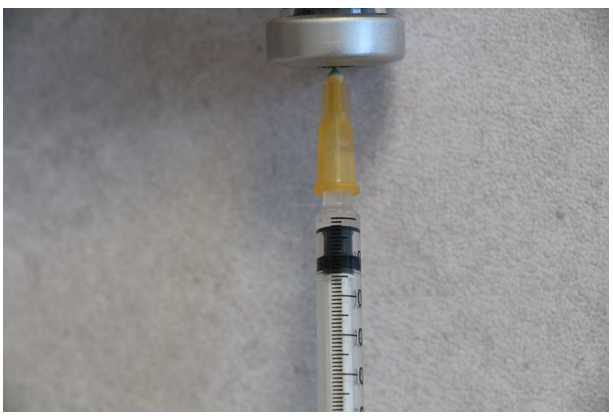

FIGURE 1 | Tuberculin syringe with a 25-G needle and a flat-end plunger drawn up with 0.02-ml solution.

TABLE 2 | Results of Questionnaire Survey of Drug Intradermal Test (IDT) methods used in 20 European allergy centers.

Questions	Answers
Number of IDT done per year	30–6,000 IDT
Do you use dissolved and filtered drug solution for IDT?	Yes: 5/20 (crushed pills or other non-injectable forms of the drugs, diluted in saline then filtered) No: 15/20
Which solvent do you use?	Saline or phenolated saline: 16/20 Sterile distilled water or the solvent recommended as diluent for the infusion: 4/20
What volume do you inject?	No fixed volume but a volume to raise a wheal: 12/20 A fixed volume: 0.02 ml: 1 0.03 ml: 3 0.04 ml: 3 0.05 ml: 1
Site of injection	Upper arm: 4 Forearm: 14 Back: 1 Non-specified: 1
Measurement of the Wi	Yes: 15/20 No: 5/20
What are your criteria for a positive immediate reading?	The existence of a given diameter of W20: 4 centers (3–5 mm depending on the centers) The existence of a given diameter of erythema at 20 min (E20) 3 mm ≥: 6 mm depending on the centers (2 centers also consider the W20). W20 ≥ Wi + 3 mm: 6 centers

Wi, mean diameter of the wheal (bleb or papule) immediately after ID injection.
W20, mean diameter of the wheal or the wheal developed at 20 min.
E20, mean diameter of the erythema developed at 20 min.

30-G needles. The injection sites were inspected just after the injection, with the measurement of the Wi and at 20 min post-injection for wheal (W20) and erythema (E20), respectively, and their diameter was measured as recommended (2–5).

Analysis of Wi When Standardized IDT Is Performed by Individual Tester Injecting 0.02 and 0.03 ml Saline, Respectively

As the preliminary results showed that the test volumes injected varied among allergy centers from 0.02 to 0.05 ml, it was decided to compare the most used volume of 0.03 ml with the proposed lower volume of 0.02 ml NS. This was carried out by trained operators using the standardized IDT method on volunteer subjects in six centers.

The results obtained, the Wi obtained using the local and the standardized IDT methods on different injection sites and volume injected, were subject to chi-square and Kruskal–Wallis tests and non-parametric data by Wilcoxon test. The differences in wheal sizes were considered statistically significant if the p-value was ≤ 0.05. The statistical analysis was performed using SAS software, version 9.2.

RESULTS

Questionnaires on the Methods for Doing IDT

All centers answered that they followed the ESCD and/or EAACI guidelines (2, 3), but no two centers carried out and interpreted the IDT in the same way (**Table 2**). Even if the two European guidelines recommended injecting a given volume (between 0.02 and 0.05 ml) (2, 3), 12/20 centers did not use a fixed volume but injected a volume to produce the targeted Wi diameter.

In addition to the answers summarized in **Table 2**, the centers may differ on the syringe size and the gauge of the needle used, on the way syringes are filled with the test solution, and in the training for IDT. Some centers also used crushed pills, diluted in saline then filtered, for performing IDT, which was not recommended by any previous guidelines.

Evaluating Injection Volume Obtained by Using Different Syringe Size and Syringe-Filling Method

Drawing up a larger volume and expelling excess solution to the required volume gave more reproducible result (mean 0.024 ml, SD = 0.002). This method of syringe filling was adopted into the standardized IDT method (**Figure 1**). The needle gauge did not appear to affect the injection volume obtained as shown by the results acquired in Copenhagen and Nancy. Copenhagen (27-G needle and 1-ml syringe) mean volume = 0.027 ml (range 0.019–0.037), SD = 0.0.0037. Nancy (25-G needle and 1-ml syringe) mean volume = 0.027 ml (range 0.012–0.037), SD = 0.0035. When a 0.5-ml syringe instead of a 1-ml syringe was used to draw up the solution, the mean volume was less and the standard deviation lower, 0.023 ml (range 0.017–0.027), SD = 0.0019.

Comparing Non-standardized Methods With Injection of a Standardized Volume

Seven centers performed local non-standardized NS IDT with injection volumes that ranged from 0.02 to 0.05 ml and also with a fixed volume of 0.03 ml (**Table 3**). Local center IDT methods of skin testing were based on previously published guidelines (2, 3).

There were large intercenter and interindividual variations in the Wi when local IDT methods were compared. This is likely to be due to the different ways the injection volume was measured and the injection technique. There was a statistically significant difference in the mean Wi diameter across centers even when a fixed volume of 0.03-ml injection was used for IDT ($p < 0.0001$). The overall median Wi was 5 mm ($n = 318$) with a range of 2–11 mm, variation in mean Wi of 3–8 mm, and interquartile range of 4–5 mm.

Investigation of Whether Standardized IDT Method Reduces Intercenter Variability

During an ENDA meeting, syringe filling, differences in the injection needle gauge, injection technique, and reading of the skin reaction were evaluated.

Needles of 25–30 G did not affect the Wi produced. However, the volume of the syringe used affected the actual volume drawn up into the syringe, probably due to the dead volume of the syringe. In addition, a 0.5-ml syringe has wider spacing between markings, which enables a more accurate measurement of volume of the test solution. Unfortunately, the 0.5-ml syringe is not available throughout Europe. We also consider the variability due to syringe size to be limited. For the standardized method, we, therefore, advised that either 0.5- or 1-ml syringes can be used for IDT, subject to local availability.

To help decide on the test volume to be used in the study, NS IDT was performed on the skin of the coauthors of this study. It was noted that larger injection volumes (>0.02 ml) tended to produce very big wheals. A standardized IDT method with a description of each step, using 0.02-ml injection, was proposed and agreed by the study group (**Table 4**).

Eight centers performed IDT using 0.02-ml saline, and six of these, in addition, performed IDT with 0.02-ml AX (20 mg/ml).

IDT performed with standardized method syringe filling (**Figure 1**) and 0.02-ml saline on 1,096 patients (**Table 5**) showed that a significant difference in the Wi persisted between centers ($p < 0.0001$). However, differences in the mean and median diameters between centers were reduced, with variation in the mean Wi (4.5–5.4 mm) in the range 2–10 mm, with a median diameter of 5 mm (IQR 5–5 mm).

IDT with standardized syringe filling with 0.02-ml AX performed on 240 patients gave a mean Wi diameter of 5.2 mm and a median Wi of 5 mm (range 2–9 mm, IQR 4.5–5 mm). The difference in Wi between centers remained statistically significant ($p < 0.0001$) (**Table 5**).

There was no correlation of wheal size with age or sex with either local methods or standardized IDT.

TABLE 3 | Median injection wheal (Wi) diameter for intradermal tests with saline using non-standardized individual protocols, demonstrating large inter-center variations.

Center	Porto	Vilnius	Ankara	Paris (Necker)	Firenze	Nancy	Munich	Total
Mean age (yrs.)	8	47	43	9	45	55	32	42
Site								
UA (*n*)			15	9		24	24	72
FA (*n*)	6	32	11		99	22	24	194
B (*n*)			28				24	52
Injected volume								
0.02 ml (*n*)			3				36	39
0.03 ml (*n*)	6	22	48	9	99	46	36	266
0.04–0.05 ml (*n*)		10	3					13
Total (*n*)	6	32	54	9	99	46	72	318
Median Wi diameter (R) mm by center	6 (5–8)	5 (3–11)	3 (2–4)	8 (7–9)	5 (4–6)	5 (3–7)	4.5 (3–7)	Wi diameter Median 5 mm Range (2–11) IQR 4–5 $^{**}p < 0.0001$

UA, Upper Arm; FA, Forearm; B, Back; n, number of tests; R, range minimal and maximal; IQR, interquartile range.
***The variables were subject to a chi-squared test for the qualitative variables, a Kruskal-Wallis test for the quantitative variables.*

TABLE 4 | Summary of proposed ENDA protocol for performing and reading drug intradermal test.

1. IDT must be performed, following negative prick tests, using pharmaceutical grade human drugs in injectable form. IDT is contraindicated in severe cutaneous adverse drug reactions
2. Syringe size and needle gauge:
- Tuberculin syringe of preferably 0.5 ml or if not available, 1-ml volume
- Needle gauge of 25, 27, or 30 G
- Same fixed or new needle can be used for test IDT
3. Injection technique:
- Adopt sterile techniques.
- Fill syringe with test solution; change the needle if not fixed. Tap the barrel of the syringe to make the air bubble rise to the needle end of the syringe. Expel air bubble and excess volume pushing the plunger to the 0.02-ml mark on the barrel (**Figure 1**). A syringe with a flat end plunger is better than one with a tapered end to help measure the volume of the test solution drawn into the syringe.
- With the bevel of the needle facing upward, pierce skin tangentially in the upper dermis (at about 10° angle to the skin surface).
- Then slowly inject the measured volume intradermally.
4. Control:
- After intradermal injection of 0.02 ml of saline or test solution at recommended concentration, an injection wheal measuring 4.5- to 5.5-mm diameter should form.
- If no clear wheal forms, repeat injection.
5. Record all injected solutions, batch number, and map of injection sites. Draw around and/or measure the diameter of the immediate injection wheal (Wi). If you surround the wheal with ink, always measure the inner diameter.
6. Read the IDT after 20 min. Measure wheal (W_{20}) and surrounding erythema (E_{20}).
7. If the wheal is not round, measure the length (L), then the width (w) taken perpendicularly, in the middle of the axis length Wi = (L+w)/2.
8. In patient records and publications, IDT results must be recorded as follows: Wi, W_{20}, and E_{20}.
9. At 20 min, the IDT is considered positive only if there is a wheal, $W_{20} \geq Wi + 3$ mm and surrounding erythema, E_{20}.
10. For delayed reactions read at 24, 48 h, or later (please specify the time interval). IDTs are considered positive when there is an erythematous induration or swelling at the injection site.

Analysis of Wi When Standardized IDT Is Performed by Individual Tester Injecting 0.02- and 0.03-ml Saline, Respectively

The Wi obtained by an individual tester injecting 0.02- and 0.03-ml NS, respectively, using the standardized IDT was compared in six different centers (**Table 6**). With 0.02-ml NS injection volume, the Wi mean diameter was 5.1 mm (range 3–8 mm) and median = 5 mm (IQR 4.5–5 mm). When 0.03-ml NS was injected, the mean Wi was 6.2 mm (range 3–8 mm) and median = 6 mm (IQR 5.5–6 mm) (**Table 6**).

Standardization of IDT produced larger Wi. For the 0.03-ml injection volume, the mean Wi was 6 mm (**Table 6**) compared to 5 mm before standardization (**Table 3**). However, the smaller injection volume of 0.02 ml produced smaller Wi (5 mm) (**Table 6**).

Injection Site Does Not Affect Wi Readings

The Wi diameter was not affected by the injection site when the standardized IDT method was used. NS IDT was performed on two or more injection sites (UA, FA, B) in three centers (Ankara, Nancy, and Munich). There was no significant difference in the Wi diameter obtained in the different injection sites ($p > 0.05$) (**Table 3**). In addition, the Wi diameter obtained using the standardized IDT with NS and AX at the recommended

TABLE 5 | Diameter of IDT injection wheal (Wi) after injecting 0.02 ml saline and amoxicillin (20 mg/ml) using standardized method for drug intradermal tests.

	Porto	Vilnius	Ankara	Firenze	Nancy	Malaga	Graz	Groningen	Total
Saline number tested	26	87	78	118	734	6	24	23	1,096
Age	10	51	48	46	59	28	28	44	43
Site									
UA	0	0	0	0	409	0	8	0	417
FA	26	87	78	118	133	6	8	23	479
Back					192		8		200
Median Wi diameter (R) mm	5 (4–6)	5 (3–10)	5 (4–6)	5 (3–6)	5 (2–7)	5 (4–6)	5.4 (4–7)	4.5 (3–6)	Wi diameter Median 5 mm Range (2–10) Q1 = 5, Q3 = 5, IQR = 0, $^{**}p < 0.0001$
Amoxicillin number tested	3	23	0	86	88	17	0	23	240
Age	5	51		45	53	35		44	48
Site									
UA	0	0	0	0	88	0	0	0	88
FA	3	23	0	86	0	17	0	23	152
Median Wi diameter (R) mm by center	5 (5–6)	6 (4–9)		5 (3–6)	5 (2–7)	6.4 (4–7)		5 (4–7)	Wi diameter Median 5 mm Range (2–9) Q1 = 4.5, Q3 = 5, IQR = 0.5, $^{**}p < 0.0001$

*R, range minimal and maximal; IQR, interquanrile range; Wi, injection wheal; UA, Upper Arm; FA, Forearm; B, Back. **The variables were subject to a chi-squared test for the qualitative variables and a Kruskal-Wallis test for the quantitative variables.*

TABLE 6 | Comparison of diameter of IDT injection wheal (Wi) after injection of saline (0.02 ml or 0.03 ml) by trained operators using the standardized method and syringe size used.

	Groningen	Firenze	Porto	Nancy	Malaga	Munich	Total
Number of volunteers	23	11	10	15	10	8	77
Mean age	44	44	34	30	48	30	30
Site ($p = 0.4735$)							
UA (*N*)	0	0	0	30	0	0	30
FA (*N*)	46	22	20	0	20	16	124
Wi diameter (mm) with injection volume (ml)							
0.02 ml	5 (3–6)	5 (4–7)	5 (4–6)	5 (5–6)	6 (5–7)	5 (5–8)	5 (3–8)
0.03 ml	6 (3–6)	6 (5–8)	6 (6–7)	6 (6–6)	7 (6–8)	7 (6–7)	6 (3–8)
Syringe size (ml)							
0.5 ml (*n*)	46	0	20	0	0	0	66
1 ml (*n*)	0	22	0	30	20	16	88

N, number of test; M, median; R, range; SD, standard deviation; UA, Upper arm; FA, Forearm; IQR, interquartile range; No difference of Wi according to the site of injection ($p = 0.4735$).

concentrations (8–10) performed on the UA, FA, and B was not significantly different and suggested that variation of the test results between centers was independent of the injection site and the drug used. Differences in Wi in different sites in the same individual patients were not compared.

DISCUSSION

IDTs are essential for the diagnosis of DH. This study demonstrates significant differences in the IDT methods used, in the volume injected, and in the Wi diameter obtained by the study centers. It shows that standardization of the IDT procedure and injection volume produced improved, reproducible, and more comparable skin test results. Tester training had a positive influence on precision and reduced variability. The identification of several causes of variability in the performance of IDT enabled an IDT standardized method to be proposed (**Table 4**). The adoption of the proposed method and training should lead to more reproducible and comparable results between centers and clinical studies.

In spite of the many guidelines published, our questionnaire survey of 20 allergy centers in ENDA showed that the majority did not follow published guideline recommendations. Indeed, most of the centers (12/20) did not inject a fixed volume as recommended by all guidelines (2–6), but injected a volume to achieve the target Wi. Using a fixed volume of an IDT drug solution of known concentration means injecting a known and fixed quantity of the tested drug. Drug IDT could induce immediate or delayed flare reactions in addition to the wheal (11, 12). The incidence of these flares may depend on the method, the concentrations, and the volume injected. Three flares were observed among 30 patients with IDTs for cutaneous adverse drug reactions (11). The incidence of systemic reactions in patients with positive skin tests to penicillin varies from 0.7 to 9.4% (12, 13). Even with a fixed injection volume, the diameter of Wi could be affected by the age of the individual and the degree of skin atrophy. Injecting a fixed test volume would allow more robust comparison of IDT results between individuals and centers. As illustrated in **Table 1**, the five guidelines vary in the volume of drug injected, the target Wi diameter, the time before reading the immediate skin reaction, the criteria for an immediate and delayed positive test reading, and the site for performing IDT. The differences in the Wi diameter following injection of test volumes used in the guidelines and in our standardized method are stark.

With the ENDA IDT standardized method, the 0.02-ml injection volume produced a mean Wi of 5.1 mm (range 3–8 mm), whereas 0.03 ml produces a mean Wi diameter of 6.2 mm (3–8 mm), median = 6 mm. With the ESCD guidelines showing that injecting 0.04 ml results in raising a Wi of 4–6 mm (2), the EAACI guidelines show that injecting 0.02–0.05 ml produced a Wi of 3 mm (3), and the SFAR guidelines show that injecting 0.02–0.05 ml caused a Wi $\leq$4 mm (5). Such differences make the comparison of IDTs performed following different guidelines of doubtful value. The BSACI guideline uses a fixed volume of 0.03 ml. The resulting wheal, a median Wi of 6 mm, is similar to that obtained by the ENDA standardized method. However, there is a difference in the time when the immediate test reading is taken, 20–30 min with the BSACI guideline and 20 min with the ENDA standardized method.

Macy et al. (6), in their IDT protocol, used 0.02 ml and took the reading of the immediate reaction 15 min after the injection, which was considered positive when the wheal is $\geq$5 mm. In a recent paper, the similarities and differences between Europe and North America in the approach to the diagnosis of DH reactions have been highlighted (14). However, the method for doing and reading IDT, which we have shown to be different among ENDA allergy centers, is not in the list of differences between the two continents.

With the standardized IDT method, the 0.02-ml injection volume produced a mean Wi of 5.1 mm, whereas 0.03 ml and above produced a Wi diameter of 6.2 mm (3–8 mm). Due to the small risk of sensitization and anaphylaxis induced by IDT (9), it is considered good practice to inject a small as possible volume of potential drug allergen that will produce a test wheal that enables accurate reading of the diameter. That is why we have recommended that 0.02 ml of non-irritating test allergen solution should be used for IDT.

Variability could also be caused by differences in measuring wheal sizes and differences in the depth of injection. The somewhat surprising but interesting finding that the injection site did not significantly affect Wi needs further evaluation. It appears that IDT is a more complicated and variable method than previously acknowledged and that detailed recommendations and training are needed for method consistency and reproducible results and interpretation. Intertester variability could be reduced by having designated trained members of staff to perform IDT (15).

We hope that these highly detailed ENDA guideline for performing IDT will help to standardize the IDT method. We envisage that further studies will be necessary using this standardized method to determine if Wi could be affected by the age of the patient, the test site, and skin atrophy. It would also be interesting to determine the negative predictive value of IDT in using different criteria for their positivity W20 $\geq$ Wi+ 3 mm, W20 $\geq$ Wi $\times$ 2 or W20$\geq$ a fixed diameter of 10 or 5 mm.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AB, MW, ST, VK, SB, LG, HM, EG, WA, HE, MT, CP, JG, and KB: collecting, data, and corrections. AB, MW, and JG: writing the document. CA: statistics.

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Biological Therapies of Severe Asthma and Their Possible Effects on Airway Remodeling

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Asthma is a chronic and heterogenic respiratory tract disorder with a high global prevalence. The underlying chronic inflammatory process and airway remodeling (AR) contribute to the symptomatology of the disease. The most severely ill asthma patients may now be treated using a variety of monoclonal antibodies aiming key inflammatory cytokines involved in asthma pathogenesis. Although clinical data shows much beneficial effects of biological therapies in terms of reduction of exacerbation rates, improvement of lung functions, asthma control and patients' quality of life, little is known on the effects of these monoclonal antibodies on AR—a key clinical trait of long-term asthma management. In this review, the authors summarize the data on the proven effects of monoclonal antibodies in asthma on AR. To date, in terms of reversing AR, the mostly studied was omalizumab. However, some studies also addressed this clinical issue in context of other severe asthma biological therapies (mepolizumab, benralizumab, tralokinumab). Still, data on effects of particular biological therapies on AR in severe asthma are incomplete and require further studies. According to the American Thoracic Society research recommendations, future research shall focus on AR in asthma and improve drugs targeting AR, including the available and future monoclonal antibodies.

Keywords: asthma, airway remodeling, airway remodeling in asthma, biological therapy, omalizumab, mepolizumab/reslizumab, benralizumab

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INTRODUCTION

Asthma is a chronic, heterogeneous and inflammatory respiratory condition characterized by shortness of breath, cough, wheezing, and chest tightness. It belongs to the group of obstructive diseases for which the variable airflow limitation is characteristic (1). Various asthma phenotypes differ in causes and mechanism of symptom formation, and thus in severity and frequency of symptoms and exacerbations (2). Currently, asthma affects 1–18% of the population in various countries (1, 3, 4). It occurs in all age groups, with new diagnoses mostly made in children aged 0–9 [early-onset asthma, usually atopic (5)] and in adults aged 40–49 [late-onset asthma, often with eosinophilic phenotype (6)]. Noteworthy is that not only asthma, but also other allergic diseases'–urticaria, allergic rhinitis or food allergies—prevalence increase worldwide (4, 7, 8). The causes of this epidemiological phenomenon mainly include: environmental changes associated with the modification of the surrounding microbiome affecting microbiological and immunological changes in the human respiratory tract from the earliest years of life (the so-called "hygiene theory"), past respiratory infections, exposure to allergens, air pollution, and other pollutants (9, 10).

Currently, "asthma" is considered an umbrella term, which encompasses several, both clinically and pathophysiologicaly different variants of the disease. The two main divisions concern the type of inflammation: T_h2-predominant and non-T_h2. Further, phenotypes are distinguished as either eosinophilic asthma or non-eosinophilic (11). In particular, among T_h2-predominant phenotypes, the most prevalent endotype is the allergic asthma. It develops on basis of atopy, in particular in response to inhaled allergens such as house dust mites, grass pollen, trees, and pets (6). Apart from classical childhood-onset allergic asthma, late-onset eosinophilic asthma is now one of the best-defined phenotypes (12). Several other endotypes of asthma include obesity-associated asthma, neutrophilic asthma, very-late onset asthma and other.

In the pathogenesis of the disease, mediators of the T_h2-dependent reaction play a key role, including: IgE, IL-3, IL-4, IL-5, IL-13, IL-33, TSLP, and other (13). In non-allergic asthma, although the cellular pathomechanism is different, most of the mediators remain the same, with main variations including IL-17 and PGD_2. The underlying immunopathological mechanisms of asthma lead to chronic airway inflammation resulting in number of consequences for the bronchi. The airways become hypersensitive and constrict when subject to stressful stimuli. Another result of this ongoing inflammation is airway remodeling (AR), a process of structural changes of bronchi walls. The chronic airway inflammation thus leads to reduced airway airflow and clinical symptoms—wheezing, shortness of breath cough, chest tightness. Unfortunately, those symptoms are few and non-specific, thus the differential diagnosis of asthma is often difficult. Additionally, asthma is often associated with comorbidities, including: other allergic conditions (rhinosinositus, nasal polyps, atopic dermatitis), obesity, diabetes, gastroesophageal reflux, depressive and anxiety disorders, and other (14).

In clinical practice, we distinguish 3 levels of asthma severity (mild, moderate and severe) and 5 Global Initiative for Asthma (GINA) treatment steps (1). Mild asthma comprises of GINA steps 1. and 2. Moderate asthma, characterized by more severe symptoms and more frequent exacerbations is GINA step 3. Severe asthma are GINA steps 4. and 5. The most severely ill patients, i.e., those who do not achieve asthma control despite using high doses of inhaled steroids, are qualified for step 5. biological treatment with monoclonal antibodies against key asthma mediators. The overall clinical goal in asthma is disease control, i.e., a therapy that provides optimal symptom reduction. Drugs and their doses are modified depending on the symptoms, severity of the disease and exacerbation frequency. Treatment may be intensified in absence of control or reduced if long-term, optimal disease control is achieved.

AIRWAY REMODELING IN ASTHMA

In the pathogenesis of asthma symptoms, bronchospasm under the influence of external stimuli plays a key role (15). Simultaneously, the inflammation that occurs in the bronchi is responsible for the onset of symptoms. Currently, another distinguished disease component is AR, i.e., a process of reconstruction of the bronchi wall. Much research is currently focused on AR, the understanding of which will allow to search for new therapeutic possibilities of asthma (16, 17).

TABLE 1 | Key molecular factors contributing to airway remodeling (in particular—the factors that are aimed by currently available or investigated biological therapies of asthma).

Factor	Key effect(S) on airway remodeling	References
IgE	1. Indirect contribution—IgE stimulates production of cytokines involved in airway remodeling (IL-4, IL-5, IL-13, TGFβ1, and other) during the late phase 2. Direct contribution—induction of ASM proliferation *in vitro*	1. (25) 2. (26)
IL-4	1. Increased synthesis of α-smooth muscle actin and collagen III 2. Induction of TGF-β release by airway epithelial cells	1. (27) 2. (28)
IL-5	1. Promotion of subepithelial and peribronchial fibrosis by eosinophil recruitment and subsequent production of TGFβ1	1. (29, 30)
IL-13	1. Induction of TGF-β release by airway epithelial cells 2. Changes in goblet cell density	1. (28, 31) 2. (32)
IL-17	1. Promotion of ASMC migration 2. Increase of matrix metaloproteinases 3. Cross-talk with TGFβ1 resulting in EMT 4. Stimulation of inactive fibrocytes maturation to fibroblast, which deposit collagen within ECM	1. (33) 2. (34) 3. (35) 4. (36)
TSLP	1. Promotion of collagen deposition 2. Goblet cells hyperplasia 3. Local eosinophil recruitment in airway 4. Increase in type-I collagen and α-SMA expression in human lung fibroblasts	1, 2, 3. (37) 4. (38)

Chronic respiratory epithelium inflammation leads to changes in microvascularization, thickening of the airway walls and impaired airflow through the bronchi, and consequently to impaired ventilation (18). Thus, AR is a change of composition, content and distribution of cellular and molecular components in the airway wall (19). In asthma, it is associated with many structural changes–epithelial damage, subepithelial fibrosis, angiogenesis, hypertrophy and proliferation of myofibroblasts and myocytes and increased number of smooth muscle fibers in airway smooth muscle cells (ASMC), that increase airway smooth muscle (ASM) mass (20, 21). A number of inflammatory molecular factors are involved in these structural changes, either directly or *via* further induction of inflammatory reaction, namely eosinophilic (22). Starting with the local epithelium-derived factors, the key AR mediators include: PDGF (platelet-derived growth factor), TGFβ (transforming growth factor β, with particular emphasis on $TGFβ_1$, among its three isoforms), FGF (fibroblast growth factor), EGF (epidermal growth factor), prostaglandin D2 (PGD2), CXCL2, CXCL3, IL-8, eotaxin, TSLP, CCL1, and other, which all promote ASMC migration (23, 24). The cytokines produced by T_h2 (IL-4, IL-13) and T_h17 cells (IL-17, IL-21, IL-22, TNFα) share the same effect. All of the inflammatory factors that are targeted by currently available and investigated biological therapies contribute to AR. The summary of their effects on particular components of AR is available in **Table 1**.

Currently, the possible role of epithelial-mesenchymal transition (EMT) in AR is also strongly discussed. EMT is a transformation of epithelial cells into mesenchymal-like cells by loss of their epithelial traits (39). Features of EMT in AR are currently intensively studied and emerging studies confirm that EMT occurs in AR in asthma (40, 41). A major mediator of that process is $TGF\beta_1$, which has been proven to induce EMT of airway epithelial cells–this process occurs to a greater extent in cells of asthmatic than of non-asthmatic patients (42). It is thus worth to pay attention to the inflammation mediators which are targeted by biological therapies of severe asthma and their effect on $TGF\beta_1$-mediated EMT [eg. IL-4, IL-17 (35, 43)]. *In vitro* studies also show that neutrophils from severe asthmatics induce EMT in healthy bronchial epithelial cells *via* $TGF\beta_1$ dependencies (44). A need for further research in this area is suggested in the literature (40, 45, 46).

As a result of AR, patients may experience irreversible airway obstruction which leads to worsening of lung function, airway dilatation and response to bronchodilators. AR thus significantly contributes to the development and long-lasting persistence of asthma symptoms (16, 47–49).

SEVERE ASTHMA AND ITS BIOLOGICAL THERAPY

Severe asthma affects 3.6–10.0% of patients with asthma (50–52), which corresponds to around 4 million patients globally. Currently, much research is focused on pathomechanisms of severe asthma and development of its new biological therapies (53). Although it is much less prevalent than mild and moderate asthma, severe asthma contributes to about 60% of costs associated with this disease, mainly due to drug costs (54, 55).

The ground-breaking achievement in severe asthma treatment was the introduction of its first biological treatment—anti-IgE monoclonal antibody omalizumab. The following years brought further biological agents aimed at different factors, including IL-5, IL-5R, IL-13, IL-4R, and other. Each of these drugs blocks a certain immunological pathway triggering and controlling the allergic or non-allergic airway inflammation. With the now-available monoclonal antibodies in asthma, clinicians may select a drug according to asthma phenotype. Currently, approved by the FDA and available on the market are: omalizumab, mepolizumab, benralizumab, reslizumab, and dupilumab (56).

Omalizumab is a humanized IgG1/κ monoclonal antibody that binds to the IgE immunoglobulin Fc fragment (57). Thus, it inhibits the main mediator of type I reaction pathway. By binding blood-circulating free IgE molecules, it inhibits the activation of mast cells and basophils (58). Launched in 2003, omalizumab has been used in severe allergic asthma and, since 2014, in chronic urticaria. Omalizumab is the very first monoclonal antibody included in the GINA recommendations (in 2004) on step 5. as an addition to standard therapy with high doses of inhaled steroids, β2-agonists and other drugs. Clinical and observational studies conducted over several years of using omalizumab have proven that it improves asthma control and relieves its symptoms, reduces exacerbation risk and improves lung function (59–61). Long-term safety of this drug was demonstrated in adults in terms of oncological safety and pregnancy (62–64) and in children (65).

Mepolizumab–another biological drug for severe asthma treatment—was registered in 2015. This antibody binds IL-5, which prevents it from binding to the IL-5R α subunit on eosinophils. This drug is thus used in patients with eosinophilic asthma as by blocking the IL-5 signaling, the patient's eosinophil population is reduced, which leads to clinical improvement (66). Clinical and observational studies confirmed that mepolizumab used in the treatment of severe eosinophilic asthma improves asthma control, reduces the number of exacerbations and doses of steroids used and improves lung function (67, 68). Importantly, both mepolizumab and omalizumab exhibit a comparable safety profile (69).

Whilst having achieved greatly beneficial clinical effects with biological treatment, as research in severe asthma progressed, further drugs were introduced by FDA, including: benralizumab (which targets IL-5R α subunit), dupilumab (which inhibits IL-4 and IL-13 signaling) and reslizumab (anti-IL-5 antibody) (56, 70, 71). The summary of their biological and clinical effects is available in **Table 2**.

EFFECTS OF PARTICULAR BIOLOGICAL DRUGS ON AIRWAY REMODELING

AR in asthma is mainly caused by long-term, uncontrolled airway inflammation (16). With the duration of the disease and its symptoms, structural changes in the bronchi progress, which may lead to a significant and long-term impairment of lung function (49). Considering the above and the immunomodulatory effect of biological therapies, it may be assumed that these drugs may significantly affect AR. However, data on this subject are limited and few studies cover this clinical aspect. A summary of research covering biological therapies' impact on AR is available in **Table 2**.

Omalizumab

Roth et al. described the effects of IgE-contained serum from allergic asthma patients on ASM cells. The effects of such incubation were: ASM cells proliferation, deposition of type-I collagen in 48 h and of fibronectin in 24 h. A 1 h pre-incubation of ASM cells with omalizumab, prevented these three effects. The addition of allergens did not increase the IgE-dependent effects on cells incubated in omalizumab (73). Another interesting study on omalizumab was published by Huang et al. (72), in which the authors analyzed of omalizumab on allergen- and IL1β-stimulated proinflamatory cytokine and nitric oxide production in human bronchial epithelial cells (BECs) and compared them to those of budesonide. In that study omalizumab shared similar effects as budesonide in decrease of TNF-α, TGFβ and IL-4 production (72).

In 2012 Hoshino and Ohtawa compared 16 patients on omalizumab to 14 patients with conventional severe asthma treatment and measured their airway dimensions with high-resolution computed tomography (HRCT). A 16-week

TABLE 2 | Summary on biologic therapies for the treatment of severe asthma and with their clinical effects and confirmed effects on AR.

	Drug	Form	Target	Biological effects	Clinical effects	Effects on airway remodeling	Other fda-approved indications
FDA—approved monoclonal antibodies for treatment of moderate-to-severe asthma	Omalizumab	Humanized IgG1/κ, monoclonal antibody	IgE	◦ ↓ circulating total IgE ◦ Down-regulation of FcεRI receptors on basophils, mast cells, and dendritic cells	◦ Improvement of lung function (FEV1) ◦ Improvement of quality of life (AQLQ) ◦ Improvement of asthma control (ACT) ◦ ↓ oral and inhaled corticosteroid use ◦ Reduction in exacerbation and hospitalization frequency (59)	◦ Reduction of production of TNF-α, TGFβ and IL-4 in bronchial epithelial cells (72) ◦ Prevention of ASM cell remodeling *in vitro*(73) ◦ Reduction of airway wall thickness in computed tomography (74, 75)	◦ Chronic idiopathic urticaria
	Mepolizumab	Humanized IgG1/κ, monoclonal antibody	IL-5	◦ Blockage of IL-5/IL-5R binding on eosinophils ◦ ↓ blood eosinophils ◦ ↓ sputum eosinophils	◦ Reduction in exacerbation frequency vs placebo ◦ Improvement in AQLQ vs placebo ◦ No significant effect on FEV1, PEF, PC_{20}(76)	◦ Reduction of airway remodeling markers (tenascin, lumican, and procollagen III) and airway eosinophils expressing TGFβ1 in bronchial reticular basement membrane and reduction of TGFβ1 in bronchioalveolar lavage after mepolizumab treatment (77) ◦ Reduction of AR observed in computed tomography (78)	NA
	Benralizumab	Humanized IgG1/κ, monoclonal antibody	IL-5 Receptor alpha subunit (IL-5Rα)	◦ ↓ eosinophils and basophils *via* antibody dependent cell mediated cytotoxicity (ADCC)	◦ Reduction in exacerbation frequency ◦ No significant effect on FEV1 ◦ Mixed data on quality of life and asthma symptom scores (79)	◦ Decrease in airway smooth muscle mass (predicted using computational modeling approach) (80)	NA
	Dupilumab	human IgG4 monoclonal antibody	IL-4 Receptor alpha subunit (IL-4Rα)	◦ Blockage of IL-4/IL-4Rα binding ◦ Blockage of IL-13/ IL-4Rα binding	◦ Reduced rate of severe asthma exacerbations and improved lung function (FEV1), asthma control and quality of life (81, 82)	Studies on *in vitro* or *in vivo* effects of dupilumab on airway remodeling are currently non-available	◦ Eczema ◦ Moderate-to-severe atopic dermatitis in adolescents ◦ Chronic rhinosinusitis with nasal polyps
	Resliuzmab	humanized IgG4/κ mAb	IL-5	◦ Blockage of IL-5/IL-5R binding ◦ ↓ circulating eosinophils ◦ ↓ sputum eosinophils	◦ Reduced exacerbations, improved FEV1, forced vital capacity, the 7-item Asthma Control Questionnaire (83)	Studies on *in vitro* or *in vivo* effects of reslizumab on airway remodeling are currently non-available	NA
Drugs investigated (currently or previously) in severe asthma treatment	Secukinumab	human IgG1κ monoclonal antibody	IL-17A	◦ Blockage of IL17A -, −17F -, −17A/F heterodimer -, and −17E–(IL-25)/IL-17RA binding	NA	Studies on *in vitro* or *in vivo* effects of secukinumab on airway remodeling are currently non-available	◦ Plaque psoriasis ◦ Psoriatic arthritis ◦ Ankylosing spondylitis ◦ Discontinued in asthma
	Brodalumab	human, IgG2 monoclonal antibody	IL-17 receptor A (IL-17RA)	◦ Blockage of IL17A -, −17F -, −17A/F heterodimer -, and −17E–(IL-25) /IL-17RA binding	◦ No significant improvement in lung function (FEV1) and asthma control in subjects with inadequately controlled moderate to severe asthma (84)	Studies on *in vitro* or *in vivo* effects of brodalumab on airway remodeling are currently non-available	Plaque psoriasis
	Tralokinumab	Human IgG4 monoclonal antibody	IL-13	◦ Blockage of IL-13/IL-13Rα1 ◦ Blockage of IL-13/IL-13Rα2 binding	◦ Inconsistent effects on annualized asthma exacerbation rate (85)—development of tralokinumab in severe asthma was discontinued by the producer after this study (86) ◦ No significant improvement of lung function (FEV1) (87)	◦ No significant effect on bronchial eosinophilic count ◦ No significant reduction of airway remodeling in bronchial biopsy features–Airway smooth muscle area, RBM thickness, collagen type IV, periostin, TGFβ and other (87)	◦ None available (possibly in atopic dermatitis in the future) (88) ◦ Discontinued in asthma
	Secukinumab	Humanized IgG4 monoclonal antibody	IL-13	◦ Blockage of IL-13/IL-13Rα1 ◦ Blockage of IL-13/IL-13Rα2 binding	◦ Decrease in asthma exacerbations incidence ◦ Improved lung function (FEV1%) (89)	◦ Greater clinical effects (decrease in exacerbation rate and improvement in lung function) in patients with high serum periostin levels – a protein contributing to airway remodeling (90)	◦ None available (possibly in atopic dermatitis in the future)(91) ◦ Discontinued in asthma
	Tezepelumab (AMG 157)	human, IgG2 monoclonal antibody	TSLP	◦ Blockage of TSLP/TSLP-receptor binding	◦ Inhibition of late allergen-induced asthmatic response (FEV1) (92) ◦ Reduction of annualized asthma exacerbation rate (93)	◦ Studies on *in vitro* or *in vivo* effects of tezepelumab on airway remodeling are currently non-available	NA

The table includes approved and emerging therapies with published human data. NA, none available.

omalizumab treatment significantly reduced the airway wall thickness measures: airway wall area corrected for body surface area (WA/BSA)−13.7 to 12.1 mm^2/m^2, percentage wall area (WA%)−71.1 to 64.7% and wall thickness (T)/$\sqrt{}$BSA−1.21 to 0.92 mm/m. Luminal area (Ai/BSA) at the right apical segmental bronchus significantly increased (4.8 to 6.4 mm^2/m^2) and the percentage of sputum eosinophils significantly decreased. These effects were not observed in the conventional therapy group (74).

Tajiri also studied effects of omalizumab in regard to AR in a 48-week follow-up of omalizumab in 26 patients (CT measurements were analyzed in 14 patients). A significant reductions of WA% was observed (57.1 vs. baseline 62.0) and a small, but significant, increase in Ai/BSA (12.1 vs. baseline 12.0) (75).

However, Przybyszowski et al. reached slightly different results to the above. The authors analyzed changes of HRCT airway dimensions in 12 patients before and after at least 4 months of omalizumab treatment. They observed a decrease in airway wall area and WA/BSA, but no changes in WA% nor in luminal area to total bronchial area ratio (94).

Mepolizumab

Flood-Page published in 2003 a study on the effects of mepolizumab on AR markers in bronchial biopsies of 24 atopic asthmatics from a randomized, double-bind, placebo-controlled study, which were obtained before and after three mepolizumab infusions. Compared to placebo, treatment with mepolizumab significantly reduced the expression of three extracellular matrix proteins: tenascin, lumican and procollagen III in the reticular basement membrane. Moreover, mepolizumab significantly reduced the number and percentage of airway eosinophils expressing $TGF\beta_1$ mRNA and decreased $TGF\beta_1$ in BAL fluid (77).

Only one clinical study (12-month mepolizumab vs. placebo trial, 61 subjects) analyzed the effect of mepolizumab on AR. The mean change in CT measured wall area and total area corrected for body surface area was significantly greater in treatment group than in placebo group. In fact, in mepolizumab group the values decreased whereas in placebo group an increase of these parameters was observed (78).

Benralizumab

A very interesting approach in assessing effects of benralizumab on AR was taken by Chachi et al. (80). The researchers used bronchial biopsies of 15 patients on benralizumab and 10 patients receiving placebo, which were collected from subjects with eosinophilic asthma during a previous phase I multicenter, randomized, double-blind, placebo-controlled trial (95). The eosinophil count in airway lamina propria was assessed in pre- and post-treatment biopsies. It decreased significantly in benralizumab group by 66.4% and by 88% relative to placebo. Knowing the mechanism of action of benralizumab, and with the observed mean change in eosinophil count, the authors used a computational model to predict effects of this drug on AR. They concluded that in benralizumab group the drugs pro-apoptosis efficiency was 47%, corresponding to a consequent 29% relative reduction of ASM mass. Additionally, in the benralizumab group, a non-significant reduction in the number of tissue myofibroblasts was observed. The authors suggest that as ASM cells do not express IL-5R, the effects of benralizumab on ASM mass are an indirect effect of reduced eosinophilic inflammation. They proposed an assumption that depletion of local eosinophils results in decrease of airway TGFβ–a major growth factor contributing to AR, which is majorly expressed in lungs by the eosinophils.

Other Antibodies (Dupilumab, Reslizumab, Secukinumab, Brodalumab, Tralokinumab, Lebrikizumab, Tezepelumab)

To authors knowledge, no study covered any aspect of AR alleviation (either *in vitro*, *in vivo* in animal models or *in vivo* in humans) in therapy with FDA-approved dupilumab and reslizumab. The issue was also not addressed regarding secukinumab, brodalumab, and tezepelumab—which were clinically studied in asthma, but are now discontinued in this indication.

Fragmentary data can be found regarding tralokinumab and lebrikizumab. In a phase 2 trial of tralokinumab in asthma, features of AR in bronchial biopsies (ASM area, RBM thickness, collagen type IV, periostin, TGFβ and other) were not reduced, neither was the bronchial eosinophilic infiltration (87). Lebrikizumab in turn showed greater clinical effects (decreased exacerbation rate and improved lung function) in asthmatic patients with high serum periostin levels (protein contributing to AR) in a phase 2 trial (90). However, the two drugs' development in asthma is also discontinued.

SUMMARY

According to the American Thoracic Society (ATS) 2017 expertise—and other researchers' opinions (16, 96)–it should be further investigated whether, and if so, to what extent, biological therapy of asthma significantly affects AR and what are the clinical consequences of such an effect. Moreover, research on AR was indicated by ATS as crucial in the development of knowledge about asthma and its treatment. The need to monitor AR in future clinical studies is pointed out as an important aspect of response to modern biological treatment of severe asthma. These recommendations also highlight the need to study the impact of currently available biological preparations on AR and to look for new drugs that alleviate it (97).

The development of biological therapies has opened a new chapter in treatment of severe asthma. Since the introduction of the first monoclonal antibody in this disease—the anti-IgE drug omalizumab in 2004—a new range of biology-oriented therapy emerged. With the arrival of the subsequent antibodies targeting other molecules involved in asthma pathophysiology, it became possible to treat the most severely ill patients using phenotype-oriented drugs. However, as only since recent years new drugs in this field arrive, little is known about their effect on the AR—a key clinical feature of severe asthma and its long-lasting consequences. The data available to date confirms with a high degree of probability only the beneficial role of omalizumab

in reversing AR. Some promising studies cover this topic in regard to mepolizumab and benralizumab. Yet, future research of available and upcoming biological therapies in severe asthma shall address this clinical issue as an important feature of long-term severe asthma management.

AUTHOR CONTRIBUTIONS

GK and MP created the concept of the paper. GK conducted the literature research and wrote the manuscript. PK and MP revised the paper.

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Formulations for Allergen Immunotherapy in Human and Veterinary Patients: New Candidates on the Horizon

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Allergen immunotherapy is currently the only causal treatment for allergic diseases in human beings and animals. It aims to re-direct the immune system into a tolerogenic or desensitized state. Requirements include clinical efficacy, safety, and schedules optimizing patient or owner compliance. To achieve these goals, specific allergens can be formulated with adjuvants that prolong tissue deposition and support uptake by antigen presenting cells, and/or provide a beneficial immunomodulatory action. Here, we depict adjuvant formulations being investigated for human and veterinary allergen immunotherapy.

Keywords: immunotherapy, adjuvant, allergy, allergen, veterinary, human

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INTRODUCTION

Allergen immunotherapy (AIT) is currently the only causative treatment for allergic diseases of animals and man. Subcutaneous administration of allergen extracts—with or without an aluminum hydroxide adjuvant—historically has proven efficacious for many allergic patients. However, recent studies suggest that desensitizing properties of the allergen potentially can be enhanced by alternate adjuvants or delivery systems, while maintaining freedom from adverse effects.

A number of delivery systems for AIT are currently being investigated (1–3) and applied in animal models, but rather few human or veterinary clinical studies exist. For nano- (NP) or microparticle (MP) preparations, various particulate compositions are complexed or filled with allergens (3). The particulate materials must be biocompatible (resulting in no adverse reaction) and can either be biodegradable (broken down in the organism) or non-biodegradable. Several non-biodegradable materials tested as delivery systems for allergens *in vitro* as well as in animal models, such as dendromers/dendrosomes (4), polyethylenimine (5), polypropylene sulfide (6), multiwalled carbon nanotubes (7), gold nanoparticles (8), or fullerenes (9) have been comprehensively reviewed (2). However, their fate in the organism is not absolutely clear and thus must be carefully studied.

We selected here the most promising novel AIT formulations, encompassing both modifications of allergen and inclusion of immunomodulators, and describe their performance in human and veterinary trials.

FORMULATIONS WITH VEHICLES THAT PROTECT IMMUNOGENICITY

For an allergen-specific and prolonged effect of AIT, allergens must reach the immune system in a recognizable form, and be released from any carrier in an optimal, perhaps gradual manner. Thus, one general approach to enhance response to AIT is to protect allergens from degradation, and/or ensure optimal release by packing them into resistant carrier materials.

Methylmethacrylate Coating

Grass pollen allergen was coated with a co-polymer of methacrylic acid and methylmethacrylate, called Eudragit L-100®, to protect against gastric degradation, and administered orally to Guinea pigs (10). The secondary antibody response was greater than with an aqueous solution of the allergen. An encapsulated ragweed allergen extract given to people with hay fever led to an increase of anti-ragweed IgG antibodies, a dampened increase of IgE antibodies, and decreased symptom-medication scores without systemic reactions (11).

Plant Cell-Wall Fusion Proteins

Plant cell-expressed or complexed allergen proteins delivered orally are protected from gastric acid and enzymatic degradation, but are then digested by gut microbes in the colon and release the allergens to the immune system (12). Transgenic rice expressing the major house dust mite (HDM) allergen Der p 1 was developed as an edible AIT product (13). Several other proteins have been used in this fashion to induce tolerance in mice. After oral prophylactic administration of transgenic rice expressing modified Japanese cedar pollen allergens Cry j 1 and Cry j 2 (14, 15) to BALB/c mice or HDM allergen Der p 2 in transgenic tobacco in a murine asthma model (16), a decreased allergic response was uniformly seen. Chemically modified ragweed pollen shells fed to BALB/c mice were incorporated in the subepithelial tissue (17). In addition, bone-marrow derived macrophages and dendritic cells cultured with this pollen increased expression of CD40, CD80, CD86, and MHC class II molecules and secreted proinflammatory cytokines TNF-alpha and IL1-beta. Such studies have not been performed in human or veterinary patients.

Polyanhydrides

Particles made of amphiphilic polyanhydrides are biodegradable and show a favorable safety profile. Poly[methyl vinyl ether-co-maleic anhydride] (Gantrez® AN 119) has been investigated in mouse models for oral immunotherapy against peanut allergy (18–20), cashew nut allergy (21), and *Lolium perenne* pollen allergy (22). Three doses of nanoparticle-coated peanut allergens were able of protecting CD1 mice against severe anaphylaxis induced by a peanut challenge (18). Similarly, in CD1 mice presensitized to peanut, AIT with nanoparticle-encapsulated peanut allergen was associated with significantly lower concentrations of mMCPT-1, and an increased survival rate after challenge, compared to AIT with free peanut extract (20). Similar results were seen with allergens of *L. perenne* combined with Gantrez nanoparticles and LPS of *Brucella ovis* (22). Oral administration of cashew nut-loaded nanoparticles to BALB/c mice led to a decrease in splenic Th2 cytokines, and an enhancement of pro-Th1 and regulatory cytokines with an increased expansion of T regulatory cells compared to mice immunized with free allergens (21). Despite promising results in these murine models, no published studies in human or veterinary patients exist.

Evidence thus indicates that formulations protecting the allergens are beneficial, and show Th1- and Treg-inducing capacity.

ALLERGENS ADMINISTERED WITH NOVEL ADJUVANTS

A different approach incorporates adjuvants along with the allergen, with the goal of enhancing a desirable, non-allergic immune response, optimally counteracting an allergy-immune milieu.

Monophosphoryl Lipid A (MPLA)

Monophosphoryl lipid A (MPLA) is a compound derived from Gram-negative bacteria and effectively applied in human allergic patients since 1975 (23). *In vitro* studies indicate that it may also induce the secretion of Th1 cytokines from equine cells, thus making it a candidate for the treatment of insect bite hypersensitivity (IBH) (24). Twelve healthy Icelandic horses were immunized with *Culicoides nubeculosus* allergens adjuvanted with MPLA plus alum, or alum alone (25). When their peripheral blood mononuclear cells (PBMCs) were stimulated, the MPLA/alum-immunized horses produced more IFN-gamma and IL-10, both preferable in allergy.

Gelatin-CpG-ODN

Gelatin particles combined with CpG-ODN (GbpCpG) are among the few preparations already studied in veterinary allergy patients, including canine atopic dermatitis (26, 27) and equine recurrent airway obstruction (RAO, an analog of human asthma) (28–30). Uptake of these particles by canine PBMCs could be demonstrated with confocal laser scanning microscopy, and an increase of IL-10 secretion could be shown when cells were incubated with GbpCpG compared to CpG-ODN alone (27). Atopic dogs improved clinically after subcutaneous administration of GbpCpG, while their IL-4 expression decreased (26). Bronchoalveolar lavage cells from RAO horses were incubated with different CpG-ODN sequences; IL-10 and IFN-gamma release was increased, while IL-4 decreased (30). When nebulized with a gelatin nanoparticle-based CpG-ODN formulation, horses with RAO improved clinically and the IL-10 concentration increased in their bronchoalveolar lavage fluid

Abbreviations: AIT, allergen immunotherapy; FDA, U.S. Food and Drug Administration; HDM, house dust mite; MP, microparticle; MPLA, monophosphoryl lipid A; NP, nanoparticle; ODN, oligodeoxynucelotides; OVA, ovalbumin; PEG, polyethylene glycol; PGA, poly-glutamic acid; PHEA, poly(hydroxyethyl)-aspartamide; PLGA, poly-lactic-co-glycolic acid; TLR, Toll-like receptor; VLP, virus-like particles; RAO, recurrent airway obstruction; IBH, insect bite hypersensitivity; PBMC, peripheral blood mononuclear cells; WGA, wheat germ agglutinin; SLP, S-layer protein; LT, heat-labile toxin; SHAS, Strontium-doped hydroxyapatite porous spheres; TADM, Triacedimannose.

(28). In a subsequent placebo-controlled trial, this treatment caused a persistent decrease of allergic clinical variables in horses treated with nebulized GbpCpG (29). A later study described lyophylisation of GbpCpG facilitating its storage and use (31).

Triacedimannose (TADM)

Incubation of the synthetic trivalent glycocluster TADM with birch-stimulated PBMC of allergic rhinitis patients suppressed the production of all Th2-type cytokines (32). TADM suppressed IgE production and enhanced IFN-gamma production in a mouse model of OVA-induced allergic asthma (32). Intranasal application of TADM and timothy grass pollen extract to sensitized BALB/c mice led to a much greater decrease in lymphocyte and eosinophil counts in blood, BALF, and lung biopsies compared to CpG-ODN and MPLA, and (in contrast to CpG-ODN alone) did not increase neutrophil counts (33).

Polysaccharide Polymers

Carbohydrate-based particles complexed with Phl p 5 grass pollen allergen or cat allergen Fel d 1 were successfully used in several studies of AIT in mice (34–37).

The polyaminosaccharide chitosan (poly-D-glucosamine) is approved for use in human wound healing, but is not yet evaluated for AIT. Chitosan particles were used with ovalbumin as a mucoadhesive to promote uptake by oromucosal dendritic cells *in vitro* (38), and also with allergens from HDM and peanut in mouse models to augment AIT (39–41).

Other polysaccharides used for preparation of particulate delivery systems are dextran, alginate, starch, and cellulose derivates. Amylopectin-based microparticles were formulated with Bet v 1 from birch pollen for sublingual treatment of allergic mice (42). Mannan-dextran-maltodextrin covalently attached to OVA and papain were intradermally injected into BALB/c mice, leading to elevated humoral immune responses, and an IgE-to-IgG-shift (43). Another potentially useful polysaccharide is pullulan, a polysaccharide which, coupled to HDM allergen Der p 2, and administered to dogs, effectively reduced clinical signs of atopic dermatitis (44). Carbohydrate-modified ultrafine ceramic-core based nanoparticles, so-called aquasomes, are not biodegradable, and have been applied in the mouse model with ovalbumin (OVA) as model allergen preparation for intradermal application (45).

Heat-Labile Toxin (LT) From *E. coli*

A patch delivery system for birch pollen allergen rBet v 1 with and without heat-labile toxin (LT) from *Escherichia coli* was compared to subcutaneus alum-adsorbed rBet v 1 in a guinea pig model (46). Only the rBet v 1-LT was able to induce allergen-specific blocking IgG antibodies comparable to subcutaneous immunization.

Miscellaneous Particulate Formulations

Strontium-doped hydroxyapatite porous spheres (SHAS) have been used with OVA subcutaneously in a mouse model and led to a sustained stimulation of both CD4+ and CD8+ T cells (47). AIT with SHAS-OVA showed a higher efficacy as assessed by symptom scores compared to soluble OVA. This approach was not tested clinically in human or veterinary patients.

Poly(epsilon-caprolactone; PCL) is a biocompatible adjuvant, and in mice sensitized to OVA led to lower IgE, fewer anaphylactic reactions, and higher survival rate compared to alum-adjuvant treated animals (48). Studies in human and veterinary patients are lacking.

Modified difunctional water-soluble PEG dimethacrylate (PEG-acetal-DMA) macromonomers have cleavable acetal units (49), and when those were filled with allergen (OVA, grass pollen allergen, HDM allergen) and encapsulated into liposomes, they could avoid IgE-dependent activation of basophils *in vitro*, but were taken up by dendritic cells (50).

Poly-glutamic acid particles (PGA) were used with *Phleum pretense* pollen extract *in vitro* and increased allergen-specific IL-10 production and proliferation of autologous CD4+ memory T cells (51). Other investigators have shown that PGA *per se* is an allergen in fermented soybeans, which causes hypersensitivity reactions and even late-onset anaphylaxis (52–54). To the authors' knowledge, there are no studies evaluating PGA in animals.

Protamine-based nanoparticles are biodegradable and biocompatible arginine-rich peptides. When complexed with Ara h 2 from peanut and CpG-ODN, they could counteract a Th2-dominated allergen-induced immune response in mice (55). A combination of liposomes with protamine and DNA was also proven effective in combating *Chenopodium album* allergy in a mouse model (56). At this point, there are no published clinical studies with protamine-based nanoparticles.

Mesoporous silica nanoparticles were successfully used in allergy models (57) with HDM allergen Der f 2 for subcutaneous prophylactic treatment of mice (58). However, when applied epicutaneously with mite extract in the form of agglomerates, they induced AD-like skin lesions and promoted IgE-responses (59). Studies in human and veterinary patients are lacking.

Taken together, many of novel adjuvants have shown Th1-promoting capacity *in vitro* and *in vivo* in murine models and even veterinary patient studies for horses and dogs. They were capable of counter-acting IgE, inducing preferentially IFN-γ, and/or IL-10 and also resulting in reduced symptom scores, being more effective than their non-adjuvanted controls.

ALLERGENS COUPLED TO IMMUNOMODULATORS

Efforts have also been made to enhance an overall shift in the immune response away from Th2, while at the same time presenting the offending allergen. Some approaches incorporate elements that can redirect the overall immune response from an allergy-prone Th2-IgE-milieu to a more Th1-IgG-dominated reponse.

Modified Adenine Conjugates

Der p 2 allergen bound to 8-OH-modified adenine (nDer p2-Conj) forms an allergen-TLR7 agonist conjugate. When injected subcutaneously, it reduces allergen challenge-induced murine

airway inflammation (60–62), triggers TLR7, redirects allergen-specific Th2 responses, and promotes a Th1 response as well as an increase in IL-10 with prolonged effects.

Mannan-Modified Allergens and Allergoids

Mannan preparations, alone or allergen-conjugated, appear capable of downregulating IgE responses. Konjac glucomannan (Amorphophallus konjac) fed to BALB/c mice suppressed IgE class switching in B cells and inhibited Th1 and Th2 responses (63). It also suppressed IgE production and clinical signs in a mouse model of allergic rhinitis (64). Administration of neoglycocomplexes of mannan with ovalbumin and papain to sensitized mice led to a class switch from IgE to IgG, and to a decrease in basophil degranulation *in vitro* (43).

Polymerized allergoids have been coupled to non-oxidized mannan from *Saccharomyces cerevisae* (PM-allergoids); this is one of the few modified allergen preparations that has been studied in dogs (65). Dendritic cells capture PM-allergoids better than native allergens and enhance Th1/Treg cell responses upon subcutaneous or sublingual administration (66, 67). Interestingly, the addition of alum may impair their tolerogenic properties (68).

DNA Engineered Hybrids With Copolymers

Hybrid allergen molecules are obtained by combining the epitopes of several allergens. Subsequently, their immunogenicity can be enhanced by coupling with copolymers. Engineered hybrids expressing the major allergens from *Parietaria* pollen allergens Par j 1 and Par j 2 were prepared as nanoaggregated copolymers with poly (hydroxyethyl)-aspartamide (PHEA). They are biodegradable, water-soluble and showed low cytotoxicity, no effect on hemolysis, and no non-specific activation of basophils. Basophil activation properties were, however, maintained in cells from *Parietaria*-allergic subjects, indicating preserved crosslinking capability of the hybrid allergen (69). No *in vivo* studies have been reported with this preparation.

Allergen Linked to CpG Oligodeoxynucleotides (CpG-ODN)

CpG-ODN are short, single-stranded synthetic DNA molecules with immunostimulatory properties that induce a Th1-based immune response (70), which prevents Th2-mediated hypersensitivity in mouse models of allergic diseases such as allergic rhinitis (71), asthma (72), conjunctivitis (73), and anaphylactic shock (74). Purified Amb a 1 from *Ambrosia artemisiifolia* pollen linked to CpG-ODN was successfully tested subcutaneously in humans and resulted in a shift from Th2 to Th1 with an increase of IFN-γ and a decrease in IL-5, proving suitable as an agent for immune redirection in immediate hypersensitivity diseases (75).

Siderophore-Bound Iron or Retinoic Acid as Immunomodulatory Ligands

Bos d 5 cow milk allergen is capable of binding iron via siderophores. The immunomodulatory properties of iron-bound allergen were tested *in vitro* with human PBMC (76). The empty *apo*-form of Bos d 5 increased CD4+ cells, IL-13, and IFN-gamma, whereas the complexed *holo*-form decreased CD4+ cells and induced apopotosis. Similarly, only the *apo*-form of birch pollen allergen Bet v 1 led to an increase in IL-13, while IFN-gamma was increased with both formulations when incubated with human PBMC (77). Accordingly, spiking of Bet v 1 or Bos d 5 with iron may be an effective approach to improve the efficacy of AIT against birch pollen and cow milk allergy, respectively (76, 77).

The major allergen Bos d 5 was also complexed with the vitamin A metabolite retinoic acid (78). IgE binding was not influenced, but PBMCs from healthy people stimulated with the complex led to a decrease of CD4+ T cells as well as IFN-gamma, IL-13 and IL-10, although induction of CD4+CD25+Foxp3+ regulatory T cells was not seen (79). In contrast to *apo*-Bos d 5, a highly allergenic molecule, *holo*-Bos d 5 thus seems to have reduced immunogenicity.

Expression of Allergens by Bacterial Vectors

Streptococcus thermophilus (ST) expressing rBet v 1 was evaluated in a mouse model (80). BALB/c mice were sensitized with rBet v 1 and then treated orally with either ST, ST and rBet v 1, or ST expressing rBet v 1. After aerosol challenge, T regulatory cells, IL-10, and IFN-gamma were increased with the expressed-allergen preparation; bronchial eosinophilia, allergen-induced IL-4, and the rBet v 1-specific IgE/IgG2 ratio were decreased, indicating a shift from Th2 to Th1 and Treg immune responses (80).

Profilin (Che a 2), the major allergen of *C. album*, was expressed in *Lactobacillus lactis*, and was bound by human anti-profilin IgE (81). However, bacterial survival was greatly reduced with low pH and simluated gastric and intestinal juices. Oral vaccination with recombinant *Lactobacillus plantarum* expressing the Japanese Cedar pollen allergen Cry j 1 led to a suppressed allergen-specific IgE response and decreased nasal symptoms in a murine model of allergic rhinitis (82).

Allergens Conjugated to Bacterial Products

Bacterial surface S-layer proteins (SLPs) are two-dimensional crystalline arrays of glycoprotein subunits present on the outermost layer of many bacteria, and have strong adjuvant properties. Conjugating recombinant allergens with SLPs leads to strongly reduced IgE-binding activity and promotes the induction of allergen-specific Th0/1 cells and regulatory T cells. This type of allergen modification has been attempted with inhalant allergens (83). Subsequently, bacterial S-layers have been studied as carriers for peanut allergen-derived peptides (84, 85). A fusion protein of an Ara h 2-derived protein and an S-layer protein was recognized by Ara h 2-specific IgE of human patients but was not able to degranulate sensitized rat basophils *in vitro* (84). The A20, tumor necrosis factor-induced protein 3 (TNFAIP3), is a ubiquitin-modifying protein playing a defensive role in the pathogenesis of allergic diseases. A DNA vaccine coexpressing Der p 2 and ubiquitin A20 encapsulated into nanoparticles used intranasally in a murine model of allergic

rhinitis was able to inhibit allergen-specific IgE, IL-4, IL-10, and IL-17 secretion and to increase IgG1, IgG2a, and IFN-γ (86, 87).

A genetically engineered inhalative cholera toxin B subunit/allergen fusion molecule, CTB-Bet v 1, was shown to improve the immunomodulatory capacity of the mucosal delivery system better than chemically coupled products (88).

Overall, the concept of redirecting the immune response from a Th2 to a Th1-bias as part of AIT has promise. However, most immunomodulatory components—except for CpG-ODN—have been tested in murine models only, and need to be further tested in human and veterinary patients.

COMBINATION AND MISCELLANEOUS APPROACHES

Several formulations combine the enhancing and modulating effect on the immune response, in parallel to protecting the antigen from degradation or digestion, and further releasing it in a delayed manner. Different particulate formulations together with immune-cell targeting substances have been used for these attempts.

Liposomes

Liposomes are bilayers of phospholipids, forming vesicles which can transport aqueous substances inside. They are biocompatible, biodegradable, and can be co-formulated with oligomannose coats, a preparation that was tested in human HDM-allergic asthma patients (89). Mouse models were used to study the efficacy of liposomes in treating allergy against Japanese cedar pollen (90), HDM (91), cat (92), OVA (93), or cockroach (94). Lipid nanoparticles together with *Parietaria* allergen Par j 2 were characterized biochemically and biophysically (95). Liposome complexes with CpG-DNA and individual allergen extracts were used intradermally for treatment of canine atopic dermatitis after failure of conventional AIT (96). Pruritus improved and IL-4 production decreased with treatment (96). Chronic rhinitis in adult cats could be treated with feline IL-2-filled liposomes plus DNA, although a Th2 bias could not be identified in those cats (97). Liposomes with HDM allergens Der p 1 or Der p 2 reduced clinical and medication scores, skin test responses, and bronchial challenge responses in asthmatic patients (89).

Poly-Lactic-Co-Glycolic Acid Particles (PLGA, PLG, PLA)

These polyesters are approved for use in people as absorbable surgical suture. In mouse models for birch allergy, they were successfully administered subcutaneously with Bet v 1 (98, 99). In addition, PLGA-microparticles were used orally with different plant lectins e.g., *Aleura aurantia* lectin, wheat-germ agglutinin or *Ulex europaeus*-I, or *Vibrio cholerae* neuraminidase to target mucosal cells for enhanced uptake (100–103). Other allergens used with PLGA-particles in animal models via different routes are the *Chenopodium* allergen rChe a 3, as sublingual immunotherapy in a mouse model of allergic rhinitis (104, 105), Ole e 1 from olive pollen or T cell epitopes thereof for intranasal prevention (106, 107), bee venom allergen PLA2 (108), pollen-profilin from palm *Caryota mitis* (109), Der p 2 from HDM (110), peanut extract (111), or beta-lactoglobulin from milk whey (112). PLGA locally induced a regulatory T cell response via the incorporated mediator substances TGF-beta-1, rapamycin, and IL-2 to prevent a subsequent contact dermatitis reaction (113). In addition to complexing PLGA-particles with allergens, PLGA were complexed with immune-modulating substances such as CpG-ODN for allergy and asthma prevention (114) and with Der p 2-A20 DNA in allergic rhinitis (87) in mouse models. There are no studies in companion animals with PLGA.

Virus-Like Particles (VLP)

Virus-like particles are used as carriers for allergens, or without antigen for antigen-independent immunomodulation (115). Particles consisting of bacteriophage coat proteins and a TLR-9 agonist, but without allergen, were injected into HDM-allergic patients and led to lower symptom-medication scores, higher quality of life and better allergen tolerance (116). A second study with A-type CpG-ODN and HDM-extract showed similar results; allergen-specific IgG increased as well (117). Recently, equine IBH was safely treated with IL-5-linked VLP made from cucumber mosaic virus to induce auto-antibodies against IL-5 (118–121). Clinical signs of treated horses improved and their eosinophilia was decreased compared to controls. The same principle was used successfully with IL-31-linked VLP for treatment of IBH in horses and for atopic dermatitis in dogs (122, 123). A very interesting approach is the immunization of cats with Fel d 1-VLPs (HypoCat^{TM}) to induce a neutralizing antibody response in the animal against its own Fel d 1-protein for protection of humans against cat allergy (124, 125). In BALB/c mice, adeno-associated VLP were also tested with an OVA-derived B cell epitope (126), with Art v 1 from mugwort (127) and with peanut allergens Ara h 1 and Ara h 2 (128). Fel d 1 displayed on VLPs failed to induce human mast cell activation *in vitro* (129). The peptide HDM allergen Der p 1 was coupled to a virus-like particle derived from a bacteriophage and injected in healthy volunteers. Significant IgG responses against the allergen were observed and the vaccine was well-tolerated (130).

Aleuria Aurantia Lectin (AAL)

AAL is derived from the edible orange peel mushroom *A. aurantia*. When birch pollen-sensitized BALB/c mice were fed with birch pollen-AAL-microspheres, the birch pollen-specific IgG2a, but not IgG1 or IgE increased, as well as IFN-gamma, IL-10, and IL-4 (101). Oral administration of birch pollen-AAL-MS led to an IgG2 antibody response in naive BALB/c mice (102). AAL microspheres may have the potential to serve as a vehicle and adjuvant for oral immunotherapy, potentially stimulating specific mucosal immune responses via M-cell targeting (100).

Wheat Germ Agglutinin (WGA)

Birch-pollen allergens were entrapped in poly(D,L-lactic-co-glycolic acid) microspheres, further coated with WGA to target enterocytes used for oral immunotherapy of type I allergy to protect allergens from digestion and to support intestinal uptake (131). The antigenicity of the birch pollen was maintained at ∼60% even after 2 h of simulated gastric digestion, and allergen-specific IgG serum concentrations increased in BALB/c mice fed with the WGA-birch pollen-microspheres (131).

With these approaches, VLP, liposomes, and PLGA particles seem to have promise, and are already tested in human, canine, feline, and equine patients.

SUMMARY

Allergen immunotherapy is the only treatment for allergic diseases that is truly causal and modifies the course of the ongoing disease. As this review discusses, many dozens attempts have been made to identify adjuvants, immunomodulators, physical packaging, conjugates, and combinations of the above to modify allergenic proteins, making them safer, and more efficacious in AIT. Many of the formulations have scarcely progressed beyond *in vitro* studies, though some show great promise in rodent models. Our task is now to select the most promising candidates, and carry them forward into preclinical studies that can more carefully predict which will translate into clinical benefit. Because many human allergic diseases are found nearly identically in animals, veterinary studies could serve as an elegant precursor to the same investigations in human patients.

AUTHOR CONTRIBUTIONS

IP-S and EJ-J designed and drafted the manuscript, wrote abstract and introduction. IP-S wrote part on particulate delivery systems. CA worked on allergen modifications. AS contributed immune modulators/activators. RM worked on all the parts completing results and references. DD wrote summary and edited, formatted and finalized MS. All authors contributed to the article and approved the submitted version.

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Therapeutic Potential of the Intestinal Microbiota for Immunomodulation of Food Allergies

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Food allergy is an atopic disease that is caused by the immune system targeting harmless food antigens that can result in life-threatening anaphylaxis. As humans and microbes have co-evolved, inevitably commensal microbes have a tremendous impact on our health. As such, the gut with its enormous microbial richness reflects a highly tolerogenic environment at steady state, in which immune cells are educated to react in a well-calibrated manner to food and microbial antigens. Recent evidence indicates that the susceptibility to food allergy is critically linked to microbial dysbiosis and can be transmitted by microbial transfer from humans to mice. Experimental work and epidemiological studies further point toward a critical time window in early childhood during which the immune system is imprinted by microbial colonization. Particularly, Foxp3-expressing regulatory T cells turn out to be key players, acting as rheostats for controlling the magnitude of food allergic reactions. An increasing number of bacterial metabolites has recently been shown to regulate directly or indirectly the differentiation of peripherally induced Tregs, most of which co-express the RAR-related orphan receptor gamma t (RORγt). Genetic ablation provided additional direct evidence for the importance of RORγt+ Tregs in food allergy. Future strategies for the stratification of food allergic patients with the aim to manipulate the intestinal microbiota by means of fecal transplantation efforts, pre- or probiotic regimens or for boosting oral immunotherapy may improve diagnosis and therapy. In this review some of the key underlying mechanisms are summarized and future directions for potential microbial therapy are explored.

Keywords: intestinal microbiota, food allergy, regulatory T cells, Foxp3, oral tolerance, anaphylaxis, bacterial metabolites

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INTRODUCTION

The enormous collection of microorganisms living in and on us is collectively referred to as microbiota. Bacteria, archaea, eukaryotes, and their associated viruses compose a highly complex microbial ecosystem (1). The microbiota has co-evolved during the evolution of all multicellular organisms and has become a *de facto* and even necessary "organ" in all modern vertebrates, fulfilling basic functions like the provision of nutrients and essential vitamins (2, 3). Today, there is

strong evidence that correct physiological functioning of this organ is dependent on a harmonious host-microbiota relationship (4).

On the other hand, vertebrates have evolved complex innate and adaptive immune functions, responsible for detecting, containing, and eliminating a large array of microbial pathogens (5, 6). Even some of the simplest forms of multicellular organisms, such as hydrozoans, exhibit innate immune pathways responsible for the recognition, and maintenance of certain bacterial associations (7). The discrimination between beneficial and pathogenic microorganisms poses a major challenge for the immune system that we are only beginning to understand.

Humans are no different in this respect: we have been surrounded by a great number of microorganisms for the majority of human history co-evolving with our microbiota (1). Today, abundant evidence indicates that the microbiota is essential for the correct functioning of human physiology (8). However, recent human development has shifted our relationship with microorganisms in a short time period, evolutionarily speaking, and these rapid changes were not accompanied by the necessary adaptations to our changing microbiota (**Figure 1**). Improvements in our way of life have extended our life spans, through medicine, sanitation, and industrialization of our food production system. With these changes, a large array of microbial infections is no longer a death sentence for humanity. Simultaneously, there was a marked increase in prevalence of several immune-related disorders, such as Crohn's disease, asthma, and food allergies (9). This relationship was already noticed in the late 1980's and early 1990's, eventually being named the Hygiene Hypothesis: we removed the infections, but in the process, the immune system lost something as well (10).

Today, we understand that not the removal of pathogens itself, as initially proposed, has an effect on our immune responses, but the processes that eliminated dangerous pathogens have also eliminated other microbial and fungal bystanders and eventually multicellular parasites such as helminths (e.g., *Ascaris*, *Trichuris,* or *Schistosomes*) from our microbiota, breaking a beneficial relationship selected over eons of evolution (11). Eventually, modern humans and particularly those living in developed countries have irretrievably lost certain ancient microorganisms that have been instrumental to set up a healthy host-microbial homeostasis (12). If this homeostasis cannot adequately be reached during colonization in childhood, an enhanced risk of atopic diseases including food allergy may be one long-term consequence.

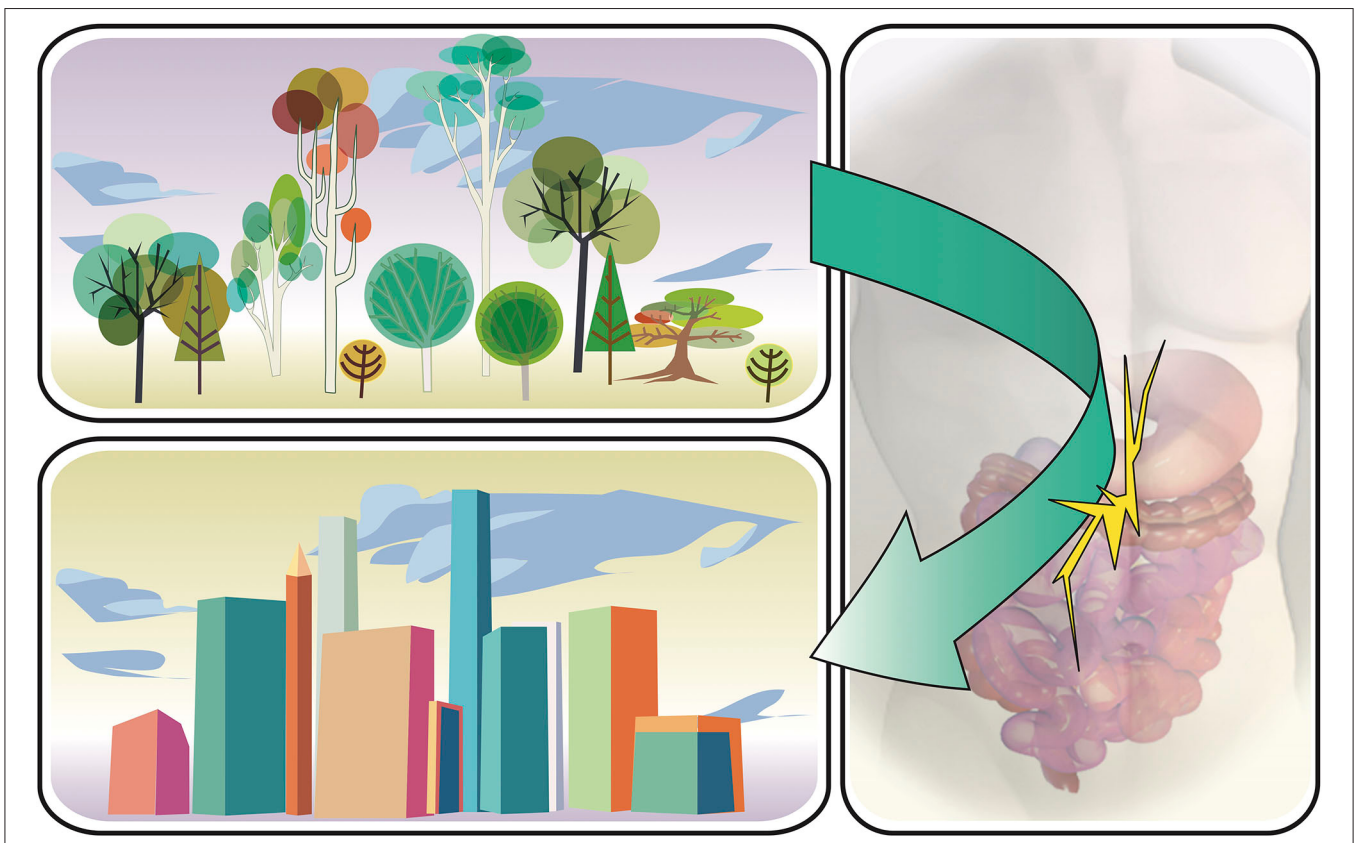

FIGURE 1 | Illustration of changes in life style factors having an impact on the intestinal microbiome. Humans have shifted very quickly from an ancestral to a modern way of living, evolutionarily speaking. These changes have impacted the balance between the intestinal microbiota and the immune system.

ALTERATION OF THE GUT MICROBIOTA IN FOOD ALLERGIC PATIENTS

Some studies have directly related food allergies and the gut microbiome in patients (13, 14). Still, the current understanding for the implication of the microbiota in human allergies is mostly based on epidemiological studies including birth cohorts. The current use of bacterial 16S rRNA sequencing, as well as metagenomic sequencing, allows the characterization of the microbial composition of both the environment and the intestinal microbiome. Several large cohort studies conducted in children raised in a traditional farming environment revealed a protective effect of this environment and this has been positively correlated to bacterial and fungal diversities found in these environments (15–19). Similarly, distinct communities of bacteria have been found in stool samples of neonates which preceded later signs of allergic asthma (20). Importantly, such communities led to an enhanced allergic lung inflammation after transplantation into germfree mice that provided direct evidence for the underlying causality. In another study, analysis of stool samples from neonates revealed three different microbial patterns one of which being associated with multiple sensitizations to various allergens: such microbiomes showed lower relative abundance of *Bifidobacterium*, *Akkermansia*, and *Faecalibacterium* and increased levels of the metabolite 12,13-dihydroxy-9Z-octadecenoic acid (12,13-DiHome), which has been confirmed to aggravate allergic lung inflammation in murine models (21, 22).

In prior studies, the amount of exposure to conserved bacterial products such as Lipopolysaccharide (LPS) from the environment has already been negatively correlated to atopic sensitization and this was associated with reduced production of cytokines by peripheral blood leukocytes (16). Another more recent large cohort study tracked the intestinal microbiome composition within the first years of life. Although initially intended for observing the microbiome's influence on the incidence of diabetes, the cohort provided the opportunity to show HLA matched class risks in geographically distinct locations (23). Despite the initial non-allergic focus of the study, it found that growing up with a comparatively modern lifestyle is associated with an increased risk of atopy and autoimmune manifestations. Children from Finland showed a relatively high sensitization rate toward typical allergens such as milk and egg, Russian children only showed a relatively low frequency of sensitization and Estonian children were intermediate. Fecal samples of Finish and Estonian children showed a relative high abundance of *Bacteroides*, while Russian children had a higher abundance of *Bifidobacterium*. Interestingly, metagenomic analysis revealed that LPS synthesizing gene clusters were among the most differentially expressed pathways between the three groups. The authors then demonstrated that the origin of the LPS (*Bacteroides* spp. vs. *E. coli*) leads to a structural difference with a strong effect on the immunostimulatory capacity of these variants on primary human peripheral blood monocytes (23). This revealed how the structural variation of a single component sensed by pattern recognition receptors (PRRs) due to differences of microbial compositions early in life may contribute to enhanced sensitization rates and thus risk of food allergy in children. It is possible that much more structural variations of such microbial determinants sensed by the PRRs of the immune system exist and educate the microbiota-host homeostasis in children.

MECHANISTIC INSIGHTS FROM MURINE MODELS

While human studies are often limited due to being correlative, murine models provide a defined system for the determination of the mechanistic basis of the microbiota's impact on health and disease in the host. However, it has to be acknowledged that there are profound differences between the digestive system and microbiota of humans and mice as reviewed in (24).

Experimental evidence from murine models has shown that the intestinal microbiota protects the host from allergic inflammation by: (I) contributing to the establishment of antigen-specific oral tolerance, (II) preventing excessive inflammation, (III) impacting on various aspects of host physiology, such as intestinal barrier function, degradation of xenobiotics, production of metabolites, and, (IV) directly contributing to the development of both the innate and adaptive immune system, such as basophil hematopoiesis (25).

Mice housed under germ-free conditions or treated with broad-spectrum antibiotics gave the first direct hints that the microbiota is essential to maintain a balanced (and thus a non-type 2 immunity prone) immune system. Such mice typically show elevated serum Immunoglobulin E (IgE) levels while all other immunoglobulins are downregulated (25–27). The microbiota maintains IgE at basal levels which requires microbial exposure during early live (28). In the absence of microbial colonization during this time window, Th2-skewed follicular helper T cells (Tfh) may develop that support class-switching to IgE in B cells which is directed against food antigens (29). A recent study has identified a special subset of allergen-specific Tfh cells that secrete IL-4 and particularly IL-13 which are instrumental to induce anaphylactic IgE (30). Interestingly, another study found expression of RAG proteins in B cells within the intestinal lamina propria exclusively around the time of weaning (31). As the positive and negative selection of B cells is thought to occur primarily in the bone marrow these results point toward another step of B cell education in response to microbial colonization. This could be particularly important in the case of food allergy as IgE specific for both bacteria and food antigens has been found in patients and mice with food allergy (32).

Antibiotic treatment in mice can also result in exaggerated basophil-mediated Th2 cell responses and allergic inflammation, indicating that the microbiota directly restrains the size of circulating basophil populations by limiting the proliferation of bone marrow resident precursor populations (25). Recolonization of germfree mice with specific bacterial strains (such as *Clostridia* mixtures) leads to decreased allergen-specific IgE and reduced susceptibility to anaphylactic reactions in a model of peanut allergy (33). Recolonization with *Clostridia* in this model also reduced the uptake of the allergen by affecting

the intestinal barrier permeability. This effect can be mimicked by genetically limiting intestinal barrier integrity, e.g., through knockout of the transcription factor retinoic acid receptor-related orphan receptor gamma t (RORγt). RORγt is a major regulator of intestinal IL-22-producing immune cells such as ILC3s or γδ T cell subsets known to enforce the intestinal barrier (33, 34). Still, permeability in the intestinal tract is most likely not the only decisive factor as exposure to intestinal microbes generally leads rather to a Th1- and Th17-dominated immune response.

Furthermore, transplantation of gut microbiota samples from children with food allergy into germfree animals led to more severe anaphylactic reactions when such xenotransplanted mice were challenged with allergen in a food allergy model (32, 35). This provides direct evidence that a human intestinal microbiota from allergic children can confer this susceptibility to another species, representing a key step in understanding the underlying causality. Other barrier sites apart from the intestine also show exaggerated allergic reactions in the absence of microorganisms. For example, germfree mice or very young mice with incomplete colonization show an increased Th2 immunity and worsened allergic lung inflammation, supporting the beneficial role of the microbiota also in the lung (27, 36).

THE MICROBIOTA AND TREGS

Regulatory T cells (Tregs), characterized by the expression of the transcription factor Foxp3, and Tr1 cells, characterized by the expression of immunoregulatory cytokines such as IL-10 in the absence of Foxp3, are critical for regulating immune responses, dampening inflammation and for general homeostasis of barrier surfaces (37, 38). The microbiota can directly impact on the frequency of Tregs, as oral administration of murine and human *Clostridia* strains transferred into germfree mice leads to a drastic increase in Treg frequencies within the colon (39, 40). Surprisingly, germfree mice do not harbor less Tregs in the small intestine which may be due to an altered de-differentiation process of Tregs at this site (41). Similar to the intestinal microbiota, colonization of the skin was shown to recruit Tregs during a specific time window and these Tregs are most likely specific for such commensal microbes (42).

Two types of Tregs can be distinguished according to the origin of differentiation: Thymic-derived Tregs (tTregs) that are selected within the thymus probably due to recognition of self-antigens, and peripherally induced Tregs (pTregs) that differentiate in peripheral organs from naïve T cells. In the intestine, microbial colonization is responsible for inducing the differentiation of pTregs (43). Preventing pTreg differentiation by knocking out the CNS1 (conserved non-coding sequence 1) region next to the Foxp3 promotor revealed that these immune cells prevent a spontaneous type 2 immune bias at mucosal sites (44). Dietary antigens from solid foods are also tolerized by inducing a population of short-lived pTregs in the small intestine where uptake of nutrients including food allergens most likely takes place (45). Interestingly, germfree animals raised in the absence of macronutrients revealed that in the absence of both dietary and microbial antigens (and therefore the majority of intestinal pTregs), the adaptive intestinal T cell response is heavily skewed toward a Th1-dominated response whereas the absence of microbes alone heavily skews the intestinal T cell responses toward a Th2-dominated and therefore pro-food allergy immune response (45). Therefore, immunological tolerance of dietary antigens is of pivotal importance but the intestinal microbiome most likely is a key driver in preventing the Th2-skewing after recognition of dietary antigens and limiting the susceptibility toward food allergy. We and others have demonstrated that microbiota-induced pTregs share features with intestinal Th17 cells, such as the expression of the transcription factor RORγt (46, 47). Their induction can be mediated by a diverse range of bacterial species and the lack of RORγt+ Tregs leads to exacerbated Th2 and Th17 pathology in the intestine (46, 47). Noteworthy, RORγt+ Tregs have also been detected upon oral exposure to food antigens using transgenic T cells recognizing an epitope from chicken ovalbumin (45, 46). Due to the highly artificial nature of such T cell receptor (TCR) transgenic T cell transfer approaches and the observation that germfree mice raised in the presence of solid food and thus food antigens still show a dramatic reduction in RORγt+ Tregs, the relevance of this observation remains to be investigated in more physiological conditions.

In order to exploit the induction of pTregs by the microbiota for therapeutic purposes, the underlying mechanisms for this induction needs to be understood on a molecular level. Recent evidence indicates that bacterial metabolites, such as short-chain fatty acids (SCFAs) (48, 49) and cell surface polysaccharides from typical commensals, such as *Bifidobacterium bifidum*, are capable of inducing pTregs (50), further confirming the impact of the microbiota on this cell population. More recently, secondary bile acids were shown to induce Foxp3 expression in naïve T cells either directly or in a dendritic cell-dependent manner (51–53). In particular, Isodeoxycholic acid producing bacteria increased colonic RORγt+ Tregs *in vivo*, which was not observed when transplanting bacteria unable to generate this secondary bile acid (53).

Furthermore, RORγt+ Tregs have been shown to have a protective role in a model of food allergy, and the expression of RORγt is indispensable for this function (32). The same group reported that RORγt-expression in Tregs can also be detrimental for allergic inflammation of the lung, as it may drive the expression of pro-inflammatory cytokines in different conditions (54). These studies relied on an elegant murine model of enhanced signaling via the interleukin 4 receptor (IL-4R). It is based on a point mutation within the intracellular domain of the IL-4R that has also been found in a subset of patients with food allergy (55). As a consequence, intestinal Tregs start to (over-)express the transcription factor Gata3 and secrete the cytokine IL-4, making this Treg population rather a pathogenic driver of food allergy than an immune regulator (56). Such type 2 prone Tregs are less stable but most likely their differentiation is independent from microbiota effects, as Gata3+ Tregs can be found in germfree animals and Gata3 and RORγt expression are usually mutually exclusive (46, 57). Still, transplantation of fecal samples from IL-4R mutated mice subjected to food allergy

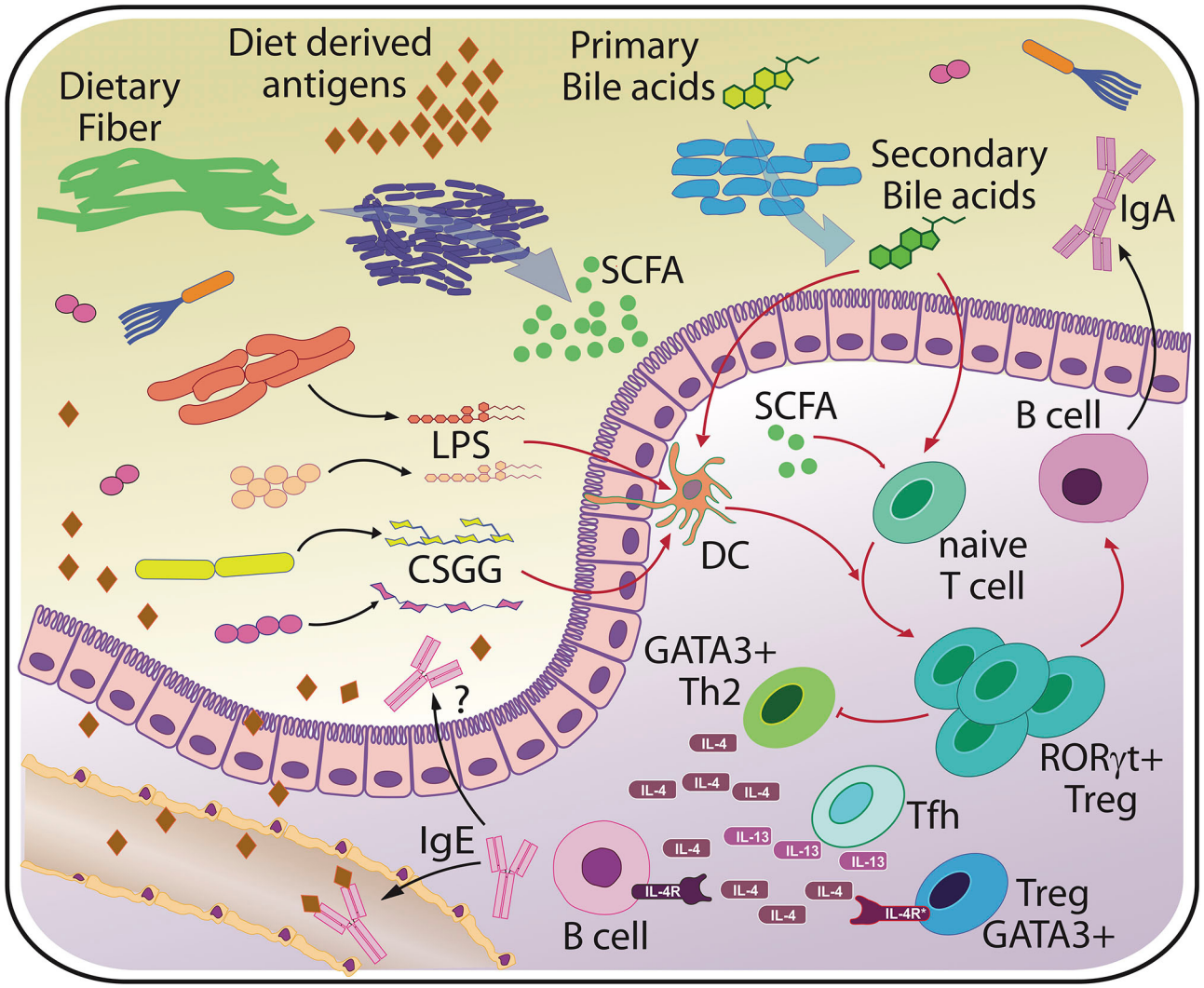

FIGURE 2 | Basic principles of host-microbiota interaction relevant for food allergy. The scheme indicates major bacterial molecules from various bacterial sources that have been linked to (RORγt+) Treg induction and protection from sensitization and/or food allergy. Protective microbial factors may include but are not limited to variants of lipopolysaccharide (LPS) from different bacterial species, cell surface polysaccharides from typical commensals such as *Bifidobacterium bifidum* (CSGG, Cell-surface β-glucan/galactan polysaccharides), short chain fatty acids (SCFA) and secondary bile acids that all act directly on T cells or on accessory cells such as dendritic cells (DC). Microbiota-dependent RORγt+ Tregs are thought to protect against excessive accumulation of T cells secreting type 2 cytokines such as interleukin 4 (IL-4) and interleukin 13 (IL-13). Ultimately, a tight restriction of B cells secreting IgE specific for food and bacterial derived antigens, which is thought to be mediated by T follicular helper cells (Tfh), must be achieved to prevent systemic reactions, such as anaphylaxis. Regulation of the intestinal microbiota may be accomplished through bacterial coating by host or maternally-derived luminal IgA and controlled barrier function.

can confer the enhanced Th2 skewing and allergic reactions to wildtype animals suggesting that excessive IL-4R signaling also has a strong impact on the microbiota (58). In other contexts, Gata3 expression in Tregs has been proposed as a general hallmark of Tregs residing within different tissues as compared to secondary lymphoid organs making it questionable whether Gata3+ Tregs can generally be considered pathogenic in patients without mutations in the IL-4R pathway (59).

Altogether, the discovery of different Tregs subsets with unique functions offers a cellular and molecular link to how microbial compositions may modulate the risk for allergic inflammation (**Figure 2**). Whether these microbial effects act directly on T cells, and how much other cellular players known to regulate T cell fate, such as dendritic cells, contribute to microbiota-mediated effects remains a matter of current investigation (53, 60).

DISCUSSION

Current treatment for food allergies mainly comprises rescue medication treatment after exposure and allergen avoidance. Desensitization can only be achieved with specific oral

immunotherapy (OIT), however it is a lengthy process of several years and full remission only occurs in up to 50% of the subjects who have undergone OIT for milk, egg and peanut and adverse reactions are common (61). Treatment options utilizing a preventive effect of the microbiota have been few and limited to probiotics and fecal microbial transplantations (FMTs) with no clear overall effective results (62). The integration of RORγt+ Treg frequencies for patient stratification and assessment of OIT and FMT effectiveness could lead to better success rates in the future. As more and more bacterial strains and bacterial metabolites have been identified to regulate RORγt+ Treg homeostasis, more patient-tailored treatment options may be developed. For instance, colonization of mice and humans with certain *Clostridiales* taxa has already been linked to protection from food allergy, yet the efficacy of a therapeutic application in humans remains to be investigated (32, 35). Furthermore, the clinical application of microbe-based therapeutics may be complicated by several factors including the variability of the host microbiome composition in FMTs, stably imprinted intestinal microbial ecosystems, unforeseen side effects on host physiology and the impossibility to reprogram existing long-lived immune cells (e.g. T-and plasma B cell populations). However, not only microbe- but also parasite-derived molecules may be used in the future for the treatment of food allergies as particularly helminth parasites have been part of our intestinal microbiota over long periods of human evolution. Currently, such treatments are already tested for therapeutic purposes in allergic airway inflammation models and it has already been established that they can act on several cell types, including myeloid cells, and on the differentiation/proliferation of Tregs (63–65).

In general, RORγt+ Tregs may serve as an indicator for the patient's microbiota in a "dysbiotic" state and the respective patient being at elevated risk of allergic or other inflammatory diseases (66). Still, the assessment of this cellular parameter is currently not possible in clinical settings and exemplifies the medical need for the identification of surrogate measurements. As long as the microbiota host interaction and the metabolic pathways of certain species remain only partially understood, the interventions to manipulate the microbiota will remain largely unspecific, and the potential of such microbiota-based therapies are limited. Ultimately, the exploitation of metabolic pathways and other RORγt+ Treg-stimulating agents by changing microbial compositions to enhance oral tolerance and protect from sensitization and food allergy is nevertheless highly attractive. Many questions in this regard remain unanswered, for example, how the generic induction of (RORγt+) Tregs by microbial consortia or their products can induce an antigen- or allergen-specific tolerogenic B and T cell response. Alternatively, a polyclonal and/or unspecific TCR repertoire within the Treg population may be sufficient to suppress allergen specific Th2 cells in a bystander suppression or by acting on accessory cells, e.g., dendritic cells. One may ask why evolution did not select for a general higher level of (RORγt+) Tregs to avoid overt intestinal inflammation and the risk of food allergy. It is perhaps because generalized suppression of adaptive immunity at mucosal sites through excessive pTreg generation could be dangerous, as contact with facultative pathogens are frequent and risk of infections are high. Setting the correct bar for RORγt+ Tregs and homeostasis with the developing microbiota needs to occur in childhood, and seems to also be imprinted from the mother to the next generation by a double-negative loop involving maternally transmitted IgA (67, 68). While this mechanism ensures that the next generation benefits from a maternal experience on the correct bar for a beneficial host-microbiota equilibrium, microbial adaptations are still able to fine-tune the level of RORγt+ Tregs throughout life and offer therapeutic options. Thus, a well-calibrated balance between pro- and anti-inflammatory signals needs to be integrated by accessory cells such as dendritic cells, or even directly by activated T cells at the right time point of colonization. One long-term goal is therefore to better understand this complex integration in order to combine this knowledge with OIT regimens or for diagnostic purposes to determine food allergic risk patterns ideally already early in life.

AUTHOR CONTRIBUTIONS

All authors contributed to the writing of this review and have read and approved the final version.

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Basophil Activation as Marker of Clinically Relevant Allergy and Therapy Outcome

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For some years now the basophil activation test (BAT) using flow cytometry has emerged as a powerful tool and sensitive marker that can be used to detect clinically relevant allergies, provide information on the severity of an allergic reaction, and monitor therapies. Compared to other *in vitro* diagnostic tests, BAT seems to have a better informative value in terms of clinical relevance. In general, the BAT can be used for the diagnosis of the most common forms of IgE-mediated allergy such as hymenoptera venom allergy, inhalant allergy, food allergy, and drug allergy. Various basophil markers and parameters have been established which, depending on the trigger of the respective allergy, can provide information on the clinical relevance of sensitization, on the development of natural tolerance, on trigger thresholds, and on the severity of the allergic reaction. The BAT also serves as a suitable follow-up instrument for various therapeutic approaches such as specific immunotherapy, desensitization protocols, or use of anti-IgE-antibodies for the various diseases. Quality controls for routine use, standardization, and automatization are expected to expand the range of applications for the above-mentioned indications.

Keywords: basophil activation test, basophil parameters, food allergy, hymenoptera venom allergy, inhalant allergy, drug allergy, immunotherapy, anti-IgE-treatment

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INTRODUCTION

Cellular *in vitro* tests can be used for the allergy diagnosis of type I allergies and serve for the detection of indirect sensitization on basophils (due to their easier availability compared to mast cells). In recent years the basophil activation test (BAT) which measures activation markers after incubation with allergens or other triggers by flowcytometry has emerged as the most widely used test for this purpose.

In most studies the activation marker CD63 was favored, occasionally also CD203c. CD63, a membrane component of the basophil granules, is not a basophil-specific marker and is also expressed on other blood cells. Therefore, further labeling is necessary for the identification of basophils. Possible markers include anti-CCR3, anti-IgE, anti-CRTH2, CD203c, or anti-CD123. CD203c, an ectoenzyme located both on the plasma membrane and in the cytoplasmic compartment of basophils, is a basophil-specific marker and is expressed constitutively. The test can be performed with full blood, washed basophils, or donor basophils. This and various protocols are the main differences between the BATs used in different laboratories. CD203c and CD63 markers are upregulated after IgE receptor aggregation but have partially different metabolic pathways and follow different kinetics. Interleukin-3 potentiates the allergen-induced CD63 expression without upregulating CD63 itself, whereas it increases CD203c expression even without allergen.

Results of the BATs are usually expressed as percentages of activated basophils (% CD63+ cells), sometimes also as MFI (mean fluorescent intensity). This basophil reactivity measures the number of basophils that respond to a given stimulus. Maximal basophils reactivity is the maximal activity induced by a given stimulus. Additionally, further parameters such as results of the determination of the half-maximal concentration (EC50, CD-sens, basophil sensitivity), the calculation of a ratio (CD63 ratio), of allergen-induced CD63 activation in comparison to an IgE-dependent positive control (anti-IgE of anti-FcεRI), or of the area under the curve (AUC) in dose-response curves turned out to be of value for the assessment of clinically relevant allergies and therapy outcomes (1–4). Details can be found in an EAACI position paper (1).

ELUCIDATION OF CLINICALLY RELEVANT ALLERGY

Food Allergy

For food allergies, the sensitivity of the BAT varies between 62 and 90% and the specificity between 80 and 100% depending on the allergen. In general, cellular tests are useful to detect the trigger of an IgE-mediated reaction to food if conventional diagnostics is negative or not available and a provocation test is expected to be potentially life-threatening. In recent years, more and more studies have been published which see the basophil activation test as a diagnostic tool prior to oral provocation being only necessary in remaining unclear cases (1).

In 2014, Santos et al. could show that the BAT discriminates between allergy and tolerance in peanut-sensitized children. Receiver operator curves (ROC) showed that the BAT with a peanut extract was better than skin prick test (SPT) and sIgE to Ara h 2 and peanut for this purpose. The application of BAT as a second or third step in the diagnostic workup dramatically reduced the need for oral provocation tests. It was recommended to perform oral food challenges in cases with equivocal BAT as well as in BAT-negative patients (5). Other authors showed that a negative CD-sens to peanut of Ara h 2 excluded an allergy (6). Certain parameters of the BAT using a peanut extract correlated with the severity of the reaction (CD63 ratio) and with the amount of eliciting allergen (CD-sens) (2, 7). Interestingly, only the use of a peanut extract and not of Ara h 2 in the BAT was associated to the eliciting dose of peanut in allergic patients (8).

In milk allergic children BAT helped in deciding when to reintroduce cow's milk in their diet showing that CD63 ratio reflected the severity of reaction to oral challenge (9). This parameter was also significantly higher among patients with milk allergy who reacted to baked milk than among those who tolerated it (10). As a consequence, the BAT reduced the need for a food challenge in children suspected of IgE-mediated cow's milk allergy (11).

Baked egg-reactive children had significantly increased basophil activation in response to intermediated stimulation levels of egg white protein compared to tolerant children, but there was a great overlap in basophil activation between these groups, which made it difficult to use it in clinical practice (12).

CD63 and CD203c expression at several allergen concentrations differed between individuals allergic or sensitized to hazelnut, too. In this study, EC50 of allergen-induced CD203c expression displayed a better discrimination compared to CD63, but there was no significant difference between patients with oral allergy syndrome and systemic reactions (13).

Similarly, basophil activation with peach extract was higher in mugwort pollen-related peach allergic patients than in tolerant subjects, but the BAT results were comparable in patients with oral allergy syndrome and systemic reactions, limiting its utility in predicting severity. In contrast, the basophil activation with Pru p 3 correlated not only with clinical allergy but also with the severity of symptoms having the best diagnostic performance compared to determination of sIgE (14).

Also for rare food allergies, e.g., the alpha-gal syndrome, it could be shown that the BAT differentiates between patients with a clinically relevant allergy and asymptomatic alpha-gal sensitization. Especially the parameter CD63 ratio for low concentrations of alpha-gal turned out to be a reliable basophil parameter and was better than sIgE to alpha-gal (4).

In another study it was shown that the BAT using hydrolyzed wheat protein and ω-5 gliadin was highly useful for diagnosing the subtypes of hydrolyzed wheat protein WDEIA (wheat-dependent exercise-induced anaphylaxis) and conventional WDEIA indicating an IgE-response to different protein components (15). Despite a tendency to higher wheat CD-sens values, only the combination of CD-sens and sIgE to wheat or wheat components was useful in the prediction of wheat challenge outcome (16).

Due to good results of CD203c sesame-induced basophil expression joint utilization of BAT and skin prick test with a high protein concentration sesame extract, this approach may also obviate the need for oral food challenge in most patients with sesame food allergy (17).

Hymenoptera Venom Allergy

For hymenoptera venom allergies, the sensitivity for the BAT varies between 85 and 100% and the specificity between 83 to 100% (1). There is no correlation between basophil activation and the clinical severity of the sting reaction reported by patients (18).

Because diagnostic sting challenges for insect venom allergies are not performed routinely for ethical reasons, this cellular test can be used in diagnostics for the detection of an IgE mediated reaction, especially if skin tests and specific IgE antibodies to insect venom extracts are negative (19). Although the component resolved diagnosis has made significant progress in specific IgE determination for insect venom allergic patients, there are still individuals in which only the BAT showed positive results (20). The use of recombinantly produced CCD-free hymenoptera venom allergens also lead to an improvement of the BAT results compared to the total hymenoptera venom extracts, both in terms of the number of positive results and the level of activation (21).

The BAT turned out to be helpful also in cases of double sensitization to bee and vespid venom and a clinical reaction to only one insect species or in cases of insect stings that cannot be clearly assigned to a particular insect species from the clinical history. In about one third of the patients information about

the clinically relevant insect could be obtained by the BAT incubating the cells with bee and wasp venom extracts and, if necessary, by calculating the half-maximum concentration of the dose-response curves and forming a ratio (22–24). The clinical relevance of such BAT results could be confirmed in patients with double sensitization (skin test and specific IgE antibodies) and exclusive monosensitization to vespid venom in the BAT: 92% of the patients tolerated a sting challenge test with the bee (BAT negative) without systemic reaction, and 7% suffered from a mild systemic reaction (25). Thus, unnecessary specific immunotherapy can be avoided.

Inhalant Allergy

The sensitivity of the BAT for house dust mites, pollen, latex, or cat hair is 91–100% for both extracts and recombinant major allergens, and the specificity is between 96 and 100% (1).

Due to the good sensitivity of conventional diagnostics, cellular tests are used less for diagnostic purposes in routine, but the usefulness of the BAT and component-resolved diagnosis in distinguishing between symptomatic allergic rhinitis patients and asymptomatic sensitization to house dust mite could be demonstrated. Symptomatic patients showed a lower threshold for *in vitro* basophil activation and a higher AUC. There was also a positive correlation between the number of recognized house dust mite allergens and the AUC of basophil activation (26).

BAT seems to be advantageous in the diagnosis of local allergic rhinitis (LAR) because it was able to diagnose at least 50% of these cases allergic to house dust mite extracts and was more sensitive than detection of nasal specific IgE and less time-consuming than nasal provocation tests (27, 28). Similar results were shown for LAR patients with olive tree pollen (29). Based on these studies BAT has been shown to have a sensitivity of 50.0–66.6% and a specificity of 90.0–91.7% in LAR. These results reinforce the usefulness of BAT, a rational step of a diagnostic approach in LAR before nasal provocation tests.

Drug Allergy

In general, sensitivity of the BAT for most drugs is significantly lower than the sensitivity of the allergens mentioned above. The sensitivity of the BAT for beta-lactam antibiotics is about 50% with a positive predictive value of about 90%. In order to obtain relevant information about the sensitization of a patient by this test, the BAT should be carried out within half a year after the clinical reaction, since the cells' reactivity to the antibiotics decreases thereafter. Sensitivity for quinolones is slightly better (about 64%) with a positive predictive value of about 90% (30).

The sensitivity of BAT in hypersensitivity reactions to NSAIDs being independent of IgE-/FcεRI cross-linking is very low (20–40%) with specificities of 40–100%; only BAT with pyrazolones showed better results (sensitivity about 54%, specificity about 95%) (30, 31).

For radio contrast media the sensitivity is about 60% with positive predictive values of about 97%. The sensitivity for muscle relaxants varies between 54 and 92% for BAT (specificity: 100%) with a positive predictive value of about 96% (30). Algorithms for allergy workup in perioperative hypersensitivity reactions include the BAT before considering drug provocation tests: Negative skin testing and BAT results might increase confidence in performing drug provocation tests (32–34).

The studies to date show that cellular tests with drugs should only be used as a supplement to existing diagnostics, and they are not a substitute for provocation tests (30).

THERAPY OUTCOME

Over the last few years, it has become apparent that the BAT can serve as a suitable follow-up instrument for various therapeutic approaches such as specific immunotherapy, desensitization protocols, or use of anti-IgE-antibodies for various allergic diseases.

Immunotherapy in Food Allergy

During a 12-months sublingual immunotherapy (SLIT) for peanut allergy in children a significantly decreased basophil activity after stimulation with the two lowest concentrations of a crude peanut extract could be demonstrated (35). Others showed that 2-years responders of a SLIT had significantly lower percent CD63+ basophils than non-responders for the lower peanut stimulant levels, but there are also studies demonstrating that peanut-induced basophil response was most reduced in the immune tolerant group after 24 months of oral immunotherapy (OIT), although differences between immune tolerant and non-tolerant participants did not achieve statistical significance (36, 37). Using the CD63 ratio with a crude peanut extract, a significant decrease of this parameter at all concentrations after 3 to 5 years of peanut SLIT was observed (38).

In a pilot study the utility of BAT for monitoring the acquisition of clinical tolerance after oral desensitization to cow's milk over 12 months was shown (39). Furthermore, milk-induced %CD63 and %CD203c expression was significantly lower in patients >24 months of oral immunotherapy vs. in patients <24 months of treatment (40).

Also, a decrease in antigen-specific CD63 basophil expression (egg white, ovomucoid, ovalbumin) was associated with the development of tolerance to egg by specific oral tolerance induction after 15 days and 1 month, respectively, of the buildup phase (41, 42).

In contrast, a 6 month or 12 month SLIT with a peach extract lead to an increase in basophil activation following stimulation with rPru p3 (43, 44).

Immunotherapy With Hymenoptera Venoms

A basophil activation decrease using mostly submaximal concentrations of insect venoms was only observed in part of the studies up to 18 months after beginning of venom immunotherapy (VIT), but was found throughout all studies after 2 years of treatment, and maintained until the completion of a 3–5-years immunotherapy period (45–50). A significant difference was also shown for submaximal concentrations of bee venom in patients reacting to a sting challenge compared to patients not reacting at the end (mean 4.4. years) of VIT (51). The depression of allergen-specific basophil response also lasted 1 year after completing 4–6.5 years of immunotherapy (47).

In a BAT inhibition assay incubating blood of donor patients with insect venom allergy with sera from patients undergoing VIT for at least 1 year, the basophil response was almost completely inhibited at submaximal allergen concentrations (52). It was shown that patients who reacted after discontinuation of immunotherapy in field re-stings had a persistence of high basophil activation at submaximal concentrations in contrast to protected patients (53).

Immunotherpy With Inhalant Allergens

First indications of the benefit of BAT for the monitoring of specific immunotherapy (SIT) with pollen were shown in patients with Japanese cedar pollinosis. Significant reductions in the allergen-induced CD203c response in basophils were observed in part of the subjects already 1 month after beginning of a rush immunotherapy (54). CD-sens dropped significantly after reaching the maintenance dose of SIT for birch or grass allergy compared to before (55). Similarly, a decrease in allergen-induced basophil activation at submaximal allergen concentrations was demonstrated at the end of a short-term preseasonal immunotherapy over 7 weeks and additionally at the peak of the grass pollen season after immunotherapy (56). CD63 expression decreased also 8 months after an immunotherapy with an olive pollen allergoid compared to baseline values (57). Basophil sensitivity was significantly lower after 1 month of treatment with subcutaneous immunotherapy (SCIT) to grass pollen when compared to SLIT-tablet treatment, and although the differences diminished towards the end of the study (15 months), they remained significant (58). Interestingly, a decrease in basophil sensitivity after 3 weeks of treatment predicted long-term improvement in seasonal combined symptom and medication scores during 3 years of treatment in grass pollen allergic patients (59). Grass pollen immunotherapy induced sustained suppression of the allergen-specific basophil response during initiation and after 1–2 years after completion of treatment (60). In contrast to these studies, a significant decrease

TABLE 1 | Overview over possible current applications of BAT for discrimination between clinically relevant allergy and tolerance, monitoring immunotherapy, and follow-up of anti-IgE treatment for food, hymenoptera, and inhalant allergies according to the literature.

Allergy	Allergen	Discrimination between allergy and tolerance/sensitization	Monitoring immunotherapy (IT)	Follow-up of anti-IgE treatment	Comments	References
Food						
	Peanut	Yes	Yes (1–5 years IT)	Yes		(5, 6, 35–38, 74, 75)
	Milk (baked)	Yes	Yes (1–2 years IT)	Yes		(9–11, 39, 40, 76)
	Egg, baked	Partially[a]	Yes (15 days to 1 month IT)		[a]Great overlap between groups	(12, 41, 42)
	Hazelnut	Yes[b]			[b]No discrimination between OAS and systemic reaction	(13)
	Peach	Yes[c]	No		[c]No discrimination between OAS and systemic reaction	(14, 43, 44)
	Alpha-Gal	Yes				(4)
	Sesame	Yes (together with SPT)				(17)
	Wheat	Yes (together with sIgE)			Discrimination between subtypes of WDEIA possible	(15, 16)
Hymenoptera						
	Bee and wasp venom	Yes (in terms of IgE-mediated reaction and of double sensitization)	Yes (1.5–5 years IT and > 1 years after the end of IT)			(20–25, 45–53)
Inhalant						
	Pollen (Japanese cedar, grass, olive pollen allergoid)	Yes[d]	Yes, in most studies[e,f]	Yes	[d]Especially for LAR [e]SCIT better than SLIT [f]Not for a five-grass pollen tablet	(27–29, 54–61, 77)
	House dust mite	Yes	Yes, in most studies			(26, 62–64)
	Cat			Yes		(78)
	Latex		Yes			(66)

IT, immunotherapy; LAR, local allergic rhinitis; OAS, oral allergy syndrome; SCIT, subcutaneous immunotherapy; SLIT, sublingual immunotherapy; WDEIA, wheat dependent exercise induced anaphylaxis.

in the basophil activation to various grass allergens was not found after 2 or 4 months of a SLIT with a five-grass-pollen tablet vs. placebo using a defined allergen challenge chamber (61).

For house dust mite (HDM) allergy a significant decrease in BAT results in the course of specific immunotherapy with HDM allergens in children was shown. CD-sens seemed to be a better monitoring parameter than the plain percentage of CD63-expressing basophils (62). Another study demonstrated that after the first and second year of HDM immunotherapy, CD63 expression was lower in atopic dermatitis active group than in the atopic dermatitis control group (63), but others did not find a significant change of basophil reactivity to HDM during 24 months of immunotherapy nor a significant association between the change in clinical symptoms and a change in basophil reactivity (64). A phase I study with timothy grass and dust mite dual-SLIT for pollen allergy showed that basophil activation for these two allergens decreased after 24 months of SLIT compared to baseline (65).

During a latex sublingual immunotherapy in children BAT determinations showed significant decreases in recombinant and natural latex allergens in the active group at 6 months, but not at 12 months (66).

Desensitization of Drugs

It was shown in single cases that desensitization protocols can be monitored by the decrease of basophil sensitivity to the eliciting drug. This was published for insulin, pertuzumab, adalimumab, and brentuximab (67–70). For other drugs, e.g., etanercept and platinum compounds, this could not be constantly demonstrated (71, 72).

Anti-IgE Treatment

In patients with chronic urticaria in whom omalizumab is licensed there was no significant difference in activation of donor basophils incubated with patients' serum before and after 3 months of treatment (73).

In contrast, in patients with peanut allergy, individually dosed omalizumab *in vivo* could be monitored by CD-sens based on peanut induced basophil activation *in vitro* and facilitated peanut oral immunotherapy (74, 75). In severe cow's milk allergy, CD-sens monitoring during omalizumab treatment helped in the decision for performing food challenge (76).

Timothy allergic patients who received omalizumab for 3 months had a decline in CD-sens during the treatment and stayed below the starting value for at least 3 months after the treatment (77). A decrease of CD-sens after a 4-months treatment with omalizumab was also seen in cat allergic patients (78). Furthermore, 12–14 months after closing of 6-years omalizumab treatment, a downregulation of basophil reactivity was still seen (79).

CONCLUSION AND PERSPECTIVES

This overview showed that the flowcytometric measurement of allergen-induced basophil activation and the calculation of basophil parameters from the dose-response curves could help to gain better estimates of *in vivo* reactions in a number, but not all type-I allergic diseases in comparison to conventional diagnostics (**Table 1**). Especially the consideration of results in the submaximal allergen range proved to be particularly relevant and should be pursued further. A thorough characterization of the patients which were not completely transparent in all studies is a prerequisite. Furthermore, quality controls for routine use, standardization, and automatization are expected to expand the range of applications for the above-mentioned indications.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Predicting Success of Allergen-Specific Immunotherapy

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The immune response to antigens is a key aspect of immunology, as it provides opportunities for therapeutic intervention. However, the induction of immunological tolerance is an evolving area that is still not sufficiently understood. Allergen immunotherapy (AIT) is a disease-modulating therapy available for immunoglobulin E (IgE)-mediated airway diseases such as allergic rhinitis or allergic asthma. This disease-modifying effect is not only antigen driven but also antigen specific. The specificity and also the long-lasting, often life-long symptom reduction make the therapy attractive for patients. Additionally, the chance to prevent the onset of asthma by treating allergic rhinitis with AIT is important. The mechanism and, in consequence, therapy guiding biomarker are still in its infancy. Recent studies demonstrated that the interaction of T, B, dendritic, and epithelial cells and macrophages are individually contributing to clinical tolerance and therefore underline the need for a system to monitor the progress and success of AIT. As clinical improvement is often accompanied by decreases in numbers of effector cells in the tissue, analyses of cellular responses and cytokine pattern provide a good insight into the mechanisms of AIT. The suppression of type-2 immunity is accompanied by decreased levels of type-2 mediators such as epithelial CCL-26 and interleukin (IL)-4, IL-13 produced by T cells that are constituting the immune memory and are increasingly controlled by regulatory T and B cells following AIT. Immune tolerance is also associated with increased production of type-1 mediators like interferon-gamma, tissue-homeostating factors like indoleamine 2,3-dioxygenase (IDO) expressed by macrophages and dendritic cells. Although these individual genes were convincingly demonstrated to play a role immune tolerance, they do not predict therapy outcomes of AIT on an individual level. Therefore, combinations or ratios of gene expression levels are a promising way to achieve predictive value and definition of helpful biomarker.

Keywords: allergen-specific immunotherapy, tolerance, biomarker, immune cells, epithelial cells, tissue homeostasis, rhinitis, asthma

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INTRODUCTION

Allergen-specific immunotherapy (AIT) is practiced for more than 100 years now, but the understanding of the cellular and molecular mechanisms just evolved in the last 20 years. Good news is that several studies convincingly demonstrated clinical efficacy of injection as well as of sublingual treatment designs. While quite a few improvements in dosing, adjuvants, allergen extraction, allergen expression, and allergen modification were explored, the main treatment principle is the administration of the antigen into the host. In contrast to conventional vaccination

against pathogens, the main target in allergen vaccination is to inhibit an already ongoing immune response. Thus, although the exact opposite reaction is intended, the applied intervention is the same: inject the antigen/allergen into the organism. In order to induce tolerance by the allergen vaccination, several adjustments have been used to obtain a tolerogenic effect such as the route of administration, extended vaccination schemes for several years, antigen doses, antigen modifications, tolerogenic adjuvants, and use of tolerogenic cell populations (1–5). Biomarker that monitor the antigen-specific induction of an immune response are available for conventional vaccination such as antigen-specific immunoglobulins (Ig's). In contrast, markers of tolerance induction are more difficult to identify, since the absence of inflammation and thus measurable markers are lacking. Ideal would be a marker that predicts whether a patient benefits from AIT before it evens starts. While the identification of such a marker is very difficult, research has taken smaller steps to approach this predictive biomarker that can be used to decide whether to apply ASIT or not. The smaller steps include marker that indicate whether allergen has been successfully administered (1.1), monitoring of anti-inflammatory effects (1.2), induction of immunosuppressive mechanisms (1.3), and prediction of treatment success following initiation of the treatment (1.4).

Tracking Allergen Vaccination

Key diagnostic marker for any antigen-specific immune response is the Ig response and the conversion of initial IgM to high affinity maturated IgGs that demarcates the successful vaccination process. Exposure to environmental antigens as well as allergen-specific immunotherapy results in the induction of IgG4. This Ig isotype is distinguished from other Ig's by its constant region that it is not bound by complement factors and is bound only by low affinity to Fc receptors (6) and therefore not trigger proinflammatory responses. IgG_4 is very efficiently induced even against those epitopes that were not recognized by patients' IgE (7). For this reason, IgG_4 is currently the only generally accepted biomarker of AIT that demonstrates that the patient has received the therapeutic antigen (8). In context of allergy, it was demonstrated that IgG_4 can block IgE binding sites and thereby mediates a welcome anti-inflammatory effect. This effect is particularly welcome in insect venom immunotherapy, where the prevention of IgE-mediated anaphylaxis is of particular value. Therefore, the potential of IgG_4 in relation to IgE to bind to an allergen has been investigated intensively as a tolerance biomarker; however, its relationship to clinical symptoms is only visible in larger cohorts. Resolution of IgG_4 responses to distinct epitopes revealed differential patterns in the recognition of allergens for insect venoms but did not show clinical outcomes (9). In addition, the ratio of IgG_4 and IgE only correlates marginally to clinical symptoms (10). The inclusion of the IgG_4 avidity demonstrated by serum-inhibition assays (facilitated antigen binding FAB) provides an improved view on the competition of IgE and IgG_4. In this assay, it can be visualized that the ability to bind and compete with IgE is improving in AIT. A minor but significant correlation of facilitated allergen binding with combined daily symptom and medication scores was shown over a 3-week peak season at the first maintenance dose (week 8 of treatment); however, placebo samples were included in this analysis as well (11).

Due to differences in the components of the allergens, the picture can be very complex (12). In substitution therapies (e.g., hemophilia), the therapeutic recombinant protein (in this case factor VIII) is neutralized by antibodies of the IgG_4 isotype (13) and destroys its catalytic activity. It is therefore the "bad guy" in this therapy as it inactivates the therapeutic agent. This example outside of AIT highlights the need for administration schemes that do not generate an immune response or at least promote tolerogenic recognition of the therapeutic antigen. Development in this direction could also be relevant for AIT and in particular for immunotherapies that use peptides rather than proteins. Peptide-specific immunotherapy does not induce IgG_4, as it lacks three-dimensional epitopes for Ig binding and yet gives rise to a regulatory T cell response (14, 15). The clinical sustainability of peptide-induced tolerance could yet not be demonstrated convincingly, as high placebo signals prevented a successful conclusion of these trials.

Another interesting immunoglobulin isotype is IgA2, which is selectively transported through the epithelial surfaces. Allergen-specific IgA2 is increased by AIT, but in contrast to IgG_4, these differences become only apparent 2 years after initiation of the treatment (16). IgA together with IgE and IgG_4 is also present in salivary fluids and could be particularly interesting for sublingual immunotherapy (17).

Detecting Decreasing Inflammation

Allergen-specific immunotherapy decreases local inflammatory, particularly type-2 cytokines such as IL-4, IL-5, or IL-13 following AIT; however. initial therapy even increases a broad spectrum of inflammatory responses including IL-36G, IL-8, CXCL-1, CXCL-2, and IL-1α, which was shown in nasal brushings (18). In contrast, interferon-gamma (IFN-γ) was found to be increased by AIT following 3 years of therapy (19). Clinical symptoms (FEV1, FVC, and FEF25–75%) correlate partially with bronchial lavage levels of IL-4 as well as IL-8 and most favorable with eosinophil counts (20). Several studies reported an initial increase in allergen-specific IgE. In particular, the initial increase in IL-4 may be counterproductive and act against tolerance induction. Inflammatory cytokines and transcription factors, specifically IL-4-induced GATA3, can bind and block the activation of the *FOXP3* promoter and thereby prevent the differentiation of AIT-induced regulatory T cells (Tregs) (21, 22). Cytokines induced in the vaccination phase can therefore be envisionaged as negative biomarker in AIT, which was also the basis for a clinical trial, where anti-IL-4 was used in the up-dosing phase to prevent antitolerogenic effects of IL-4 and thereby promote tolerogenic vaccination. In fact, the rise in allergen-specific Th2 cells in the up-dosing phase of AIT could be successfully reduced (5, 23). In addition to IL-4, also other inflammatory cytokines such as TNF family members, IL-1 or IFNs, may prevent tolerance induction (24–27). In order to limit these mediators as well, inhibitors could be imagined that block the activity and signal transduction of these mediators and thereby provide "tolerogenic adjuvants" for the vaccination. One idea is to use already clinically

approved immune suppressors that temporarily block signal transduction of these cytokines. As these proinflammatory mediators, such as IL-4, often trigger the Janus kinase (JAK) pathway, the JAK inhibitor Tofacitinib represents one potential candidate for this approach. This was the background of an experimental immunotherapy where a JAK inhibitor was improving experimental tolerance induction, when it was used to cover the vaccination phase (1, 28). However, also other clinically used immunosuppressants such as glucocorticoid were suggested to support Treg cells at least in *in vitro* models (29), while cyclosporine A is counteracting the induction of these cells (30).

Indicators of Immune Tolerance

Clinical unresponsiveness is often not identical with immunological tolerance; however, in other diseases, a relationship of induction of regulatory B cells (Bregs) in spontaneous clinical tolerance against kidney transplants was demonstrated (31). Bregs are able to suppress cells of the immune system by secretion of IL-10 (18, 32), IL-35 (33) and transforming growth factor beta (TGF-β) (32). IL-10^{+} B cells are a heterogenous group that can be separated to different subsets that demarcate distinct maturation phenotypes such as CD1d^{hi}CD5^{+} (34, 35), CD24^{hi}CD27^{+} (36), CD24^{hi}CD38^{hi} B cell subsets (37), and CD25^{+}CD71^{+}CD73^{-} (38). The latter

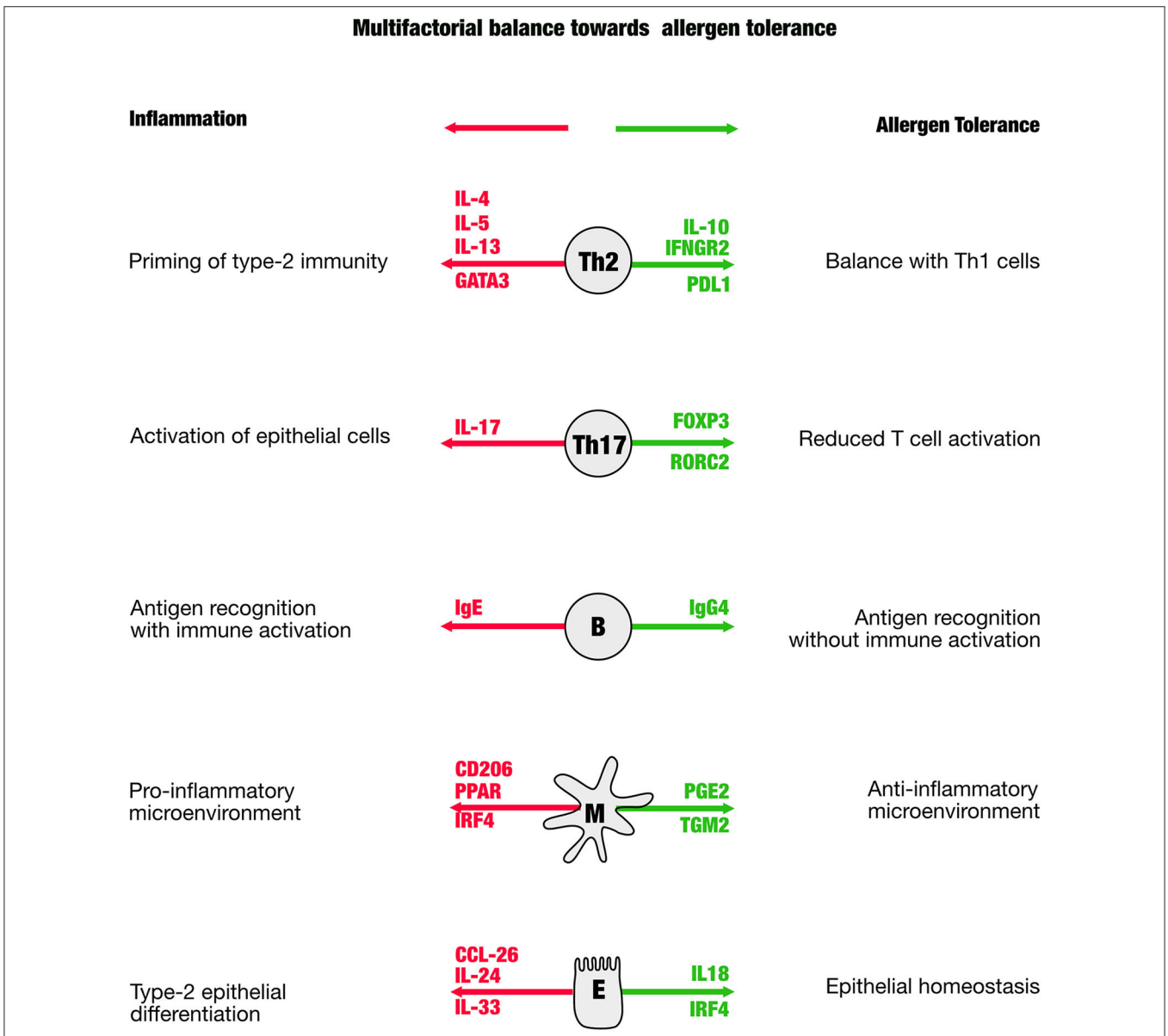

FIGURE 1 | The scheme is illustrating distinct cellular mediators that have been shown to be involved in the immune regulation in allergen-specific immune tolerance. In green are shown genes that appear in the process of tolerance induction, in red are those that indicate a proallergic, inflammatory direction.

subset has been shown to be induced following AIT inhibiting antigen-specific $CD4^+$ T cell proliferation and production of anti-inflammatory IgG_4 antibodies. Induction of IL-10^+ B cells is an early event in AIT, observed most abundantly within weeks of the up-dosing period (18). In the same study, $FOXP3^+$ Tregs increased only after 3 years of therapy, and also the decrease in Th2 cells took as long thus much later as the induction of Bregs. At this time point, Bregs are back to baseline levels. In contrast, Th17 cells appeared also relatively early but are mainly considered as proinflammatory cells, despite the fact that they require the presence of the rather anti-inflammatory cytokine TGF-β (39, 40). Depending on certain circumstances and anatomic locations, T cells are described, which express FOXP3 and IL-17 at the same time and inversely correlated with Th2 cells (41). These cells or cells of a similar phenotype ($FOXP3^+$IL-17^+) also occur transiently in the first year of AIT (18). It may represent a transitory "Tr17" population, which possibly originates from Th17 cells and may further differentiate into fully regulatory T cells.

Predicting Therapy Success: Hopeful or Helpful?

The prediction of therapy success, and in particular to support the physician to manage AIT to make it successful, is a major aim in biomarker research. The difficulty is that multiple proinflammatory players are in balance with those that act anti-inflammatory (**Figure 1**). As a consequence, a reliable biomarker will not be based on a single molecule, but rather need to cover multiple analytes that better represent the balance of the players contributing to tolerance. The first blood-borne biomarker that fulfills this idea is a ratio of Bregs and Th17 cells taken after the up-dosing phase, thus in the early phase of the therapy (18). This ratio correlates with the therapy success [*Retrospective Assessment of Seasonal Allergic Symptoms* (RAAS)] after 3 years and embraces the idea to include regulatory and inflammatory elements into account. The Bregs in the equation showed a high spread as well as the proinflammatory Th17 cells that were previously not considered in context of other AIT studies. Th17 cells are known to be very "plastic," which means that they may not be fully differentiated and may have enough stemness to further differentiate even into regulatory T cells. This biomarker is awaiting to be validated in larger cohorts to be able to answer the question whether this marker is not only hopeful, but also helpful.

Discussion and Outlook

The role of the tissue and tissue biomarker in the regulation of tolerance is still insufficiently investigated in AIT, while increasingly recognized as key factor in transplantation tolerance via mechanisms of amino acid consumption [auxotrophy; (42)]. The tissue interacts with the specific immune system either directly or indirectly via tissue-resident macrophages or dendritic cells. Differentiation of tissue-resident macrophages may provide important information and biomarker of how effective the allergen tolerance has been corrected by allergen-specific immunotherapy (43). Dendritic cells are key orchestrators of both the innate and the adaptive immune responses and are essential for the regulation of $CD4^+$ T cell responses. When triggered by an allergen, immature DCs polarize into DC1s, DC2s, DC17s, or DCregs, which in turn can differentiate T cells into Th1 cells (DC1s), Th2 cells (DC2s), Th17 cells (DC17s), or regulatory T cells [DCregs; (44)]. Changes in expression of five combined DCreg/DC2-associated markers (CD141, C1Q, GATA3, FcγRIIIa, RIPK4) in peripheral blood mononuclear cells (PBMCs) correlated with clinical efficacy of sublingual immunotherapy (SLIT) at 2 and 4 months (44). The link between tissue cells, T cells, and macrophages or dendritic cells is regulated by $CD8^+$ T cells, which recognize specific antigens on any cell via major histocompatibility complex class I (MHCI), while $CD4^+$ T cells recognize allergen peptides only on MHCII expressed by dendritic cells. Consequently, CD8 cell may also play an important in direct tolerance induction (45), by interacting between tissue cells and antigen-presenting cells. Both $CD4^+$ and $CD8^+$ T cell cells produce interleukins that selectively act on tissue and epithelial cells that can mediate specific epithelial responses and can thereby contribute to clinical tolerance (46, 47). In turn, epithelial cells interact with all these cell types and innate lymphoid cells. Epithelial cells are directly responding to environmental influences and can therefore influence immune tolerance and immune homeostasis (48). Specifically, epithelial tissues respond to allergic inflammation or viral infections with distinct differentiation processes and commit toward E2 and E1 cells, respectively (49). These phenotypes can be detected in allergen-specific immunotherapy and are noninvasively detectable in secretions of the upper and lower airways (50). The important role of epithelial cells in tolerance regulation was also demonstrated in studies showing that airway epithelial cells can break immunotolerance upon recognition of bacteria and FcγRIII-mediated activation of the cells (51).

In conclusion, AIT induces clinical allergen tolerance that depends on multiple mechanisms across different immune and tissue cells. Therefore, an effective biomarker will consist of multiple analytes that cover different cellular processes. Whether multiple biomarkers as opposed to a single marker are helpful depends on simple devices that need to be developed and convert the hopeful experimental results into an helpful clinical routine.

AUTHOR CONTRIBUTIONS

UZ and CS-W wrote the manuscript and prepared the figure. All authors contributed to the article and approved the submitted version.

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REVIEW
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Research Progress in Atopic March

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The incidence of allergic diseases continues to rise. Cross-sectional and longitudinal studies have indicated that allergic diseases occur in a time-based order: from atopic dermatitis and food allergy in infancy to gradual development into allergic asthma and allergic rhinitis in childhood. This phenomenon is defined as the "atopic march". Some scholars have suggested that the atopic march does not progress completely in a temporal pattern with genetic and environmental factors. Also, the mechanisms underlying the atopic march are incompletely understood. Nevertheless, the concept of the atopic march provides a new perspective for the mechanistic research, prediction, prevention, and treatment of atopic diseases. Here, we review the epidemiology, related diseases, mechanistic studies, and treatment strategies for the atopic march.

Keywords: atopic dermatitis, asthma, food allergy, allergic rhinitis, atopic march

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INTRODUCTION

In recent decades, the incidence of allergic diseases has continued to increase, affecting ∼20% of the worldwide population, especially children (1). Cross-sectional and longitudinal studies have suggested that allergic diseases occur following a time-based order: from atopic dermatitis (AD) and food allergy in infancy to gradual development into allergic asthma (AA) and allergic rhinitis (AR) in childhood. In terms of anatomic structure, it follows the spatial evolution of skin–gastrointestinal tract–respiratory tract, and this phenomenon is defined as the "atopic march" (2).

Among the allergic diseases mentioned above, some resolve gradually to disappear with age, whereas others continue for many years (3). Some studies have shown that the atopic march does not progress completely in a temporal pattern with genes and the environment (4). Nevertheless, the concept of the atopic march provides a new perspective for the mechanistic research, prediction, prevention, and treatment of allergic diseases.

Here, we review the epidemiology, related diseases, mechanism of action, and treatment strategies of the atopic march.

EPIDEMIOLOGY OF THE ATOPIC MARCH

AD: The First Manifestation of the Atopic March

AD is a chronic recurrent skin disease. Its clinical manifestations are chronic inflammation of the skin, itching, and an impaired skin barrier. AD affects 3% of adults and ∼30% of children, and its prevalence tends to increase with age (5). AD occurs in the early years of life. Some epidemiology studies have shown that 45% of affected children had the condition before 6 months of age, 60% before 1 year of age, and up to 85% before 5 years of age (6, 7).

AD etiology is a combination of various factors involving genes and the environment (8). Once external allergens contact a damaged skin barrier, keratinocytes are stimulated to secrete thymic stromal lymphopoietin (TSLP) and other factors in conjunction with Langerhans cells (LCs) to stimulate T-helper type 2 (Th2) immune responses. Then, the body is stimulated to produce non-specific immunoglobulin (Ig)E (if children are exposed to allergens such as mites for a long time, specific IgE may appear). Subsequently, T cells, eosinophils, macrophages, mast cells, and type 2 innate lymphoid cells (ILC2s) infiltrate to secrete cytokines, resulting in local inflammation of the skin (9). AD patients can be classified into two types based on whether the IgE level is increased: intrinsic (normal IgE and non-allergic) and extrinsic (high IgE level associated with increased disease severity). Studies have shown that extrinsic AD increases the risk of developing the atopic march (10, 11).

AA and AR: The End Progression of the Atopic March

AA is a common chronic airway disease characterized by the inflammation, hyperresponsiveness, and remodeling of airways (12–15). With modernization and industrialization, AA incidence has increased year by year. This may be because of lifestyle alterations, changes in environmental factors (e.g., increase in indoor dust mites and outdoor pollution), changes in dietary habits, and many other factors. AR involves inflammation of the nasal mucosa (16) and diminishes the quality of life of sufferers (17).

Epidemiologic evidence has revealed a link between AA and AR. A retrospective follow-up study reported the incidence of AR to be higher in AA patients than in non-AA persons (18). In another cohort study, Leynaert et al. demonstrated that 74–81% of AA patients reported AR. Also, they found that AA occurred in 2% of non-AR persons, but in 18.8% of AR patients upon exposure to pollen or animal dander (19).

AR may lead to changes in the function of the lower airways through three main mechanisms. Firstly, stimulation of the nasal mucosa contracts bronchial smooth muscle through the nasal–tracheal reflex. Secondly, various chemical mediators and cytokines released by antigenic stimulation causing nasal mucosa allergy are absorbed into blood, are transported to the lung through circulation, and then act on the trachea and bronchi, causing smooth muscle spasm. Thirdly, nasal inflammatory mediators and secretions are discharged through the nasal passage to the lower airways, resulting in a reduced β-adrenergic receptor functional response (20).

Epidemiology of the Atopic March: Linking AD With AA or AR

Dharmage et al. found, in infants who have AD within 2 years of age, that the incidence of AA and AR increased significantly during age 6–7 years. In particular, early-onset, persistent, and IgE-positive AD led to a higher risk of developing AA and AR (9). A longitudinal study on a Canadian birth cohort (2,311 children) has shown that AD with sensitization at 1 year of age increased the prevalence of AA and AR at 3 years of age more than 11- and 7-fold, respectively (21). In a recent report from Thailand, 102 children with AD (diagnosed at 1.5 years of age) were reviewed, and subsequently, AR and AA were diagnosed in 61.8 and 29.4%, respectively. Concomitantly, 67% of the AA patients also suffered AR (22). A prospective cohort study (3,124 children aged 1–2 years) reported that, compared with children with no history of AD, those once having AD, particularly moderate-to-severe, early, and persistent AD, were more inclined to develop AA and AR (23).

The discoveries mentioned above strongly support the natural process of the atopic march.

Roles of Food Allergy

IgE-positive food allergy commonly coexists with AD in early childhood as the earliest manifestation of the atopic march. In 2011, Japanese scholars conducted a retrospective questionnaire survey on freshmen, and they found that AD occurred earlier in those with accompanying food allergy. Also, having food allergy was regarded as the biggest risk factor for the atopic march (24). A family-based cohort study from Chicago revealed that symptomatic food allergy, especially severe or multiple food allergies, was closely related to AA in children aged ≥6 years. Children with food allergy developed AA earlier than those without food allergy (25). A survey of 2,222 infants with AD aged 11.5–25.5 months showed that 64% of children diagnosed with AD within 3 months of birth exhibited an IgE-mediated sensitivity to milk, peanuts, or eggs. Also, in infants <12 months of age, the proportion of infants with sensitivity to eggs, milk, or peanuts increased with AD severity, but this phenomenon was not manifested in children with AD after 1 year of age (26). Among adults with AD, food allergy is relatively rare (27–31). In addition, studies have shown that children sensitive to milk in infancy subsequently exhibited aggravated airway inflammation and increased airway responsiveness to histamine (32, 33). Remarkably, food allergy commonly exists together with AD in infants. Therefore, it is worth exploring whether the link between food allergy and AA or AR is related to AD or is a direct consequence of the food allergy itself.

EoE: A New Manifestation of the Atopic March?

Eosinophilic esophagitis (EoE) is a chronic esophageal inflammatory disease induced by pollens or food allergens (34). EoE patients are sensitive to allergen avoidance and glucocorticoid therapy. Genome-wide association study (GWAS) data have indicated that EoE shares some susceptible genetic loci with other manifestations of the atopic march, including polymorphisms in the signal transducer and activator of transcription 6 gene (*STAT6*) and *TSLP* (35). In addition, epidemiology studies have demonstrated EoE to be associated with other allergic diseases. For example, Mohammad et al. found that, of 449 EoE patients, the prevalence rates of AR, AA, and AD were 61.9, 39, and 46.1%, respectively, and that up to 21.6% of EoE patients developed these three atopic diseases (36). Another study involving 35,528 people reported that those with IgE-positive food allergy were at a higher risk of EoE (37). A birth

cohort study involving 130,435 children determined a positive association between EoE and other allergic manifestations (34).

The studies mentioned above suggest the potential of EoE as the fifth "member" of the atopic march, but this hypothesis is controversial. For example, EoE occurs not only in childhood but also after childhood. In addition, EoE can occur in individuals without a history of atopy. Therefore, larger cohorts are needed to study the epidemiologic relationship of EoE with other manifestations of the atopic march and the mechanisms involved (38).

POSSIBLE MECHANISMS UNDERLYING THE ATOPIC MARCH

Dysfunction of the Skin Barrier

The skin is the foremost barrier for defense against external stimuli, such as pathogens, environmental pollutants, and ultraviolet light. As a component of the innate immune system, the skin has several defensive functions, including microbial, chemical, physical, and immune barrier. These different functions of the skin barrier coordinate with each other to resist external stimuli and maintain skin homeostasis.

Allergens can enter the body through damaged skin to cause sensitization, which is defined as "transcutaneous sensitization" (39). Transcutaneous sensitization can cause AD and, subsequently, AA and AR (5). Studies have shown that epicutaneous disruption induces sensitization after exposure to peanut and egg allergens (40, 41). Spergel et al. demonstrated that repeated cutaneous exposure to egg allergens induced AD-like skin inflammation and AA-like bronchial hyper-responsiveness in a mouse model (41). Emerging studies now suggest that the skin barrier protein filaggrin and epithelial cell-derived cytokines such as TSLP, IL-25, and IL-33 might be related to the progression of the atopic march.

Filaggrin

Filaggrin, a barrier protein, has important roles in the integrity of the stratum corneum in terms of structure and composition. Mutations in the filaggrin gene (*FLG*) can impair the barrier function of the skin and induce an allergic response (42, 43). Several studies have shown patients with impaired or reduced levels of filaggrin to be more susceptible to food sensitization (44–46). Moreover, *FLG* mutations increase the risk of early and severe AD and of AA in individuals who have had AD (47–49).

Thymic Stromal Lymphopoietin

TSLP is an interleukin (IL)-7-like epithelial cell-derived cytokine which regulates the Th2 response (50). Zhang et al. found that TSLP overexpression in keratinocytes aggravated AA-like airway inflammation in mice subjected to ovalbumin (OVA) sensitization intraperitoneally and OVA challenge intranasally (51). Another *in vivo* study demonstrated that keratinocytic TSLP was essential to induce a Th2 response in the skin and to trigger aeroallergen-challenged AA phenotypes (52). In addition, Noti et al. found that the effect of TSLP was enough to develop experimental EoE-like phenotypes in mice (53). Also, they found that TSLP in skin facilitated food allergy (54).

Interleukin-33

IL-33 is derived from epithelial cells and acts on macrophages, ILC2s, Th2 cells, mast cells, and basophils through the suppression of tumorigenicity 2/IL-1 receptor accessory protein heterodimer (*ST2/IL1RL1*) (55–60). Several studies have explored the roles of IL-33 in allergic diseases and found high expressions of IL-33 in the skin or airway epithelial cells in AD or airway inflammation (61–63). Blockade of ST2 expression can alleviate food allergy in peanut- and OVA-challenged models (64, 65).

Interleukin-25

IL-25 is also an epithelial cell-derived cytokine (66–68). Kim et al. found that IL-25 inhibited filaggrin expression in the skin and aggravated skin inflammation by coordinating with Th2 cytokines (69). Lee et al. reported OVA/alum-sensitized allergic diarrhea to be inhibited in mice lacking IL-17RB, the receptor of IL-25, whereas IL-25 overexpression in the intestine accelerated the development of allergic diarrhea (70). Kang et al. found that the mRNA expression of IL-25 was upregulated in rat lungs in a TiO_2-induced model of airway inflammation (71).

In conclusion, allergens (including food and aeroallergens) enter the skin through the damaged skin barrier. Then, they stimulate skin epithelial cells to release TSLP, IL-25, and IL-33. This action activates some immune cells in the dermis [e.g., basophils, mast cells, dendritic cells (DCs), eosinophils, ILC2] to secrete cytokines, and subsequently, Th2 cells are generated and IgE production in local lymph nodes occurs. Th2 cells can secrete more type 2 cytokines (e.g., IL-4) to activate more ILC2 and eosinophils, and IgE can act on mast cells and basophils. This positive feedback causes skin inflammation and AD (72). Furthermore, IgE, Th2, TSLP, IL-25, and IL-33 might enter the digestive and respiratory tracts through blood circulation to facilitate the development of AA, AR, and food allergy if allergens are re-encountered (73, 74) (**Figure 1**). Therefore, skin barrier dysfunction might be a potential mechanism underlying the atopic march.

Microbiome Alteration

Many microorganisms are colonized in the intestine, skin, and respiratory tract (75) and influence health and disease. Several studies have suggested that microbiome alteration plays roles in atopic diseases locally or peripherally.

Kennedy et al. observed the skin microbiome dysbiosis in early life of AD patients, and they also found that colonization with commensal *Staphylococci* at 2 months was related to a lower risk of AD at 1 year of age (76). Forno et al. and Abrahamsson et al. reported that children who had AD at 6 months (77) and 2 years (78) of age had decreased intestinal microbial diversity at 1 month of life. A study of the KOALA birth cohort demonstrated that infants with *Clostridium difficile* colonization in the gut at 1 month of life were inclined to develop AD and other atopic diseases (79). Azad et al. demonstrated that infants who had a positive skin prick test for food sensitization at 1 year had lower gut microbial richness at 3 months (80). Abrahamsson et al. reported that infants with low diversity in intestinal flora at 1 month of age were inclined to develop AA at school age (81). In addition, Teo et al. determined that microbiome alteration

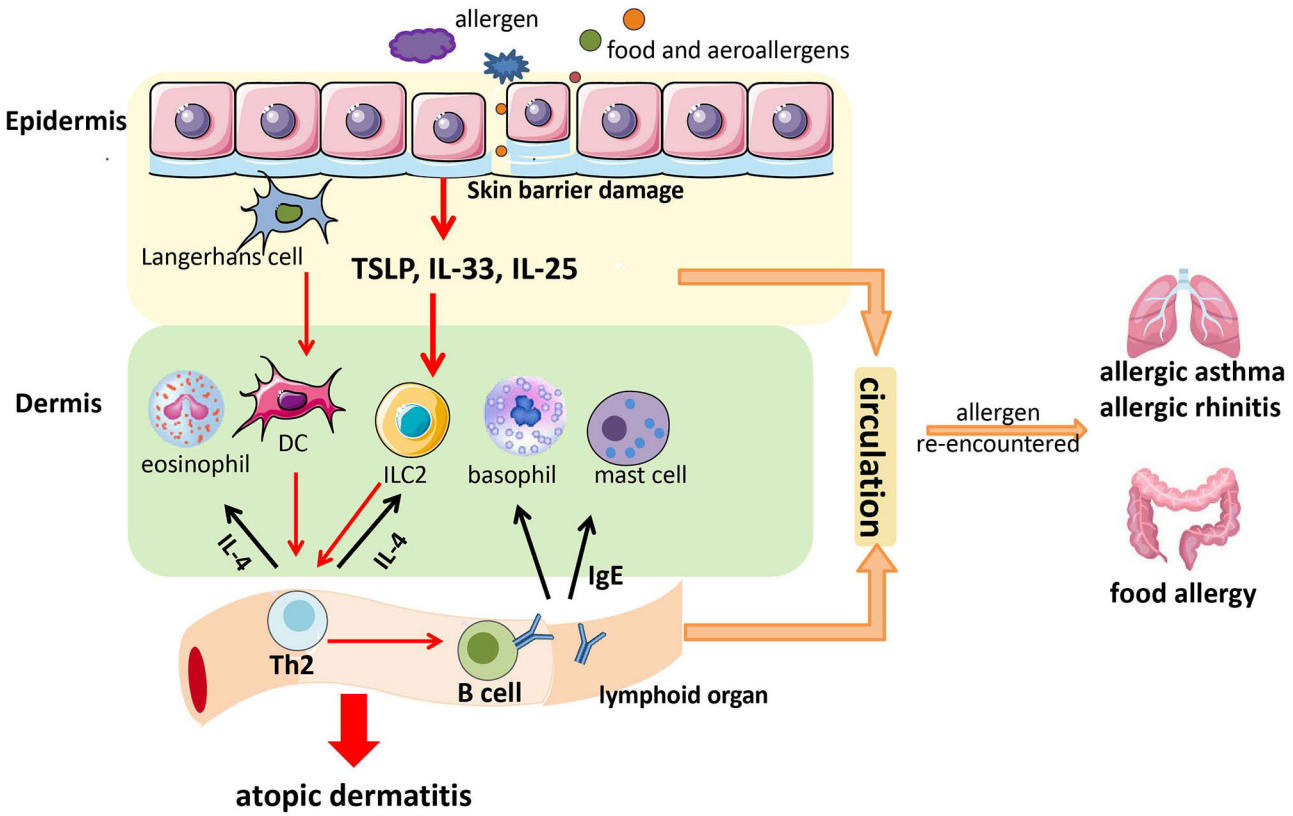

FIGURE 1 | A possible model of the contribution of skin barrier dysfunction to the atopic march. Allergens (including food allergens and aeroallergens) enter the skin through the damaged skin barrier. Then, they stimulate epithelial cells in the skin to release thymic stromal lymphopoietin (TSLP), interleukin (IL)-25, and IL-33. This action activates some immune cells in the dermis (e.g., basophils, mast cells, DCs, eosinophils, and ILC2) to secrete cytokines, followed by the generation of T-helper type 2 (Th2) cells and immunoglobulin E (IgE) production in local lymph nodes. Th2 cells can secrete more type 2 cytokines (e.g., IL-4) to activate more ILC2 and eosinophils, and IgE can act on mast cells and basophils. This positive feedback causes skin inflammation and atopic dermatitis (AD). Furthermore, IgE, Th2 cells, TSLP, IL-25, and IL-33 might enter the digestive tract and respiratory tract through blood circulation to facilitate the development of allergic asthma (AA), allergic rhinitis (AR), and food allergy if allergens are re-encountered.

following respiratory infections during infancy might contribute to the development of AA (82).

Moreover, some studies have shown that microbes regulate atopic diseases by secreting metabolites. Furusawa et al. reported that the short-chain fatty acids produced by several intestinal microorganisms induced the proliferation of colonic regulatory T cells (Tregs) and further ameliorated colitis and allergic responses (83). Dysbiosis of *Faecalibacterium prausnitzii*, as observed in AD, was found to reduce the production of butyrate and propionate and further destroyed the intestinal mucosa. Then, some toxins permeated into the circulation and induced a Th2 immune response to facilitate skin inflammation and AD development (84). Johnson et al. found that the polysaccharides derived from *Bacteroides fragilis* induced $CD4^{+}Foxp3^{-}$ T cell activation and further prevented AA onset (85).

The studies mentioned above strongly suggest that microbiome alteration may be involved in the atopic march. However, further studies are needed to determine whether microbiome shifts are a cause or a consequence of the atopic march.

Epigenetic Factors

Epigenetic mechanisms can regulate gene expression and constitute the cause of diseases. Several epigenome-wide studies have revealed DNA methylation in blood to be related to food allergy (86, 87) and AA (88). Recently, Peng et al. undertook DNA methylation analyses on the cohorts of the Generation R Study (343 at mid-childhood and 839 newborns) in the Netherlands and Project Viva (396 at mid-childhood and 232 newborns) in the USA. Meta-analyses linked the differential methylation profiles of the peripheral blood of mid-childhood children with food allergens, environmental/inhalant allergens, and atopic sensitization. Multiple methylation site-related genes were enriched to AA pathways, including eosinophil peroxidase (*EPX*), *IL4*, interleukin 5 receptor A (*IL5RA*), and proteoglycan 2 (*PRG2*). Furthermore, Peng et al. identified several methylation sites of cord blood to be related to allergic phenotypes in mid-childhood and that some methylation sites of cord blood were also present in mid-childhood (89), which suggested a longitudinal time trend.

The findings mentioned above suggest that epigenetics may have roles in allergic diseases. However, these studies show only a correlation between epigenetics and the atopic march. Whether epigenetic change is a cause or a result of the atopic march warrants large and detailed longitudinal studies.

"Social" Dysfunction of Cells and Molecules

Allergic reactions occur not only in the regions where allergens are in contact directly but also in long-distance, non-contact sites. This may be a systemic reaction of the body, and the mechanisms are incompletely understood. Through literature search, Luo et al. proposed a model of "social events" of cells and molecules to explain the atopic march (90). Epithelial cells, such as epidermal keratinocytes and airway epithelial cells, are the first line of defense against allergen exposure and initiate the inflammatory response by releasing proinflammatory cytokines. Thus, epithelial cells are considered to be key participants in allergic diseases. In this model, Luo and colleagues considered that it is the atopic factors produced by epithelial cells locally, not in the circulation, that drive allergy at different sites and that certain allergens are the irritants that trigger the release of atopic factors at different sites. Zhang et al. reported that TSLP overexpression in keratinocytes induced AD-like symptoms and also aggravated OVA-induced AA manifestations in mice. However, they also found that increased TSLP expression in the skin and, subsequently, peripheral blood was not sufficient to induce lung inflammation (51). The atopic reaction in the lung might be induced by the TSLP derived from the lung epithelia themselves. Therefore, the atopic reaction in the skin and the lung might be the consequence of the "social dysfunction" of homologous epithelia and molecules such as TSLP. Despite its rationality and interpretability, the theory of social events needs sufficient evidence from *in vivo* and *in vitro* studies.

Interference of Other Predicted Genes

Marenholz et al. performed GWAS on 2,428 cases with AD in infancy and AA in childhood and on 17,034 controls. They identified seven susceptible sites associated with the atopic march: *FLG* [1q21.3], *AP5B1/OVOL1* [11q13.1], *IL4/KIF3A* [5q31.1], *IKZF3* [17q21], *C11orf30/LRRC329*, *EFHC1* [6p12.3], and *rs99322* [12q21.3] (91).

Bioinformatics analyses by Gupta et al. revealed that the atopic march involved 16 common pathogenic genes: *IL4*, *IL5*, *TSLP*, *RNASE3*, *IL13*, *IL10*, *IGHG4*, *IFNG*, *CCL11*, *FCER2*, *RNASE2*, *FOXP3*, *KCNE4*, *CD4*, *IL4R*, and *CCL26* (92). These genes were predicted through large-scale and high-throughput bioinformatics analyses, and their roles in the atopic march need to be determined through further experimentation.

Summarily, Paller et al. have reviewed the multifactorial etiology of the atopic march, including skin barrier damage, microbiome alteration, and epigenetic factors (93), and we consider that "social" dysfunction of cells and molecules, and the interference of other predicted genes, may also contribute to the atopic march (**Figure 2**). However, further studies are required to detail the relevant mechanisms.

ANIMAL MODELS FOR STUDIES ON THE ATOPIC MARCH

The modeling process of Leyva-Castillo et al. consisted of two phases. In the first phase, wild-type (WT) BALB/c mice were treated with calcipotriol MC903 plus OVA through epicutaneous sensitization. This led to increased levels of Th2 cytokines, Th17 cytokines, and OVA-specific IgE and IgG1 in serum. In the second phase, MC903-treated (epicutaneous) OVA-sensitized mice underwent intranasal challenge with OVA. These mice exhibited AA-like symptoms with increased mucus secretion, eosinophil infiltration, and expression of Th2 cytokines (52).

In a model established by Han et al., WT BALB/c mice were first treated with OVA plus TSLP *via* the intradermal route (four times within 2 weeks). After 9 days, the mice were challenged by OVA *via* the intranasal route for four consecutive days. Consequently, the mice exhibited increased OVA-specific IgE in serum as well as cellular and eosinophil infiltration in the bronchoalveolar lavage fluid. Histopathology showed severe inflammatory infiltrates in mouse lungs. In addition, periodic acid–Schiff staining showed excessive goblet cell metaplasia and mucus secretion (94).

Moreover, one study showed that epicutaneous exposure to *Aspergillus fumigates* aeroallergens followed by intranasal challenge with *A. fumigates* induced an allergic nasal response in BALB/c mice (95).

In conclusion, the models mentioned above have one similarity: the skin is used as a sensitization site, consistent with the feature that AD is the initial manifestation during the atopic march. These animal models facilitate the studies of the mechanisms underlying the atopic march.

REFUTATIONS OF THE ATOPIC MARCH

Despite substantial epidemiologic and experimental evidence, some scholars argued that the prevalence of the atopic march may be overemphasized (96).

First, the methods of the data collection and disease identification initiate one main concern. Considering the cost and time required to make physician diagnoses, allergic disease identification was often based on "yes" or "no" questions. In existing epidemiologic surveys, the diagnosis of AD, AA, and AR simply used "yes" or "no" questionnaires, and some even lacked further physician identification (97–99). In addition, deviations and over-reporting in questionnaire surveys from some individuals led to an overestimation of the disease prevalence (100). Another rebutted criticism of the atopic march is the failure to consider disease heterogeneity or variations. Martinez et al. found that AD patients were at a higher risk of developing transient early AA and persistent AA, not late-onset AA (97). This indicates that the association between AD and AA may be restricted to specific AA subpopulations, not universal. Moreover, some individual-level analyses did not support the typical temporal pattern. At an individual level rather than a large-scale population level, Belgrave et al. demonstrated that only 3.1% of children followed the classical

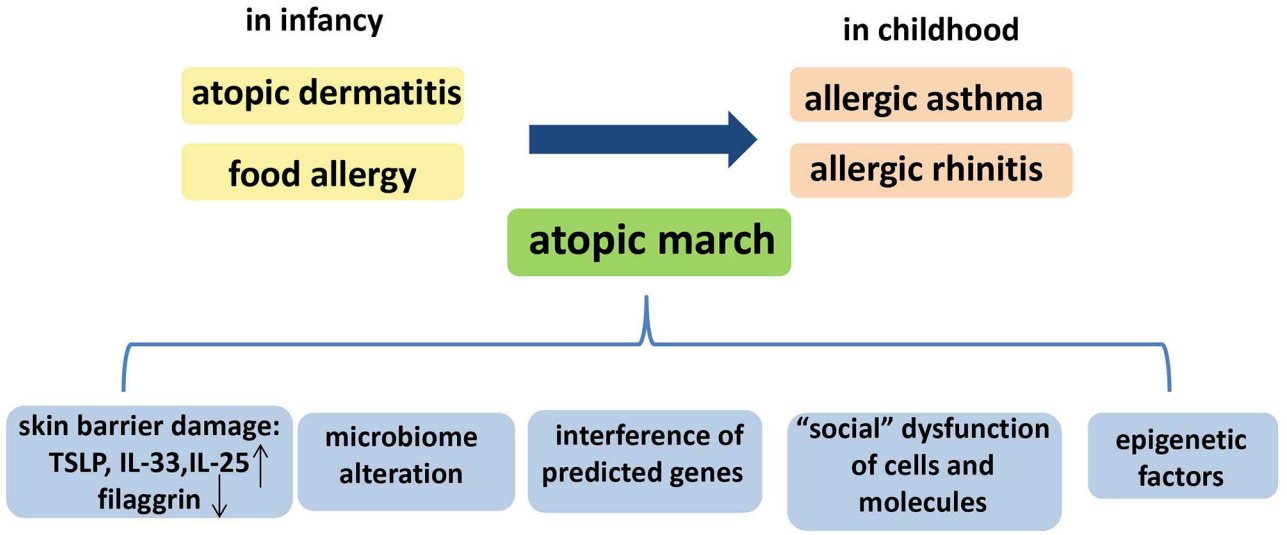

FIGURE 2 | The temporal pattern and the possible mechanisms of the atopic march. The temporal pattern of the atopic march is, in general, from atopic dermatitis (AD) and food allergy in infancy to gradual development into allergic asthma (AA) and allergic rhinitis (AR) in childhood. Several mechanisms could underlie the atopic march: skin barrier damage, microbiome alteration, "social" dysfunction of cells and molecules, epigenetic factors, and interference of other predicted genes.

atopic march procession (AD first, followed by AA and then AR) and more than 90% of children with atopic manifestations did not (101).

Unusually, a study from Italy found an evidence of a "reverse" atopic march. The study included 745 children aged 6–9 years with AA only, without a history of food allergy or AD. After a 9-years follow-up, 20% of the children were found to have developed AD (102). In addition, the prevalence of the atopic march differed in distinct countries. Colombian scholars followed up 326 mother–infant pairs in a birth cohort study, and they found that AA was the most common manifestation by 24 months. The prevalence of recurrent AA was 7.1% at 12 months and reached 14.2% at 24 months. However, allergic symptoms induced by milk, egg, or other food allergens were scarce, only 1.8%, and AD was not observed in any cases (103).

Although these studies refute the concept of atopic march to a certain extent, we cannot deny the contribution of the theory of atopic march to the early prevention, diagnosis, and treatment of allergic diseases. Future research on the atopic march should improve the current data collection and disease identification methods, not only relying on "yes" or "no" questionnaires, take disease subtypes into account, and perform the study in an individual level rather than only in a group level.

PREVENTION AND TREATMENT STRATEGIES FOR THE ATOPIC MARCH

Several measures used to prevent and treat allergic diseases are expected to interfere with, delay, and block the natural process of the atopic march.

Food Interventions

In most studies, breastfeeding for > 6 months has been recommended because it reduces not only the incidence of AD but also of other allergic diseases (104). A 15-years follow-up study of the German Infant Nutritional Intervention (GINI) cohort has shown that, if breastfeeding is not possible, compared with standard cow's milk formula (CMF), the interventional use of partial whey hydrolyzate (pHF-W) formula and extensive casein hydrolyzate (eHF-C) formula in the first 4 months of life has significant preventive effects on AD, and the eHF-C formula also reduced the prevalence of AA and AR (105). However, the mechanisms underlying the preventative effects of hydrolyzed formulas are unknown. In addition, the assessment of the effects of hydrolyzed formulas was based on parental reports of physicians' diagnosis, not on the clinical examinations. These are the criticisms against the use of hydrolyzed formulas for the prevention of allergic conditions.

Moreover, Wickens et al. found that supplementation with *Lactobacillus rhamnosus* for the first 2 years of life reduced the prevalence of AD by about half (106). However, further studies are required to handle the uncertainties about whether other probiotics are equally effective and how probiotics exert their effects on allergic diseases.

Furthermore, the Learning Early About Peanut Allergy (LEAP) trial demonstrated that, compared with children who avoided peanut, sustained peanut consumption, beginning in the first 11 months of life, significantly decreased the prevalence of peanut allergy at 60 months of age in infants with high atopic risk (107). In addition, a large-scale population-based prospective study showed that early introduction of cow's milk protein as a supplement to breastfeeding might promote tolerance, reducing the incidence of IgE-mediated cow's milk protein allergy (108).

Environmental Prevention

Exposure to several environmental factors is closely related to having allergic diseases. Studies have shown that smoke in the environment increases children's risk of allergic sensitization and AA (109). Therefore, it is strongly recommended that all parents should stop smoking tobacco. Dust mites, pollen, cockroaches, pet fur, and fungi are common allergens, and avoiding exposure to these allergens can reduce the sensitization of children at high risk. However, it has also been proposed that there was no correlation between house dust mite (HDM) exposure and AA (110), and keeping pets (cats or dogs) in the home in the first year after birth reduced the risk of sensitization to multiple allergens during childhood, but it impaired lung function once cat or dog sensitization has occurred, particularly in children with a family history of AA (111). These controversial views are initiating further research to evaluate their relevance.

Medical Treatment

Symptomatic Treatment

Antihistamines are used to relieve itching in AD patients and to prevent skin damage aggravated by scratching. Ketotifen, an H1 antihistamine, significantly lowered AA risk in infants with AD or other pre-asthmatic conditions (112, 113). A double-blind, randomized, placebo-controlled trial showed that compared to placebo, cetirizine significantly reduced the incidence of AA in AD patients sensitized to grass pollen or to HDM (114). However, considering the side effects of antihistamines, a large number of clinical trials are needed to evaluate the security of antihistamines and the effectiveness of interventions in the natural course of allergic diseases.

Glucocorticoid is also an effective anti-inflammatory treatment for allergic diseases, and inhaled glucocorticoids has now become the first-line treatment for AA (115). Although the symptoms of AD and AA can be significantly improved by glucocorticoids, it is prone to relapse after withdrawal. In addition, there are many side effects. Therefore, glucocorticoids should be prescribed with caution by the physicians.

Allergen-Specific Immunotherapy

Allergen-specific immunotherapy (ASIT), also known as "desensitization therapy," can alleviate allergy symptoms for a long time and change the natural course of allergic diseases (116). Several ASIT routes have been documented in preclinical studies, including subcutaneous immunotherapy (SCIT), sublingual immunotherapy (SLIT), epicutaneous immunotherapy (EPIT), and oral immunotherapy (OIT). The recognized mechanism of specific immunotherapy is stimulation of the secretion of IL-10 and transforming growth factor-β from Tregs, promotion of the balance of Th1 cells/Th2 cells, and conversion of IgE to IgG to block the IgE-mediated immune cascade (117, 118). Zhong et al. found that the clinical symptoms and quality of life of AD patients with HDM sensitization could be improved after 2 years of ASIT (119). Besh et al. demonstrated that combining basic therapy with SCIT acquired significantly better results in AA patients compared to basic therapy only (120). Karakoc-Aydiner et al. found that the nasal symptom scores of children with AR were significantly reduced after receiving dust mite allergen vaccine through SCIT or SLIT (121). However, the lack of security greatly limits the development of ASIT. For example, the adverse reactions of SLIT mainly focus on local reactions, such as oromucosal pruritus and gastrointestinal reaction (122). In addition, almost all clinical trials related to OIT are accompanied by one or several serious adverse reactions, such as severe gastrointestinal reactions, systemic allergic reactions, etc. (123). Long-term follow-up of milk OIT patients showed that the complete immune tolerance rate after OIT treatment was only 31% (124, 125). Therefore, further research on ASIT should be directed at the improvement of not only its efficacy but also security.

Targeted Therapy

Omalizumab is a human monoclonal antibody against IgE. In 2003, it was approved for the treatment of severe AA in adolescents and adults. Esquivel et al. demonstrated that omalizumab inhibited rhinovirus infections, illnesses, and exacerbations of AA through specific binding to IgE (126). Dupilumab is a human IgG4 monoclonal antibody against IL-4 receptor subunit alpha (IL-4Rα), and it can inhibit IL-4 and IL-13 signaling pathways by interacting with IL-4Rα (127). Dupilumab has been approved by the US Food and Drug Administration to treat infants with moderate-to-severe AD with poor results from conventional treatment (128). Tezepelumab (AMG 157) is a monoclonal antibody (G2λ) against TSLP. In one clinical trial, tezepelumab treatment for 5–12 weeks blunted inhaled allergen-induced AA attacks (129). Of note is that these targeted therapy medicines are only licensed for use in certain allergic diseases. Although the off-label uses or adjunct to treatment for numerous allergic conditions have acquired encouraging results, their potential efficacy still needs to be evaluated through clinical trials.

BIOMARKERS OF THE ATOPIC MARCH

Although there are no reliable biomarkers to identify subjects with high risk of atopic march, Davidson et al. have proposed some recommendations recently for future research to explore biomarkers, which would provide some possibilities to examine the atopic march.

The relevant proposals are as follows: (1) to look at the protein, RNA, and lipid signatures in infants before and after AD using multi-omics approaches; (2) to analyze transcriptomics, proteomics, metabolomics, and the cell types of infant blood sequentially; (3) to perform sequential immune profiling of the blood, including serology, cytokine profiles, and the evolution of specific B and T cells; (4) to investigate the microbiomes in the skin and gut from birth; and (5) to consider potential maternal delivery effects for atopy (130).

CONCLUSION

The global increase of atopic diseases greatly lowers the quality of life. The theory of atopic march facilitates our understandings of the pathophysiology of atopic diseases and further promotes the early detection, prevention, and treatment of children at

risk of allergic diseases. Future studies on atopic march would be directed at the following points. Firstly, the methods for data collection should be improved and disease heterogeneity or variations should be considered when performing substantial epidemiologic surveys. Secondly, more detailed and logical mechanisms, including genetic and environmental aspects, should be explored to account for the temporal pattern, which would pave the way for novel approaches for the prevention and timely early treatment of the clinical manifestations, ultimately reducing the allergy burden.

AUTHOR CONTRIBUTIONS

LY contributed to collection of references and manuscript preparation. JF and YZ contributed to manuscript modifications. All authors contributed to the article and approved the submitted version.

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REVIEW
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Structural Aspects of the Allergen-Antibody Interaction

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The development of allergic disease involves the production of IgE antibodies upon allergen exposure in a process called sensitization. IgE binds to receptors on the surface of mast cells and basophils, and subsequent allergen exposure leads to cross-linking of IgE antibodies and release of cell mediators that cause allergy symptoms. Although this process is quite well-understood, very little is known about the epitopes on the allergen recognized by IgE, despite the importance of the allergen-antibody interaction for the allergic response to occur. This review discusses efforts to analyze allergen-antibody interactions, from the original epitope mapping studies using linear peptides or recombinant allergen fragments, to more sophisticated technologies, such as X-ray crystallography and nuclear magnetic resonance. These state-of-the-art approaches, combined with site-directed mutagenesis, have led to the identification of conformational IgE epitopes. The first structures of an allergen (egg lysozyme) in complex with Fab fragments from IgG antibodies were determined in the 1980s. Since then, IgG has been used as surrogate for IgE, due to the difficulty of obtaining monoclonal IgE antibodies. Technical developments including phage display libraries have contributed to progress in epitope mapping thanks to the isolation of IgE antibody constructs from combinatorial libraries made from peripheral blood mononuclear cells of allergic donors. Most recently, single B cell antibody sequencing and human hybridomas are new breakthrough technologies for finally obtaining human IgE monoclonal antibodies, ideal for epitope mapping. The information on antigenic determinants will facilitate the design of hypoallergens for immunotherapy and the investigation of the fundamental mechanisms of the IgE response.

Keywords: allergy, allergen, IgE antibody, structure, X-ray crystallography, nuclear magnetic resonance, cryo-electron microscopy, mass spectrometry

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INTRODUCTION

The interaction between allergens and IgE antibodies is at the core of the allergic response. Epitopes could potentially be located on any part of the allergen surface. However, evidence shows that antibodies are very specific about the epitopes that they recognize and certain areas on the allergen seem to be preferential for antibody binding. The identification of epitopes recognized by IgE is valuable for the design of hypoallergens or other therapeutics. However, allergen-epitope

information has been difficult to obtain. This review will discuss various methods to probe epitopes and the knowledge that has been gained from available studies on allergens.

HISTORICAL PERSPECTIVE TO IgE EPITOPE MAPPING

Since the 1980s, efforts to identify antigenic determinants on allergens have been pursued, but progress in the area has been slow due to technical limitations. Original epitope mapping studies were based on the synthesis of overlapping peptides covering the full sequence of the allergen, and the selection of the peptides that bound IgE (1, 2). This approach led to the identification of linear epitopes that comprise a sequential or continuous set of amino acids. However, allergens are proteins or glycoproteins with a defined three-dimensional structure that determines the molecular surface and epitopes recognized by antibodies. Therefore, most allergenic epitopes are conformational, involving amino acids that are close in space due to the protein folding, but non-contiguous in the allergen sequence (3). Technologies that consider the three-dimensional structure of the allergens were necessary to analyze conformational epitopes.

In the absence of complete structural information, most of the original approaches to epitope mapping were indirect, based on the reduction of IgE antibody binding to modified allergen molecules in dot blots or enzyme-linked immunosorbent assays (ELISA) (4). They were possible thanks to peptide synthesis or to the development of recombinant technology, with *in vitro* expression of either allergen fragments, mutants, or allergen chimeras, and their subsequent testing for IgE antibody binding. The development of microarrays or bead-based epitope assays facilitated the investigation of the relevance of linear epitopes, using large sets of linear peptides (5, 6). Microarrays have been especially useful for food allergens because they have mainly linear epitopes due to food processing and/or digestion (6–9). Several IgE/IgG4-binding peptide epitopes were suggested as biomarkers for predicting clinical reactivity and severity to certain foods (10, 11). Another approach uses information from the allergen structure, and hybrid or chimeric allergens are designed by combining the sequences of homologous allergens from different species (12–14). Patches on the allergen surface associated with binding of IgE (from sera of subjects allergic to one of the allergens in the chimera) indicate the presence of epitopes (most likely conformational) on those regions. Another approach to epitope mapping is the identification of mimotopes, which mimic the structure of an epitope (15). It is based on the use of phage display libraries for the selection of peptides that, in combination with a computational algorithm, allow the identification of patches on the allergen surface that mimic conformational epitopes (16, 17). A knowledge of the allergen structure is needed, but the mimotope resulting from the analysis is not necessarily the same as the real epitope. Each of these technologies has provided valuable information on epitope mapping.

X-ray crystallography and nuclear magnetic resonance have determined the three-dimensional structure of many allergens, which helps immensely in interpreting epitopes. Allergens have a wide variety of three-dimensional structures, despite belonging to a limited number of protein families (18–20). Only 1.3% of the total Pfam domains are present in allergens (http://www.meduniwien.ac.at/allfam/). Once the allergen molecular surface is defined, certain amino acids can be selected for site-directed mutagenesis to analyze allergen-antibody interactions (21). Experimental IgE binding and cross-reactivity data can be compared for homologous allergens in conjunction with the molecular structure to understand the approximate location of IgE-binding epitopes (22, 23). Ultimately, the structures of allergen-antibody complexes provide the most detailed information of the epitope-paratope interaction. These precise technologies, although more laborious, directly identify the residues involved in allergen-antibody interactions. This review primarily covers X-ray crystallography and NMR approaches to epitope mapping (**Table 1**). Additional technologies that also consider the three-dimensional structure of proteins for epitope mapping are cryo-electron microscopy (cryo-EM) and chemical protection assays combined with mass spectrometry (MS). These will also be briefly discussed.

STATE-OF-THE-ART TECHNOLOGIES FOR EPITOPE MAPPING

X-Ray Crystallography

Over 88% of experimental models of macromolecules that are deposited in the Protein Data Bank (PDB) were determined using X-ray crystallography (**Table 1**). This technique is often used to generate experimental models of antigen-antibody complexes, and allows for a detailed description of epitopes, paratopes and their chemical interactions. Structural analysis by X-ray crystallography provides the most detailed description of interactions between allergens and antibodies, but is not always easy to perform (24, 25). This approach requires the generation of: (1) significant quantities (mg amounts) of pure and homogeneous protein preparations, specifically the allergen-antibody complex, and (2) a well-diffracting crystal to perform an X-ray diffraction experiment. An additional difficulty in studying epitopes is that highly flexible molecules, like antibodies, are typically recalcitrant to the process of crystallization. To our knowledge, there is not currently a single structure of an antigen in complex with an intact antibody. Fragments derived from monoclonal antibodies (Fab, Fab′) or antibody constructs (single-chain variable fragment -scFv-, scFab, rFab) are used for crystallization because they have significantly reduced conformational flexibility in comparison with intact immunoglobulins. Success in obtaining well-diffracting crystals is not guaranteed, even when sufficient quantities of pure and homogeneous allergen-antibody complexes are available. As crystallization conditions cannot be predicted, hundreds or thousands of trials using different solvent conditions are tested, as well as modifications to the allergen and antibody (26). Once a well-diffracting crystal is obtained, the process of

TABLE 1 | Comparison of four epitope mapping techniques that consider the three-dimensional structure of the allergen: X-ray crystallography, NMR, cryo-EM and mass spectrometry.

X-ray crystallography	Nuclear magnetic resonance
• Crystalline state, however, the crystals contain ∼30–70% of disordered solvent	• Solution conditions (requires weeks of stability for data collection).
• Theoretically no structure size limit	• High resolution structures up to ∼30 kDa.
• Proteins purified from natural sources can be used	
• Expression with isotope is typically not required for proteins or DNA. Sometimes selenomethionine is incorporated instead of Met.	• Protein/DNA samples usually require ^{13}C and ^{15}N labeling (stable isotopes). Cost of expression is prohibitive except in prokaryotes.
• X-rays diffraction data are recorded, and the diffraction patterns are used to calculate initial electron density maps. The maps are used to trace a model of the macromolecule, that is later refined and validated	• Data is nuclear resonance frequencies of primarily ^{1}H, ^{13}C, and ^{15}N. Distances between ^{1}H atoms are used to build ensembles of possible structures.
• Highly flexible/disordered regions of proteins cannot be modeled and are absent in the final models	• Motion and disorder can be directly measured on many time scales.
Mass spectrometry	**Cryo-electron microscopy**
• Typically used in protection assays for epitope mapping.	• Can determine atomic resolution structures frozen from solution in vitreous ice.
• High sensitivity/low sample requirements.	• Low sample requirements.
• Atomic resolution identifies specific residues for protection from modification.	• Resolution occasionally as good as X-ray crystallography.
• Residues that are convenient to modify in protection assays are not always useful for epitope mapping.	• Performs better on very large samples with high symmetry, typically 100's of kDa, so it is currently not easily or generally applicable to allergen epitope mapping.
• Chemistry of modification procedures can have off target effects.	

structure determination can be very fast, as currently available software allows to determine initial models very quickly after collection of diffraction data. Therefore, taking into account the many advances in molecular biology, instrumentation, and software development, it is not surprising that the number of experimental structures deposited to the PDB and determined by X-ray crystallography continuously increases. Currently, 145,000 models have been determined using this technique. However, very few are structures of allergen-antibody complexes (see section Structures of Allergen-Antibody Complexes by X-Ray Crystallography).

Nuclear Magnetic Resonance (NMR)

NMR approaches to observe antibody complexes utilize molecules in solution as opposed to crystallization that attempts to coax molecules out of solution and into a crystal lattice. NMR detects the resonant frequencies of atoms in a magnetic field. These frequencies are primarily influenced by the type of atom (^{1}H, ^{13}C, or ^{15}N) and secondarily by the chemical environment of particular atoms. These data provide a rich source of atomic structure when the resonant frequencies can be specifically attributed to individual atoms (**Table 1**).

The primary struggle with NMR is sensitivity, which is why large powerful magnets are required. An additional difficulty in observing macromolecules is that the signals become exponentially more difficult to observe as molecular weight increases. NMR methods can determine macromolecular structures but are typically limited to molecules of <20 kDa for high resolution structures. All of the atoms in small allergens (approximately <20 kDa) can be readily observed, while much larger complexes (such as IgE, 190 kDa, combined with two allergens) require specific labeling of certain chemical groups that provide high sensitivity. It is important to realize that, in contrast to crystallography that directly determines the structure of the complex, the NMR data on epitopes requires a careful comparison of the atomic frequencies or intensities in the allergen before and after complexation. Therefore, the NMR results are potentially subject to interpretation in the context of previously known structures or epitope mapping data.

Cryo-Electron Microscopy (Cryo-EM)

Another emerging methodology that may become applicable to epitope mapping is cryo-electron microscopy (Cryo-EM) (**Table 1**). Due to technical improvements in the detectors, and secondarily computational methods, cryo-EM has demonstrated the ability to determine macromolecular structures at resolutions occasionally as good as X-ray crystallography, but frequently reasonable for epitope mapping (27). In July 2020 there were 63 Cryo-EM structures with <2 Å reconstruction resolution out of more than 5,000 reported in the PDB. Some attractive advantages of Cryo-EM include that samples are flash frozen, so they don't require crystallization and much less sample is typically required, frequently less than a mg. However, in the sample, the molecules still need to be relatively homogeneous in purity and conformation so the inherent flexibility of antibodies may preclude high resolution analysis. Cryo-EM is the opposite of NMR, regarding its preference of larger molecules for higher resolution information, whereas NMR yields more detailed information on smaller molecules. Although smaller antibody constructs such as Fv or scFv are presently too small for structural analysis by Cryo-EM, this technique may improve to facilitate the use of smaller proteins (28, 29).

There have been several papers on epitopes mapped by Cryo-EM, which are worth noting. For example, Fab fragments from monoclonal antibodies were localized on the spike protein of SARS-CoV-2, and on Zika virus particles (30, 31). More intriguing was the characterization of multiple epitopes simultaneously using polyclonal Fab from sera in a study of

neutralizing antibodies of the HIV envelope trimer (32). The study was able to characterize several epitope sites from a small blood volume derived from an immunized animal. Notice that the antigens in all three cases were very large proteins or particles, which is favorable for Cryo-EM characterization but is not typical of allergens which are usually small proteins. However, in this rapidly developing field, studies like these may be feasible in the future for allergens.

Protection Assays Combined With Mass Spectrometry (MS)

Alternative methods for epitope mapping that rely on mass spectrometry are described in this section (**Table 1**). They differ from the methods described above that are traditionally associated with structural biology and determination of experimental models of macromolecules. One of the MS approaches is called paratope or epitope "excision" (33). The excision procedure includes enzymatic proteolysis that allows for generation of peptides forming epitopes or paratopes, which later are identified using, for example, a combination of MALDI (matrix-assisted laser desorption/ionization) and ESI (electrospray ionization) mass spectrometry (33–35). This approach requires a small sample that does not need to be labeled. However, as most often the epitopes of interest are discontinuous/conformational in nature, excision is usually combined with chemical modification of the studied complexes. The most common method of the chemical modification involves hydrogen-deuterium exchange (HDX). During this modification the antigen-antibody complex is placed in heavy water and protein backbone amide hydrogen atoms (^{1}H) can be exchanged for deuterium (^{2}H). The rate of the ^{1}H-^{2}H exchange (HDX) depends on solvent accessibility and dynamics of a particular protein fragment. Generally, hydrogens that are buried within the protein core or shielded from the solvent, such as hydrogens buried in an antigen-antibody interface will have a low rate of ^{1}H-^{2}H exchange. After the incubation in heavy water the complex undergoes enzymatic cleavage and the resulting peptides are identified by the change in mass, using MS. It is expected that surface residues forming epitopes and paratopes will have a relatively low level of incorporated ^{2}H. This information combined with the molecular models of the antigen and the antibody allows for mapping of the interacting molecular surfaces. Therefore, HDX-MS became a very successful technique that not only has found application in analysis of antigen-antibody complexes, but also in mapping of other protein-ligand interactions (36, 37). Moreover, HDX-MS can be used for studies of protein conformational dynamics, and was successfully used in characterization of the dynamic behavior of antibodies (38, 39).

^{1}H-^{2}H exchange is not the only chemical modification that can be applied in protection assays combined with mass spectrometry. For example, various surface exposed amino acids can be oxidized by H_2O_2 or modified by photochemically induced reactions (40, 41). The modifications to the allergen before and after complexation with the antibody can be compared for epitope information.

STRUCTURES OF ALLERGEN-ANTIBODY COMPLEXES BY X-RAY CRYSTALLOGRAPHY

The X-ray crystallographic structures of allergen-antibody complexes were first determined for egg lysozyme with fragments of murine IgG monoclonal antibodies (mAb) (42–47). Subsequently, other structures were reported for other allergens, where murine IgG mAb were selected as surrogates for human IgE, due to their capacity to inhibit binding of human IgE antibody to the allergen (**Table 2**) (48–53, 56, 58, 60–64). These studies involved the purification of an allergen either from the natural source or from *in vitro* cultures expressing recombinant allergens. The IgG mAb were cleaved using pepsin or papain, which resulted in F(ab′)$_2$ -that was reduced to F(ab′)- or Fab, respectively. These antibody fragments contain the paratope and were purified and combined with the allergen to form a complex, which was purified for crystallography.

Although there are <20 different allergens that have their structures determined in complexes with antibodies (**Table 2**) their analysis provides interesting insights into epitopes and paratopes. Chicken lysozyme (Gal d 4) is often used as a test molecule and there is a vast amount of literature on the use of this protein to study interactions with antibodies. Therefore, to avoid bias that a large number of lysozyme-antibody complex may cause we selected for analyses only some representative structures. While allergens have a wide variety of structures, antibodies of the same isotype have the same structure, formed by immunoglobulin-fold domains of about 100 amino acids. Light chains have an N-terminal variable domain (V_L) followed by a constant domain. Similarly, heavy chains have a variable N-terminal domain (V_H), but it is followed by either 3 (in IgG) or 4 (in IgE) constant domains. The central part of these domains is made of anti-parallel β-sheets, in which β-strands are linked to form the so-called Greek-key motifs. For example, IgG V_H domain is made of anti-parallel β-sheets composed of nine β-strands that are linked by eight loops (**Figure 1**) (65). Of the four apical loops, only loops 1, 2, and 4 interact with the antigen, and contain the complementary determining regions (CDRs): CDR1, CDR2, and CDR3, respectively. The loops 1, 2, and 4 are between beta-sheets B-C, C′-C″, and F-G, respectively (**Figure 1**). A similar β-sheet configuration occurs in the light chain. The 6 loops involved in paratope formation form the following CDRs: H CDR1, H CDR2, H CDR3 in the heavy chain, and L CDR1, L CDR2, and L CDR3 in the light chain. The CDRs contain the amino acids that form the paratope. The H CDR3 is sufficient for most antibody specificities (66), although exceptions have been found (67). In a few cases, additional residues outside the CDR, located in the "framework" of the antibody, can also contribute to antibody binding. While epitopes can be located on different parts of the allergen surface, paratopes are always at the apical region of the variable domain of the antibody, formed by the 6 CDRs. The CDR boundaries have been historically defined in different ways (68–70), and currently the ImmunoGenetics website (www.imgt.org) utilizes a consensus for their estimation.

TABLE 2 | Structures of allergen-antibody complexes by X-ray crystallography.

Allergen	Allergen source	Allergen expression system	Antibody	Antibody expression system	PDB code
Allergen in complex with IgG antibody construct					
Api m 2	Honeybee	Insect cells (high five)	Fab; mIgG1 mAb 21E11	*Mus musculus* hybridoma cells	2J88 (48)*
Bet v 1	Birch	*E. coli*	Fab′; mIgG1 mAb BV16	*Mus musculus* hybridoma cells	1FSK (49)*
Bla g 2	German cockroach	*P. pastoris*	Fab′, mIgG1 mAb 7C11	*Mus musculus* hybridoma cells	2NR6 (50) (51)*
Bla g 2	German cockroach	*P. pastoris*	Fab, mIgG1 mAb 4C3	*Mus musculus* hybridoma cells	3LIZ (52)*
Der f 1	House dust mite	*D. farinae* mite culture	Fab; mIgG1 mAb 4C1	*Mus musculus* hybridoma cells	5VPL (53) (54)*
Der p 1	House dust mite	*D. pteronyssinus* mite culture	Fab; mIgG1 mAb 4C1	*Mus musculus* hybridoma cells	1) 3RVW (53) 2) 3RVX (53) (55)*
Der p 1	House dust mite	*D. pteronyssinus* mite culture	Fab; mIgG1 mAb 5H8	*Mus musculus* hybridoma cells	4PP1 (56) (57)*
Der p 1	House dust mite	*D. pteronyssinus* mite culture	Fab; mIgG1 mAb 10B9	*Mus musculus* hybridoma cells	4PP2 (56) (57)* (55)*
Der p 2	House dust mite	*P. pastoris*	Fab; mIgG1 mAb 7A1	*Mus musculus* hybridoma cells	6OY4 (58)* (59)*
Fel d 1	Cat	CHO	Fab; IgG4 mAb REGN1909	CHO[c]	5VYF (60)
Gal d 4 (lysozyme)	Chicken	Not specified (most likely *Gallus gallus*)		*E. coli* (1FDL) or *Mus musculus* hybridoma cells	(1) (42) (2) (43) (3) 3HFM (44) (4) 1FDL (45) (5) 1MLC (46) (6) 1YQV (47)
Gal d 4 (lysozyme)#	Chicken	Not specified	Human V_H domain; VH H04 Phage displayed	*E. coli* BL21 Gold	(1) 4PGJ (61) (2) 4U3X (61)
Phl p 7	Timothy grass	*E. coli* BL21 star DE3	Fab; hIgG1 mAb 102.1F10 was expressed based on a hIgG4 that was generated from matched heavy- and light-chain sequences by single B cell cloning from allergic individuals	FreeStyle 293F	5OTJ (62)
Allergen in complex with IgG antibody constructs containing human IgE variable regions					
Bos d 5	Cow	*E. coli*	Fab; hIgG1 mAb D1: C_κ and CH1 of IgG1 cloned with IgE V_H/V_L isolated from human IgE derived from a combinatorial library	*E. coli* RV308	2R56 (63)
Phl p 2	Timothy grass	*E. coli* BL21	Fab; hIgG1 mAb huMab2: C_κ and CH1 of IgG1 cloned with IgE V_H/V_L isolated from human IgE derived from a combinatorial library	CHO-K1	2VXQ (64)*

**Manuscripts that report inhibition of IgE antibody binding by the antibody used in the X-ray crystal structure (or viceversa).*

#Only selected complexes with lysozyme are listed. For example, complexes of human V_H domains with lysozyme were chosen to compare them with complexes formed by Fabs.

A significant fraction of the available allergen-antibody structures corresponds to complexes of Group 1—the best studied—and Group 2 house dust mite allergens, and the cockroach allergen Bla g 2 (**Figure 2**). Groups 1 and 2 comprise the most important major allergens from house dust mites. A major allergen is one to which >50% of subjects allergic to the allergen source are sensitized. Group 1 includes Der p 1, Der f 1, Blo t 1, and others, and are cysteine proteases. Group 2 includes Der p 2, Der f 2, Blo t 2, and others with an MD-2-related lipid-recognition (ML) domain (www.allergen.org). Three X-ray crystal structures of Der p 1 have been determined in complexes with three different murine IgG mAb (4C1, 5H8, and 10B9), from which mAb 4C1 is a cross-reacting antibody that also binds to Der f 1 (53, 56). Comparison of Der f 1 and Der p 1 structures with 4C1 revealed that the cross-reactive mAb binds to a conserved surface patch that is present on both allergens (53). Unexpectedly, this patch is not a part of the largest conserved surface area in common for both Der f 1 and Der p 1, and which includes the active site of the enzymes. The majority of the amino acids forming the central part of the epitope are conserved, and in very similar conformations. Interestingly, the epitopes for 10B9 and 4C1 partially overlap, but 10B9 is

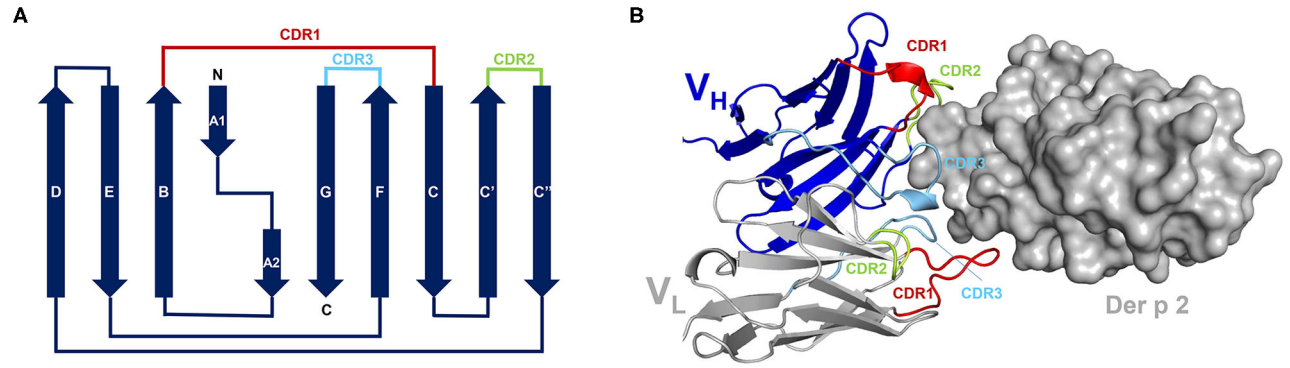

FIGURE 1 | (A) A topological diagram of IgG V_H. β-strands are shown as arrows that indicate direction of the peptide. N- and C-termini, as well as individual β-strands are labeled. Loops corresponding to three CDRs are highlighted using different colors. The figure was prepared based on a diagram presented by Bodelón et al. (65). **(B)** Complex between 7A1 and Der p 2.0103. Only variable domains of the 7A1 antibody are shown. CDRs are marked using the same colors as used for the topological diagram. Der p 2.0103 is shown in surface representation.

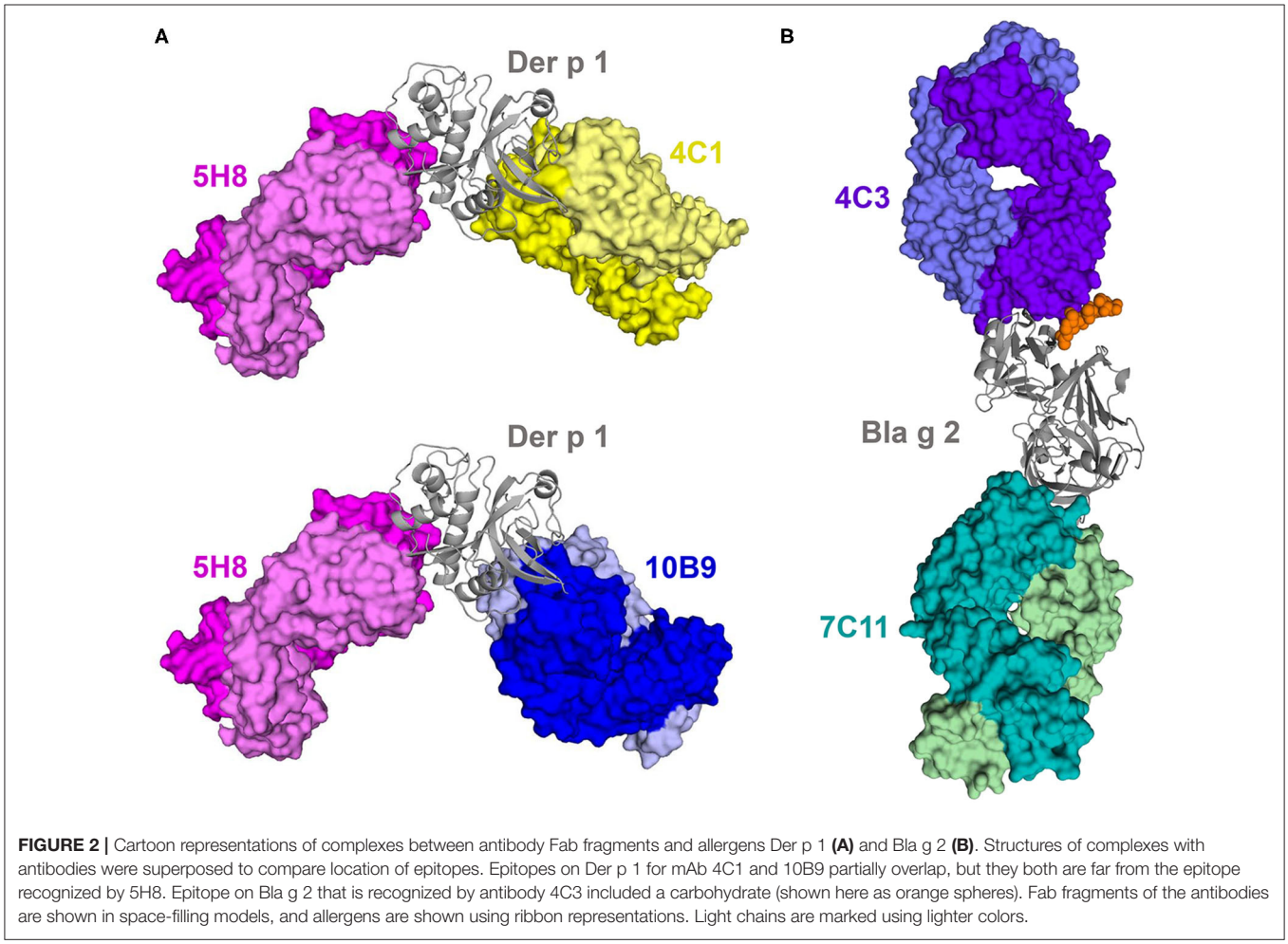

FIGURE 2 | Cartoon representations of complexes between antibody Fab fragments and allergens Der p 1 **(A)** and Bla g 2 **(B)**. Structures of complexes with antibodies were superposed to compare location of epitopes. Epitopes on Der p 1 for mAb 4C1 and 10B9 partially overlap, but they both are far from the epitope recognized by 5H8. Epitope on Bla g 2 that is recognized by antibody 4C3 included a carbohydrate (shown here as orange spheres). Fab fragments of the antibodies are shown in space-filling models, and allergens are shown using ribbon representations. Light chains are marked using lighter colors.

not able to bind to Der f 1. The epitope for 4C1 is "rotated counterclockwise" by ∼90° in relation to the position of the 10B9 epitope on Der p 1. On the other hand, the 5H8 binding epitope is located at a significant distance from both 4C1 and 10B9 epitopes. The image of the structure of three Der p 1-antibody complexes clearly illustrates that mAb 5H8 and 4C1

or 10B9 can simultaneously bind to the same allergen molecule (**Figure 2**) (56). Bla g 2 has a bilobal structure typical of aspartic proteases, but it is enzymatically inactive due to substitutions in the catalytic site (71, 72). Two structures of Bla g 2 with mAb 7C11 and 4C3 have been determined, showing their binding to opposite lobes of the molecule (50, 52). The complex with mAb 4C3 was unique because it showed that carbohydrates contributed to the interactions with Bla g 2 (**Figure 2**). The murine IgG mAbs used in these crystallographic studies were chosen as surrogate IgE antibodies, because they inhibit binding of IgE to the allergen. Once the IgG epitope was identified, site-directed mutagenesis of the allergen residues involved in antibody recognition was performed, followed by IgE antibody binding analysis of the mutants, to identify IgE antibody binding sites. This approach resulted in the design and production of allergen mutants with decreased capacity to bind IgE, which are being investigated as future candidates for immunotherapy (51, 58, 67, 73).

The antibodies in the majority of allergen-antibody complexes that have their structures determined are IgG1. However, three of the structures reported in **Table 2** are different from the IgG1 isotype: 1) IgG4 (in complex with Fel d 1), 2) an IgG1 construct engineered to combine the constant domains of human IgG1 heavy and kappa light chains with variable regions of a human IgE construct derived from an scFv combinatorial library (in complex with Bos d 5), or 3) an Fab isolated from a combinatorial library, which is a hybrid of the variable domain of the IgE Fab and the constant domain of human IgG1 (in complex with Phl p 2) (60, 63, 64). The IgG4 (REGN1909) binding Fel d 1 is a fully humanized antibody that was derived from mice immunized with recombinant dimeric Fel d 1 (60). REGN1909 is able to partially block IgE binding to natural Fel d 1, with a maximum inhibition of 51%. REGN1909 together with another IgG4 (REGN1908), which binds to a different non-overlapping epitope, was able to block up to 83% of IgE binding to natural Fel d 1. A combination of X-ray crystallography and HDX-MS was used to elucidate information on the antibody binding epitopes for REGN1908 and REGN1909. Only the crystal structure of Fel d 1 in complex with REGN1908 was obtained (60).

While most often the antibodies that are used in studies of allergens are composed of light and heavy chains with six total CDRs, there are also examples of heavy chain only antibodies. These can contain two heavy chains only (and therefore have 3CDRs for recognition per chain) or single domain antibodies, which have a single antigen binding domain (74–76). Heavy chain only antibodies are present in nature and are produced by camelids and sharks. The paratopes formed by the single chain antibodies have a very similar amino acid composition to that observed in conventional antibodies (77, 78). The heavy chain only antibodies, and especially their V_H domains, are relatively easy to produce and their biophysical, as well as structural properties, allow for easy application in biotechnology and therapeutics (77, 79). Single domain antibodies (specific for lysozyme) were isolated years before discovery of heavy chain antibodies in camelids (76), and were proposed as alternative to conventional monoclonal antibodies. Later on, camelids' V_H domains also became a model for the generation of their human equivalents. Fully human V_H single domains were used to generate complexes with Gal d 4 (**Table 2**) (61).

A new type of allergen-antibody interaction was recently reported thanks to the determination of a Phl p 7-antibody crystal structure (**Figure 3**) (62). An IgG4 originally generated from single B cell cloning was converted into an IgG1 for structure determination of the allergen-antibody complex. The structure revealed that two antibodies bind simultaneously to Phl p 7 in two different ways: (1) the classical mode that involves both heavy and light chains of the antibody, and (2) an unusual non-standard way, involving only binding of the light chain to the allergen to a separate Phl p 7. This resulted in trapping two monomeric allergen molecules between two molecules of the same antibody (62). While Phl p 7 was not a dimer, the stoichiometry of the complex still required two Phl p 7 molecules. Therefore, this Phl p 7-antibody structure has changed the prior view that one antibody is able to recognize only a single epitope on an allergen/antigen.

The allergens that we have described are proteins, and their interactions with antibodies are the same as for other proteinaceous antigens. It also has to be stressed that X-ray crystallography provides generally a static picture of the interacting molecules. However, both antigens and specifically antibodies display a great level of conformational flexibility (80, 81). It was shown that conformational flexibility and local structural dynamics of antibodies play a very important role in recognition and binding (82, 83). A higher level of conformational flexibility usually is attributed to antibodies that are not matured, and the flexibility allows them to recognize more antigens and/or altered antigens (84). During an antibody's maturation the increase of specificity is often achieved at the cost of the conformational flexibility, and a more rigid antibody binds better to one antigen (85, 86). Therefore, studies of the CDR conformations are critical for understanding the process of recognition and binding of antigens by antibodies (87–90), and these studies are most often performed using NMR, HDX-MS and various computational methods.

LESSONS LEARNED ABOUT ALLERGEN-ANTIBODY INTERACTIONS USING X-RAY CRYSTALLOGRAPHY

An analysis of 16 allergen-antibody structures selected from **Table 2** revealed a detailed description of the interface formed by epitopes and paratopes (45–50, 52, 53, 56, 58, 60–64). Typically, the interface area falls in the 650–920 Å^2 range (an average of 813 Å^2; **Figure 4A**) (91). In the complex with Phl p 7 mentioned above, the interface area is larger, and can be divided between a "classic" interface (∼820 Å^2) with one antibody, and an additional interface (∼380 Å^2) responsible for the non-standard interaction with the light chain from the second antibody (62). In most cases, the antibody heavy chain provides the largest contribution toward the total area of the interface, but this contribution is not always significantly bigger than the light chain share (**Figure 4A**). The light chain provides between 23 and

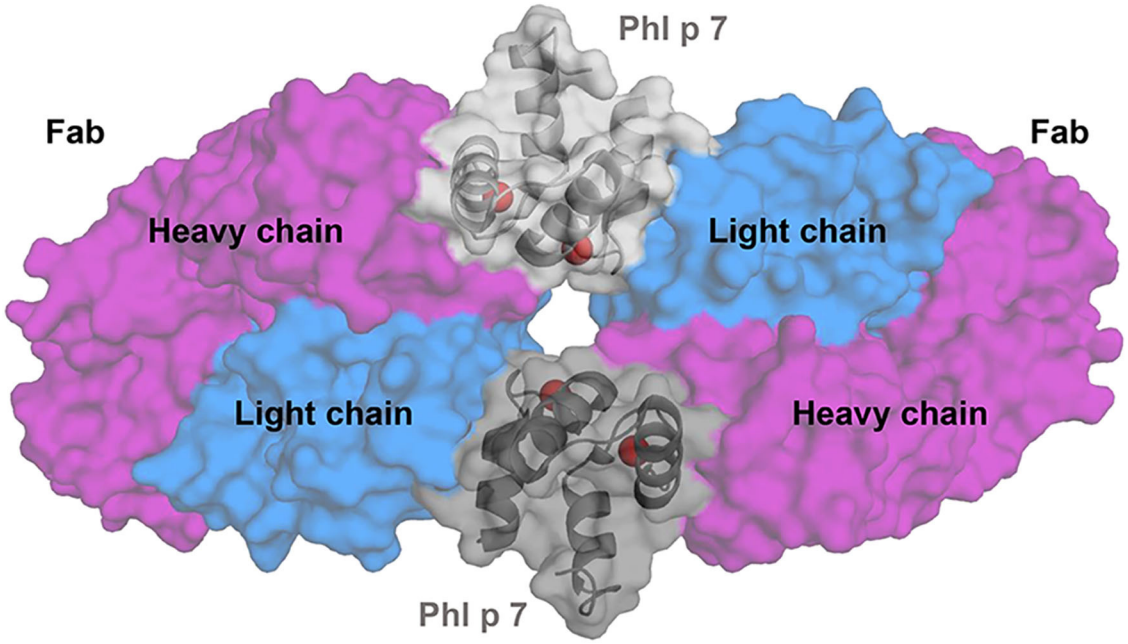

FIGURE 3 | Superantigen Phl p 7 interactions with Fab. Cartoon representation of a complex between two Fab fragments of a human antibody and two molecules of timothy grass pollen allergen Phl p 7 (PDB code: 5OTJ). The crystal structure revealed an unusual binding of two molecules of the monomeric allergen and two molecules of the antibody. Phl p 7 molecules are shown in gray. Light chains of the antibody are shown in blue and heavy chains in purple. Calcium ions bound by the allergen are presented as red spheres.

53% of the interface area. In the Api m 2 and Bla g 2 (2NR6) complexes, the light and heavy chains contribute almost equally to the interface area (48, 50). However, it is worthwhile to note that in two complexes of antibody V_H domains with Gal d 4 (PDB codes: 4PGJ and 4U3X), the allergen-antibody interface areas are quite large (810 and 826 $Å^2$, respectively), perhaps to compensate that the paratope is formed only by the heavy chain.

Analysis of the allergen-antibody interfaces at the amino acid level shows that paratopes are formed by 18–28 residues that interact with epitopes composed of a similar number of amino acids (12–25 in the set of 16 complexes analyzed here) (**Figure 4B**, **Table 2**) (45–50, 52, 53, 56, 58, 60–64). In addition, it is possible to examine the distribution of various amino acids in the epitope and paratope areas. While the amino acid composition of epitopes is barely different from the overall composition of the allergen surface residues (93), there is a significant bias in amino acid composition of paratopes (**Figures 5**–**7**). Namely, the paratopes have a very high content of tyrosine, serine, and glycine residues, with relatively low content of isoleucine, leucine, lysine, methionine, and proline (94–97). The paratopes also tend to have a relatively high content of aromatic residues (Tyr, Trp, Phe, and His). Unfortunately, the relatively small number of determined structures of allergen-antibody complexes does not allow for generalizations on the compositional bias of allergen epitopes, especially when among the 16 structures analyzed here, three contain Der p 1, three contain Gal d 4, and two contain Bla g 2. However, in large datasets of protein-protein interactions, aromatic residues are also generally favored (98, 99). No obvious differences were observed between allergen-antibody interactions and the antibody recognition of other non-allergen proteins.

The chemical interactions that drive allergen-antibody formation include covalent (H-bonds) as well as non-covalent binding interactions (e.g., hydrophobic, van der Waals, charge-charge, and cation-π interactions). Hydrophobic and electrostatic interactions are most important for a primary contact between antigens and antibodies (100). However, once the distance between antigen and antibody is shortened, van der Waals interactions and H-bonds start to play a significant role. H-bonds are especially important, as they quite often are associated with specificity of the binding. The analysis of 16 structures in **Table 2** indicates that there are between 7 and 16 H-bonds that mediate contacts within the epitope-paratope interface (**Figure 8**). Heavy chains of the antibodies are responsible for the majority of the hydrogen bonds that are formed. While most often atoms that are hydrogen donors or acceptors in the H-bonds belong to the side chains of amino acids forming paratopes or epitopes, there are also hydrogen bonds formed by main chain atoms (**Figure 8**).

Amino acids with charged side chains also play an important role in mediation of epitope-paratope interactions (101). For example, salt bridges in the interfaces are formed between positively charged amino acids (Arg or Lys) and negatively charged side chains of Asp or Glu. Enrichment of epitopes in such

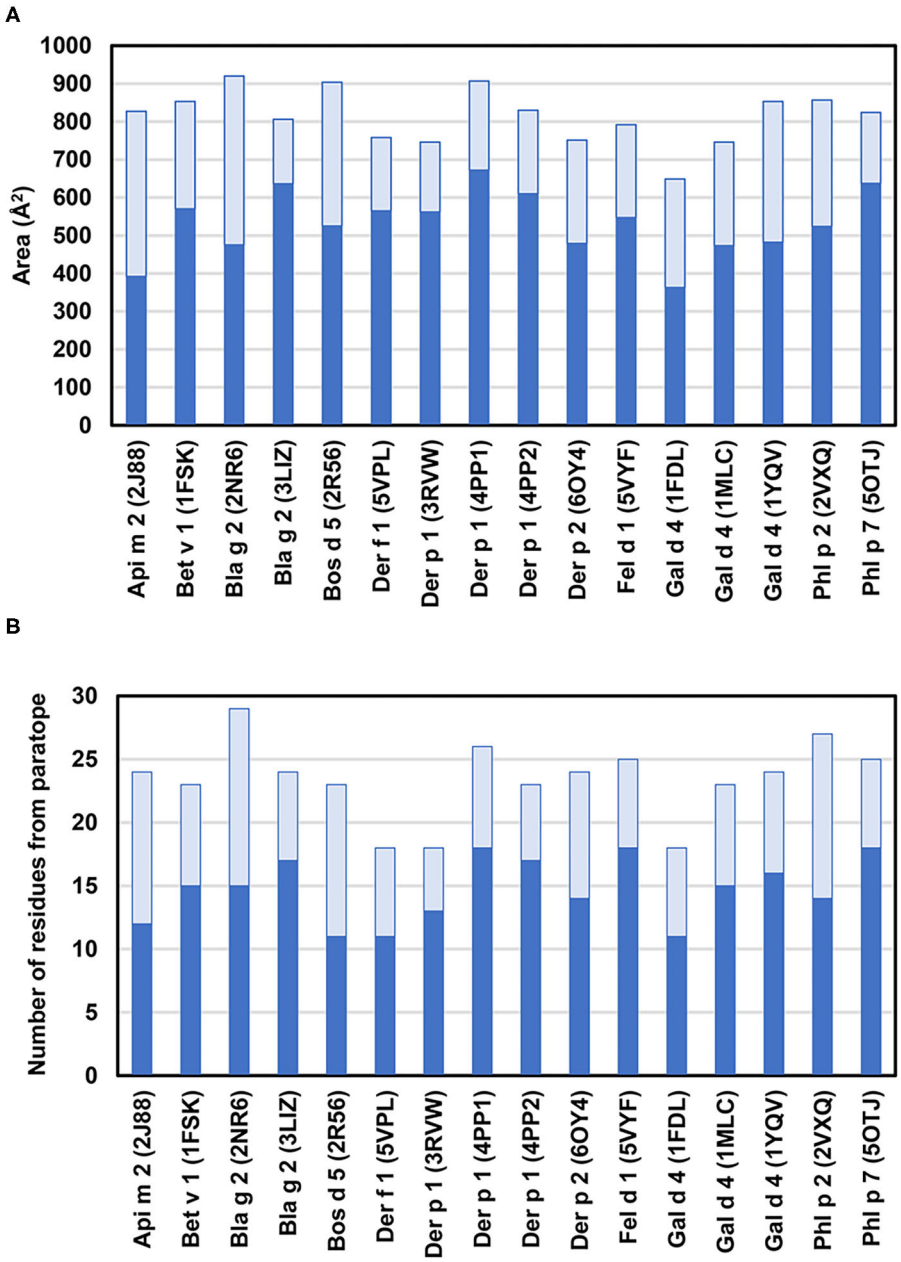

FIGURE 4 | (A) Allergen-antibody interface areas. Dark blue color corresponds to the area of the interface that corresponds to heavy chain and light blue color indicates the area of interaction with light chain. In the case of Phl p 7 (PDB code: 5OTJ) the only area corresponding to the standard mode of binding is reported. **(B)** Number of residues from heavy chain (blue) and light chain (light blue) that participate in interactions with allergens. Only residues that contribute at least 2.0 Å^2 to the interface area (as calculated with PDBePISA) (92) are counted.

amino acids is illustrated in **Figures 5–7**. It has been shown that electrostatic interactions increase the binding specificity between antigens and antibodies (102, 103). In addition, positively charged side chains may participate in cation-π interactions (50, 104, 105). This type of interaction is relatively common in antigen-antibody interfaces, as it is formed by aromatic residues (e.g., Phe, Tyr, Trp) and side chains of Arg or Lys, which are over-represented in epitopes and paratopes. For example, cation-π interactions were observed in interfaces formed between Bla g 2 and mAb 7C11, Der p 1 and 5H8, as well as between Der p 2 and mAb 7A1 (50, 56, 58). Side chains of aromatic residues may be also involved in various π-π interactions (56, 105).

Besides protein-protein contacts at the allergen-antibody interface, other chemical moieties can form contacts between

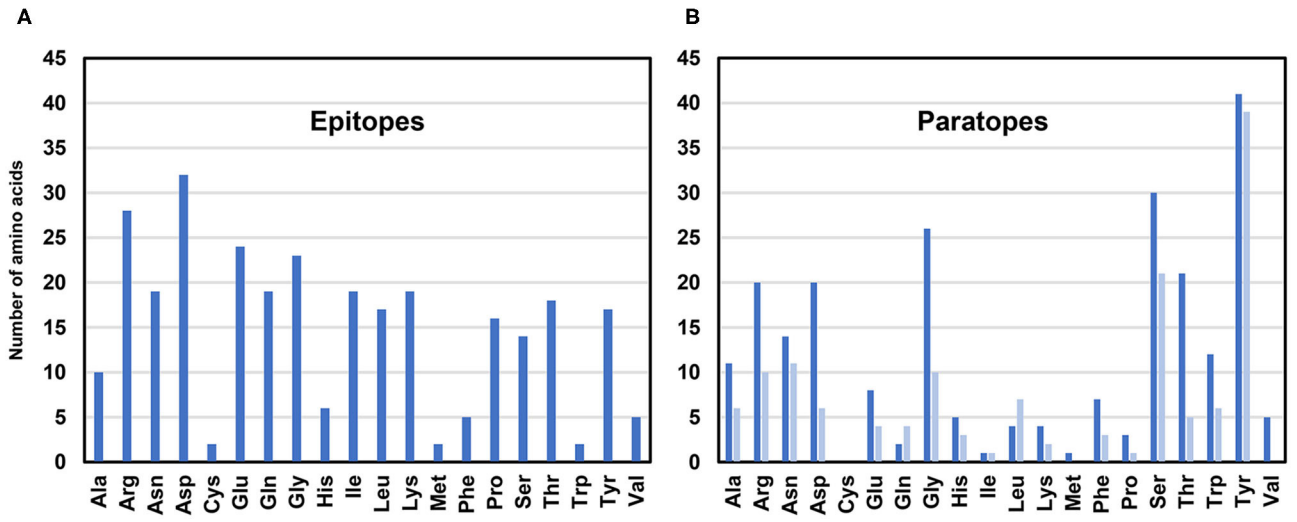

FIGURE 5 | Number of amino acids in epitopes **(A)** and paratopes **(B)**. Data for paratopes is shown for heavy chains (blue) and light chains (light blue). In the case of the Phl p 7 (PDB code: 5OTJ) only residues participating in the standard mode of binding are counted.

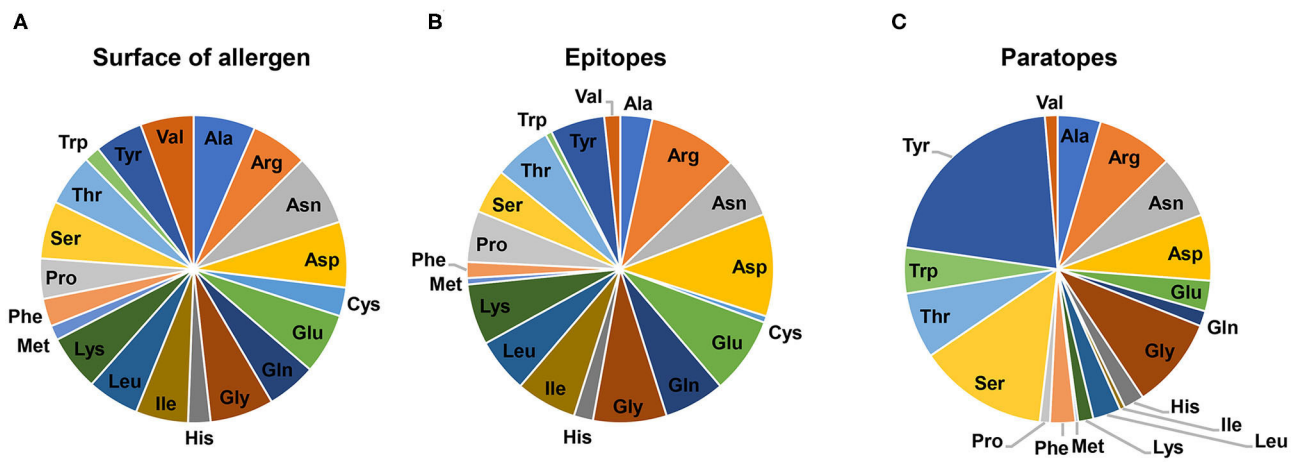

FIGURE 6 | Distribution of amino acids on the surface of allergens listed in **Table 2 (A)**, in epitopes **(B)** and paratopes **(C)**.

the two molecules. One of them is carbohydrates. An epitope on Bla g 2 that is recognized by mAb 4C3 includes a glycan (**Figure 2**) (52). The role of this glycan as part of the epitope has also been demonstrated in relation to IgE binding and basophil histamine release (106). This observation stresses the potential importance of post-translational modifications of the allergens for their interactions with antibodies (107, 108). Production of recombinant proteins in some systems, such as *E. coli*, which do not add carbohydrates to expressed proteins, may lead to a lack of proper recognition by antibodies from allergic individuals raised against the glycosylated natural allergen. Nevertheless, the allergen recognition might still occur, if the antibody recognizes the protein part of the epitope.

During the process of antigen-antibody recognition many water molecules that were on the surface of the interreacting molecules are displaced. However, this process does not always lead to their complete displacement. In fact, water molecules can play an important role in allergen-antibody interactions, in purely protein-protein complexes, and the aforementioned protein-carbohydrate complex (52). Very often, the water molecules are buried between the allergen and the antibody and mediate the contact between the macromolecules through hydrogen bonds (52, 58, 109). In some cases, the presence of buried water molecules significantly improves the fit between allergen and antibody surfaces, allowing for stronger binding (75).

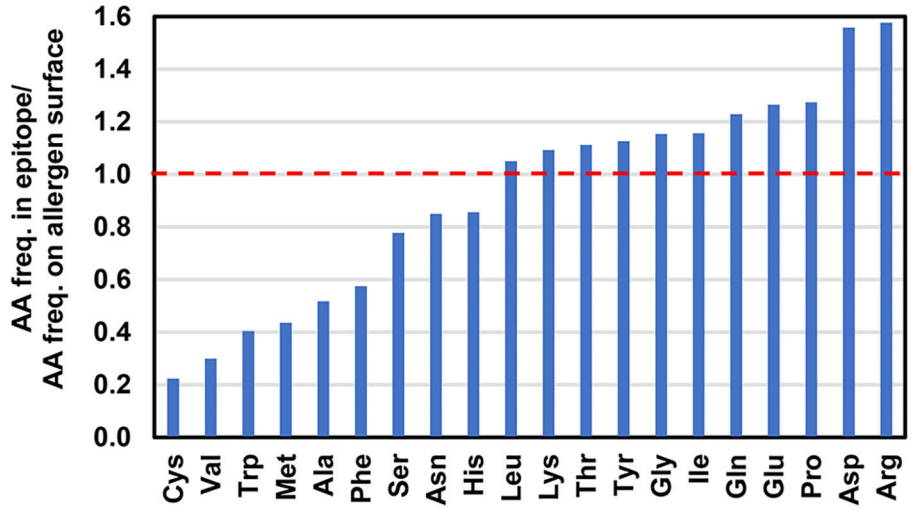

FIGURE 7 | Ratio of amino acid frequencies (AA freq.) in the epitopes to the amino acid frequencies on allergen surfaces. A ratio value above 1 indicates that a particular amino acid is observed more often in the epitopes in comparison with the allergen surfaces.

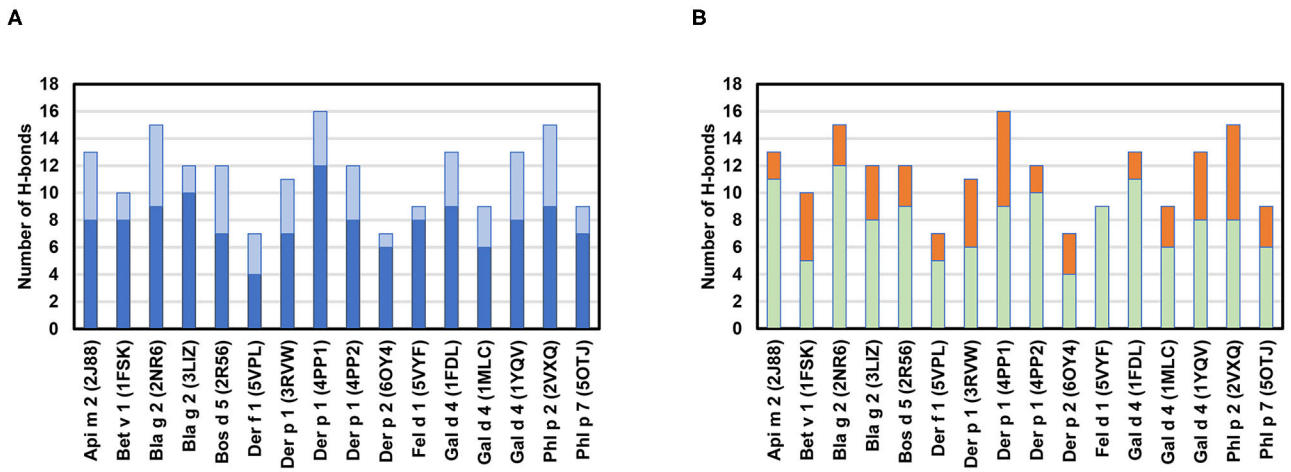

FIGURE 8 | (A) Number of H-bonds between paratopes and epitopes. H-bonds formed by residues from heavy chains are indicated in blue and H-bonds formed by residues from light chains are in light blue. **(B)** Number of hydrogen bonds formed by side chain (green) or main chain atoms (orange) of the antibodies. Calculations were made with PDBePISA (92), and only H-bonds for which distances between donor and acceptors were below 3.3 Å are taken into consideration.

IDENTIFICATION OF IgE ANTIBODY BINDING EPITOPES

The most interesting complexes for allergy research are with human IgE, but they are also the most challenging to obtain. One of the main limitations to defining epitopes for human IgE has been the difficulty of obtaining human IgE monoclonal antibodies in the amounts required for crystallography or NMR. IgE is polyclonal and present in low concentrations in blood (ng/mL). B cells expressing IgE circulate in low frequency in peripheral blood (3×10^{-7} to 7×10^{-6}) (110), which makes it difficult to isolate and grow them in primary cultures. Historically, several alternative methods were developed.

One approach to study the human IgE repertoire is to isolate IgE antibody constructs from phage display combinatorial libraries prepared using peripheral blood mononuclear cells (PBMC) of allergic subjects (111–113). Basically, antibody heavy chains are combined with light chains from the same or a different subject, to form IgE antibody constructs that are displayed by phagemids. These constructs are then isolated based on their allergen specificity in a selection process called panning. Such technology relies on the fact that antibody specificity largely resides in the heavy chain variable domain and its third hypervariable loop (H CDR3) (66). Phage display technology led to the isolation of IgG1 antibody constructs with IgE variable domains against Bos d 5 from cow and Phl p 2 from timothy

grass pollen, and the allergen-antibody Fab complexes were determined by X-ray crystallography (**Table 2**) (63, 64). Since the antibodies were isolated using IgE combinatorial libraries, it is not known whether light and heavy chain pairing corresponds to that observed in antibodies produced by allergic individuals. Both structures are useful, as they currently provide the closest picture of the interactions between allergens and IgE that take place in humans.

The structure of the Bos d 5-antibody complex illustrates an additional important phenomenon, namely the importance of the oligomerization state or quaternary structure of the allergen. Bos d 5 is a dimer in the reported structure (114). Dimerization of an allergen allows for cross-linking of IgE receptors with the same antibody. Localization of IgE epitopes clearly illustrates why, for allergens forming homo-oligomers, only one epitope per protein chain is sufficient for the allergic reaction to be triggered (115, 116). For example, a cockroach Bla g 2 mutant with amino acid substitutions that prevented dimerization induced less β-hexosaminidase release from mast cells than the dimeric wild-type Bla g 2, suggesting a functional role of dimerization in allergenicity (50). Dimerization of an allergen also provides an opportunity to use a single mAb binding for capture and detection in a "sandwich" ELISA (115, 117).

Other studies addressed allergen epitope mapping using indirect approaches. A cluster of several IgE antibody binding epitopes was located on the C-terminal domain of Phl p 1 using human IgE obtained by phage display technology. In combination with site-directed mutagenesis, the authors designed a hypoallergenic group 1 grass pollen allergen fragment (118). Two other studies used IgE constructs from phage display libraries to map epitopes on Phl p 5 and Bet v 1 (119, 120). Four independent epitope clusters on Phl p 5.0101 and two on Phl p 5.0201 were identified (119). Four Bet v 1-specific IgE (for one of which the structure was determined) were identified that targeted two non-overlapping epitopes in Bet v 1, as assessed by immunological assays (120).

Recently, a house dust mite Der p 2-specific IgG mAb overlapping with IgE was mapped by X-ray crystallography and site-directed mutagenesis analysis. A Der p 2-specific IgE construct isolated from a single-chain variable fragment (scFv)-encoding phagemid library recognized the same main residues as the IgG, further confirming the relevance of this epitope to human health (58). These studies underline the utility of using constructs derived from phage display technology to investigate the antigenic determinants relevant to allergy.

Alternative approaches to isolate antibodies are based on sorting single B cells for amplification of mRNA that encodes for the antibody. They have proven effective for identifying the exact pairing of IgG heavy and light chains, but not for B cells expressing IgE due to their low frequency in blood (121). A study used single B cell RT-PCR to obtain allergen-specific IgG antibody pairings (122). In addition, heavy chain variable gene sequences of IgE antibodies were obtained by deep sequencing PBMCs, but this study did not lead to the production of allergen-specific native pairs for IgE. One recent publication reports single B cell sorting combined with RNAseq as an approach to obtain human IgE mAb against peanut allergens (123). However, large amounts of sequencing (currently at very high cost) would be required to obtain sequences of the full IgE repertoire using this technology.

A new approach to isolate human IgE monoclonal antibodies has emerged using hybridoma technology (124). Individuals are selected according to their specific IgE sensitization, and their B cells are screened for allergen-specific IgE reactivity before fusion with myeloma cells to create hybridomas. This is an advantage versus the RT-PCR approach, in which the allergen-specificity is not known until recombinant antibodies are expressed based on the sequences obtained. Using this technology, several allergen-specific antibodies were isolated and are being used for IgE epitope mapping by X-ray crystallography and NMR (125–127). It should be noted that this method is still labor intensive, but compared to the other approaches, the clones contain the natural pairing of the heavy and light chains increasing the relevance of this technology.

EPITOPES DEFINED BY NUCLEAR MAGNETIC RESONANCE (NMR)

Because of the limitations in the size of proteins for which NMR can determine structures, NMR experiments to determine allergen epitopes necessarily involve clever experimental design and accurate interpretation. In well-designed experiments, the data provides atom-specific information on the epitope region, which can be readily understood in the context of the allergen structure. The following section describes the design and range of applicability of various NMR designed experiments.

NMR Protection Assays

The earliest NMR epitope mapping experiments designed by Yvonne Paterson and co-workers were protection assays that measured the exchange rate of amide protons for deuterons in an antigen with and without the antibody present, similar to the HDX-MS (128). Instead of measuring a change in mass, the approach takes advantage of the fact that protons and deuterons resonate at very different frequencies. The exchange of protons for deuterons leads to a disappearance of observable ^{1}H frequencies in the antigen. In the Paterson design, the antibody was covalently linked to beads to make an affinity column. Subsequently, the antigen in solution was added to the column and allowed to bind the antibody. The buffer was then easily changed from $^{1}H_2O$ to $^{2}H_2O$, so the ^{1}H-amide protons on the antigen surface could be exchanged for deuterons, except in the epitope that was protected by the antibody. Finally, the antigen was eluted at low pH to quench or stop further amide exchange. The cleverness of this design is that the antigen (smaller than the antibody, and therefore with better NMR properties) retains information about the protection. Paterson applied this method to the model antigen cytochrome c, and it was subsequently adapted for the allergens hen-egg lysozyme (Gal d 4) and Der p 2 (59, 129).

Each of these protection studies provided useful epitope mapping information for the antibodies analyzed. Paterson showed that one antibody protected from amide exchange 11

residues that were derived from 3 discontinuous peptides (128). The 3 peptides were all in close proximity on the crystal structure. The Der p 2 studies probed the epitopes of 3 murine mAb, one of which (epitope for mAb 7A1) was recently corroborated with a crystal structure, and further NMR data (see below) (58). However, only the protection assays for mAb 7A1 gave discontinuous epitope information. The absence of protection information for the other two antibodies does not imply that the other epitopes are linear. Instead, it was probably due to unfortunate circumstances where the exchange rate for protected versus non-protected was too fast to measure using the antibody column technology.

Additionally, in the lysozyme studies, it became apparent that not only could amides in the epitope be protected from exchange, but more distal atoms could show differences in exchange rate (129). This is understood to be due to conformational changes in the antigen, or changes in the folding-unfolding rate of the protein due to the formation of the complex with the antibody, which was also noticed by Paterson et al. (130). Interestingly, similar distal changes in exchange rate were observed for binding of the Fv fragment of the lysozyme antibody and the mAb 7A1 (58, 131). This is an important lesson for all NMR studies: proximal and distal changes in antigen conformation upon antibody binding can similarly influence the data, and it might be difficult to differentiate *a priori* which changes occur within the epitope. Therefore, it is frequently important to support the observed changes in the NMR data with additional information. This additional data could be proximity in the structure of atoms that experience NMR spectral changes, or data from mutant proteins and complimentary immunoassays to prove or disprove antibody binding.

The HDX-NMR protection assays were successful but have fallen out of favor for several reasons. First, creating an antibody column with enough capacity for an NMR experiment, typically 5–10 mg of antibody, can be cost prohibitive. Second, not all antigens survive the low pH required to quench the exchange. Third, NMR instrumentation improved and labeling techniques (132) with newer experiments [called TROSY (133, 134)] were developed to better observe larger complexes directly, obviating the need of using the antibody column and the measurement of exchange rates.

NMR Direct Observation of Complexes

The "antibody"-allergen complex can now be directly observed with careful choices of the labeling scheme. Complete ^{15}N backbone labeling of the allergen or antibody fragment such as Fab (∼50 kDa) or scFv (∼25 kDa) is sometimes possible. These amide detection techniques using antibody fragments have been successfully applied to the allergen Blo t 5 in complex with a Fab. A discontinuous epitope was identified by comparing the ^{1}H-^{15}N chemical shift perturbations of the bound and free allergen (135, 136). This epitope was shown to overlap with binding sites of patient polyclonal IgE. However, in our experience, the use of smaller forms of the whole antibody, such as Fab or scFv, in an attempt to increase NMR signals, has not always been successful. Some of these smaller antibody constructs are hard to produce, and surprisingly, do not always maintain the high affinity of the full antibody for the antigen. Therefore, other techniques such as those below have been explored for NMR detection of allergen-antibody complexes.

A similar labeling scheme, but in a subtly different experiment, was used to map the Der f 2 epitopes of two full length murine IgG (150 kDa) (137). The authors again utilized ^{1}H-^{15}N labeled allergen, which would typically not be detectable at this large size when bound to antibody, assuming tight binding to the IgG. In this case, detergent was added to the sample to reduce the antibody affinity. As a result, in the NMR experiment the researchers were observing the allergen ^{1}H-^{15}N chemical shift perturbations between bound and free, with the smaller molecule in the free state being the one that was detected. The ratio of bound to free was tuned with the concentration of detergent so that there was a differential reduction or broadening in the NMR signals of those residues in proximity to the antibody compared to the free protein. The broadening is due to increased relaxation of the NMR signal due to the large fully ^{1}H labeled antibody binding to the antigen (138). This same effect is noted below in other experiments. In the Der f 2 study, the differential exchange broadening data provided results that mapped the epitopes to two disparate regions of the protein, consistent with the simultaneous binding of the two antibodies. A potential disadvantage of this technique is that it requires empirical tuning of the solvent conditions, which may or may not be applicable to all systems.

Instead of looking at fragments of the antibody-allergen complex, or the free allergen in exchange with complex, it is also possible to utilize whole antibodies, but this requires another compromise in the labeling scheme. At the very high molecular weights of allergen-antibody complexes, usually only methyl groups are still observable in a background of otherwise ^{2}H labeled proteins (132). Focusing on only labeling methyl groups in the allergen restricts the number of probes available for epitope mapping to the methyl groups of Val, Leu and Ile. The effectiveness of this was demonstrated for [U-^{2}H, ^{1}H,^{13}C-methyl Val, Leu, Ile] Der p 2 bound to an scFv fragment of mAb 7A1 (58). The data showed relaxation broadening for Ile-97 in the allergen, which was directly in contact with the scFv, and chemical shift perturbations for V63 and L61, which were adjacent to the epitope as described in a crystal structure (**Figure 9**). This is again consistent with previous observations that close proximity to the ^{1}H antibody causes broadening or a disappearance of signal, and distal residues can also experience chemical shift perturbations (138).

Methyl labeled Der p 2 as described above was successfully applied to study 4 human IgE mAb epitopes and 3 murine IgG mAb epitopes (127). Similar data observations of broadening and chemical shift perturbations were analyzed to map the epitope regions. Since the epitope data is sparse, being only derived from a few methyl probes, it needs to be interpreted with caution. For each antibody, the data indicated that few residues in close proximity were broadened or perturbed, and this was consistent with previous mutagenesis studies or data from Der p 2 isoforms. The NMR data were in agreement with the relative epitope

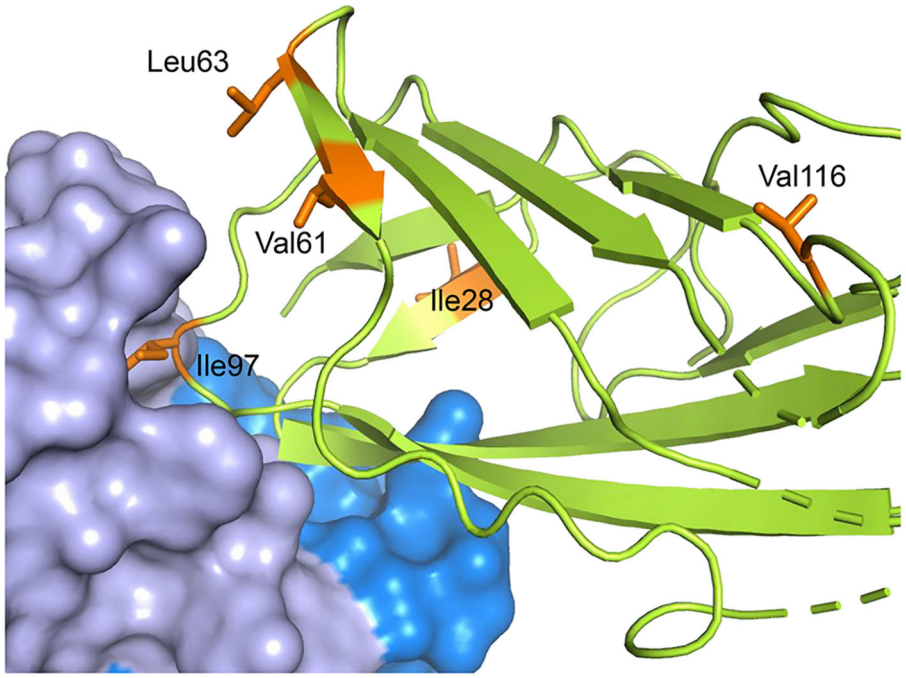

FIGURE 9 | Residues both near and far from the epitope can be affected by antibody binding. The crystal structure of Der p 2 in complex with the murine IgG mAb 7A1 is shown with Der p 2 rendered in green with specific methyl residues highlighted in orange. The mAb 7A1 heavy and light chains are rendered as blue and lavender surfaces, respectively. The shift of methyl resonances of the orange residues upon complex formation were measured by NMR. The figure shows that residues proximal and distal from the epitope can be affected by binding to an antibody.

mapping obtained using competitive and direct antibody binding immunoassays, which demonstrated which epitopes did or did not overlap.

While these data are an impressive first mapping of human IgE epitopes, three important drawbacks of the technique need to be mentioned. First, it requires monoclonal antibodies, of which human IgE are very difficult to clone from patients. Although this technique may be applicable to polyclonal antibodies, its effectiveness remains to be demonstrated in this case. ^{1}H-^{15}N labeling of Art v 1 and Bet v 1 was combined with either pooled allergic sera, or individual allergic sera, respectively, but the NMR results were nebulous (139–141). Second, the methyl labeling is expensive, typically $1,000 per liter of bacterial expression culture. Thus, high expression levels of the protein are needed to be cost effective. And third, as mentioned above, the distribution of sparse methyl groups may not be ideal for all allergens to get good epitope data. The paucity of data also requires a rather generous interpretation of which residues might be directly involved in the epitope. Hence, the epitopes proposed from these NMR data likely include more residues than the ones that are directly observed contacting the antibody in a crystal structure and should be further tested for functional importance.

In summary, a variety of NMR techniques and labeling schemes have been applied for allergen epitope mapping. In each case, atoms or residues specific to the epitope were successfully identified.

FUTURE DIRECTIONS

Experimental epitope mapping of IgE antibodies on allergens originated ∼30 years ago with the identification of mostly linear epitopes. Several breakthroughs have allowed the identification of conformational epitopes. These epitopes are the most common on allergens, especially on allergens for which exposure occurs by inhalation. Techniques such as recombinant technology, X-ray crystallography and nuclear magnetic resonance were developed and used for the determination of structures of allergen-antibody complexes. These advances required preparation of pure and homogeneous allergens and monoclonal antibodies. Initially, mostly IgG antibodies that inhibit IgE antibody binding were used as surrogates of IgE and fragmented for epitope mapping. Another approach led to the isolation of IgE antibody constructs using phage display technology. Only more recently, single cell antibody sequencing and human hybridoma technology are opening a new era of epitope mapping that will allow direct visualization of allergen-IgE antibody interactions in detail. Other technologies such as cryo-electron microscopy and labeling with mass spectrometry will also contribute to epitope mapping with less demanding protein amounts. Moreover, the experimental results allow for a significant development of many computational approaches to identify and/or analyze paratopes and epitopes (142). For example, approaches used in image recognition, like Zernike moments, were shown to be very promising in predicting B-cell epitopes (143–145). Therefore,

we expect that computational methods will start to play a more important role in studies of interactions between antibodies and allergens. Ultimately, identification of IgE antibody binding epitopes associated with the human IgE repertoire will contribute to understanding the immune response to allergens and will lead to the design of modified recombinant allergens for safer and more effective immunotherapy.

AUTHOR CONTRIBUTIONS

AP designed, wrote, and edited the review. She especially wrote the sections about IgE epitope mapping. GM wrote the sections associated with NMR and cryo-EM. MC analyzed crystal structures of allergen-antibody complexes and wrote sections about X-ray crystallography and mass spectrometry. GM and MC also contributed to editing the manuscript. All authors contributed to the article and approved the submitted version.

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In vivo Induction of Functional Inhibitory IgG Antibodies by a Hypoallergenic Bet v 1 Variant

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Allergic sensitization to the major allergen Bet v 1 represents the dominating factor inducing a vast variety of allergic symptoms in birch pollen allergic patients worldwide, including the pollen food allergy syndrome. In order to overcome the huge socio-economic burden associated with allergic diseases, allergen-specific immunotherapy (AIT) as a curative strategy to manage the disease was introduced. Still, many hurdles related to this treatment exist making AIT not the patients' first choice. To improve the current situation, the development of hypoallergen-based drug products has raised attention in the last decade. Herein, we investigated the efficacy of the novel AIT candidate BM4, a hypoallergenic variant of Bet v 1, to induce treatment-relevant cross-reactive Bet v 1-specific IgG antibodies in two different mammals, Wistar rats and New Zealand White rabbits. We further analyzed the cross-reactivity of BM4-induced Wistar rat antibodies with the birch pollen-associated food allergens Mal d 1 and Cor a 1, and the functional capability of the induced antibodies to act as IgE-blocking IgG antibodies. Enzyme-linked immunosorbent assay (ELISA) was used to determine the titers of rat IgG1, IgG2a, IgG2b, and IgE, as well as rabbit IgG and IgE antibodies. To address the functional relevance of the induced IgG antibodies, the capacity of rat sera to suppress binding of human IgE to Bet v 1 was investigated by using an inhibition ELISA and an IgE-facilitated allergen-binding inhibition assay. We found that the treatment with BM4 induced elevated Bet v 1-specific IgG antibody titers in both mammalian species. In Wistar rats, high BM4-specific IgG1, IgG2a, and IgG2b titers (10^4 to 10^6) were induced, which cross-reacted with wild-type Bet v 1, and the homologous allergens Mal d 1 and Cor a 1. Rat allergen-specific IgG antibodies sustained upon treatment discontinuation. Sera of rats immunized with BM4 were able to significantly suppress binding of human IgE to the wild-type allergens and CD23-mediated human IgE-facilitated Bet v 1 binding on B cells. By contrast, treatment-induced IgE antibody levels were low or undetectable. In summary, BM4 induced a robust IgG immune response that efficiently blocked human IgE-binding to wild-type allergens, underscoring its potential therapeutic value in AIT.

Keywords: hypoallergen, Bet v 1, birch pollen, AIT, IgG, blocking, allergy, PFAS

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INTRODUCTION

Birch pollen (*Betula verrucosa*) represents the major elicitor of tree pollen-associated allergic symptoms in Europe, with sensitization patterns ranging from 54 up to 92% among patients allergic to tree pollen (1–3). The peculiarity that the IgE-mediated pathology is dominantly triggered by the recognition of the allergen Bet v 1 – reactivity rates around 95% – prompted researchers world-wide to develop therapeutic approaches targeting this protein (4). Besides seasonal occurring symptoms, such as rhinoconjunctivitis and allergic asthma, birch pollen allergic patients frequently report oral and food allergy symptoms, creating a complex disease endotype. These symptoms are elicited by plant food sources including fruits, vegetables and nuts, and occur due to the recognition of similar structural motifs shared between food allergens and the primary sensitizing pollen allergen. The estimated prevalence of symptoms triggered by such food allergens ranges between 50 and 90% among birch pollen allergic patients (5–9). This clinical manifestation is described as the pollen food allergy syndrome (PFAS) (5). Apples and hazelnuts belong to the most common allergenic sources amongst plant foods eliciting birch pollen-associated PFAS symptoms (8, 10, 11). Here, the culprit allergens responsible for triggering PFAS are the Bet v 1-homologous pathogenesis-related protein-10 (PR-10) class proteins Mal d 1 (apple, *Malus domestica*) and Cor a 1 (hazelnut, *Corylus avellana*). Although the adverse health effects caused by birch pollen are efficiently treatable by allergen-specific immunotherapy (AIT), the concomitant treatment of PFAS symptoms induced by Bet v 1-associated food sources remains debatable, highlighting strengths, and possibilities for therapeutic improvements (12–16). Other disadvantages of AIT are long treatment duration, the risk of side-effects and, in case of subcutaneously applied AIT, repeated injections into the patient's skin, resulting in a poor treatment compliance (17).

In order to address these challenges in AIT, we have developed a hypoallergenic variant of Bet v 1, termed BM4, possessing beneficial characteristics including decreased allergenicity (reduced binding by Bet v 1-specific IgE) and ameliorated immunogenicity (enhanced T cell-activating capacity and proliferation) (18–20). BM4 was genetically engineered through the substitution of five amino acids in the wild-type Bet v 1 sequence by corresponding residues in the Mal d 1 sequence, resulting in a general collapse of the otherwise globular PR-10 fold. In turn, these structural changes altered conformation-dependent IgE epitopes (18). The EU-funded project "BM4SIT – Innovations for Allergy" aimed to evaluate the efficacy of BM4 as an AIT vaccine candidate in a first-in-men clinical trial. Drug toxicity studies, preceding human clinical trials, are used to investigate the safety profile of vaccine candidates in various mammalian species in order to estimate patients' tolerability, and, thus, are a mandatory step toward drug approval. In the course of the BM4 toxicity studies, conducted in Wistar rats and New Zealand White (NZW) rabbits, we undertook a detailed profiling of the BM4-induced humoral immune response in a naïve setting. It is well established that the reduction of symptoms in AIT, and consequently the improvement of the patient's quality of life, are mainly accomplished by the induction of immunotolerance, which is maintained by allergen-specific blocking IgG4 and partially by IgG1 antibodies able to neutralize IgE-mediated allergen binding (21–24). Therefore, the objective of the present study was to perform a pre-clinical evaluation of the Bet v 1-specific IgG as well as IgE immune response induced by immunizations with BM4 in both Wistar rats and NZW rabbits. We further analyzed the functionality of the induced IgG antibodies regarding their capability to inhibit human IgE-facilitated binding of Bet v 1-IgE complexes to B cells, a confirmed biomarker for AIT efficacy (25, 26). Additionally, we investigated the cross-reactivity of the serum antibodies toward the Bet v 1-associated food allergens Mal d 1 and Cor a 1.

Herein, we show that immunizations of Wistar rats and NZW rabbits with BM4 resulted in high levels of IgG antibodies cross-reactive to wild-type Bet v 1. In Wistar rats, functional Mal d 1- and Cor a 1-cross-reactive IgG antibodies were induced, however, to a lesser extent compared to the Bet v 1. In rats receiving repeated immunizations with BM4, sustained IgG antibody titers remained even upon treatment discontinuation. In addition, BM4-induced IgG antibodies displayed a functional inhibitory activity toward the binding of human IgE to the wild-type pollen allergen (Bet v 1) and the associated food allergens (Mal d 1 and Cor a 1).

Abbreviations: AIT, allergen-specific immunotherapy; BSA, bovine serum albumin; CD, circular dichroism; ELISA, enzyme-linked immunosorbent assay; EBV, Epstein–Barr virus; FSC, forward scatter; FTIR, Fourier transform infrared; HRP, horseradish peroxidase; hS, human serum; inhibition FAB, inhibition IgE-facilitated allergen binding; L/D, live/dead; LOQ, limit of quantification; NZW rabbits, New Zealand White rabbits; PR-10, pathogenesis-related protein-10; PFAS, pollen food allergy syndrome; SCIT, subcutaneous immunotherapy; SSC, side scatter; SFP, specific pathogen-free; SEM, standard error of the mean; s.c., subcutaneous.

MATERIALS AND METHODS

Recombinant Proteins

BM4 (designated BM41 by the manufacturer Biomay AG, Vienna, Austria) was produced recombinantly and endotoxin-free under GMP conditions based on the described protocol (18). Both BM4 and placebo were formulated using aluminum hydroxide (Alu-Gel-S, Serva, Heidelberg, Germany). Expression, purification, physicochemical characterization, and determination of endotoxin contamination (<0.3 ng/mL) of recombinant Bet v 1.0101, Mal d 1.0108, and Cor a 1.0401 (called Bet v 1, Mal d 1, and Cor a 1 in the following) were performed as previously described (27, 28). A representative SDS-PAGE image showing the different recombinant proteins can be found in **Supplementary Figure S1**.

Analysis of Secondary Structural Elements

The structural composition of the recombinant proteins was determined using circular dichroism (CD) and Fourier transform infrared (FTIR) spectroscopy. The CD spectra were recorded at 20°C between 190 and 260 nm using a JASCO J-815 spectropolarimeter (Jasco, Tokyo, Japan). All proteins were

diluted in a 10 mM potassium phosphate buffer to a final concentration of 0.1 mg/ml. For the structural analysis using FTIR, the spectra in the range of the amide 1 and amide 2 peaks (1500–1700 cm^{-1}) were recorded at a constant temperature (25°C) for each protein at concentrations of 1.5–2.0 mg/ml using an AquaSpec transmission cell adapted to a Tensor II FTIR system (Bruker Optics Inc., Billerica, MA, United States). The data were analyzed using the OPUS spectroscopy software 6.0 (Bruker Optics Inc., Billerica, MA, United States). The second derivative of the amide 1 spectra was calculated after vector-normalization (25 smoothing points) using the Savitzky–Golay algorithm.

Animal Immunization Model(s)

Both species, NZW rabbits (Lidköpings kaninfarm, Lidköping, Sweden) and Wistar rats (RjHAN:WI, Janvier Labs, France), received a single subcutaneous (s.c.) injection (hereinafter "single immunization model") of either 320 μg of BM4, formulated as 320 μg BM4/2 mg aluminum hydroxide/0.9% NaCl/ml, or the respective amount of adjuvant without antigen, termed "placebo" in the following (n = 5 male and n = 5 female per group). Since in human allergen-specific subcutaneous immunotherapy (SCIT) AIT vaccine is administered via the s.c. route of administration, BM4 was administered likewise. Treated animals were daily monitored in a specific pathogen-free (SPF) environment on a frequent basis for 14 days post-injection for clinical signs of test item effects, such as changes in skin and fur, respiration, eyes and mucous membranes, circulation and behavior patterns, and then sacrificed. Blood samples were collected pre- and 14 days post-injection and stored at −20°C until analysis. Sera of the single immunization model were only used for an initial screening of BM4-specific and Bet v 1-cross-reactive antibodies, whereas a further functional characterization of these sera was not pursued.

According to the OECD Guidelines for the testing of chemicals no 420, the rat is the preferred species. Therefore, the repeated toxicity study analyzing potential toxic effects of the hypoallergen was only conducted in Wistar rats (Charles River, Sulzfeld, Germany). Injections containing either 160, 80, 40, or 20 μg of BM4 (formulated as xx μg BM4/1 mg alum/0.9% NaCl/500 μl) were administered s.c. on a bi-weekly schedule over a period of 6 months (12 injections/animal in total); termed "repeated immunization model" in the following. This model was conducted in order to provide a immunization schedule relatable to a human AIT protocol. The animals of the main group (n = 10 per gender per treatment group) were sacrificed 1 week after the last injection and those in the recovery group (5 per gender per treatment group) 6 weeks after the last injection. The dose level used in the study was based on the doses employed in human AIT protocols.

All animal studies were conducted in compliance with the "OECD Principles of Good Laboratory Practice" (ENV/MC/CHEM(98)17) and the standard operating procedures (SOPs) of UTUCAL. All procedures and protocols were approved by the National Animal Experiment Board of Finland in accordance with the EU Directive 2010/63/EU on the protection of animals used for scientific purposes under the license numbers ESAVI/7217/04.10.03/2012, and ESAVI/8528/04.10.07/2015. UTUCAL animal facilities operate in compliance with the OECD Principles of Good Laboratory Practice (GLP) and have Animal Welfare Assurance by the Office of Laboratory Animal Welfare (OLAW), PHS, NIH. Assurance identification number is A5040-01.

Enzyme-Linked Immunosorbent Assay

Endpoint titer of BM4-, Bet v 1-, Mal d 1-, or Cor a 1-specific IgE, IgG1, IgG2a, and IgG2b levels in rat sera were determined by enzyme-linked immunosorbent assay (ELISA). Either BM4, Bet v 1, Mal d 1, or Cor a 1 were coated in a concentration of 2 μg/ml diluted in 1× phosphate-buffered saline on Nunc MaxiSorp® flat-bottom 96 well plates (Thermo Fisher Scientific, United States). Serial dilutions of rat sera were incubated with the respective antigen overnight at 4°C. Horseradish peroxidase (HRP)-conjugated mouse anti-rat IgG1, IgG2a, IgG2b (clones G1 7E7, 2a 8F4, and 2B 10A8, respectively, purchased from SouthernBiotech, Birmingham, United Kingdom), and IgE (clone MARE-1, Thermo Fisher Scientific, Rockford, IL, United States) antibodies diluted 1:5000 were used as detection antibodies. The SureBlue TMB (3,3′,5,5′-Tetramethylbenzidine) Microwell peroxidase substrate (KPL, Gaithersburg, MD, United States) was used for detection. Since NZW rabbits only possess a single IgG subclass, the BM4- and Bet v 1-specific rabbit IgG ELISAs were performed using a HRP-conjugated goat anti-rabbit IgG antibody, Fc Fragment (Jackson ImmunoResearch Inc., Suffolk, United Kingdom) for detection (29). Plates were measured at a wavelength of 450 nm using an ELISA Reader Infinite 200 PRO (Tecan, Switzerland). The limit of quantification (LOQ), defined as the sum of the mean plus the 10-fold standard deviation of the detection antibody controls, was used as cut-off for endpoint titer determination. The final antibody titers were calculated by plotting the experimental absorbance values against the dilution of a serum as previously described (30). For the determination of rabbit total IgE, an ELISA kit from BlueGene Biotech (Putuo District, Shanghai, China) was used according to the manufacturer's instructions.

Inhibition ELISA

For the inhibition ELISA, 2 μg/ml of either Bet v 1, Mal d 1, or Cor a 1 were coated on Nunc MaxiSorp® flat-bottom 96 half-area well plates (Thermo Fisher Scientific, United States). After blocking with 0.5% BSA, the immobilized antigen was incubated with rat sera in dilutions of either 1:32 for Bet v 1 or 1:2 for Mal d 1, and Cor a 1 for 2h at room temperature. To exclude any potential interference of IgE antibodies, the rat sera were heat-inactivated at 56°C for 1h prior to the experimental procedure. After the inhibition step, the samples were incubated with different human reference serum pools per antigen (derived from birch allergic patients suffering from PFAS caused by apples and/or hazelnuts), diluted 1:2 for Bet v 1 and Mal d 1, and 1:8 for Cor a 1 (depending on the presence of specific IgE in the serum pools), overnight at 4°C. An alkaline phosphatase-conjugated mouse anti-human IgE antibody (clone B3102E8, SouthernBiotech, Birmingham, AL, United States), diluted 1:1000, and the alkaline phosphatase substrate p-nitrophenyl phosphate (Sigma-Aldrich, St. Luis, MI,

United States) were used for detection. The absorbance was recorded at 405 nm after 3h of incubation at room temperature. The data were normalized to the uninhibited control (100%). The percentage of inhibitory activity of each individual serum was calculated by inverting the normalized absorbance value (100 minus normalized absorbance value) after background subtraction (detection antibody control).

Inhibition IgE-Facilitated Allergen Binding (Inhibition FAB) Assay

We performed inhibition FAB assays to determine the capacity of BM4-induced rat antibodies to prevent the formation of Bet v 1-IgE complexes using a defined human indicator serum pool obtained from six individual birch pollen allergic patients. The indicator serum pool contained high levels of Bet v 1-specific IgE antibodies, as determined by ImmunoCAP assays (31). For this purpose, we used an adapted version of the protocol published by Shamji et al. (32). In short, IgE of rat sera was inactivated by heat treatment at 56°C for 1h prior to the experimental procedure. For the inhibition FAB assay, sera of animals receiving repeated immunizations of either 0 (placebo), 20, or 40 μg BM4 were used (main group: $n = 20$, recovery group: $n = 10$). A total of 15 μl of each rat serum was incubated with 10 ng/ml Bet v 1 for 1h at 37°C, followed by the addition of 1 μl of the human serum pool and further incubation for 1h at 37°C. Subsequently, 1×10^5 Epstein–Barr virus (EBV)-transformed B cells expressing CD23, the low IgE-binding receptor (also called FcεRII), were added to the antibody allergen reaction mix. The cells were stained using a PE-labeled anti-human CD23 (BD Biosciences, San Jose, CA, United States) and a FITC-labeled anti-human IgE (KPL/medac GmbH, Wedel, Germany) in the dilutions 1:40 and 1:100, respectively. The experimental conditions, including antigen concentration, amount of human reference serum and the dilution of the FITC-labeled anti-human IgE antibody, were defined prior to the experiment (**Supplementary Figure S3**). To avoid false-negative results by dead cells, SYTOX™ Red Dead Cell Stain (Thermo Fisher Scientific, Waltham, MA, United States) in a dilution 1:100 was used for live/dead (L/D) discrimination. As positive control, the uninhibited human serum pool (no rat serum, only Bet v 1 and human serum) was used for IgE-Bet v 1 complex formation. The cells were analyzed using a Cytoflex S (Beckman Coulter, Brea, CA, United States) and FlowJo v 10 (FlowJo, LLC, Ashland, OR, United States). Doublet discrimination was performed by gating the side scatter area (SSC-A) versus the SSC height (SSC-H). Only living EBV-transformed B cells were considered for the data analysis. The detailed gating strategy is shown in **Supplementary Figure S4**. The data were normalized to the mean of the uninhibited control (100%). The percentage of inhibitory activity of the sera was calculated by inverting the percentage obtained for the IgE-Bet v 1 complex formation (100 minus complex formation value) after baseline subtraction (mean of untreated cells). Compensation was performed and isotype as well as single stained controls were included in the experimental set-up. Ethical approval for using human serum of allergic patients was obtained by the Dutch ethical committee (number: NL65758.018.18).

Statistical Analysis

Data in the text are presented as mean ± standard error of the mean (SEM). Statistics were calculated on transformed data [Y = Log(Y)]. A paired *t*-test was used to compare pre- and post-immunization sera (**Figures 1**, **3A–B**) and BM4-specific with Bet v 1-specific titers (**Figure 3C**), whereas an unpaired t-test was performed for the comparison of Mal d 1- and Cor a 1-specific antibody titers of the 80 μg BM4 treatment group with the 160 μg group (**Figure 5**). For comparing more than two sample groups, a one-way ANOVA was performed (**Figures 2**, **4**, **6**, **7**). For the correlation of the inhibition ELISA and inhibition FAB assay data with the transformed Wistar rat IgG titers a Pearson correlation test was performed. All statistical analyses were performed using the GraphPad Prism 8.0 software (GraphPad Software, San Diego, CA, United States). The *p*-values were reported in the following way; ns > 0.05; $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$; $^{****}p \leq 0.0001$.

RESULTS

A Single Immunization With BM4 Induces High Levels of Antigen-Specific IgG Antibodies in Wistar Rats and NZW Rabbits

To investigate in course of an initial screening the ability of the hypoallergenic molecule to induce an efficient antigen-specific IgG immune response in two different mammalian species, we determined the IgG antibody titers 2 weeks post-immunization with 320 μg BM4 (**Figure 1A**). In NZW rabbits, BM4 induced a 44.5-fold increase ($p < 0.0001$) of the mean antigen-specific IgG level post-immunization compared to the levels of the placebo control that remained unaltered (**Figure 1B**, mean titer: 8,828.7 pre-immunization and 393,177.8 post-immunization). In Wistar rats, we determined the antigen-specific IgG1, IgG2a, and IgG2b antibody titers in the pre- and post-immunization sera and compared them to the placebo control receiving only the adjuvant without antigen (**Figure 1C**). In the BM4 treatment group, the titers of the rat IgG antibody subclasses were significantly elevated following injection with the hypoallergen ($p < 0.0001$). The mean titers increased 24,721-fold for IgG1, 1,560-fold for IgG2a, and 402-fold for IgG2b (mean post-immunization titers: 271,932, 107,199, and 27,264, respectively). In comparison, the IgG titers of the placebo group, pre- and post-immunization, showed no change. Additionally to the IgG antibody titers, we sought to monitor the antigen-specific IgE levels in order to evaluate possible IgE-mediated side effects caused by the treatment. The treatment did not affect the total IgE level within rabbit sera (**Figure 1B**). In Wistar rats, the treatment with BM4 did not induce BM4-specific IgE (**Figure 1C**).

Elevated Specific IgG Titers Persist in the Sera of Wistar Rats After Discontinuation of Repeated Immunizations With BM4

In order to mimic a standard AIT protocol using BM4 and to evaluate its consequences on the humoral immune response, we

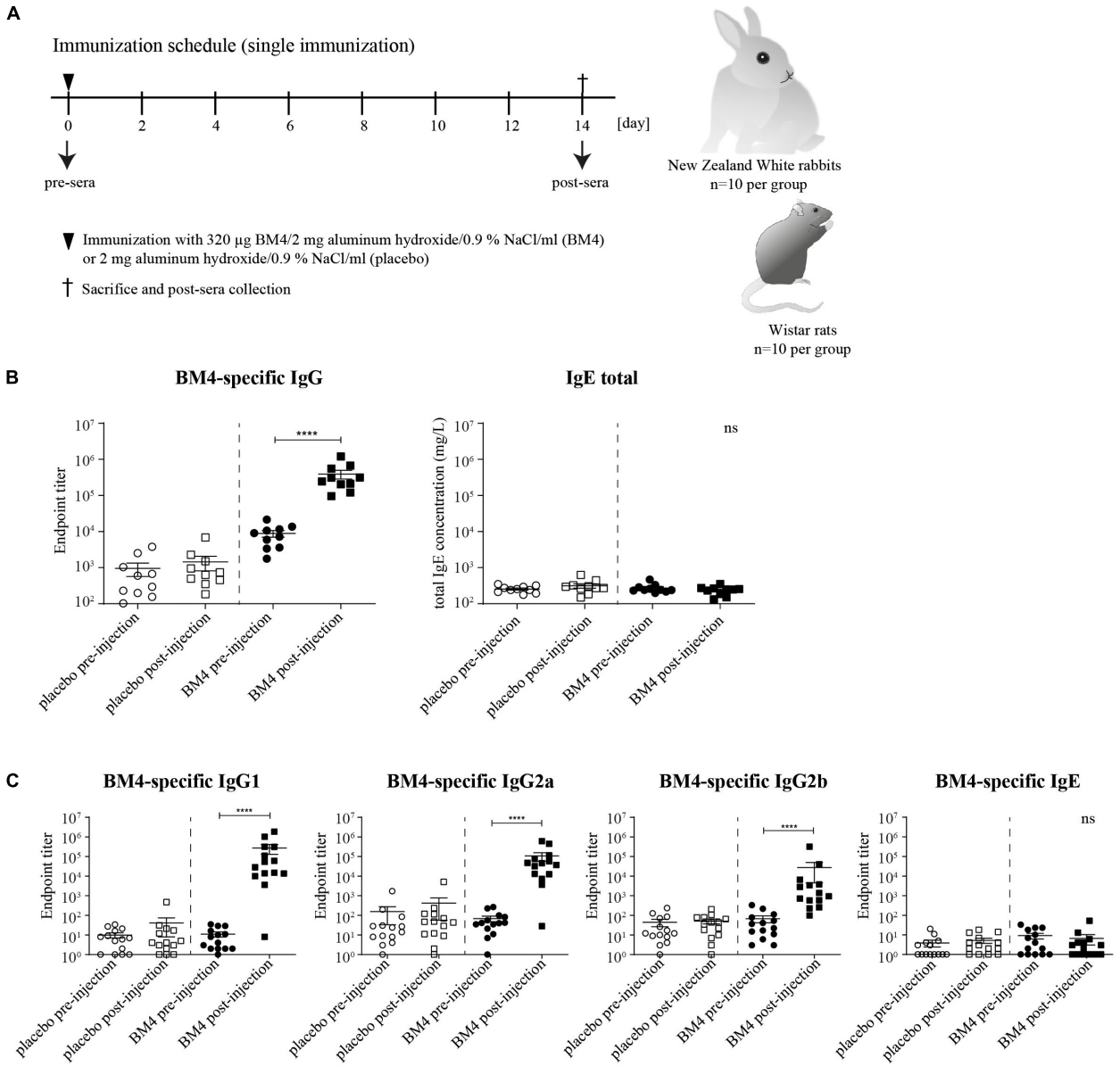

FIGURE 1 | Immunization schedule of the single immunization model **(A)**. Wistar rats or NZW rabbits (each $n = 10$ per group) were immunized subcutaneously either with 320 µg BM4 or a corresponding adjuvant control (placebo). Rabbit BM4-specific IgG antibody titers and total IgE pre- and post-immunization were determined **(B)**. Rat BM4-specific IgG1, IgG2a, IgG2b, and IgE endpoint titers pre- and post-immunization **(C)**. A paired *t*-test was performed to compare the pre- versus post-immunization values of each group.

immunized naïve Wistar rats with BM4 bi-weekly over a period of 6 months (**Figure 2A**, main group). To assess the levels of IgG antibodies after treatment discontinuation, we analyzed the BM4-induced IgG antibody titers 6 weeks after the last injection (recovery group). The animals were immunized with either 0 (placebo), 20, 40, 80, or 160 µg BM4. In contrast to the placebo group, all individual animals of the main group receiving the hypoallergen showed a dose-independent increase of IgG titers comparable to the single application of 320 µg presented in **Figure 1C** (**Figure 2B**, $p < 0.0001$). In contrast to the single immunization model, the highest doses of BM4 (80 and 160 µg) in the repeated immunization model also caused a slight elevation of BM4-specific IgE antibodies compared to the placebo group (mean IgE titer: 389 and 169, respectively, $p < 0.0001$). On average, IgG1 and IgG2a levels were still 500 to 1,000-fold higher than IgE. Even the IgG2b titers, which increased less compared to IgG1 and IgG2a, were still 63.4-fold higher than the IgE levels. Interestingly, it appears that there are two types of responses among animals, one with increased IgE and the other with no alterations in the IgE levels.

Regarding the recovery group, the mean of BM4-specific IgE levels of each treatment group dropped below 1.5 resembling

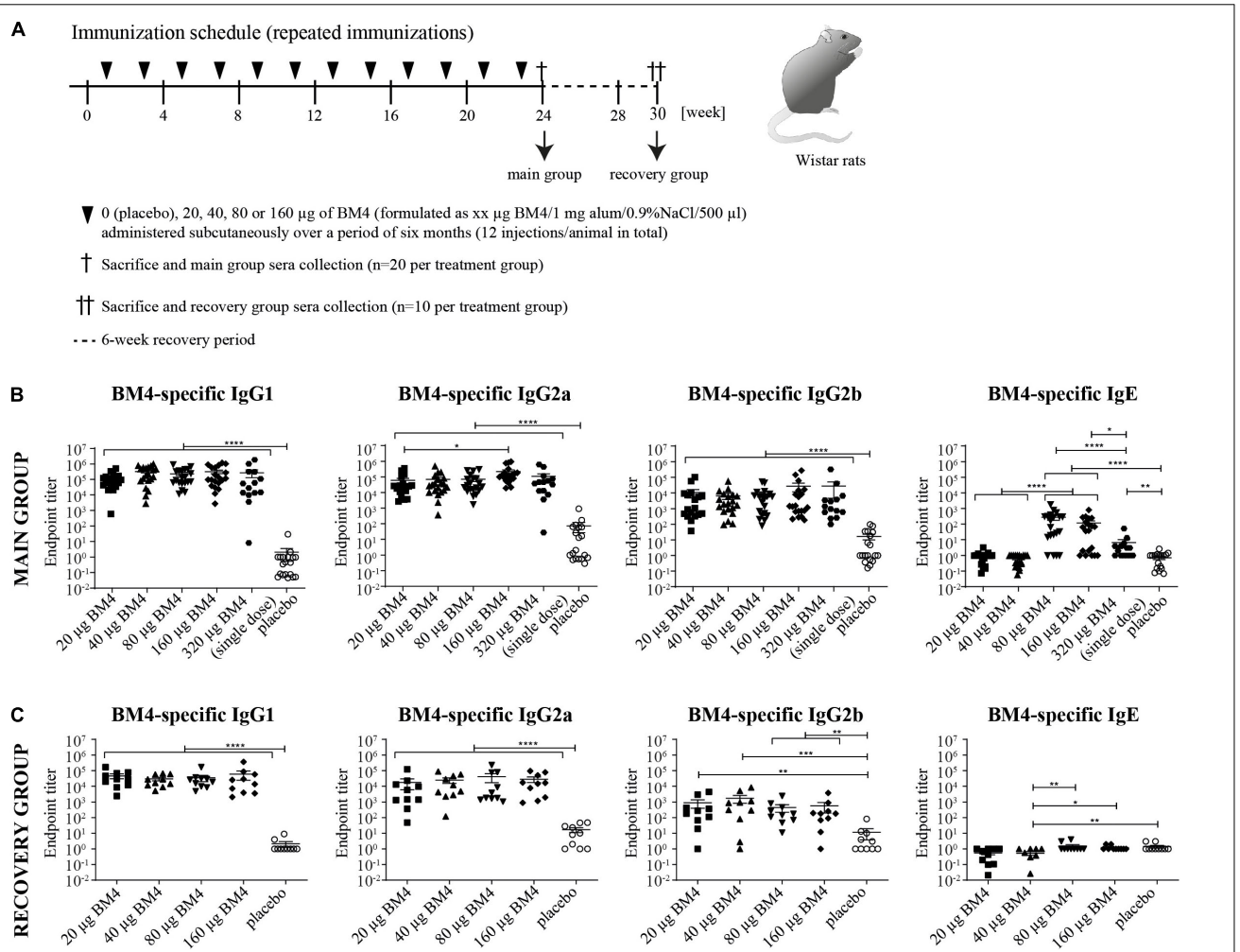

FIGURE 2 | Immunization schedule of the repeated immunization model **(A)**. Wistar rats received bi-weekly s.c. immunizations with either 0 (placebo), 20, 40, 80, or 160 µg BM4 over a period of 6 weeks. Animals of the main group (n = 20 per treatment) were sacrificed 1 week after the last application, whereas animals of the recovery group (n = 10 per group) were sacrificed after a 6-week recovery period. Determination of BM4-specific IgG1, IgG2a, IgG2b, and IgE levels within rat sera of the main **(B)** and the recovery group **(C)** by ELISA. The single immunization of 320 µg BM4 (of the single immunization model) was only used for comparative purposes. The scatter plot depicts the mean of each treatment group and the SEM. Statistics were calculated on transformed data [Y = Log(Y)] using a one-way ANOVA. A Tuckey multiple comparison test was used to compare all groups with each other. ns >0.05; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$.

the placebo group. Of note, the 40 µg BM4 group was even significantly lower than the placebo group (p = 0.0069, **Figure 2C**). By contrast, all IgG levels of each BM4 treatment group remained significantly increased compared to placebo. The mean IgG1 titers ranged from 29,713 for 40 µg up to 59,722 for 160 µg BM4, and exceeded the placebo titer (2.1) by 14,149-fold to 28,439-fold ($p < 0.0001$). The determined mean IgG2a antibody titers were 17,969, 24,764, 40,608, and 28,456 for 20, 40, 80, and 160 µg BM4, respectively, and, thus, exceeded the placebo group (16.8) on average 1,663-fold. The IgG2b levels induced by the treatment with BM4 were approximately 30-times lower than the corresponding IgG1 and IgG2a titers. The highest antigen-specific mean IgG2b titer compared to placebo (11.6) was observed for the 40 µg BM4 (1,725, p = 0.0006) treatment group, followed by 20 µg (879, p = 0.0022), 160 µg (559, p = 0.0064), and 80 µg (446, p = 0.0019). A statistical comparison of the main and the recovery group of BM4-specific antibody titers, as well as of the following Bet v 1-, Mal d 1-, and Cor a 1-cross-reactive antibodies can be found in **Supplementary Table S1**.

IgG Antibodies Induced by Immunization With BM4 Are Cross-Reactive With Wild-Type Bet v 1

For a potential application of the hypoallergen as an AIT vaccine in humans a functional confirmation of BM4-induced IgG antibodies recognizing the wild-type allergen is mandatory. In this respect, we first determined the Bet v 1-specific IgG antibody titers of sera derived from the single immunization (**Figure 1A**) as well as the repeated immunization model (**Figure 2A**). The IgG antibodies induced by a single immunization with BM4 were cross-reactive with Bet v 1 in both mammalian

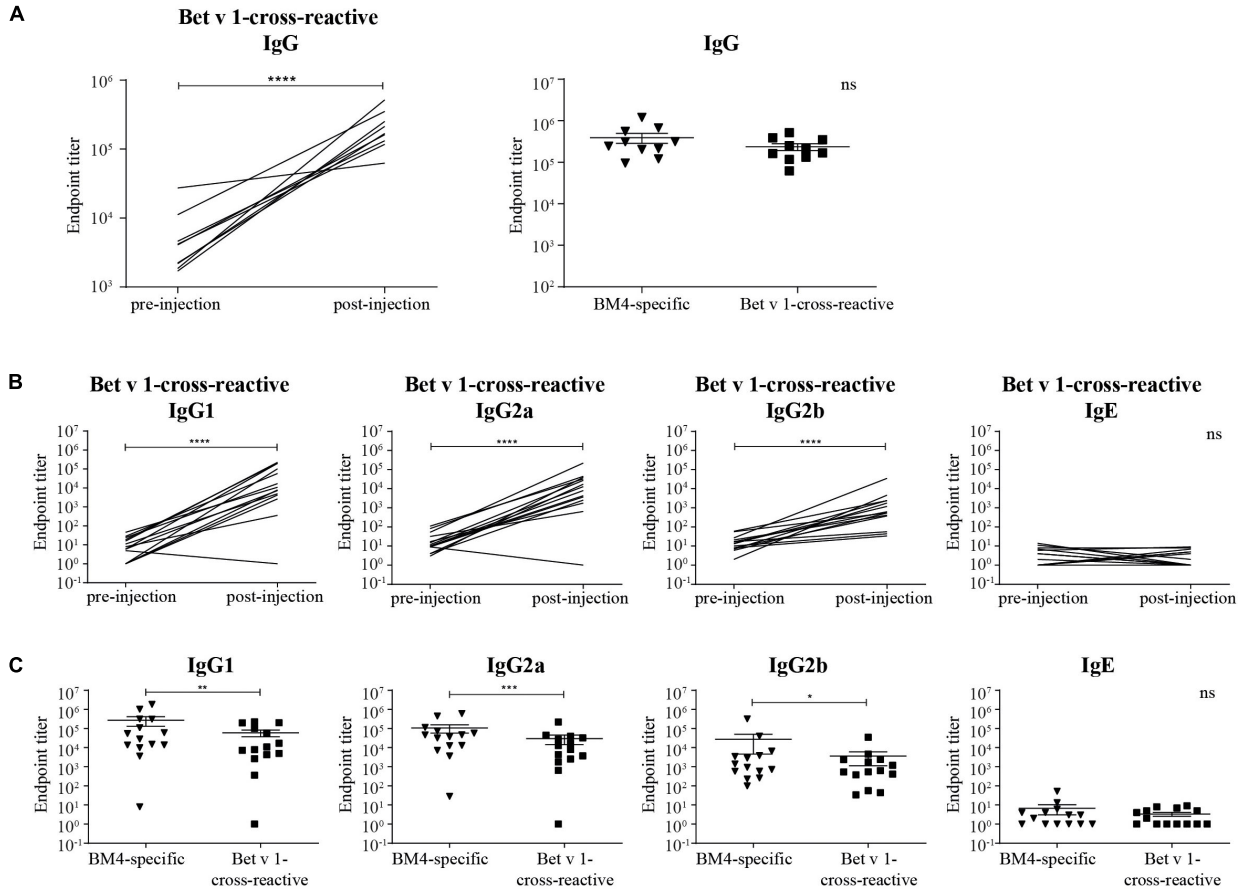

FIGURE 3 | Rabbit Bet v 1-cross-reactive IgG antibody titers pre- and post-immunization (**A**, left) and direct comparison of rabbit BM4-specific and Bet v 1-cross-reactive IgG titers (**A**, right). Rat Bet v 1-cross-reactive IgG1, IgG2a, IgG2b, and IgE endpoint titers pre- and post-immunization **(B)** and pairwise comparison of BM4-specific and Bet v 1-cross-reactive IgG1, IgG2a, IgG2b, and IgE levels **(C)**. For statistical analysis, a paired *t*-test was performed. ns >0.05; $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$; $^{****}p \leq 0.0001$.

species (**Figures 3A,B**). The induction of Bet v 1-cross-reactive antibodies in the post-sera was significantly different for NZW rabbit IgG and Wistar rat IgG1, IgG2a, and IgG2b compared to the sera before immunization (all $p < 0.0001$). Side-by-side comparison of BM4-specific and Bet v 1-cross-reactive titers of the post-immunization sera revealed that the Bet v 1-cross-reactive IgG1, IgG2a, and IgG2b levels were marginally but significantly lower than the corresponding BM4-specific values (**Figure 3C**, $p = 0.0022$, $p = 0.0003$, and $p = 0.0101$, respectively). In contrast, no difference was observed in NZW rabbit post-sera (**Figure 3A**). In Wistar rats, a more ambiguous pattern was found for the Bet v 1-specific titers. Again, the Bet v 1-cross-reactive antibody titers for all three IgG subclasses of the main group were significantly increased compared to placebo (**Figures 4A**, **6**), but in contrast to the single immunization model, the Bet v 1-specific IgG1 titers of the repeated immunization model, using 20 ($p = 0.0011$), 40 ($p < 0.0001$), and 160 μg BM4 ($p = 0.0271$), were actually significantly higher than the corresponding BM4-specific titers. In all three investigated IgG subclasses the repeated immunizations with 40 μg BM4 resulted in the highest mean Bet v 1-cross-reactive titers (IgG1: 5937,601.5, IgG2a: 1104,869.1, and IgG2b: 78,436.6), followed by 20 μg BM4 (IgG1: 1355,412.6, IgG2a: 139,285.0, and IgG2b: 9,798.1). The mean titers induced by 80 and 160 μg BM4 were comparatively lower. A similar pattern was observed for IgG1 and IgG2a titers of the recovery group (**Figures 4B**, **6**). In contrast to the main group, Bet v 1-cross-reactive IgG2b titers of animals receiving repeated immunizations with BM4 did not differ from the placebo group. Compared to the induction of slight BM4-specific IgE levels, neither in the single immunization model (**Figure 3B**) nor the repeated immunization model (**Figures 4A,B**) elevated Bet v 1-cross-reactive IgE titers were observed.

Wistar Rat IgG Antibodies Induced by Repeated Immunization With BM4 Are Cross-Reactive With the Bet v 1-Homologous Food Allergens Mal d 1 (Apple) and Cor a 1 (Hazelnut)

To investigate if treatment-induced antibodies bind to other allergens of the PR-10 family, we determined the titers of Mal d 1- and Cor a 1-cross-reactive IgG and IgE in rat sera derived

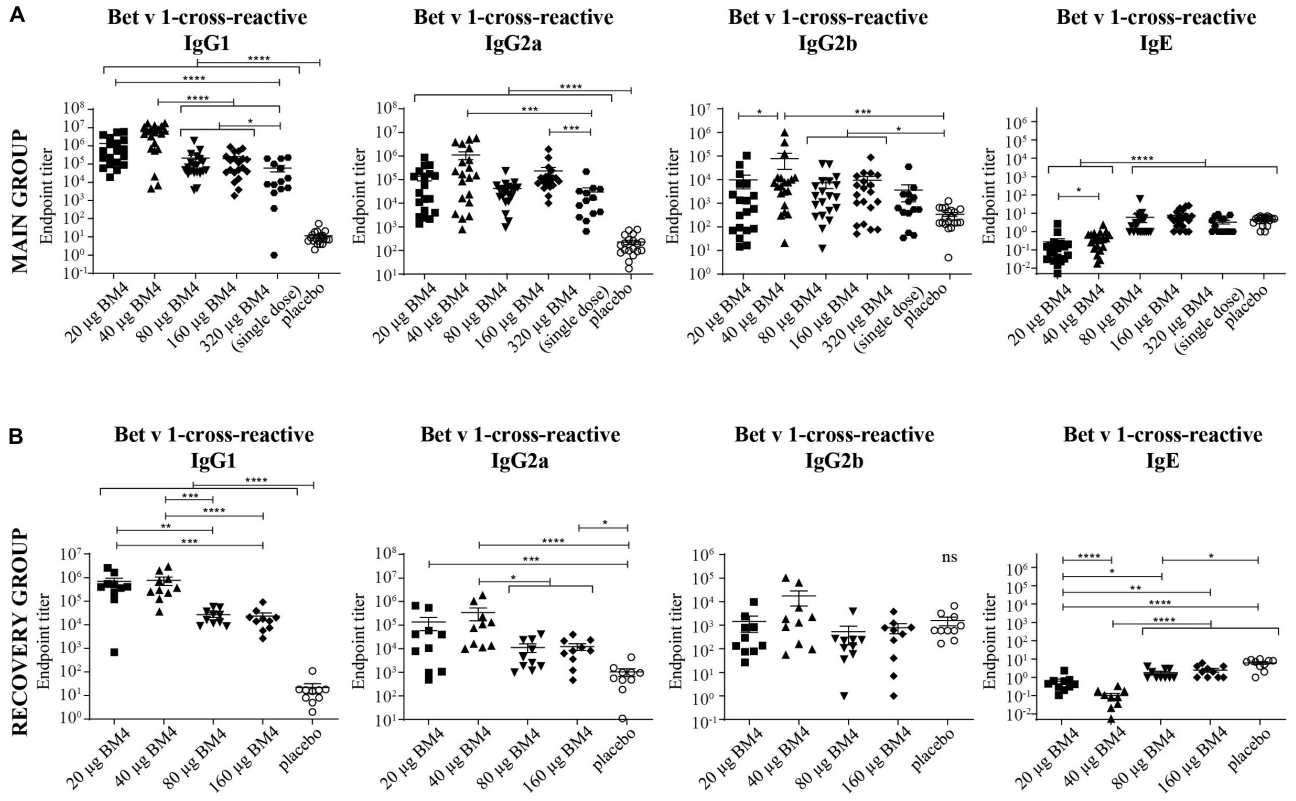

FIGURE 4 | Combined data of Bet v 1-cross-reactive IgG1, IgG2a, IgG2b, and IgE titers of rat sera of the main **(A)** and the recovery group **(B)** of the repeated immunization model evaluated by ELISA. The mean and SEM is shown in each scatter plot. Statistics were calculated on transformed data [Y = Log(Y)] using a one-way ANOVA and a Tuckey multiple comparison test. The single immunization of 320 µg BM4 (of the single immunization model) was used for comparative reasons. ns >0.05; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$.

from the repeated immunization model receiving the two highest BM4 dosages (80 and 160 µg). These food allergens share a sequence similarity of 56.6% (Mal d 1) and 67.3% (Cor a 1) with Bet v 1 and exhibit the common PR-10 fold. The structural integrity and similarity of the recombinant proteins used in this study with wild-type Bet v 1 was assessed by determining the secondary structural elements of each protein by CD and FTIR (**Supplementary Figure S2**). A high structural similarity between Bet v 1 and its homologous food allergens was observed. In contrast, BM4 exhibited a rather unfolded state using CD, whereas some structural features of the hypoallergenic molecule were still detectable when using FTIR for the analysis.

In direct comparison with the induced mean Bet v 1-specific IgG titers, it was apparent that all investigated Cor a 1- as well as Mal d 1-specific IgG subclasses were markedly lower (**Figures 5A,C**, **6** and **Supplementary Table S2**). Depending on the BM4 immunization dose, Mal d 1-specific IgG1 was either 18.6- to 28.9-fold lower than its Bet v 1-specific counterpart, and Mal d 1-specific IgG2a and IgG2b titers were 10.8- to 16.3-fold reduced. Notably, the induced Cor a 1-specific IgG titers were higher compared to their Mal d 1-specific corresponding values. Cor a 1-specific IgG1 was only 3.6- to 5.1-fold lower than the Bet v 1-specific IgG1 titers, IgG2a 5.8- to 10.5-fold, and IgG2b 4.1- to 12.9-fold. In the recovery group, except for Mal d 1-specific IgG2b, all Mal d 1-, and Cor a 1-specific mean titers of the investigated IgG subclasses dropped compared to the main group (**Figures 5B,D**, **6** and **Supplementary Table S2**). Still, compared to the Bet v 1-specific IgG titers of the recovery group, all mean titers of the Mal d 1- and Cor a 1-specific IgG subclasses were in a similar range; except for Mal d 1-specific IgG1 and IgG2a, which were 5.7- to 10.3- and 8.0- to 12.1-fold lower, respectively. Both, Mal d 1- and Cor a 1-specific IgE titers of the main and the recovery group were in a comparable range to Bet v 1-specific IgE, and thus hardly induced by the treatment with BM4.

Summarizing the specificity of the BM4-induced antibodies of the repeated immunization model, we can conclude that the highest IgG antibody levels were induced toward the wild-type Bet v 1 allergen rather than its hypoallergenic variant (**Figure 6**). IgG antibodies against the Bet v 1-homologous food allergens Cor a 1 and Mal d 1 were also induced but comparably at a lower extent. The highest IgE antibody levels were induced by 80 and 160 µg of BM4, and the specificity of these antibodies was also mostly directed toward BM4. However, in course of the toxicity study it became evident that the higher BM4 dosages (80 and 160 µg) exceeded the no-observed-adverse-effects level (NOAEL, data not shown), whereas the lower concentrations (20 and

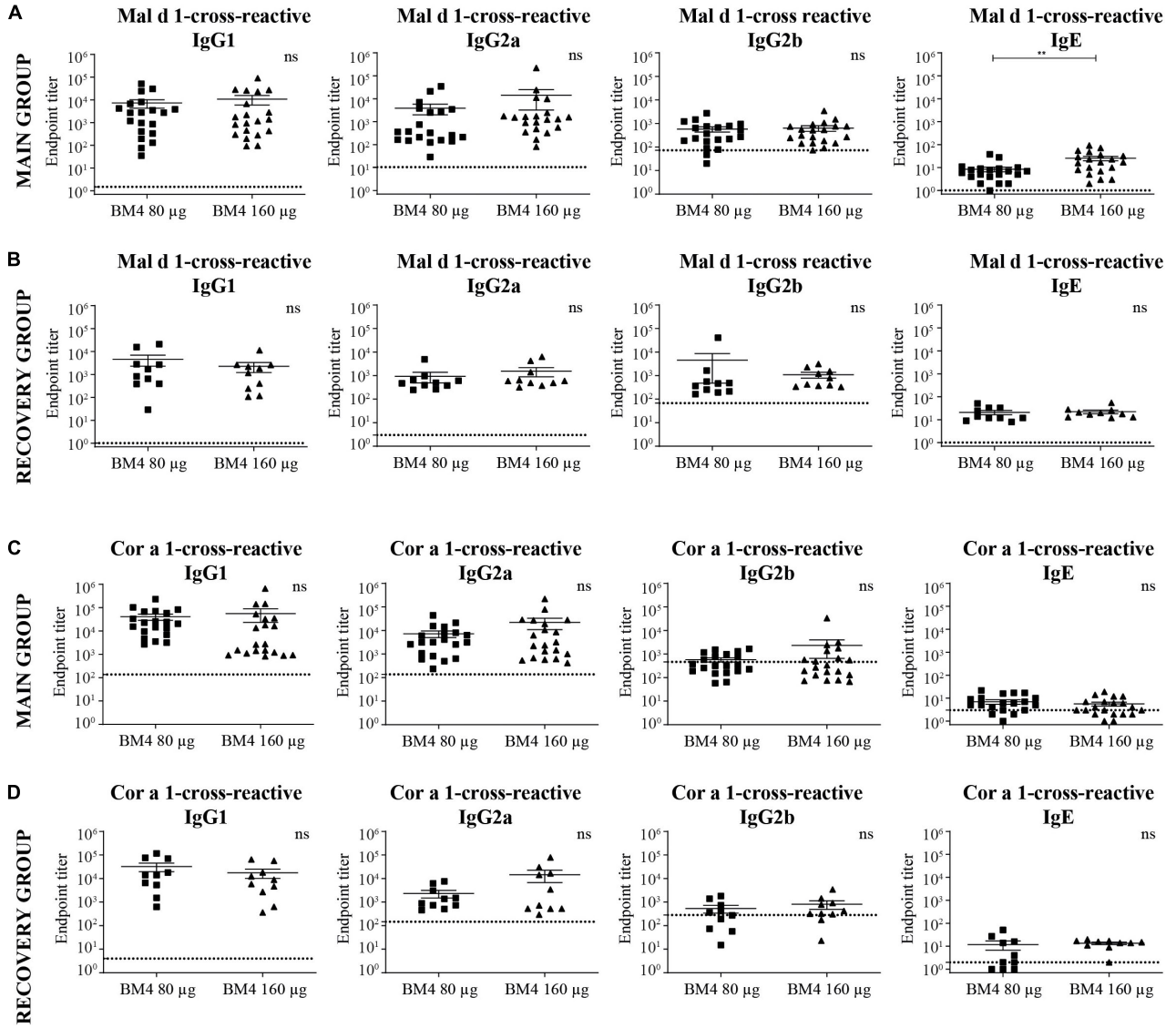

FIGURE 5 | Analysis of Mal d 1- **(A,B)** and Cor a 1-cross-reactive **(C,D)** IgG1, IgG2a, IgG2b, and IgE titers of sera derived from Wistar rats receiving repeated immunization of either 80 or 160 µg BM4 (**A,C**: main group; **B,D**: recovery group). The dotted line represents a sera pool of animals receiving placebo. For statistical analysis, an unpaired *t*-test was performed on transformed [Y = log(Y)] data. ns >0.05; $^{**}p \leq 0.01$.

40 µg) were considered "safe," enabling the further investigation of the hypoallergen in a first-in-men human clinical trial. In this respect, only sera of rats immunized with 20 and 40 µg BM4 were subjected to further functional characterization.

BM4-Induced Wistar Rat IgG Antibodies Inhibit Specific Binding of Human IgE to Bet v 1, Cor a 1, and Mal d 1

The induction of blocking antibodies (IgG4/IgG1) is a hallmark of successful AIT, thus, a sole characterization of the antibody pattern as well as of their specificity is not sufficient enough to draw conclusions of the functional relevance of the induced antibodies regarding the IgE-blocking activity of IgG antibodies. Therefore, we decided to investigate to which extent rat sera of the repeated immunization model (20 and 40 µg BM4) containing BM4-induced antibodies are able to compete with human IgE antibodies for binding of wild-type Bet v 1, Mal d 1, and Cor a 1. For this purpose, we used an inhibition ELISA (**Figure 7A** and **Supplementary Table S3**). In rat sera of the main group, immunization with BM4 (20 and 40 µg) induced a pronounced mean inhibition of human IgE binding to Bet v 1 (70.8 and 84.1%), a moderate mean inhibition to Cor a 1 (43.9 and 58%), and a low mean inhibition to Mal d 1 (27.6 and 37.6%). Whereas, placebo induced a mean inhibition of 15.6, 20.1 and 17.6% to Bet v 1, Cor a 1, and Mal d 1, respectively. Overall, the inhibitory capacity remained in sera of the recovery group (Bet v 1: 90.5%; Cor a 1: 42 and 49.5%; Mal d 1: 34.7 and 38.6%). However, for

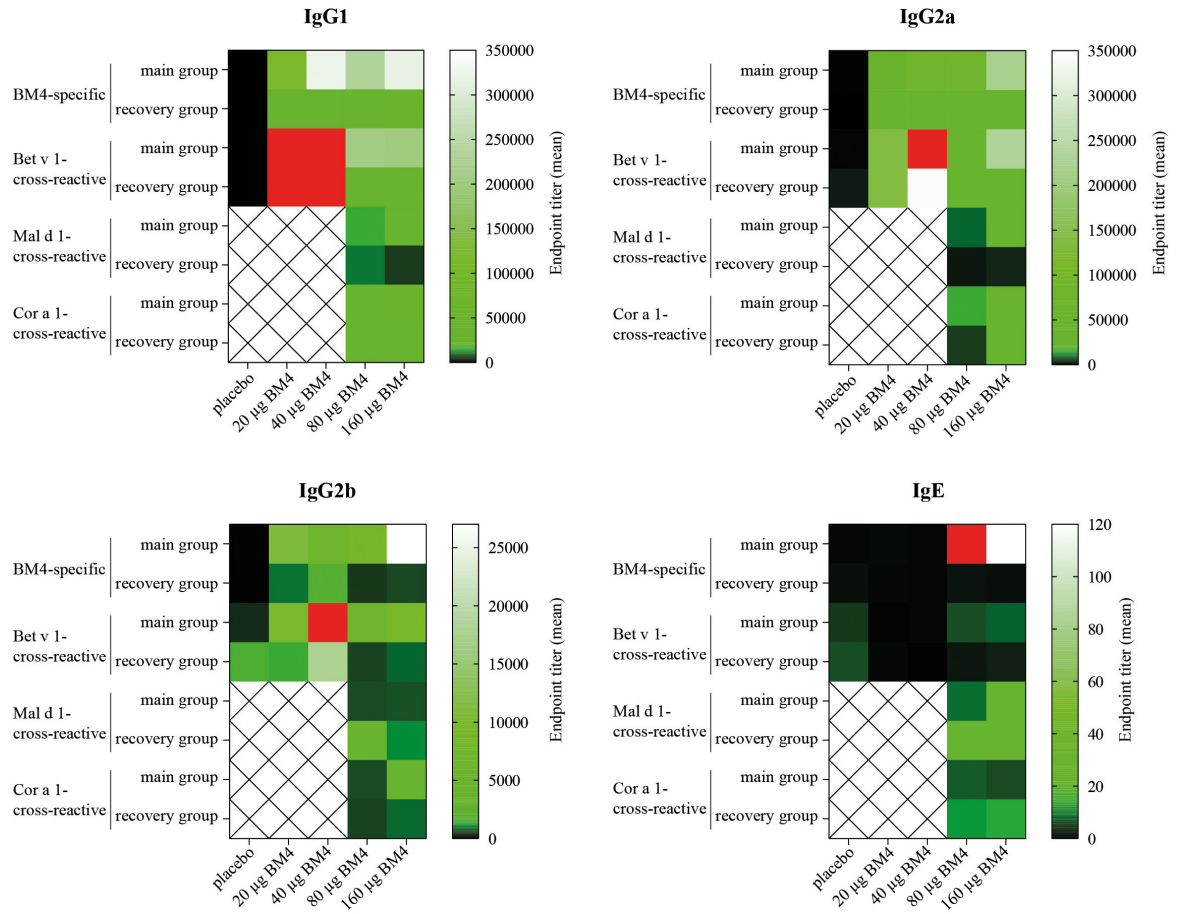

FIGURE 6 | Comparative heat map showing the mean BM4-specific, and Bet v 1-, Mal d 1-, and Cor a 1-cross-reactive titers of each treatment group (either 0, 20, 40, 80, or 160 µg BM4) of Wistar rat sera of the repeated immunization model for each IgG subclass as well as for IgE. Red squares were used to highlight values that exceeded the titer range shown in the heat map.

Mal d 1, immunization of 20 µg BM4 were not significantly different compared to placebo. For the placebo recovery group, an inhibition of 24% (Bet v 1), 13.4% (Cor a 1), and 25% (Mal d 1) was observed.

In summary, a mean difference of 61.8% (Bet v 1), 30.8% (Cor a 1), 15% (Mal d 1), and 66.4% (Bet v 1), 32.3% (Cor a 1), 11.65% (Mal d 1) was observable in the main and recovery group, respectively, compared to placebo. For Bet v 1, the inhibition results positively correlated with the respective cross-reactive IgG1 and IgG2a titers ($r = 0.8153$, $p < 0.0001$, and $r = 0.6972$, $p < 0.0001$, respectively), whereas the IgG2b titers showed a relatively low correlation with the inhibition of human IgE binding ($r = 0.3507$, $p < 0.0008$, **Figure 7B**).

BM4-Induced Wistar Rat IgG Antibodies Are Able to Effectively Inhibit CD23-Mediated Human IgE-Facilitated Bet v 1 Binding

In addition to the inhibition ELISA data, we sought to investigate whether the BM4-induced antibodies are able to interfere with the CD23-mediated binding of Bet v 1 facilitated by human IgE. Therefore, the inhibition facilitated antigen-binding (inhibition FAB) assay protocol by Shamji et al. (26) was adapted accordingly. Sera of Wistar rats receiving repeated immunizations with BM4 (20 and 40 µg) caused a significant reduction of Bet v 1-IgE complexes binding to B cells ($p < 0.0001$) compared to placebo in the main as well as in the recovery group (**Figure 8A**). A mean decrease of 86% of Bet v 1-IgE complexes was observed for sera of the main group, whereas for the recovery group a treatment with both dosages of BM4 resulted in a lowered reduction of 66% (40 µg) and 74% (20 µg). Although the IgE of rat sera were heat-inactivated in order to solely assess the capability of human IgE antibodies to provoke allergen-IgE complex formation, we screened the rat sera toward their insufficiency to cause the formation of complexes in the absence of human IgE (controls without human serum). None of the heat-inactivated rat sera was *per se* able to form allergen-IgE complexes. To determine the percentage of inhibitory activity of the BM4-induced IgG antibodies we converted the experimental values after baseline subtraction (untreated cells) by inverting the percentage of allergen-IgE complexes (mean of uninhibited

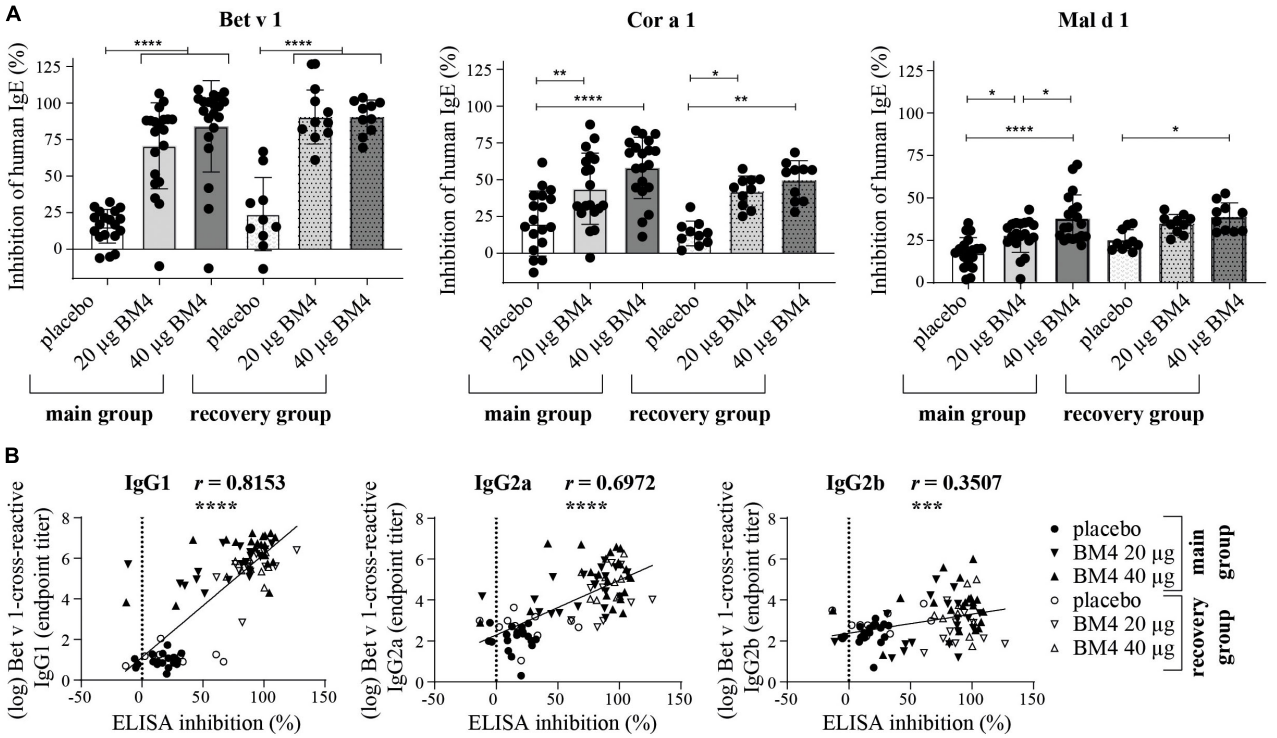

FIGURE 7 | Inhibition of human IgE binding to Bet v 1, Cor a 1, and Mal d 1 by sera derived from Wistar rats receiving repeated immunizations of either 0 (placebo), 20, or 40 µg BM4 **(A)**. The column bar charts depict the mean and standard deviation of each data set. Statistics were calculated using a one-way ANOVA and a Dunnett's multiple comparisons test comparing the individual main or the recovery groups among each other. Correlation of log-transformed Bet v 1 cross-reactive IgG1, IgG2a, and IgG2b titers with inhibition ELISA data using the Pearson correlation test **(B)**. ns >0.05; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$.

controls minus the IgE-Bet v 1 complex formation values). An efficient mean inhibitory activity of 95% was found for sera of rats receiving BM4 of the main group ($p < 0.0001$), and 74% (40 µg, $p < 0.0001$) to 82% (20 µg, $p < 0.0001$) for those of the recovery group (**Figure 8B**). A positive correlation was observed between the FAB inhibition results and the Bet v 1-cross-reactive IgG1 and IgG2a titers ($r = 0.8395$, $p < 0.0001$, and $r = 0.7102$, $p < 0.0001$, respectively), whereas there was a relatively low correlation with the Bet v 1-cross-reactive IgG2b titers ($r = 0.2947$, $p < 0.0053$, **Figure 8C**).

DISCUSSION

Allergen-specific immunotherapy is the only curative approach modulating the causative cellular and molecular origin of allergic diseases by skewing the Th2-biased IgE-mediated inflammatory response toward an anti-inflammatory immune response resulting eventually in allergen immunotolerance (22, 23). The underlying protective mechanisms involve the induction of antigen-specific regulatory T and B cells, and the secretion of immunosuppressive cytokines, such as IL-10 (23, 33, 34). Another key regulatory molecular event in the induction of immunotolerance by AIT and, consequently, the reduction of allergic symptoms, is the increased production of allergen-specific IgG4 antibodies. These antibodies are able to prevent antigen recognition by IgE and the consecutively occurring effector functions, including degranulation of mast cells and basophils, histamine release, and the recruitment of various inflammatory cells (25, 35). This allergen-neutralizing functional aspect of serum IgG4 has been reported as a sufficient predictive biomarker for AIT efficacy (25, 26, 32). Following this idea, we aimed to monitor the IgG antibody levels induced by a hypoallergenic variant of Bet v 1 in two different naïve mammalian animal models (i), and to characterize their specificity toward wild-type Bet v 1 (ii) as well as to the Bet v 1-associated food allergens Mal d 1 and Cor a 1 (iii). We further analyzed the functionality of the IgG antibodies regarding their activity to prevent human IgE-allergen binding (iv). We found that the treatment with BM4 induced elevated Bet v 1-specific IgG antibody titers in both mammalian species. In Wistar rats, high BM4- and Bet v 1-specific IgG1, IgG2a and IgG2b levels (10^4 to 10^6) were induced even upon receiving just a single immunization. Since neither Wistar rats nor NZW rabbits possess the IgG4 subclass, we sought to cover the complete IgG repertoire among these species, except for rat IgG2c that is primarily recognizing carbohydrate epitopes (36–38). While rats have four IgG subclasses (IgG1, IgG2a, IgG2b, and IgG2c), NZW rabbits only possess a single IgG subclass (29). Although an interspecies comparison of IgG subclasses is difficult, the general consensus is that rat IgG1 and IgG2a functionally resemble human IgG4/murine IgG1.

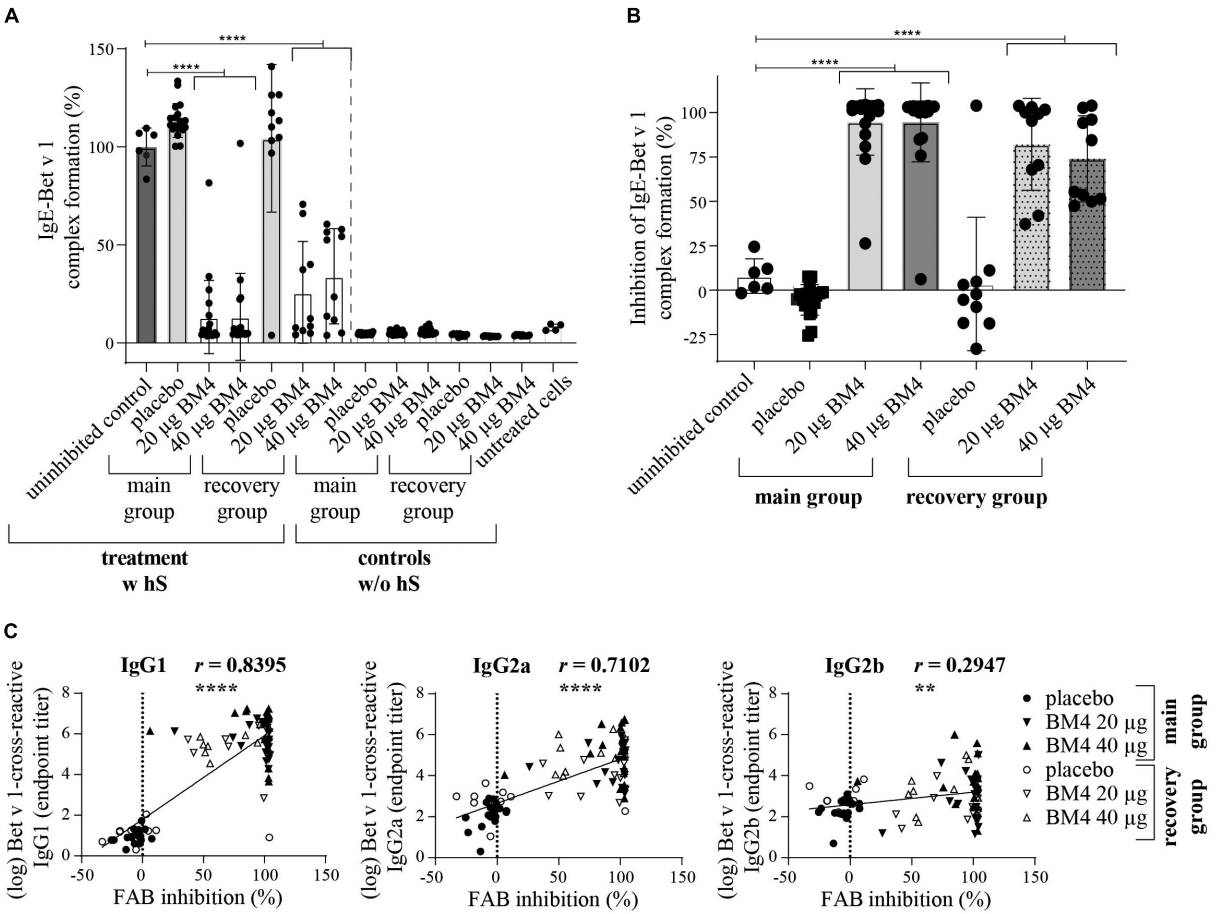

FIGURE 8 | BM4-induced rat IgG antibodies are able to sufficiently inhibit CD23-mediated complex formation of Bet v 1 and human IgE antibodies on B cells. The inhibition FAB assay was performed with sera of rats of the repeated immunization model receiving either 0 (placebo), 20, or 40 µg BM4. The percentage of IgE-Bet v 1 complex formation **(A)** was normalized toward the mean of the uninhibited control (100%). Untreated cells were used as negative controls. For the evaluation of unspecific rat IgE-Bet v 1 complex formation, controls without human serum (w/o hS) were included; w hS, with human serum. The percentage inhibitory activity was calculated by subtracting the baseline activation of untreated cells followed by subtraction of the normalized IgE-Bet v 1 complex formation values from 100 **(B)**. The column bar charts depict the mean and standard deviation of each data set. Statistics were calculated using a one-way ANOVA and a Dunnett's multiple comparisons test comparing each treatment group with the uninhibited reference. Statistics of the controls (w/o hS) were excluded due to simplicity reasons but significantly lower than the uninhibited control (positive control, $p = 0.0001$). A Pearson correlation test was performed to analyze if the determined Bet v 1-cross-reactive IgG1, IgG2a, and IgG2b titers were correlating with the inhibition of IgE-Bet v 1 complex formation **(C)**. ns >0.05; $^{**}p \leq 0.01$; $^{****}p \leq 0.0001$.

Conversely, rat IgG2b corresponds to human IgG1/murine IgG2a/2b (36–41).

In mice, immunizations with BM4 resulted in a boosted Bet v 1-specific IgG1 and IgG2a antibody secretion and were associated with a Th1-skewing effect (18). Immunizations with Bet v 1, on the other hand, hardly induced an upregulated secretion of these antibodies, which is in line with our findings of the repeated immunization model showing increased Bet v 1-specific titers compared to BM4-specific antibodies. Since the structure of BM4 differs fundamentally from wild-type Bet v 1, we hypothesize that this treatment-induced antibody cross-reactivity mainly occurs due to the recognition of sequential epitopes by the IgG antibodies. However, in contrast to our findings, where BM4 did hardly affect Bet v 1-specific IgE antibody levels, BM4 also resulted in a quick elevation of Bet v 1-specific IgE in a Balb/c immunization model (18). Especially in the case of Bet v 1, the dominant IgE epitopes mostly possess a conformational nature, which would explain why elevated BM4-specific IgE titers derived from our repeated immunization (80 and 160 µg BM4) hardly recognized Bet v 1, Mal d 1, and Cor a 1 (42). In the recovery group, no BM4-specific IgE was detectable, most likely due to the short-lived nature of IgE. Due to the non-existence of IgE memory cells, the production of IgE antibodies depends on a constantly active Th2-biased inflammatory milieu promoting the class switching of B cells to IgE-secretory plasma cells (43). In immunization models such an inflammatory milieu is provided by the co-administration of an adjuvant. Therefore, treatment discontinuation resulted in a drop of IgE titers in the recovery group. The reduced reactivity of Mal d 1 and Cor a 1 by BM4-induced antibodies compared to Bet v 1 most likely results from the differences in sequence identity between the recombinant wild-type and the mutant proteins (BM4:Bet v 1

96.86%; BM4:Mal d 1 59.75%; BM4:Cor a 1 64.78%). This explains why Mal d 1 is even less recognized than Cor a 1 in both, the main as well as the recovery group of the repeated immunization model, and why human IgE binding to Mal d 1 is also less efficiently inhibited.

By using the inhibition ELISA and inhibition FAB assay, we were able to address the functional relevance of the BM4-induced antibodies and found that sera of BM4-imunized rats were able to significantly neutralize the binding of Bet v 1 to human IgE. The inhibitory activity induced by BM4 even remained after treatment discontinuation. This and the fact that the results obtained for both assays positively correlated with the Bet v 1-cross-reactive IgG1 and IgG2a titers corroborates to our assumption that this blocking activity occurs due to the increased Bet v 1-specific IgG levels. However, further experiments are needed in order to clearly state, which rat IgG subclass is responsible for the IgE blocking activity. Also, additional *in vitro* approaches addressing the functionality of the induced IgG antibodies in other functions of effector cells, such as the basophil activation test or mediator release assays, would provide further insights on the inhibitory capacity of the BM4-induced IgG antibodies (27, 44). In a therapeutic *in vivo* model, increased Bet v 1-specific IgG1 levels induced by intraperitoneal injections of BM4 were associated with a downregulation in Bet v 1-triggered mediator release using rat basophilic leukemia cells, as well as a general decrease of Th2-mediated inflammation as judged by BALF IL5 cytokine secretion and lung infiltrating cells (20).

In general, treatment with recombinant Bet v 1 was proven successful providing a potential alternative to extract-based AIT vaccines (45, 46). By providing an efficient hypoallergen-based drug product to the market that, ideally, tackles the negative aspects of AIT (long treatment duration and treatment-induced side effects), would certainly increase the popularity of AIT and demonstrate as well as magnify its to date unexploited potential. Other hypoallergenic Bet v 1 derivatives were lacking statistical difference regarding their efficacy compared to current birch pollen extract-based AIT protocols (47). Our data, showing that BM4 induces high and sustainable IgG levels in two different mammalian species, highlight the suitability of BM4 as a hypoallergenic drug candidate for birch pollen AIT, with the potential of reducing birch-associated PFAS symptoms.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the National Animal Experiment Board of Finland. Ethical approval for using human serum of allergic patients was obtained by the Dutch Ethical Committee (number: NL65758.018.18).

AUTHOR CONTRIBUTIONS

LA devised and performed most experiments, wrote the manuscript, and created the figures. AB and MG conducted the experiments. PC, U-MJ, and EY designed, organized, and performed the *in vivo* models. FS provided the formulated GMP drug products for the *in vivo* experiments and BM4 for the ELISAs. LJ and RV devised the experiments and interpreted the data. All authors read the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.02118/full#supplementary-material

FIGURE S1 | SDS-PAGE gel of the recombinantly produced proteins used in this study. M, Pierce Unstained Protein Molecular Weight Marker (Thermo Fisher Scientific, Waltham, MA, United States).

FIGURE S2 | CD spectra of BM4, Bet v 1, Mal d 1, and Cor a 1 recorded at 20°C between 190 and 260 nm **(A)**. Amide I and II and second derivative of amide I IR-spectra of the recombinant proteins recorded at 25°C **(B)**. The percentage of α-helical and β-strand-like content of secondary structural elements was calculated from the IR-spectra, and a comparison of the experimentally collected data for Bet v 1 and Mal d 1 with the respective theoretical values deposited on PDB is shown (code: 4A88 and 5MMU, respectively).

FIGURE S3 | Definition of experimental conditions for the FAB assay. A titration of Bet v 1 concentration **(A)**, amount of human reference serum and anti-IgE antibody **(B)** was performed. Black arrows indicate the conditions used for the final inhibition FAB assay; w hS, with human serum (10 μl).

FIGURE S4 | Inhibition FAB assay gating strategy for the analysis of Bet v 1-IgE complex immobilized on CD23-expressing EBV-transformed B cells. Cells were gated based on scatter light (FSC, SSC) characteristics, followed by doublet discrimination (SSC area versus SSC height). Living B cells were gated and analyzed toward anti-IgE binding (FITC, histogram, **A**) and/or CD23 (PE) expression (dot blot, **B,C**). Only the anti-IgE$^+$ gated cells were considered for the analysis in **Figure 7**. The same gates were used for each sample. Results for positive control (uninhibited reference) and untreated cells (negative control) are shown.

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REVIEW
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The Potential of Clinical Decision Support Systems for Prevention, Diagnosis, and Monitoring of Allergic Diseases

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Clinical decision support systems (CDSS) aid health care professionals (HCP) in evaluating large sets of information and taking informed decisions during their clinical routine. CDSS are becoming particularly important in the perspective of precision medicine, when HCP need to consider growing amounts of data to create precise patient profiles for personalized diagnosis, treatment and outcome monitoring. In allergy care, several CDSS are being developed and investigated, mainly for respiratory allergic diseases. Although the proposed solutions address different stakeholders, the majority aims at facilitating evidence-based and shared decision-making, incorporating guidelines, and real-time clinical data. We offer here an overview on existing tools, new developments and novel concepts and discuss the potential of digital CDSS in improving prevention, diagnosis and monitoring of allergic diseases.

Keywords: CDSS, digital health, allergy, clinical decision support systems, prevention

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THE EVOLUTION OF CLINICAL DECISION SUPPORT SYSTEMS IN THE CONTEXT OF PRECISION MEDICINE

Origins of Digital Clinical Decision Support

Researchers and engineering technologists have been developing and exploring computerized decision support systems (DSS) for approximately 50 years (1). In their early stages, DSS were categorized according to their methodology, ranging from data-oriented approaches, whose systems merely extracted information, to model-oriented concepts, mostly focused on decision processes. Alongside technological advances, this kind of computerized tool became increasingly powerful and elaborated, offering solutions for the processing of complex data sets and their integration in decision algorithms providing data-driven suggestions to the user (2). The ability to capture expert knowledge, guidelines and reasoning techniques, together with the automation of rules via identification of key attributes led to the development of new digital support opportunities for healthcare providers in their clinical routine (3).

Abbreviations: AH, antihistamines; AIT, allergen-specific immunotherapy; AMA, American medical association; AMSS, allergy management support system; AR, allergic rhinitis; ARIA, allergic rhinitis and its impact on asthma; CDSS, clinical decision support system; COPD, chronic obstructive pulmonary disease; DSS, decision support system; eAMS, electronic Asthma Management System; EHR, electronic health record; GP, general practitioner; HCP, health care professionals; INCS, intranasal corticosteroids; PDSS, pharmacy decision support system; VAS, Visual Analogue Scale.

Methodologies

The development of computerized clinical support tools can be grouped according to the following main methodologies: (a) information retrieval tools to answer clinical questions and manage medical information (4); (b) logical models for the assignment of categories for medical standard measurements (5), characterized alerts and reminder systems (6); (c) probabilistic and data-driven prediction algorithms to improve patient outcomes (7); and (d) a modeled combination of formal and heuristic algorithms supporting physicians in their decision on the individual deployment of evidence-based solutions (8, 9). Today, these methodologies carry the potential of becoming an innovative resource for digital augmentation of clinical care, once scientifically and clinically validated. To ensure patient safety, technologies with a potential impact on medical decisions need to undergo the registration and certification process for medical devices at the respective regulatory authorities (10, 11).

Advantages of Digital Decision Support

Due to the growing amount of health data, delivering personalized precision medicine has become a challenging task (12). Large sets of information are not only derived from complex diagnostic test systems, genetic analyses and –omics approaches, but also patient-generated monitoring data, exposure information and/or the surveillance of physiological parameters via smart devices and sensors (13). In parallel, electronic health records (EHR) have become a common platform to bundle patient data and clinical decision support systems (CDSS) are frequently connected to these critical information hubs (6). This is where trained algorithms and validated computerized tools can assist health care professionals (HCP) in efficiently interpreting complex data sets and keeping the overview on the individual health status of a patient (14). Automated monitoring and alerts systems further enable a continuous personalized treatment follow-up and allow an early intervention in the case of side effects or insufficient success.

Challenges and Limitations of Clinical Decision Support

Although digital decision support tools can be potentially useful in optimizing clinical workflows, it is important to mention that they are by no means able to replace a trained healthcare professional. In addition, several challenges need to be considered. Clinical work often includes hectic situations and a broad spectrum of patients with different conditions, comorbidities, and treatment plans being treated by one doctor. Therefore, a support tool needs to be extraordinarily user-friendly, ideally adaptable to individual settings with a high level of interoperability and include a solid risk management as any medical device. Apart from technical challenges like an optimal human-computer interface there are particular aspects to be addressed within different methodologies (1). To give an example: alert systems with an high alert frequency (e.g., for potential drug interactions) may cause a alert fatigue in the user, who may decide more frequently to disregard the suggestions. As the user is a trained professional, this may not seem crucial at first sight. However, a negative impression of fatigue may lead to an adverse predisposition toward other, potentially more user-friendly, technologies. This example reflects the diversity of challenges, which need to be considered in the development of decision support tools. They are not only of technical nature but also related to adoption by different stakeholders and integration into a broad spectrum of pre-existing settings.

Adoption of Decision Support Technologies by Health Care Professionals

The prospective collection of clinical and diagnostic data provides valuable insights into disease endo- and phenotypes and has the potential to offer distinct advantages in the field of chronic diseases such as allergy and asthma (15). Digital technologies potentially allow continuous disease monitoring with agile adaptation strategies decided on by the physician to improve patient outcomes and quality of life, especially as part of a chronic disease management process. However, physicians are core to the medical decision process and accountable for their choices. If computer algorithms are aiding these, reliability and accountability are key elements to be addressed prior to a widespread adoption of any digital solution. This challenge may be one of the reasons for low adoption rates for new digital tools in clinical routine (16), although recent data from the American Medical Association show an increased interest among physicians in digital support tools (from 28% in 2016 to 37% in 2019) (17). The category CDSS in this assessment included any modules and integrated mobile applications in conjunction with EHR, also enabling the remote monitoring of patient-related parameters and automated integration of the results in the central data set. Interestingly, the adoption rates for monitoring tools alone, without integration in a CDSS, increased from 12% (2016) to 16% (2019) for remote efficiency monitoring and from 13% (2016) to 22% (2019) for remote management tools for chronically ill patients (17) (**Figure 1**). It has to be stated, though, that reported use does not necessarily reflect any improvement of patient outcomes (18). In order to enable a critical evaluation and smooth implementation of new tools in healthcare systems, it is important to ensure that professionals are adequately trained on the benefits and challenges of CDSS before applying them in clinical practice (9).

CLINICAL DECISION SUPPORT FOR ALLERGIC DISEASES

Allergic diseases are clinically and immunologically multifaceted, as well as pathogenetically heterogenous (15), which makes an evidence-based clinical diagnosis rather challenging. Moreover, the ratio of allergists per allergic patient is in general rather low and highly heterogenous in different countries (19, 20). As a first point of contact, most allergic patients see a primary care doctor, who frequently lacks the knowledge, confidence or resources to meet their specific needs due to insufficient training (21). Independently from the individual level of specific training, clinicians are confronted with a broad spectrum of clinical manifestations, such as allergic rhinitis (AR), asthma, atopic eczema, food allergies, anaphylaxis, drug allergies or occupational

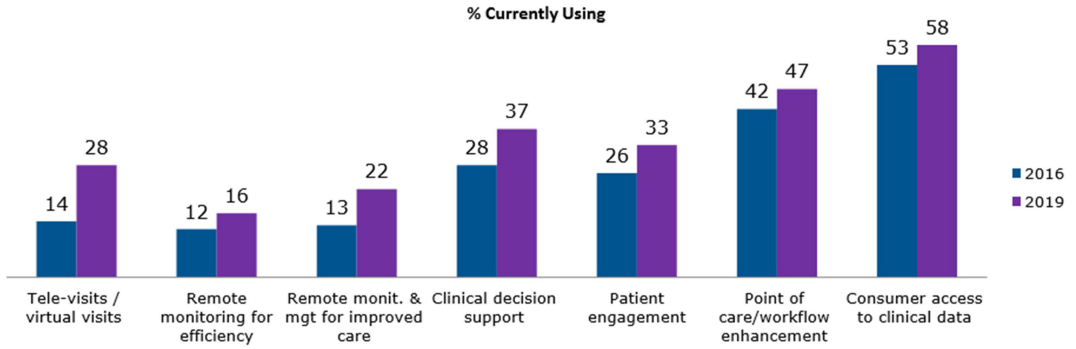

FIGURE 1 | The use of digital health tools among United States physicians in 2016 and 2019. The survey has been performed by the American Medical Association (AMA) among 1300 (1359 respectively) physicians working in different clinical settings. © 2020 American Medical Association. Reprinted with Permission [17]. https://www.ama-assn.org/.

allergies, whose pathogenesis is heterogeneous. In addition, the nomenclature and classification of these allergic diseases is being challenged (22, 23) and the guidelines for diagnostic work-ups change over time (24, 25) or differ between different regions (26, 27).

In this context, computerized decision support concepts are becoming a potentially valuable tool to support clinicians in evaluating large data sets and taking into account complex guidelines. Electronic health (eHealth) technologies, especially mobile health (mHealth) tools, have become more and more popular and provide valuable clinical information on patients. However, tools merely providing information without concrete suggestions for diagnostic and therapeutic decisions should not be considered a CDSS. The software tools and mobile solutions discussed here have the potential to enhance medical decisions at the point-of-care mainly with (A) targeted patient information, (B) guideline- and evidence-based clinical knowledge, and (C) prospectively collected data (patient-/sensor-generated). A positive effect of clinical decision support on the practitioner's performance has already been shown for several chronic health conditions (28–31). However, more studies are needed to assess the impact on short, medium-, and long-term patient outcomes. In allergy care, several concepts for CDSS have been created, addressing different diseases and settings with the aim of improving detection, diagnosis and diseases management (32, 33). In most cases, the main target is to empower not only the allergist, but also the general practitioners (GPs) and even the patient, at public contact points such as pharmacies. The following paragraphs will give an overview on existing solutions, concepts and potentials for future developments.

DECISION SUPPORT SYSTEMS FOR THE MANAGEMENT OF ALLERGIC RHINITIS

Developments of the Allergy and Its Impact on Asthma (ARIA) Consortium

Several expert groups have elaborated algorithms and support tools to facilitate screening, diagnostic precision, early optimization of therapy and user-friendly monitoring of chronic respiratory allergic diseases. Among these, the ARIA consortium elaborated a detailed decision algorithm based on clinical scenarios for AR patients treated with symptomatic drugs. The development process involved a key opinion leader consensus on specific treatment recommendations, which has been published transparently (34). The authors count on a broad experience on the collection of symptom data via mobile health technology (35–37) in which the CDSS is planned to be integrated. However, the system is not yet publicly available online and publications on its implementation are expected to be published soon.

DSS in the Pharmacy

Another ARIA initiative to provide front-line decision support has been proposed to implement integrated care pathways for AR at the level of community pharmacies (37). Patients suffering from AR often self-medicate with over-the-counter-drugs with correspondingly poor results (38); hence, pharmacists assume an important role in the care pathway for patients suffering from respiratory allergies (39). An open intervention study among German pharmacists revealed that pharmacists failed to ask several questions essential to make a diagnosis, confirm the appropriateness of self-medication and the drug choice (40). When implementing a pharmacist decision support system (PDSS), the pharmacists asked seven (78%; IQR 5.25–9) instead of two (22%; IQR 1–3) of the nine required questions. The use of the PDSS resulted in a significant improvement of patient evaluation and required only 1.5 min (40). Notwithstanding its limitations, this study pioneers the implementation of a DSS for AR symptomatic treatment at pharmacy level. The ARIA consortium also underlined the importance of pharmacists for integrated care pathways of respiratory allergic diseases (41). The authors recently proposed a CDSS supporting pharmacists in monitoring the patient's symptom control and adjusting symptomatic treatment accordingly (42) (**Figure 2**). This comprehensive approach to supporting pharmacists in their front-line role in allergy care is a promising concept. The evaluation or validation of the system in a real-life setting is therefore a research priority.

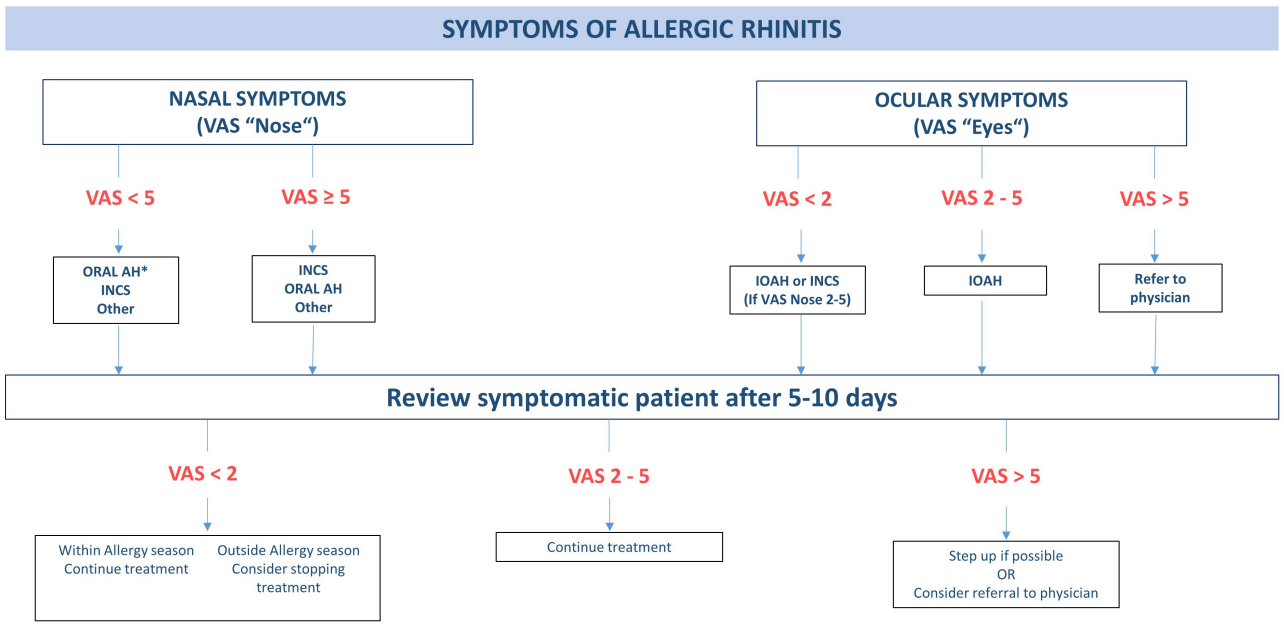

FIGURE 2 | Decision algorithm for treatment of allergic rhinitis in the pharmacy. AH, antihistamine; INAH, intranasal antihistamine; INCS, intranasal corticosteroid; IOAH, intraocular antihistamine. *INCS if coexisting asthma. Visual Analogue Scale (VAS) nose/eye: "How much are your nose/eye symptoms bothering you today?" (0 = not at all bothersome, 10 = extremely bothersome). Adapted from Tan et al. (38).

CDSS for Allergen Immunotherapy

A different concept has been followed in the development of a support system facilitating the precise prescription of allergen-specific immunotherapy (AIT), i.e., the only disease-modifying treatment for AR (33). Decision support for AIT prescription is particularly valuable in geographic areas with high rates of poly-sensitization and overlapping flowering periods of many allergenic plants; in these areas the distinction between genuine and cross-reactive sensitization is extremely difficult and the precise identification of the pollen causing allergic symptoms is crucial for AIT efficacy (43, 44). The traditional diagnostic approach, based on a retrospective clinical history and extract-based skin prick or IgE testing, is suboptimal due to several reasons. On one hand, the clinical history can be unreliable due to a recall bias, especially when seasonal symptoms occurred several months before the patient's interview. On the other hand, extract-based diagnostic tests are unable to distinguish a genuine sensitization from cross-reactivity due to poor standardization and high sequence homology. Component-resolved diagnostics (45, 46) and prospective symptom recording via eDiary application were proposed as potential solutions to support the clinician in the detection of genuine sensitizations and confirmation of their clinical relevance (47). However, both tools generate rather large data sets which are difficult to work with in a busy clinical setting, providing an optimal opportunity for digitally facilitated and guideline-oriented decision support. The strength of this particular approach lies in a blended care approach combining the expertise and experience of the doctor in personal visits with digital monitoring support. Further, a looped design, facilitates a continuous re-evaluation based on real-time patient-recorded symptom data and information on the respective allergen exposure. The physician plays a strong role in this CDSS (named @IT-DSS) as it is customizable with regard to thresholds for test positivity according to personal experience and local environmental and epidemiological conditions (**Figure 3**). The @IT-DSS has been tested in a clinical pilot and multicenter study where the combination of face-to-face visits with symptom monitoring via eDiary showed promising results in terms of patient adherence, a common challenge for most monitoring apps. Although the daily use of the app for symptom and medication monitoring declined slowly over time, the observed drop in adherence was significantly lower than in other studies based on the spontaneous download of apps from the respective app stores (47). Further results, especially on the efficacy of the CDSS are expected to be published soon.

DIGITAL DECISION SUPPORT FOR ASTHMA CARE

A multitude of tools for asthma care have been developed, ranging from reminder systems (48), monitoring apps (49, 50), smart devices (51, 52) up to comprehensive digital care platforms (53). Although many of these technologies deliver valuable information for clinical decision-making, not all classify as a CDSS. Like for AR, also for asthma care there is a great heterogeneity in concepts, user groups and outcome assessments of digital health tools and CDSS (54).

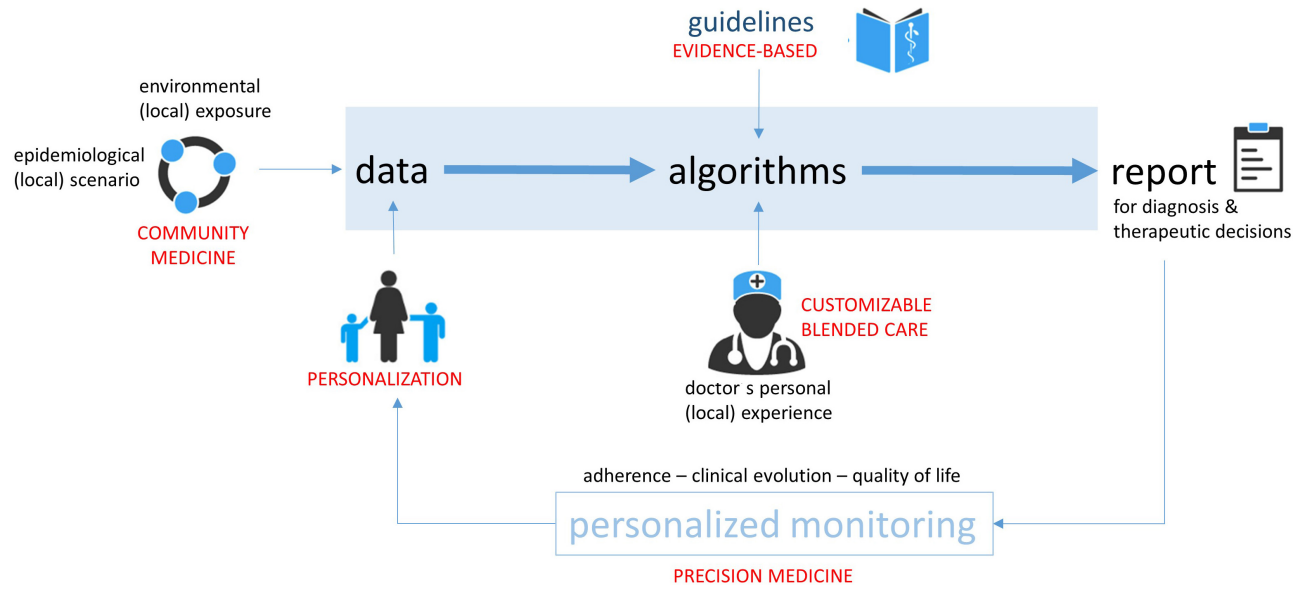

FIGURE 3 | General concept of a guideline-based, looped and customizable clinical decision support system. Thresholds can be adapted by the clinician according to his/her clinical experience and local characteristics in a blended care setting. Patient and environmental data are continuously collected, and updated reports created. Adapted from Matricardi et al. (33).

Interestingly, the impact of these systems seems to depend on the targeted user group. A systematic review on CDSS impact, when used by healthcare professionals with expertise in asthma management, reported a low effect on asthma control, mainly due to very low usage rates. The usability and user-centered design of CDSS are essential to achieve a sufficient adoption and high impact of the technologies (55). Similarly, a systematic review showed that CDSS are effective in improving care of patients with asthma and chronic obstructive pulmonary disease (COPD) when used in primary health care settings (56). The authors further underline that despite the positive impacts assessed in the randomized, controlled trials, the effects of the CDSS on user workload and efficiency, safety, costs of care, provider, and patient satisfaction remain understudied (56). Recently, the use of an electronic Asthma Management System (eAMS) improved the quality of asthma care for adult patients in a 2-year interrupted time-series study of usual care (year 1) vs. eAMS (year 2) at three Canadian primary care sites (34). However, further work on the identification of facilitators and barriers for uptake by clinicians is being done and randomized controlled trials assessing patients' outcomes are still needed (34). Other promising concepts, such as the myAirCoach system (53), combine the use of a smart adapter for inhalers, with an indoor air-quality monitor, a physical activity tracker, a portable spirometer, a fraction exhaled nitric oxide device, and an app in one platform. Although these tools have only been tested in a small number of patients and not yet been included in a CDSS, they collect valuable information for personalized decision-making and it remains to be tested whether this concept can potentially be scaled up for a broader adoption. In addition, diagnostic approaches, such as serological multiplex tests for allergen- and virus-triggered asthma (57) could deliver important information for personalized asthma management, potentially supported by a CDSS.

DIGITAL SUPPORT TOOLS FOR OTHER ALLERGIC DISEASES

Several other tools have been developed to digitally augment allergy care namely supporting diagnosis and management in a primary care setting (58) or among junior clinicians (59), as well as for specific allergic diseases such as drug hypersensitivity (60–63), food allergies (64) and urticaria (65).

Allergy Management Support for Primary Care

Based on literature review, focused interviews and testing in primary as well as secondary care patients, a group of allergists, dermatologists, GP and researchers from the Netherlands developed a guideline-based allergy management support system (AMSS) for allergy diagnosis and management in primary care settings (58). The AMSS interprets data from a 12-item multiple choice questionnaire with test results for allergen-specific IgE antibodies. After applying the algorithm to data from 118 patients, the authors identified 150 different diagnostic categories of AR, asthma, atopic dermatitis, anaphylaxis, food allergy, hymenoptera allergy, and other allergies. When comparing the AMSS outcomes with specialists' recommendations as gold standard, an agreement of 69.2% (CI 67.2–71.2) was observed. In a clinical study on the implementation of the system, GPs showed a significant improvement in allergy

diagnosis and reported a positive impact of the system on their clinical routine. However, the decision-making on medication and referral has not been affected by the use of the AMSS (66).

Diagnostic Interpretation Support

With the aim of supporting the diagnostic decisions on AR among junior clinicians, a group of developers and researchers from India established a CDSS based on the clinical history and intradermal test results for 40 locally relevant allergens. The authors developed and validated the algorithm with data from 857 allergic patients and found that the CDSS differentiated the presence or absence of AR with an accuracy of 88.31% compared to the opinion of allergy experts (gold standard). Further, the study assessed the preferred CDSS model among junior clinicians who indicated to prefer a rule-based approach for its intelligible knowledge model (59).

Drug Hypersensitivities

Several mobile applications have been created to assist doctors in the assessment of the causality, severity and preventability of adverse drug reactions (60). The use of a clinical decision support tool for non-allergists evaluating inpatients reporting penicillin allergy led to a twofold increase in penicillin or cephalosporin prescription compared to standard care (61). Similarly, a significant number of children could be de-labeled from penicillin allergy by primary care physicians following an algorithm for risk stratification and further work-up, including a telemedicine screening and single dose oral challenge (62). A systematic review has been conducted to assess the potential of computerized physician order entry systems with built-in clinical decision support for an improved management of drug hypersensitivity. The authors concluded, that the heterogeneity in recording of adverse events represents a considerable challenge for a unified interpretation of recorded data (63, 67). Further, an alert fatigue has been described in several studies due to a lack of alert specificity (67). As the alert systems are built to point out any potentially dangerous drug interaction of allergenic threat for patient safety, these alerts can become very numerous considering the large variety of drugs and potential interactions. Of course, clinical considerations of the individual patient need to be considered as well and studies showed that clinicians tend to ignore alerts in many cases. The frequent signaling of potential hazards may cause a certain fatigue among users which limits the possible impact of the system. Again, user-centered design seems to be essential, considering a balance between safety and alert frequency. This challenging task will need to be addressed in future developments and studies, recognizing particular needs, such as the support of primary care physicians in the management of chronic diseases where polypharmacy is the norm (68).

Food Allergies

An initiative to support pediatricians in the work-up of patients reporting allergic symptoms related to foods has been created consolidating complex guidelines for the management of food allergies into five key steps. The development of the Food Allergy Support Tool further involved rapid-cycle improvement methods to create a CDSS facilitating food allergy management in a primary care setting. Interestingly a pilot evaluation showed that physicians were uncertain about the benefits of the system. The authors name the necessary active user initiation as a potential barrier for implementation (64).

IMPROVING MONITORING AND PREVENTION BY CONNECTING STAKEHOLDERS: COMMUNITY ALLERGOLOGY

The strategy of making large data sets easily intelligible for specialized but also non-specialized healthcare providers, opens new opportunities for an efficient use of medical resources. In several countries, the numbers of specialized allergists are declining while there is a continuous increase of patients needing allergy care. This gap raises the pressure on primary health care workers and digital technologies represent a valuable tool for an intelligent distribution of work force and knowledge. This should ideally be supported by a digitally enabled availability of context-specific decision support, as guidelines may vary according to the clinical setting (primary care vs. specialist vs. tertiary care). By raising awareness at important community healthcare points such as pharmacies, an early identification of patients eligible for AIT can be fostered, always keeping in mind, that several significant barriers for the implementation of AIT still need to be overcome (69). Early interventions such as allergen avoidance can be efficiently implemented already at first contact with a primary health care physician and advice from specialists can be facilitated via remote consultations. But even beyond the purely medical field, a concept of community medicine can be revitalized via the use of comprehensive digital platforms. Patients can manage the access rights to their data and decide to share or donate them for research purposes or community projects, collaborating with environmental monitoring and public health institutions, a broad network of information is available for exploitation with the respective decision support tools.

Several of the above-mentioned support systems include the retrieval and storage of patient- and/or sensor-generated monitoring data. This provides the attending physician with comprehensive data sets for the evaluation of disease control during regular follow-up visits. Prospectively collected data on treatment adherence, quality of life, disease-specific symptoms and objective parameters such as sensor-recorded heart rate or sleep quality can be easily assessed in visual summaries or standardized scores (e.g., symptom and medication scores) which are generated in an automated fashion. At first sight, these digital platforms seem to clearly outperform traditional approaches. However, the impact of their use on diagnostic precision, treatment efficacy, safety, quality of life and treatment costs needs to be objectivized and studied in more detail with regard to allergic diseases as it has been already done in other specialties and areas of medical care (70–73).

PERSPECTIVES

In summary, digital technologies offer a vast potential to support clinicians in their actions for prevention, diagnosis, treatment, and monitoring of allergic diseases. Many different concepts are under development and in different validation stages, which opens a promising perspective for the next years. However, no tools are currently commercially available yet and time-consuming evaluations are necessary to enable the registration as a medical product. As CDSS may have a significant impact on key decisions for patient care, they need to be rigorously tested for applicability and usability in order to support clinicians in making the best choices for their patients. In addition, the interoperability with existing software systems and a smooth integration in clinical routine are significant challenges for a successful implementation. More real-life experiences and clinical studies will need to be conducted in order to extend our knowledge and foster a solid adoption in of digital support tools in the clinical routine.

AUTHOR CONTRIBUTIONS

SD supervised and performed the literature research and wrote the major parts of the manuscript. EP and MM contributed to literature screening, writing of the manuscript and formatting. PM carefully reviewed and corrected the manuscript. All authors contributed to the article and approved the submitted version.

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What Can Parasites Tell Us About the Pathogenesis and Treatment of Asthma and Allergic Diseases

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The same mechanisms that enable host defense against helminths also drive allergic inflammation. This suggests that pathomechanisms of allergic diseases represent evolutionary old responses against helminth parasites and that studying antihelminth immunity may provide insights into pathomechanisms of asthma. However, helminths have developed an intricate array of immunoregulatory mechanisms to modulate type 2 immune mechanisms. This has led to the hypothesis that the lack of helminth infection may contribute to the rise in allergic sensitization in modern societies. Indeed, the anti-inflammatory potential of helminth (worm) parasites and their products in allergy and asthma has been recognized for decades. As helminth infections bring about multiple undesired effects including an increased susceptibility to other infections, intended helminth infection is not a feasible approach to broadly prevent or treat allergic asthma. Thus, the development of new helminth-based biopharmaceutics may represent a safer approach of harnessing type 2–suppressive effects of helminths. However, progress regarding the mechanisms and molecules that are employed by helminths to modulate allergic inflammation has been relatively recent. The scavenging of alarmins and the modulation of lipid mediator pathways and macrophage function by helminth proteins have been identified as important immunoregulatory mechanisms targeting innate immunity in asthma and allergy. In addition, by regulating the activation of dendritic cells and by promoting regulatory T-cell responses, helminth proteins can counterregulate the adaptive T helper 2 cell response that drives allergic inflammation. Despite these insights, important open questions remain to be addressed before helminth molecules can be used for the prevention and treatment of asthma and other allergic diseases.

Keywords: helminths, inflammation, macrophage, asthma, immune regulation, allergy, helminth molecules, type 2 immunity

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INTRODUCTION

Helminth infections affect about 2 billion people worldwide, and children in developing countries are particularly susceptible (1). Depending on parasite burden, helminth infections can be asymptomatic or induce pathology in the host, with malnutrition, anemia, educational loss, and cognitive deficits as major consequences (2–4).

Helminths usually infest their host as tissue-migratory larvae, which establish niches in the lung, skin, liver, or intestine, where they develop, mate, and release new infectious offspring.

The host plays a critical role in this life cycle and represents a vehicle for the spread of the parasite. During evolution, helminths have learned to suppress host defense and establish chronic infections that can endure up to 20 years (5). Helminths typically induce a host protective type 2 cell–mediated immunity, which limits type 1 inflammation, reduces host tissue damage, and ensures parasite survival (6). Helminth-induced type 2 immune responses are initiated by the damaged epithelium, which secretes alarmins [interleukin 25 (IL-25), IL-33, and thymic stromal lymphopoietin] that activate and recruit type 2 innate lymphoid cells (ILCs2) and $CD4^+$ T helper 2 (T_H2) lymphocytes. The production of type 2 cytokines (IL-4, IL-5, IL-10, and IL-13), as well as granulocyte-macrophage colony-stimulating factor (GM-CSF), by these cells induces eosinophilia, M2 macrophage polarization, and the secretion of immunoglobulin G1 (IgG1), IgG4, and IgE (7–11).

A type 2 immune response is also a hallmark of asthma and allergy, suggesting that host defense and repair mechanisms of antihelminth immunity have implications for the pathogenesis and treatment of these inflammatory diseases. Epidemiological evidence on the reciprocity between helminthiases and chronic inflammatory diseases has implicated helminth infections in the prevention of allergy and asthma [see previous reviews (12–14)]. Helminths produce molecules with powerful immunomodulatory activities such as the anti-inflammatory protein-2 (AIP-2) in hookworms, the transforming growth factor β (TGF-β) mimic (Hp-TGM), the alarmin release inhibitor (Hp-ARI), or the enzyme glutamate dehydrogenase (Hpb-GDH) in the nematode *Heligmosomoides polygyrus* (15–18). The anti-inflammatory effects of helminth products observed in experimental models of asthma prompt a better investigation of helminth-(product)–driven regulation of type 2 inflammation and its underlying mechanisms of action in human settings. Current research aims to translate promising findings from rodent models to human disease and to ultimately develop helminth-based biotherapeutics for the prevention and therapy of allergy and asthma.

EPIDEMIOLOGICAL EVIDENCE FOR PROTECTIVE ROLES OF HELMINTHS IN ALLERGY AND ASTHMA

Helminths exert diverse effects on asthma and allergies depending on the species, parasite load, and time of infection (19, 20). Some parasites trigger or worsen asthma and allergic symptoms, whereas others tend to reduce the risk of these diseases (21).

Ascaris lumbricoides is a gastrointestinal parasite that passages through the lung. Studies in several countries have shown an association between *Ascaris* infection, asthma, and aeroallergen sensitization (22–24), which also correlated with *Ascaris*-specific IgE (sIgE) (25–27). A high prevalence of asthma and wheezing was particularly observed among *Ascaris*-infected children (28, 29). Similarly, infection with *Strongyloides* and *Toxocara* species correlates positively with allergic airway disorders. Infection with the intestinal parasite *Strongyloides stercoralis* was associated with an increased risk of asthma and its exacerbation (21, 30, 31) and *Toxocara* species infection resulted in increased allergy and asthma prevalence in children, which positively correlated with serum IgE levels (32–34). Thus, some helminth species trigger mechanisms such as the production of cross-reactive IgE or inflammatory mediators that promote allergic sensitization and/or asthma symptoms. A detailed understanding of how parasites drive allergic inflammation may provide important insights into pathomechanisms and therapeutic targets of allergy and asthma.

However, other epidemiological studies have shown a lower prevalence of asthma and allergic disorders during chronic intestinal helminth infections (35–37). Hookworm infection appears to be particularly protective (21), whereas the results for other parasites vary, depending on study design and the assessed outcomes. In several studies, deworming of chronically infected people increased allergic reactions and overall responsiveness of patients' immune cells (38–41), and long-term antihelminthic treatment increased skin prick test reactivity to mite in *Ascaris* species and *Trichuris* species–infected children, as well as in allergic rhinitis patients (38–40). However, effects on asthma or rhinitis symptoms were not assessed in these studies. Direct evidence for helminth-driven modulation of allergic diseases in humans came from a multitude of studies on *Schistosoma* species infection. Children infected with *Schistosoma haematobium* displayed reduced skin prick test reactivity to house dust mite (HDM) and other aeroallergens (42) and lower allergic responses to mite were observed in *Schistosoma mansoni*–infected individuals (43). Allergy-protective effects of helminths were related to the intensity and chronicity of the infection, as well as parasite burden (36, 44, 45). Furthermore, in the presence of *S. mansoni*, peripheral blood mononuclear cells from asthmatic patients released a lower amount of inflammatory type 2 cytokines and higher levels of anti-inflammatory IL-10 (46). A lower hospitalization rate was observed for asthmatic patients infected with *S. mansoni*, suggesting that infection may reduce asthma morbidity (47).

In summary, the detrimental or protective effects of helminthiases on asthma and allergy depend on the parasite species, the duration of the infection, and the immunological context. These diverse effects may be due to different antigen or mediator repertoires, which affect hallmark type 2 responses such as eosinophil recruitment, the activation of allergen-specific T_H2 cells, or IgE class switching. Worm molecules may also exert a different propensity for uptake by antigen-presenting cells and thus differentially regulate the induction of T cell responses. Finally, environmental factors, the presence of coinfections, and microbiota composition influence the immune response toward helminth parasites, resulting in different outcomes in helminth-infected individuals from different locations (48–50).

IMMUNOMODULATION OF ASTHMA AND ALLERGIC DISEASES BY HELMINTH MOLECULES

As helminth infection has been implicated in the prevention of allergy and asthma, experimental infection with helminths has been used in humans and animals to test potential therapeutic effects. Although rodent studies have demonstrated that helminth infection ameliorates allergic inflammation, clinical trials have

not found the same benefits (51–54). Encouraging results regarding the modulation of the immune response during asthma were observed in experimental infections with *Schistosoma* species, *H. polygyrus*, and *Nippostrongylus brasiliensis*. *S. mansoni* and *Schistosoma japonicum* are natural human parasites that showed anti-inflammatory effects in models of ovalbumin (OVA) and HDM allergy (45, 55–57). Protection against allergic airway inflammation (AAI) in *Schistosoma*-infected mice was associated with the upregulation of IL-10, downregulation of IL-5, and induction of regulatory T cells (Tregs), which together induce a modified type 2 immune response (58–60). Induction of Tregs and IL-10 production is also implicated in allergy-suppressive actions of the gastrointestinal mouse parasite *H. polygyrus* (61–64). Infection with *H. polygyrus* suppressed airway inflammation, by reducing eosinophil recruitment, and this effect was associated with Treg and Breg expansion and the upregulation of anti-inflammatory IL-10 (63, 65). IL-10–dependent prevention of allergy has also been observed with the parasite *N. brasiliensis*, in a model of OVA-induced airway hyperresponsiveness in rats. These studies suggest shared allergy-suppressive mechanisms among different parasite species (66).

Although animal models of helminth infection have contributed to the understanding of parasite-driven immune regulation in asthma and allergy, deeper insights into immunomodulatory effects of helminths have been provided by studying active molecules produced by parasites. The systematic analysis of parasite products by the help of proteomics and genomics has identified a comprehensive collection of helminth-derived molecules with immunomodulatory effects on asthma and allergic diseases (**Figure 1**). One of the best characterized helminth-derived immunomodulators is ES-62, a phosphorylcholine (PC)–containing glycoprotein secreted by the parasitic filarial nematode *Acanthocheilonema viteae*. ES-62 has shown protective effects in mouse models of asthma, lung fibrosis, and rheumatoid arthritis (67–70), with its immunomodulatory capacity depending on the PC moiety (71). Through PC modification, ES-62 can act on a variety of cells of the immune system, ranging from mast cells (MCs), macrophages, dendritic cells (DCs) to B cells, to affect intracellular pathways associated with antigen receptor and TLR signaling (67, 72–75). In MCs, ES-62 inhibits high-affinity IgE receptor (FcεRI)–induced degranulation, resulting in reduced ear swelling and hypersensitivity in a mouse model of oxazolone-induced skin inflammation. The suppression of MC activity by ES-62 further diminished airway hyperresponsiveness, lung pathology, and eosinophilia during OVA-induced AAI (67). The regulatory effects of ES-62 were mediated by the suppression of OVA-specific $CD4^+$ T cell proliferation, concomitant with decreased production of IL-4, IL-13, and interferon γ (IFN-γ) (76). The regulatory potential of ES-62 on MCs depended on the inhibition of MyD88-mediated signaling downstream of TLR4 and FcεRI3, which was partially dependent on IL-33/ST2 signaling (75, 77). The suppression of IL-33 signaling was also described as a key mechanism underlying the *H. polygyrus*–driven modulation of type 2 immune responses. This effect is mediated by the secretion

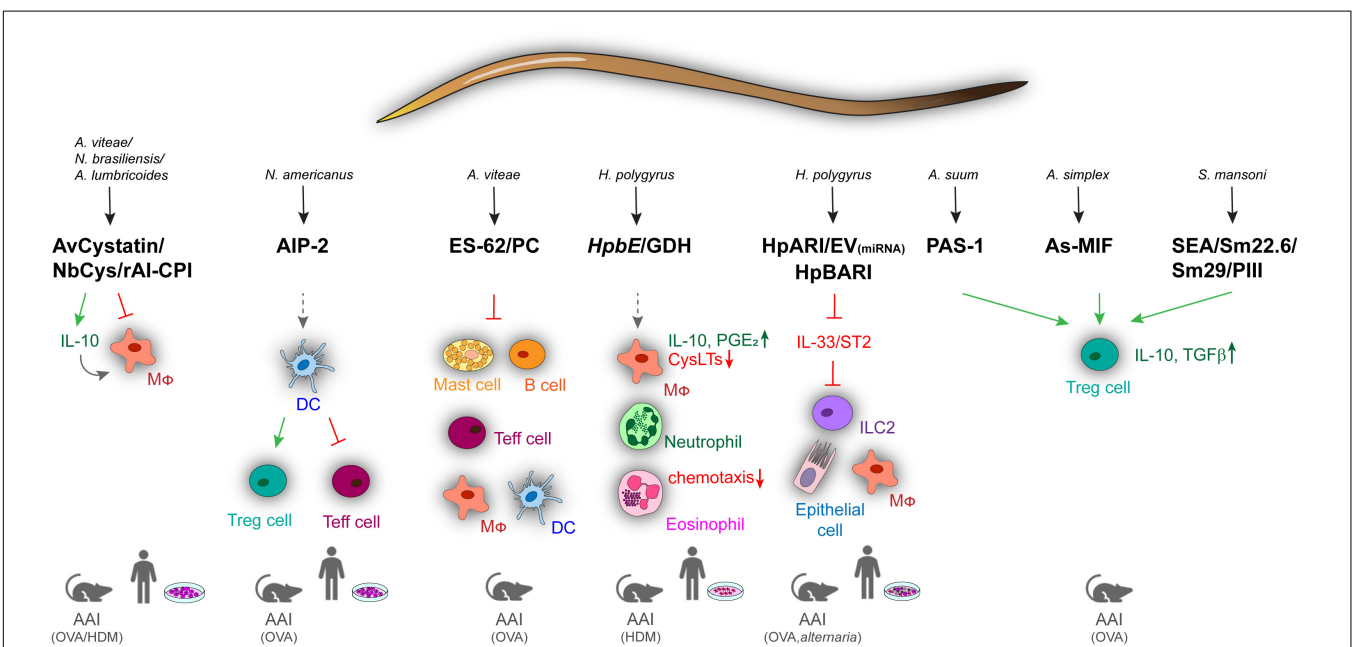

FIGURE 1 | Overview of immune regulatory helminth molecules and their mechanisms of action in mouse models of allergic airway inflammation and in human *in vitro* models. Immunomodulators from different helminths can act on a variety of cells ranging from innate to adaptive and effector immune cells. Blocking of signaling is shown by red arrows, induction by green, and modulation by spaced, gray arrows. AAI, allergic airway inflammation; AIP-2, anti-inflammatory protein 2; As, *A. simplex*; Av, *A. vitae*; Cys, cystatin; DC, dendritic cell; Ev, Extracellular vesicles; GDH, glutamate dehydrogenase; HDM, house dust mite; HpARI, *H. polygyrus* Alarmin Release Inhibitor; HpBARI, *H. polygyrus* Binds Alarmin Receptor and Inhibits; *HpbE*, *H. polygyrus* extract; Mφ, macrophage; MIF, macrophage migration inhibitory factor; Nb, *N. brasiliensis*; OVA, ovalbumin; PC, phosphocholine; SEA, schistosome soluble egg antigen; Sm, *S. mansoni*.

of an Alarmin Release Inhibitor (HpARI), which binds and blocks IL-33, and by the recently discovered Binds Alarmin Receptor and Inhibits (HpBARI) protein, which blocks the IL-33 ST2 receptor in mice and human cells (18, 78). HpARI was shown to hamper IL-33 release in human lung explants and in a human IL-33 transgenic mouse model after *Alternaria* allergen administration (18), whereas HpBARI inhibited eosinophil recruitment after *Alternaria* allergen administration (78). Another undefined *H. polygyrus* product was able to downregulate IL-33 production through the induction of IL-1β, thus promoting parasite chronicity (79). In *Alternaria*-induced AAI, *H. polygyrus* downregulated the IL-33 receptor via releasing extracellular vesicles containing microRNAs, resulting in reduced eosinophilia and improved lung function (18, 80, 81). These results indicate that vesicle release represents an efficient way to deliver immunomodulatory molecules to host immune cells. Similar to scavenging of IL-33 by HpARI, the recently identified protein p43 from *Trichuris muris* can bind IL-13 and thereby inhibit parasite expulsion (82), raising the question if this molecule can also modulate IL-13–driven airway inflammation.

Another conserved mechanism of helminth-driven immune regulation is the use of cysteine protease inhibitors (cystatins). Mammalian cysteine proteases are required for proteolytic processing of antigens, enabling presentation on MHC class II molecules and effective T cell responses. Cystatins from *A. viteae*, *Brugia malayi*, *N. brasiliensis*, *Onchocerca volvulus*, *Clonorchis sinensis*, *A. lumbricoides*, *H. polygyrus*, and *Litomosoides sigmodontis* have been shown to interfere with this process to evade antigen-induced immunity (83–94). AvCystatin from *A. viteae* mitigated airway inflammation and colitis in mice through the induction of IL-10–producing macrophages (93) and reduced pollen-specific responses in lymphocytes from allergic patients (94). Cystatin from *N. brasiliensis* (NbCys) dampened OVA-specific splenocyte proliferation, as well as IgE and cytokine production by inhibiting cathepsins L and B (89). Similar effects were observed for cystatin (rAl-CPI) from *A. lumbricoides*, which decreased T_H2 cytokine and IgE production in a mouse model of HDM-induced AAI (92).

A large repertoire of immunomodulatory molecules is also present in the egg stage of some parasites. Schistosome soluble egg antigen (SEA) from *S. japonicum* showed inhibitory effects on the development of airway inflammation in a $CD4^+$ $CD25^+$ T cell–dependent manner during OVA-induced asthma in mice (95). In the same model, antigens from *S. mansoni* (Sm22.6, Sm29, and PIII) reduced airway inflammation, eosinophilia, OVA-specific IgE levels, and T_H2 cytokine production in the BAL. The beneficial effects of Sm22.6 were due to the induction of IL-10, similar to the *S. mansoni* egg glycoprotein IPSE/α-1, which induced IL-10–producing Bregs (96). In contrast, SM22.6 and PIII triggered the expansion of $CD4^+Foxp3^+$ T cells suggesting that both Treg and Breg cells are involved in the modulation of type 2 inflammation by SEA (97).

Helminth molecules can also mimic host-derived mediators. *H. polygyrus* or administration of its excretory–secretory products (HES) induces Treg cells, suppressing effector cell proliferation *in vitro* and AAI *in vivo*. This regulatory response was mediated by Hp-TGM, a protein with TGF-β–like activity (15, 64). TGH-2 from *B. malayi* similarly activated TGF-β pathways, suggesting TGF-β signaling as a shared immunomodulatory mechanism among parasite species (98). *B. malayi*, *Ancylostoma ceylanicum*, *Trichinella spiralis*, and *Anisakis simplex* also produce homologs of the mammalian cytokine macrophage migration inhibitory factor (MIF) (99–103). MIF homologs from *B. malayi* (99) and *T. spiralis* (100) functionally reflect host MIF proteins, e.g., regarding chemotactic effects on monocytes, whereas the MIF homolog from *A. simplex* (As-MIF) showed direct anti-inflammatory activity on OVA-induced AAI, where it suppressed the production of T_H2 cytokines (IL-4, IL-5, and IL-13), as well as eosinophilia and goblet cell hyperplasia in the airways. These effects were again associated with the recruitment of $CD4^+CD25^+Foxp3^+$ T cells and the upregulation of IL-10 and TGF-β (102, 103).

Treg cell induction *in vivo* was also observed for an excretory/secretory protein of *Ascaris suum* (PAS-1), which inhibited airway inflammation in a murine model of OVA-induced AAI by decreasing eosinophilia and T_H2 cytokines in the BAL, as well as OVA-specific serum IgE (104). PAS-1 also abrogated airway inflammation and airway hyperreactivity induced by the proinflammatory *A. suum* molecule APAS-3 by reducing the production of proinflammatory cytokines in the airways and IgG1 and IgE levels in the serum (105). The amelioration of OVA-induced asthma by PAS-1 was mediated by IL-10/TGF-β–producing Treg cells ($CD4^+CD25^+$) and IFN-γ–producing $CD8^+$ T cells (104, 106). Thus, many helminth molecules target IL-10, TGF-β, and IFN-γ, which efficiently suppress type 2 cytokine and antibody responses involved in antihelminth immunity and allergic inflammation (107).

Recently, a metalloprotease (TIMP)–like protein from *Necator americanus* (AIP-2) with Treg-mediated anti-inflammatory effects on AAI was identified. AIP-2 did not suppress matrix metalloprotease catalytic activity, but modulated the activity of $CD103^+$ DCs that reduced the expression of costimulatory markers and expanded Treg cells. Thus, administration of AIP-2 reduced eosinophil recruitment, type 2 cytokine (IL-5, IL-13) production in the airways, and OVA-specific IgE in the serum. Importantly, AIP-2 also inhibited the proliferation of T effector cells from the blood of human HDM allergic patients (17).

Another recent study showed that in addition to products of the adult L5 stage of *H. polygyrus* (e.g., HES, HpARI), a preparation of the infective larval (L3) stage could protect mice against the development of AAI. The *H. polygyrus* larval extract (*HpbE*) and its active protein component, *Hpb* GDH, efficiently suppressed HDM-induced AAI *in vivo*. In particular, *HpbE* and recombinant *Hpb* GDH modulated the arachidonic acid metabolism of macrophages, inducing an anti-inflammatory, type 2 suppressive eicosanoid profile (16). *HpbE*-/GDH-treated macrophages exhibited high IL-10 and prostaglandin E_2 (PGE_2) production, but low production of proinflammatory leukotrienes, which are key mediators of AAI (16, 108). Macrophage-derived PGE_2 was particularly important for the *HpbE*-driven regulation of AAI in this study, and another study found that also helminths themselves can produce this immunomodulatory mediator (109). The *HpbE*-induced eicosanoid switch was largely mediated through nuclear factor κB, p38 mitogen-activated protein kinase, hypoxia-inducible factor 1α, and the cyclooxygenase-2 pathway.

Finally, *HpbE* reduced the chemotaxis of granulocytes from patients suffering from type 2 airway inflammation (16).

Together, these studies reveal that helminth molecules are efficient modulators of the innate and adaptive immune responses that drive AAI.

DISCUSSION

Helminths have unique immune regulatory potential, and understanding the complex array of immune responses triggered by these parasites may be instrumental for the diagnosis, prevention, and treatment of type 2 inflammatory diseases, such as allergic asthma. Identifying the molecules and mechanisms that determine whether a parasite will promote or suppress allergic inflammation may foster both the definition and targeting of pathomechanisms of chronic type 2 inflammation. Parasitic infections influence immunity and inflammation by a variety of molecular and cellular mechanisms, including the induction of Treg cells and regulatory macrophages, producing anti-inflammatory mediators, such as TGF-β, IL-10, and PGE_2, with beneficial effects in experimental models of asthma. However, the translation of these results from rodents to humans is not trivial. For instance, little is known about the correct dose or duration of parasite infection required for protective effects in humans. Safety concerns about detrimental effects of parasite infection limit clinical trials, and high immunological variation, e.g., due to different genetic background, complicates the interpretation of data from experimental helminth infection in humans. Indeed, not all studies show an impact of helminth infection or deworming on allergic inflammation (110, 111), which is in line with the lack of a therapeutic effect of intended helminth infection on AAI in humans (51–53) [for a comprehensive review, see Evans and Mitre (112)]. It is important to note that epidemiological studies commonly assess effects of helminth infection on skin prick test reactivity (e.g., atopy) rather than asthma symptoms, which may explain disparities between different studies.

Safety concerns regarding live helminth infections may be overcome by the identification and characterization of helminth-derived anti-inflammatory molecules, which may be developed as biotherapeutics. Therapeutic approaches exploiting the immunomodulatory potential of helminths, while avoiding infection-related side effects, represent an attractive treatment option for major chronic airway diseases. The identification of the cellular and molecular pathways targeted by helminth molecules (e.g., T cells, DCs, TLR-/IL-33 signaling) should aid the discovery of new worm-based drugs. Such drugs will have to be delivered preferentially locally, i.e., to the inflamed tissue at an optimal dose, route, and frequency of administration, which remains to be determined for each molecule. The recent identification of immune regulatory molecules that reduce AAI upon local delivery and simultaneously act on key human cells involved in asthma (e.g., epithelial cells, macrophages, eosinophils) (16–18) justifies the hope that effective topical helminth-based biotherapeutics can be developed. Formulation for local delivery into the airways represents a vital alternative to current biologics or oral corticosteroids that today represent the standard treatment for more severe forms of type 2 airway inflammation. However, before helminth-derived molecules can reach the clinics, there are several hurdles to be cleared. This particularly includes the immunogenicity of helminth molecules, potential proinflammatory side effects, as well as their half-life in the human organism. Reducing the immunogenicity of foreign helminth molecules represents a major challenge that may, e.g., be tackled by packaging immune regulatory proteins into nanocarriers for targeted delivery to a specific cell type or by designing non-immunogenic (humanized) mutants. Despite these challenges, significant scientific progress has been made to turn worm molecules into drug candidates. The unique and diverse modes of action of helminth-derived molecules make them promising candidates to become the next generation of biotherapeutics for the treatment of type 2 inflammatory disorders.

AUTHOR CONTRIBUTIONS

SB, FT, MR, and JE wrote the manuscript, SB and FT prepared the figures. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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Nanobodies—Useful Tools for Allergy Treatment?

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In the last decade single domain antibodies (nanobodies, V_HH) qualified through their unique characteristics have emerged as accepted and even advantageous alternative to conventional antibodies and have shown great potential as diagnostic and therapeutic tools. Currently nanobodies find their main medical application area in the fields of oncology and neurodegenerative diseases. According to late-breaking information, nanobodies specific for coronavirus spikes have been generated these days to test their suitability as useful therapeutics for future outbreaks. Their superior properties such as chemical stability, high affinity to a broad spectrum of epitopes, low immunogenicity, ease of their generation, selection and production proved nanobodies also to be remarkable to investigate their efficacy for passive treatment of type I allergy, an exaggerated immune reaction to foreign antigens with increasing global prevalence.

Keywords: allergy, allergen, nanobody, V_HH, blocking antibody, allergy treatment

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INTRODUCTION

Type I allergy, an IgE antibody mediated hypersensitivity disease, represents a common health problem affecting almost 30% of the population worldwide (1). The recognition of allergens by specific IgE antibodies that are generated after sensitization is a key event for the initiation of allergic inflammation (2). Allergic patients suffer from a variety of allergic symptoms including rhinoconjunctivitis and asthma (3) but also food allergy and skin inflammation (4). These clinical manifestations cause a major burden by reducing the quality of life of affected persons (5). While anti-inflammatory treatment based on pharmacotherapy reduces allergic symptoms and is the most commonly prescribed medication for treatment of allergic patients (6), only allergen-specific immunotherapy (AIT) represents a causative treatment of type I allergy. In fact, AIT induces a protective immunity in allergic patients based on the modification of cellular and humoral responses to the disease causing allergen (7). Besides the inhibition of IgE binding to their specific allergen, the immune deviation from a TH2 to TH1 response, and the decreases in numbers of effector cells in target organs, the generation and maintenance of allergen-specific regulatory T and B cells and the involvement of their suppressive cytokines are essential for the induction of allergen tolerance (8–10). Beyond doubt the improvement of allergic symptoms is further caused by

Abbreviations: AIT, Allergen-specific ImmunoTherapy; EBV, Epstein-Barr Virus; Fab, antigen-binding Fragment of antibodies; HCAb, Heavy Chain-only Antibody; HumAb mice, transgenic mice that produce fully human antibodies; PCA, Passive Cutaneous Anaphylaxis; scFv, single chain Fragment variable (recombinant derivative of a classical antibody); V_HH, Variable domain of Heavy chain of Heavy chain-only antibodies.

AIT-induced IgG antibodies found in serum and nasal secretions (8, 11–14). For many years AIT was conducted with aqueous natural allergen extracts and patients experienced considerable side effects due to the unpredictable composition and poor quality of the injected extracts (1). Recent developments like next-generation forms of AIT based on molecular approaches may overcome the limitations of current forms of AIT (15, 16). The last generation of improved vaccines, i.e. peptide carrier vaccines, induces an IgG response that targets IgE binding sites on allergens. Induced IgG antibodies effectively block IgE binding and are termed blocking antibodies (1, 17).

However, the efficacy of such blocking antibodies was long questioned because it revealed to be cumbersome to isolate reproducible defined, i.e. monoclonal allergen-specific antibodies comprising the capacity to inhibit allergen-induced allergic reactions.

A recent proof of concept study re-stimulated the idea to generate monoclonal allergen-specific antibodies and to evaluate their feasibility for allergy treatment. The authors could show that a single subcutaneous injection of a mixture of two human monoclonal allergen-specific IgG4 antibodies significantly reduced allergic symptoms in allergic patients (18, 19). Moreover, validated in a PCA mouse model, the mixture of these two monoclonal antibodies proved to be more potent in inhibiting mast cell degranulation than IgG antibodies purified from patients' sera who underwent successful AIT (18). Furthermore, these human monoclonal IgG4 antibodies recently completed the phase II clinical trial in treatment of cat allergic patients (https://clinicaltrials.gov/ct2/show/NCT03838731). These results proved for the first time that allergy treatment with monoclonal allergen-specific antibodies is a well-tolerated, rapid, and effective approach to reduce allergic inflammation and rekindled the blocking antibody concept (11, 20, 21).

Nevertheless, the generation and identification of blocking conventional human or humanized antibodies is connected with high costs for production, validation and application (22, 23). Therefore, cost-effective alternatives are currently sought.

The nanobody technology represents such an alternative implying a significant improvement to the laborious methods to obtain monoclonal blocking conventional antibodies. Due to their beneficial properties of small molecules and monoclonal antibodies, nanobodies in general are an attractive agent for development of novel therapeutic strategies (24, 25). The ease of their generation and production, the single domain organization, their beneficial biochemical properties and their feature to recognize small cavities on the surface of antigens and hence bind to epitopes inaccessible for conventional antibodies (26) have raised the particular interest of allergologists recently.

Can the nanobody technology provide enhanced opportunity to generate a panel of antigen-binding molecules with various epitope specificities for certain allergens different to conventional antibodies? Will these identified allergen-specific nanobodies be more efficient in blocking than conventional IgG antibodies due to their pronounced cleft recognition? Will it be possible with this technology to find single nanobodies that are able to abrogate IgE-mediated allergic inflammation? These questions and our wish to answer these questions attracted our attention. Within this review, we focus on the powerful nanobody technology to generate allergen-specific nanobodies and report on their evaluation for prospective application for passive allergy treatment.

THE COMPLEX AND LABORIOUS APPROACH TO IDENTIFY EFFECTIVE, PROTECTIVE ALLERGEN-SPECIFIC MONOCLONAL ANTIBODIES

If allergologists are asked why the search for effective protective allergen-specific monoclonal antibodies is complex and laborious, they will describe this issue by the typical quest for a needle in a haystack. Through intense and precise molecular and immunological exploration of available allergen-specific monoclonal antibodies in the past it was proven that epitope specificity and affinity are decisive for their inhibitory potential to block IgE binding and thus IgE-mediated reactions (21, 27–29). The commitment to find and isolate monoclonal antibodies with specificity and high affinity for certain allergens and even more for certain epitopes always started with several fundamental decisions. Amongst them the choice for the perfect source to gain DNA coding for antibodies and the applied technology to generate allergen-specific antibodies are two of the most critical ones. Regarding the DNA source both animals, mainly mice, and humans served as blood, spleen, tonsils and even bone marrow donors in the last decades to isolate B cells or plasma cells and thus DNA coding for antibodies (30–32). For the proof of principle, murine IgG antibodies overlapping with human IgE binding sites are valuable tools to investigate the effects to inhibit IgE epitope recognition on allergens and consequently to contribute to the design of hypoallergenic derivatives suitable for AIT (33). However, the direct therapeutic use of these murine monoclones in humans is limited by the high incidence of harmful immune responses against these administered foreign proteins (34). To mitigate this limitation numerous murine monoclonal antibodies have been re-engineered by chimerization and humanization technologies. These expensive procedures are justified for fatal diseases like different forms of cancer but were barely applied for allergen-specific murine antibodies so far with a few exceptions (35, 36). This was one of the main reasons why allergologists in the recent past endeavour to focus on human donors including allergic patients, AIT-treated patients and even healthy individuals depending on the research question (28, 37, 38).

Various methods were utilized to generate allergen-specific genuine, i.e. native antibodies with the preservation of the natural VH and VL pairing including hybridoma technology, Epstein-Barr-Virus (EBV) transformation, single B cell sorting and cloning and HumAb mice (transgenic mice that produce fully human antibodies) (18, 39–49). In parallel, versatile approaches were developed to generate non-genuine antibodies by random

combination of VH and VL chains, i.e., combinatorial Fab/ScFv libraries or (semi-) synthetic libraries (37, 38, 50–60). Based on PCR amplification as strong tool to depict large antibody repertoires and phage display to screen these large repertoires, many recombinant allergen-specific antibody fragments (Fabs or ScFvs) were isolated (37, 38, 50–56, 58–64).

All mentioned technologies have definitely contributed to the isolation and evaluation of monoclonal allergen-specific IgG, IgE antibodies and fragments thereof and furthermore to assess their feasibility for allergy treatment. Nevertheless, all mentioned technologies are also reported to have some limitations. While the hybridoma technology and EBV transformation are generally unsuitable for a comprehensive screening of large antibody repertoires because of their inefficient fusion and transformation events, the single B cell sorting was long hampered by inadequate staining technologies to clearly identify allergen-specific antibody producing cells (32, 39). The main drawback of combinatorial libraries is that they usually rely on random combination and thus most likely unnatural VH and VL antibody pairings. Additionally, it turned out independent of the applied technology to be very difficult to isolate monoclonal IgG and IgE antibodies with a broad epitope spectrum for each allergen. It also revealed that besides several blocking antibodies also many non-blocking or even enhancing antibodies were isolated (44, 63–65). While all three types of monoclonal antibodies were unambiguously supportive to study the structural requirements for efficient effector cell activation and hence contribute to elucidate the underlying mechanisms of type I allergy, non-blocking and enhancing antibodies were fully useless for the prospective application as protective antibodies.

These insights forced allergologists to look beyond the conventional antibody horizon.

THE POWERFUL NANOBODY TECHNOLOGY TO ISOLATE ALLERGEN-SPECIFIC NANOBODIES—A WELCOME ALTERNATIVE TO CONVENTIONAL ANTIBODY GENERATION

About 30 years ago, a group of Belgian scientists made an unexpected discovery, which was patented and later presented to the scientific community in the form of the well-known discovery publication in the journal Nature in 1993 (66). They found that a significant amount of non-canonical types of antibodies is naturally present in blood of Camelidae in addition to conventional antibodies. This exceptional type of antibody called Heavy Chain-only Antibody (HCAb) lacks light chains and consists of a homodimer of shortened (without CH1 domain) heavy chains. The antigen-recognition region in HCAbs is formed by only one variable domain (V_HH) that is directly linked *via* a hinge region to the Fc-domain (66). Later on, similar non-canonical HCAbs were found in some cartilaginous fishes such as sharks and ratfish (67–69). The antigen-binding variable domain of these antibodies was named VNAR as opposed to V_HH in camelids. A recombinant protein version of the V_HH-or VNAR-domain is usually called "single domain antibody" or "nanobody". The very popular term "nanobody" is the commercial name given by the Belgian biopharmaceutical company Ablynx, a pioneer in HCAb-based therapeutic applications that was acquired by Sanofi in 2018.

The nanobody generation technology was proven to be a very efficient machinery to generate nanobodies with required properties and offered crucial advantages compared to traditional techniques utilized to produce murine or human conventional antibodies. After the typical initial immunization (of camelids) step, the full repertoire of cDNA coding for functional nanobodies can be efficiently cloned from peripheral blood lymphocytes of immunized animals using PCR amplification and then a panel of nanobodies of required specificity can be easily selected using phage (or other type of) display-based methods (66, 70–72). In addition, there are different *in vitro* affinity maturation approaches to improve features of initially selected nanobodies (71, 73, 74). In some cases, especially if the antigen of interest is toxic, unstable, non-immunogenic or not available in sufficient quantity, other types of libraries (naive, semisynthetic or fully synthetic libraries) can be efficiently used instead of immune libraries for generation of nanobodies (75–79). Synthetic libraries can be made using special predesigned scaffolds such as humanized scaffolds optimized for intracellular stability (77) or optimized for bacterial expression (80). Non-immune libraries are typically much larger than immune libraries and a ribosome display was suggested for the initial selection round of such large libraries to work with higher concentrations of nanobody variants than in case of phage display (79, 80). Synthetic libraries combined with different selection procedures were successfully used to obtain conformationally selective nanobodies against G protein-coupled receptors (78), sybodies against very challenging targets such as the heterodimeric bacterial ABC exporterTM287/288 (81) or the intracellular KDEL receptor (82) to name a few examples from many others.

Nanobodies comprise unique features that distinguish them from classical antibodies. Nanobodies are the smallest known antibody fragments (4 × 2.5 x 3 nm, 12–15 kDa) of natural origin that are able to specifically bind their cognate antigens. Due to their often extended CDR3 loop they can form unusual paratopes, i.e. finger-like extensions and thus recognize special native antigenic epitopes (small cavities, concave surfaces, conformational epitopes, active sites of enzymes) that are hidden for conventional antibodies (**Figure 1**). Indeed, nanobodies have proven to be useful tools for modulating the activity of enzymes (26, 83, 84). It could be therefore speculated that allergen-specific nanobodies that modulate or inhibit the proteolytic activity of certain allergens (*e.g.*, Phl p 1, Der p 1) might reduce their penetration capacity through mucosal surfaces. Furthermore, nanobodies are able to bind small peptides with high affinity (85–89). Their high affinity, solubility and stability over a wide range of temperatures and pH, ease of producing in bacteria or other expression systems make them convenient molecules for different applications, as well as for all possible engineering modifications *e.g.*,

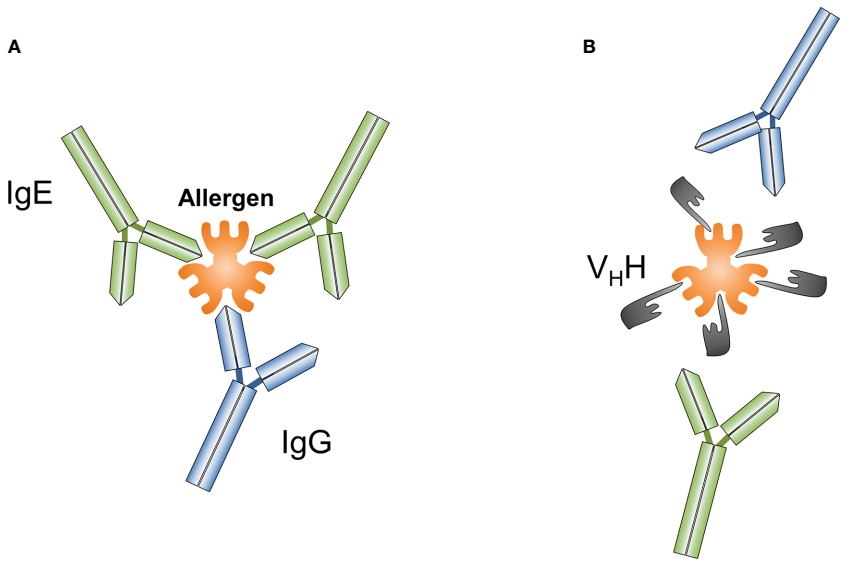

FIGURE 1 | Conventional antibodies such as IgG or IgE **(A)** and nanobodies (V_HH) **(B)** can be generated against different epitopes of targeted antigens, a particular allergen. Nanobodies overlapping with IgE binding sites on allergens prevent IgE-mediated allergic reactions.

development of complex constructs and conjugates. Nanobody-based tools are therefore increasingly used for research, molecular visualization, diagnostics and development of new treatment options for various pathologies, including cancer and other socially significant diseases (71, 72, 90–94).

So far, only one allergen-specific nanobody is described in the literature. This nanobody is reported to be specific for the major peanut allergen, Ara h 3 and was isolated from a synthetic library of humanized nanobodies *via* phage display (95). The interaction between Ara h 3 and the Ara h 3-specific nanobody resulted in a dissociation constant of 400 nM representing medium affinity binding and was further investigated by the structural determination of formed co-crystals (95). The authors acknowledged that additional work is needed to improve the affinity of the isolated nanobody to make it an attractive tool for the development of biosensors for peanut allergen detection. This finding clarifies that the selection procedure is only one part of the successful discovery of potent IgE-blocking nanobodies, thus the evaluation of selected nanobodies is critical as well.

Nevertheless, we are confident that soon more allergen-specific nanobodies will arise to be studied for their potential to abrogate IgE-mediated allergic inflammation.

EVALUATION OF THE SUITABILITY OF ALLERGEN-SPECIFIC NANOBODIES FOR ALLERGY TREATMENT

Similar to the evaluation of conventional antibodies with the focus to identify effective protective monoclones, generated nanobodies have to be assessed first for their allergen specificity, epitope recognition, cross-reactivity to homologous allergens present in related species, for their affinity to their corresponding allergens and most importantly for their ability to inhibit patients´ IgE binding to these allergens (**Figures 2A–C**). After the allergen specificity of isolated nanobodies is confirmed, the proof for cross-reactivity (**Figure 2A**) is of great importance because IgE antibodies from allergic patients often displayed cross-reactivity to allergens from other allergen sources (28, 96). High affinity and slow dissociation of formed nanobody/allergen complexes will be critical prerequisites for allergen-specific nanobodies to be chosen as suitable candidate (**Figure 2B**). However, the pivotal characteristics for an allergen-specific nanobody to be attractive for further processing will be the determination of its potential to block patients' IgE binding and hence IgE-mediated effector cell activation (**Figure 2C**). Additionally, specific nanobodies have to be tested as well for their cross-protectivity to homologous allergens. All these properties are crucial requirements for allergen-specific nanobodies to be selected for further essential investigations concerning half-life, clearance and safety.

Nanobodies are considered as proteins of weak immunogenicity due to the shared similarities with variable VH domains of human immunoglobulins (IgG3 subclass), and they can be further improved by a humanization approach (97) (**Figure 2D**). Consequently, no immune response against applied nanobodies was raised in mice or humans that were injected with nanobody-containing constructs (98–100). Safety of nanobody-based drugs is confirmed by several completed Phase 1 and Phase 2 clinical trials (101) and recent approval by the US Food and Drug Administration (FDA) and the

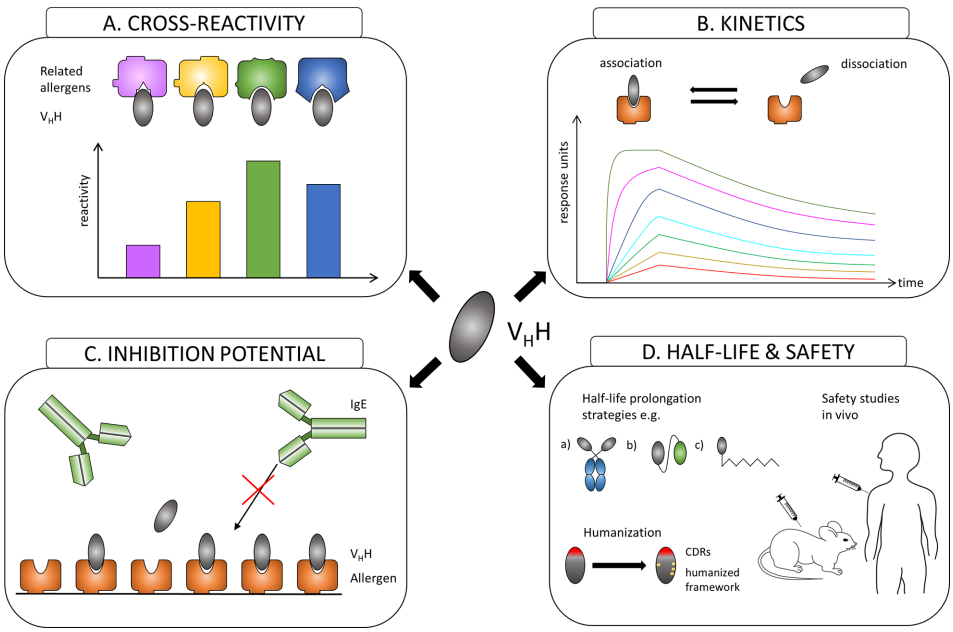

FIGURE 2 | Overview of the evaluation process of the suitability of allergen-specific nanobodies for allergy treatment. **(A)** Evaluating cross-reactivity to related allergens. **(B)** Measuring affinities of selected candidates to the allergen. **(C)** Investigating the potential to block allergen-specific IgE from binding to the allergen. **(D)** Adjusting the half-life of a suitable nanobody by e.g.: a) linking to IgG Fc region; b) oligomerization to homomers or heteromers to facilitate linking to other proteins like human serum albumin (HSA); c) PEGylation. Increasing safety by humanization of the framework and performing safety studies *in vivo*.

European medicines agency (EMA) of the first therapeutic nanobody, Caplacizumab, a bivalent nanobody designed for the treatment of thrombotic thrombocytopenic purpura and thrombosis (102).

Though advantageous for *in vivo* imaging, the small size of nanobodies could be seen as a disadvantage for passive treatment of allergy due to a quick renal clearance of nanobodies from blood (approx. 30 min). Many different strategies to extend the *in vivo* half-life of nanobody-based construct have been developed (103). They include increasing the hydrodynamic radius of a protein by attaching highly flexible and hydrophilic molecules such as polyethylene glycol (PEG) and carbohydrates or by genetic fusion with polypeptide chains mimicking the biochemical properties of PEG, fusion of V_HH to the Fc region of IgG, fusion or non-covalent binding to albumin (104) (**Figure 2D**). Nanobodies can also be used as modules to engineer larger molecules with several valencies and/or specificities, such as multivalent (105–108), bispecific (105, 109), and other (110, 111) constructs that may acquire considerably higher specificity, binding efficiency and biological activity (106, 107, 111). Nanobodies were also considered as possible ligands to design new highly specific immunosorbents (112–114).

Different types of nanobody-based tools/approaches can be envisaged to be potentially profitable for an allergy treatment: a) bispecific nanobodies for topical application to capture allergens before they penetrate epithelial mucosa in airways, b) very stable nanobodies to capture food allergen in gastrointestinal tract, c) anti-idiotypic nanobodies mimicking allergenic epitopes as a possible replacement for a complex natural allergen for a new kind of AIT vaccine development, d) multivalent nanobody-based constructs for systemical administration to efficiently block allergen interaction with IgE on mast or basophil cells, e) efficient immunosorbents to remove IgE from the blood by immune apheresis. Correspondingly, different administration approaches for nanobody-based constructs can be developed: aerosol or topical applications, oral route or subcutaneous administrations. Temporary blocking of allergen-IgE interaction (i.e. by topical or systemic administration of specific nanobodies) or a subtraction of IgE from the periphery blood (i.e. apheresis) may give a short-term treatment effect. For a long-term treatment effect we could hypothesize the use of anti-idiotypic nanobodies to IgE. Such nanobodies may represent "internal images" of an allergen and mimick hypoallergenic B cell epitopes. To efficiently induce IgG response that targets IgE binding sites on allergens, these nanobodies should be fused to a viral coat protein as it was described for next-generation forms of AIT (15).

CONCLUSION AND PERSPECTIVE

The generation of allergen-specific nanobodies unambiguously represents a reasonable progress in the field of allergy. With their well-documented qualities including their ability to recognize unusual "hidden" epitopes, high affinity binding, solubility, extreme stability and low immunogenicity, nanobodies attracted the interest of allergologists to study their suitability

for passive allergy treatment. The chance to find allergen-specific nanobodies with this powerful technology that ideally comprise high affinity and bind to epitopes partly or fully overlap with IgE binding sites on allergens is tempting. However, so far no allergen-specific nanobody fulfilling these criteria was reported indicating that it might be rather difficult to raise allergen-specific nanobodies of sufficient affinities. Whether the current lack of such nanobodies is owed to some inherent structural or functional properties of nanobodies and/or the camelid immune system or the simple reason that the current research focus in the allergy field is on AIT and its improvement has to be resolved. If allergen-specific nanobodies are identified that competitively block allergen binding to IgE and thus abrogate IgE-mediated allergic inflammation, we assume that they will represent appropriate tools for future allergy treatment. Their economic properties, i.e. low production costs encouraged researchers to elaborate antibody engineering of these single-domain antibodies for diverse applications in biotechnology and medicine. This gathered knowledge will facilitate the implementation of modified allergen-specific nanobodies tailored to the needs of allergy treatment. Nanobodies can be easily formatted for a particular application *e.g.*, modified as recognition modules in large constructs or as bi- or oligo-specific, bi- or oligo-valent derivatives.

With the availability of allergen-specific nanobodies or their derivatives with inhibitory potential, it should be possible to examine engineered candidates in proof of concept testings for efficacy and safety in experimental animal models to identify promising nanobody-based drugs for clinically relevant allergens.

AUTHOR CONTRIBUTIONS

SF, IZ and ST reviewed the literature, IZ and ST generated figures. SF and ST wrote the manuscript draft. All authors contributed to the article and approved the submitted version.

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METHODS
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IgE Epitope Profiling for Allergy Diagnosis and Therapy – Parallel Analysis of a Multitude of Potential Linear Epitopes Using a High Throughput Screening Platform

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Immunoglobulin E (IgE) is pivotal for manifestation and persistence of most immediate-type allergies and some asthma phenotypes. Consequently, IgE represents a crucial target for both, diagnostic purposes as well as therapeutic approaches. In fact, allergen-specific immunotherapy – aiming to re-route an IgE-based inflammatory response into an innocuous immune reaction against the allergen – is the only curative approach for IgE-mediated allergic diseases known so far. However, this requires the cognate allergen to be known. Unfortunately, even in well-characterized allergics or asthmatics, often just a small fraction of total IgE can be assigned to specific target allergens. To overcome this knowledge gap, we have devised an analytical platform for unbiased IgE target epitope detection. The system relies on chemically produced random peptide libraries immobilized on polystyrene beads ("one-bead-one-compound (OBOC) libraries") capable to present millions of different peptide motifs simultaneously to immunoglobulins from biological samples. Beads binding IgE are highlighted with a fluorophore-labeled anti-IgE antibody allowing fluorescence-based detection and isolation of positives, which then can be characterized by peptide sequencing. Setting-up this platform required an elaborate optimization process including proper choice of background suppressants, secondary antibody and fluorophore label as well as incubation conditions. For optimal performance our procedure involves a sophisticated pre-adsorption step to eliminate beads that react nonspecifically with anti-IgE secondary antibodies. This step turned out to be important for minimizing detection of "false positive" motifs that otherwise would erroneously be classified as IgE epitopes. In validation studies we were able to retrieve artificial test-peptide beads spiked into our

library by using IgE directed against those test-peptides at physiological concentrations (≤20 IU/ml of specific IgE), and disease-relevant bead-bound epitopes of the major peanut allergen Ara h 2 by screening with sera from peanut allergics. Thus, we established a platform with which one can find and validate new immunoglobulin targets using patient material which displays a largely unknown immunoglobulin repertoire.

Keywords: immunoglobulin E (IgE), allergy diagnosis, allergy therapy, epitope detection, combinatory peptide library, one-bead-one-compound library

INTRODUCTION

Selectively recognizing foreign matter that has entered the body is a key feature of humoral adaptive immunity. Yet, not always it is clear against which foreign matter an antibody response is directed or with which antigen an antibody will react. A substantial number of asthmatics, for instance, display high total serum immunoglobulin E (IgE) levels but do not react with the common allergens the patients usually are tested for in commercially available routine allergy diagnostic tests (1–3). In the past, those patients were assigned to suffer from "nonallergic asthma" (intrinsic asthma) (4) but recent evidence suggests that those individuals are simply underdiagnosed in terms of allergen reactivity. After all, asthmatics with regular total serum IgE account for less than 6% of asthmatic patients (5). The vast majority of asthmatics display higher total serum IgE. Consequently, including a broader panel of allergens in the testing reveals more cases of "allergic asthma" (extrinsic asthma) among asthmatics (6). So far, many patients with asthma lack proper allergy diagnosis due to the fact that *in vitro* routine allergy diagnostic tests are missing clinically relevant allergen sources, and where allergen sources are included as raw extract allergens, these often lack clinically relevant single allergenic components and, therefore, appropriate sensitivity. Still, allergy diagnostic testing has been vastly improved in the past decades due to molecular allergology providing single allergen molecules, either naturally purified from the source or obtained by recombinant DNA technology (7). The availability of single allergens for singleplex and multiplex assays (component-resolved diagnosis) has already provided the investigators with increased sensitivity, specificity, and diagnostic accuracy of the tests (8, 9). Multiplex assays in microarray format can analyze dozens of potential allergen-specific IgE reactivities in parallel (10–13), thereby allowing fine-profiling of a patient's sensitization and – together with the clinical history – his/her allergy phenotype. Molecular allergology offers further improvements to diagnostics, pinpointing sensitizations to individual allergen components on the molecular level and providing the basis for refined allergy classifications, risk predictions and personalized treatment regimens (14, 15). Yet, all these diagnostic procedures require the knowledge of at least the primary allergen source. But even nowadays, it often remains an enigma against which allergens the IgE-high-asthmatics actually are sensitized or whether all relevant allergens have been identified as yet.

Therefore, smart approaches are needed in order to determine unknown antibody reactivities and to identify the interaction sites – the so-called epitopes – on the target antigens/allergens. In most cases, these epitopes are protein-derived entities, either linear chains or three-dimensional structures composed of amino acids. A promising line of action consists in offering a broad variety of peptidic targets to the antibody (mixture) in question, and to check for reactivity.

One common approach for identifying peptidic/proteinaceous binding partners for a ligand is the use of phage display libraries (16). Here a pool of DNA sequence motifs is cloned into a permissive site of a phage surface protein. By screening the phage library with the desired ligand(s) potential binders can be isolated, propagated and sequenced in order to reveal the introduced amino acid sequence motif which interacted with the ligand(s). In allergology this technique has been used to study IgE-allergen interaction, either by cloning single chain antibody genes into the phage (17–19) and offering the gene products to a given allergen, or *vice versa* by offering a defined IgE reactivity to a pool of peptide-presenting phages created by cloning random oligonucleotides into the permissive site of the scaffold protein gene (20–23). The latter variant – defined IgE reactivity versus a broad peptide landscape – is more common as it may yield information about the epitope recognized by the IgE in question. Yet, quite often it is necessary to present purified immunoglobulin to the phage library in order to obtain meaningful results (24). This is a clear drawback of this technology as it not only increases the workload but also may cause losses in IgE reactivity due to the purification step.

As an alternative to phage libraries the use of so-called "one-bead-one-compound" (OBOC) libraries constitutes a promising approach. The OBOC technology based on the "split-and-mix" synthesis was invented by Furka et al. (25, 26) and yields a unique peptide species on each bead of the synthesis resin in pico- to nanomolar amounts per bead. Subsequent amino acid sequencing of the bead-bound peptide directly leads to the respective epitope motif. For IgE analysis, an OBOC library has been used once so far, in a study where a known IgE reactivity against shrimp tropomyosin was investigated by screening a broad peptide landscape with sera from shrimp allergics to detect IgE epitopes on tropomyosin (24).

Neither the phage display nor the OBOC technique have been used to reveal epitopes of unknown serum IgE reactivities. We therefore wanted to address this question via the OBOC strategy. In the study presented here, we have developed the methodology for the identification of hitherto unknown IgE reactivities toward unknown allergens via detection of bead-bound linear peptide epitopes.

MATERIALS AND METHODS

Materials

TentaGel S NH_2 resin was custom-made by Rapp Polymers (Rapp Polymere GmbH, Tübingen, Germany), with the following specifications: approximately 7.1 million polystyrene beads per gram dry powder, 0.36 mmol amino functions/g, bead diameter 60–70 μm under dry conditions, approximately 50 picomoles of functional amino groups per bead. These beads were used as resin material for the on-bead peptide synthesis applying the fmoc solid phase peptide synthesis technique with an automated multiple peptide synthesizer (MultiPep RS, Intavis Bioanalytical Instruments AG, Cologne, Germany).

Monoclonal, humanized IgE antibodies directed against the c-myc epitope as well as anti-human IgE antibodies with different fluorophore labels were obtained from various sources as summarized in **Table 1**. Human serum was donated by peanut allergic patients in the Allergy Outpatient Clinic of the Medical Clinic Borstel and the Interdisciplinary Allergy Outpatient Clinic, University of Lübeck. Total IgE content and specific IgE reactivity against the peanut allergen Ara h 2 were determined by ImmunoCAP assays (ThermoFisher Scientific/Phadia, Freiburg, Germany). Recognition of linear Ara h 2 epitopes by IgE from patient sera was resolved by in-house epitope mapping analysis as described before (27–30). Use of patient material for this study was approved by the ethics committee of the University of Luebeck (approval number 10-126). All patients gave written informed consent.

TABLE 1 | Commercially available antibodies used in this study.

Target	Antibody	Label	Source
c-myc	monoclonal IgE, clone 9E10, humanized	–	Absolute Antibody #AB00100-14.0
Human IgE	polyclonal (goat)	DyLight488	Agrisera #AS10758
	polyclonal (goat)	FITC	Nordic-MUBio #GAHu/IgE(FC)/FITC
	polyclonal (swine)	FITC	Nordic-MUBio #SwAHu/IgE(FC)/FITC
	monoclonal, clone BE5	FITC	ExBio #1F-324
	monoclonal, clone 4H10	FITC	ExBio #1F-326
	polyclonal (goat)	DyLight550	Agrisera #AS121901
	monoclonal, clone B3102E8	AlexaFluor555	Southern Biotech #9160-32
	rabbit/human chimeric, Omalizumab	phycoerythrin	Absolute Antibody #Ab00717-23.0
	monoclonal, clone BE5	phycoerythrin	ExBio #1P-324
	polyclonal (goat)	DyLight633	Agrisera #AS122147
	polyclonal (goat)	DyLight650	Agrisera #AS122270
	polyclonal (goat)	DyLight680	Agrisera #AS163319

Preparation of Polystyrene Beads With Peptides of Defined Sequence

Peptide sequences to be synthesized onto the beads were chosen according to known target structures recognized by human IgE antibodies. As negative control, a scrambled version of each specific peptide was produced with an online tool at http://www.mimotopes.com.

200 mg of the TentaGel S NH_2 resin (corresponding to approximately 1.5×10^6 beads) were swollen in 5 ml of a 7:3 mixture of dichloromethane (Roth, Karlsruhe, Germany) and dimethylformamide (DMF; Merck Chemicals, Darmstadt, Germany) and transferred in portions of approximately 2.2×10^5 beads (corresponding to 10 μmol amino functions) to 2 ml filter bottom reaction columns (Intavis). In the following, all reagent amounts are given per reaction column. All reaction steps were performed at room temperature. The resin was prepared for synthesis by washing three times with 800 μl of DMF. Fmoc deprotection was achieved by treating the resin two times for 8 min with 400 μl of a mixture of 20% (v/v) piperidine (Sigma-Aldrich, Steinheim, Germany) in DMF and subsequent washing seven times with 750 μl of DMF. Coupling was done by reacting a 10-fold excess of fmoc-protected amino acid building blocks (Merck or IRIS Biotech, Marktredwitz, Germany) with the resin. For this, the resin was incubated two times for 25 min each with a mixture of 77 μl of a 0.6 M fmoc amino acid building block solution in DMF, 25 μl of a 4 M solution of 4-methylmorpholine (Sigma-Aldrich) in DMF and 75 μl of a 0.6 M solution of 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (IRIS Biotech) in DMF. After washing three times with 750 μl of DMF, unreacted amino termini were capped for 5 min with 400 μl of a 5% (v/v) mixture of acetic anhydride (Merck) in DMF. Subsequently the resin was washed and extracted additional six times with 750 μl of DMF.

After synthesis the resin was treated three times for 8 min with 400 μl of a mixture of 20% (v/v) piperidine in DMF, washed seven times with 750 μl of DMF and five times with dichloromethane and dried *in vacuo*. Side chain protecting groups were cleaved off by treatment with 2 ml of cleavage cocktail [92.5% of trifluoroacetic acid (TFA; Roth), 5% of triisobutyl silane (Sigma-Aldrich) and 2.5% of water (all v/v)] for 3 h at room temperature. After incubation, the cleavage cocktail was discarded and the resin beads were washed five times with 10 ml of DMF, five times with 10 ml of pure ethanol (Brüggemann Alcohol GmbH, Heilbronn, Germany), five times with 10 ml of dichloromethane and again five times with 10 ml of DMF. Beads were treated with 30% (v/v) of H_2O/DMF, 60% (v/v) of H_2O/DMF and with neat H_2O for 5 min each at room temperature. Subsequently, the resin beads were washed ten times with 10 ml of phosphate buffered saline (PBS; 2.7 mM KCl, 1.5 mM KH_2PO_4, 136 mM NaCl, 8.1 mM Na_2HPO_4, pH 7.4), resuspended in 2 ml of PBS containing 0.05% (w/v) of NaN_3 (final concentration calculated approximately 1.1×10^5 beads/ml) and stored at 4°C. The migration of the beads from organic solvents to an aqueous buffer system results in the swelling of the beads and a final diameter ranging from 90 to 110 μm.

Synthesis of a Combinatorial/One-Bead-One-Compound (OBOC) Peptide Library via a Manual Split-and-Mix Procedure

OBOC peptides were synthesized based on the procedure published by Lam et al. (31), with some modification to adapt

the protocol to our needs. For that, 1.46 g (corresponding to approximately 10×10^6 beads and 0.5 mmol amino functions) of the TentaGel S NH_2 resin were swollen in 20 ml of a 1:1 mixture of dichloromethane and DMF for 1 h. The resin was distributed equally into 19 polypropylene vials (approximately 0.028 mmol/vial), beads were allowed to settle and the supernatants were removed carefully. To each vial, 433 µl of a 0.6 M solution of one of 19 different fmoc amino acid building blocks in DMF, 433 µl of a 0.6 M solution of 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate in DMF and 47 µl of *N*-ethyldiisopropylamine (Sigma-Aldrich) were added. Vials were incubated on an end-over-end mixer at room temperature for 2 h. This way, every proteinogenic amino acid, except cysteine, was coupled to one portion of resin beads. After that, all 19 resin portions were combined in a 20 ml reaction column equipped with a 35-µm filter bottom (Intavis), mixed well, and the supernatant was removed. The resin was washed five times for 2 min at room temperature with 10 ml of DMF each. 10 ml of a solution of 20% (v/v) piperidine in DMF were added and incubated two times for 15 min at room temperature. After that, the resin beads were washed six times for 2 min with 10 ml of DMF, and the resin was again distributed equally to 19 polypropylene vials. This cycle of splitting and mixing the resin beads was repeated eight times to create different random 8mer peptides on the resin beads (**Figure 1**).

Amino acid side chain de-protection after the last cycle of peptide synthesis, as well as migration of the beads from organic into an aqueous PBS buffer system, were performed as described above for the synthesis of beads with defined peptide sequence. The total OBOC library was suspended in 10 ml of PBS (final concentration calculated 1×10^6 beads/ml) and stored at 4°C after addition of NaN_3 to a final concentration of 0.05% (w/v), in order to prevent growth of microorganisms.

All bead numbers given in the following protocols are "calculated," referring to the starting number of beads employed in the respective synthesis. Possible losses occurring during synthesis, washing and handling steps are disregarded.

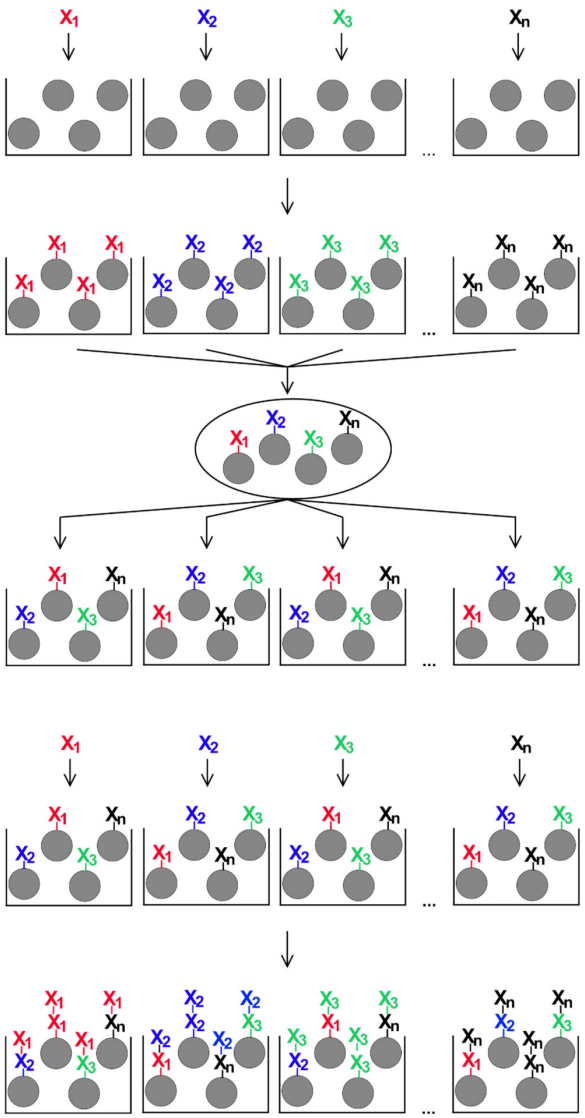

FIGURE 1 | Schematic illustration of the split-and-mix procedure for the generation of OBOC-libraries. For peptide libraries, each of n individual amino acids (X1 – Xn) is coupled to a portion of synthesis resin, then all beads are combined and mixed, distributed again in n portions, and the next coupling step with one individual amino acid per portion is performed. The number of repeated rounds of the procedure corresponds to the peptide length on each individual bead.

Screening of "Artificial" Libraries Consisting of Defined Peptide Sequences

Defined amounts of polystyrene beads carrying specific IgE target peptides (c-myc or Ara h 2, see below) were mixed with the respective scrambled version as irrelevant bead matrix. Ratios of "relevant" to "irrelevant" beads ranged from 1:10 to 1:100.

For screening experiments, mixtures of these beads (corresponding to approximately 10,000 beads in total per assay) were transferred into 2 ml reaction columns (identical to the standard columns used for peptide synthesis; Intavis) with a filter on the bottom of the vessel and a luer outlet. The reaction columns were closed with a luer sealing plug during the incubation of the beads with different reaction solutions. For washing purposes, the sealing plug was removed and the washing buffer was pressed through the beads with a fitting stamp. Contact between the tip of the stamp and the bead-covered filter was carefully avoided to prevent the loss of beads sticking to the stamp's tip.

The beads were first washed with 2 ml of PBS containing 0.1% (v/v) Tween-20 (Sigma-Aldrich) (PBST) followed by two times washing with the same amount of PBS. After washing, free areas on the polystyrene bead surface were blocked by incubating the beads in 2 ml of blocking solution (0.05% (w/v) fish gelatin (Norland Products Inc., Cranbury, NJ, United States) in PBS) for 1 h at room temperature. Constant mixing of beads in blocking solution was achieved by mounting the tube onto an overhead shaker (Intelli Mixer RM-2L, LTF Labortechnik GmbH)

performing a semicircle movement at 10 rpm. This was followed by thorough washing (six times with 2 ml of PBST and two times with 2 ml of PBS). After that, beads were ready for the incubation with the respective IgE antibody preparation used in the specific screening experiment (**Table 1**). Incubation was done overnight at 4°C on an overhead shaker (semicircle movement at 10 rpm), with IgE concentrations ranging from 1 μg/ml to 1 ng/ml in blocking solution. On the next day, unbound primary antibody was removed by three times washing with 2 ml of PBST and two times washing with 2 ml of PBS. Beads were then incubated with a secondary antibody directed against IgE and labeled with fluorophore (**Table 1**). Dilutions of the secondary antibodies normally ranged from 1:100 to 1:1,000. In some cases, higher or lower dilutions were used for special experimental purposes. Incubation with the secondary antibody was done at room temperature for 3 h in the dark with 10 rpm of semicircle mixing. Unbound antibodies were subsequently removed from the beads by thorough washing (four times with 2 ml of PBST and four times with 2 ml of PBS). Next, beads were re-suspended in the capped reaction columns using 1 ml of PBS and transferred in portions into 24- or 12-well polystyrene plates (Costar Corning, Corning, NY, United States). PBST was added (usually 5–10% of the total bead suspension volume) to decrease the surface tension of the solution and allow the beads to settle to the bottom of the well.

The beads were examined visually on a standard inverted fluorescence microscope (Nikon ECLIPSE TE2000-U; Nikon, Tokyo, Japan) equipped with a UV light source and filter systems compatible with the fluorophores used. In addition, bead fluorescence intensity and distribution was documented with a MORE life cell imaging microscope (Thermo-Fisher Scientific, Waltham, MA, United States, formerly Till Photonics, Gräfelfing, Germany) equipped with a Clara CCD camera system (Andor Technology, Belfast, United Kingdom), using appropriate filter sets to capture green, red and far red fluorescence signals (FITC-channel: detection filter wavelength: 535 ± 50 nm/rhodamine-channel: detection filter wavelength: 630 ± 75 nm/Cy5-channel: detection filter wavelength: 700 ± 75 nm). Analysis of the respective pictures (including measurement of fluorescence intensities) and image processing was conducted using the software ImageJ v1.52p (NIH, Bethesda, VA, United States) (for details, see **Supplementary Material**). Visualization of fluorescent beads for photographic documentation and publication was done by converting the 16-bit gray-scale pictures obtained with the respective filters into RGB color space using ImageJ's built-in look-up tables (LTUs) after fluorescence intensity measurements.

Separation of IgG and IgE

To separate IgE from IgG, human serum was treated with protein G-sepharose (Ab SpinTrap, GE Healthcare, Chicago, IL, United States) according to the manufacturer's instructions, with minor modifications. 300 μl of serum from an allergic donor was loaded onto a protein G-sepharose column. The flow-through of the column was collected as IgG-negative/IgE-positive fraction. IgG bound to the sepharose was eluted with 400 μl of acidic elution buffer into a tube containing 30 μl of basic neutralizing buffer, resulting in functional IgG in a buffer of neutral pH.

Screening of "Artificial" Libraries With Different Human Immunoglobulin Classes

Untreated serum from an allergic donor and the corresponding IgE-fraction obtained as flow through of the protein G-sepharose column were adjusted to the dilution of the eluted IgG-fraction by adding PBS. This results in an approximate 1.4-fold dilution and in an antibody concentration of 70% compared to the original serum. 180 μl of each of these diluted immunoglobulin preparations (total serum, IgE fraction and IgG fraction) were mixed with 20 μl 0.5% (w/v) fish gelatin in PBS, resulting in a final antibody concentration of approximately 65% compared to the untreated serum. These dilutions were then used for the incubation with pre-blocked peptide-bearing polystyrene beads as described above, using a 1:1,000 dilution of the secondary, anti-IgE antibody.

Screening of OBOC Peptide Libraries Spiked With Defined Peptide Beads

Combinatorial OBOC libraries (either untreated or depleted of anti-IgE cross-reactive beads (pre-cleaned, see below)) were spiked with beads bearing defined peptide sequences (c-myc or Ara h 2; see "Results" section for more information). The ratio "defined peptide bead to OBOC library bead" was between 1:500 and 1:1,000, some preliminary testing was done with ratios of 1:10 to 1:15. The beads were mixed as described above for the "artificial" peptide libraries, and a total of 2×10^4 to 2×10^5 beads were transferred into the reaction column used for screening. The screening protocol was identical to the procedure described for the "artificial" peptide libraries, except for the use of 400 μl of diluted serum/primary antibody and secondary antibody solution (due to higher bead numbers). Antibody concentrations and types of secondary antibodies were varied to identify optimal conditions. After the last washing step, beads in the reaction column were resuspended in 2 ml of PBS, transferred in portions into 12-well or 24-well plates and examined as described above.

Pre-cleaning of OBOC Peptide Libraries by Pre-adsorption With Fluorophore-Labeled Anti-IgE Antibody and Separation With a Large Particle Sorter

To minimize false-positive results due to binding of secondary, anti-IgE antibody directly to individual beads in the OBOC population, such anti-IgE-cross-reactive beads were identified in a pre-adsorption step and removed from the OBOC library before screening with the actual IgE samples. For this, 1×10^6 beads of an OBOC peptide library were incubated in a reaction column of 20 ml capacity (Intavis) with 15 ml of blocking solution over night at 4°C with semicircle mixing at 10 rpm. Washing steps (before and after blocking) with PBST and PBS were performed as described above, with 15 ml of washing solution per washing step. This was followed by 3 h incubation at room temperature with 3 ml of phycoerythrin-labeled anti-IgE antibody at a concentration of 500 ng/ml and another washing

two times with 15 ml of PBST and two times with 15 ml of PBS. Beads were resuspended in a total of 5 ml of PBS and transferred in portions into a 15-ml polystyrene tube. A small sample of the bead suspension was analyzed in the fluorescence microscope to verify successful staining. Afterward, the beads were stored in the 15-ml tube at 4°C in the dark, until separation of fluorescence positive and negative beads was performed, which should be done within 2 weeks after the staining procedure (personal recommendation). This separation was performed on a BioSorter (Union Biometrica, Holliston, MA, United States) equipped with a Fluidics and Optics Core Assembly (FOCA) of 500 μm, using PBS as sheath fluid. Beads were re-suspended in PBS, and concentration was adjusted until a stable event rate of 10–20 events/second was achieved. An unstained bead sample was run as control to set gating conditions. Non-fluorescent, i.e., non-IgE cross-reactive beads ("phycoerythrin-negative") were sorted with the coincidence mode set to "Pure." Data graphs were generated in FlowJo software v.10.7.1 (Becton Dickinson, Franklin Lakes, NJ, United States). The fluorescent beads were discarded, the non-fluorescent beads were stored at 4°C until use in screening experiments.

Pre-adsorption of OBOC Peptide Libraries With Fluorophore-Labeled Anti-IgE Antibody and Screening of Pre-adsorbed Libraries Spiked With Defined Peptide Beads

As an alternative approach to the pre-cleaning step, i.e., the removal of pre-adsorbed anti-IgE-cross-reactive beads from the OBOC-library via BioSorter separation before the actual screening process, the pre-adsorption step was performed using anti-IgE labeled with a different fluorophore than the one used in screening. Afterward, anti-IgE-cross-reactivity was determined in the eventual fluorescence readout.

For this, 2×10^5 beads of an OBOC peptide library, spiked with beads bearing defined peptide sequences (c-myc or Ara h 2) in a ratio of 1:1,000, were incubated in a 3-ml reaction column with 2 ml of blocking solution for 3 h at room temperature under constant mixing. After removal of the blocking solution, 400 μl of fluorescein isothiocyanate (FITC)-labeled anti-IgE (clone BE5; 1:100 in blocking solution) was added, and the incubation was continued for another 3 h. Beads were washed (four times with 2 ml of PBST, two times with 2 ml of PBS) and incubated with 400 μl of anti-c-myc IgE (200 ng/ml) or human serum (160 ng IgE/ml) in blocking solution over night at 4°C under constant mixing. Beads were washed again (three times with 2 ml of PBST, two times with 2 ml of PBS), incubated with 400 μl of phycoerythrin-labeled anti-IgE (clone BE 5; 1:1,000 in blocking solution) for 3 h at room temperature, washed and processed for microscopy as described above.

Selection and Manual Isolation of Beads

For bead selection and isolation, the standard fluorescence microscope was used. Fractions of the bead mixture which had been examined before in 24- or 12-well plates were transferred into a 6-well plate (Costar Corning) containing 1.8 ml of PBS plus 200 μl of PBST per well. The dimensions of the wells of this plate and the sample dilution allowed access to selected single beads with minimal contact to neighboring beads. This facilitated manual bead manipulation by use of a micropipette. Beads were re-evaluated and adequate beads were chosen for isolation according to their fluorescence properties (for details see section "Results"). Each single chosen bead was removed by using a standard 10 μl pipette with low retention pipette tip. Non-relevant beads were pushed aside and the chosen bead was aspired together with ≤5 μl of the surrounding liquid and transferred into a separate well until further processing for peptide sequencing.

Peptide Sequencing

After manual isolation of a fluorescence positive bead, the bead was placed onto a trifluoroacetic acid-treated glass fiber disc (Fujifilm WAKO Chemicals Europe GmbH, Neuss, Germany). Location of the bead on the filter was verified via microscopic observation. The filter was loaded into the reaction chamber of an automated peptide sequencing system (PPSQ-53A peptide sequencer, Shimadzu, Kyoto, Japan). Peptide sequences were determined by direct on-bead Edman degradation followed by HPLC separation of the step-wise cleaved-off phenylthiohydantoin-derivatized amino acids. Identification of the individual amino acids was done by reference to a phenylthiohydantoin (PTH) amino acid standard (Fujifilm WAKO Chemicals).

RESULTS

Establishing an Assay System to Detect Specific IgE-Binding With Defined Peptides Immobilized on Polystyrene Beads

One-bead-one-compound libraries have the advantage to test millions of peptides in parallel for binding of a defined target molecule. To adapt and optimize this system for the detection of new IgE epitopes, we had to establish an assay format where IgE binding to a specific peptide immobilized on polystyrene beads can be reliably detected. To achieve this, we started with an unambiguous system of a defined IgE antibody – peptide epitope pair in an "artificial peptide library" based on a matrix of irrelevant peptide-carrying beads. We decided on a commercially available, recombinant human IgE antibody directed against the Myc-protein derived peptide c-myc (peptide sequence E-Q-K-L-I-S-E-E-D-L). Beads carrying the c-myc peptide, as well as "irrelevant" matrix beads carrying a scrambled version of the c-myc epitope (sequence E-I-E-D-K-L-S-L-Q-E), were produced by classical fmoc solid phase-based peptide synthesis. These beads were used to optimize assay conditions, especially in terms of anti-IgE antibody employed (type, concentration, fluorophore label), first in order to achieve highest possible sensitivity, and second to take into consideration that the structure of the fluorescent dye coupled to the anti-IgE antibody has an influence on the screening of OBOC libraries (32).

The first experimental set-up encompassed a 1:10 mixture of c-myc beads and scrambled c-myc beads and an incubation with anti-c-myc IgE antibody in a defined blocking buffer. By successively testing a variety of monoclonal and polyclonal anti-human-IgE antibodies with different green fluorophores (either DyLight 488 or FITC, see **Table 1**) we were able to improve the assay sensitivity to a detection limit of 20 ng IgE/ml (8.3 IU/ml). Microscopic examination of the results showed that in addition to the intensity, fluorescence distribution can be taken as a criterion for a positive bead, manifested by a distinct fluorescent "corona" at the edge of the bead (**Figures 2A,B** and **Supplementary Figure 2**). This is particularly helpful for distinguishing a positive signal from the auto-fluorescence in the green channel which is an imminent drawback of polystyrene beads (33, 34), especially when they bear peptides. In general, this auto-fluorescence is equally distributed across the whole bead area – in contrast to the antibody-derived, specific signal with a fluorescent corona. However, at relatively low signal intensities, the corona is fading and may become invisible over the green auto-fluorescence. In a next step, we therefore wanted to analyze fluorescence-based detection systems with excitation/emission at longer wavelengths, where the auto-fluorescence should be less pronounced.

We used the antibody showing best results in comparative testing (**Figure 2B**), but equipped with the red fluorophore DyLight 550 (**Figure 2C**), to detect bead-bound IgE. In this set-up, anti-c-myc IgE at a concentration of $\leq$10 ng/ml could be shown to specifically label peptide-bearing beads. This was verified by isolation of the fluorescence corona-positive beads followed by peptide sequencing. When the fluorescence was moved even further into the far red range by using (polyclonal) anti-IgE antibodies labeled with DyLight 633, DyLight 650 or DyLight 680 fluorophore, the auto-fluorescence background at the respective far red emission wavelengths was considerably reduced. This improved signal-to-noise ratio allowed us to detect anti-c-myc IgE concentrations as low as 2 ng/ml (0.83 IU/ml) (**Figure 2D**), which is well in the physiological range of specific IgE in human blood (35, 36). Unfortunately, the far-red fluorescence is barely detectable for the human eye, limiting its application for the manual detection and isolation of fluorescent beads with a standard fluorescence microscope. To facilitate the manual isolation of the fluorescence positive beads, which is an integral part of this platform, we are confident that an optimal trade-off between high microscopic detectability and low auto-fluorescence background may be obtained by using a secondary anti-IgE antibody labeled with a red fluorophore such as DyLight 550 or phycoerythrin.

Detection of Specific IgE-Epitopes Within a Bead-Bound "Artificial" Library of Defined Peptides Using Serum From Allergic Patients

In addition to establishing our peptide-beads screening assay with the defined recombinant anti-c-myc IgE antibody, we wanted to capture and detect allergen-specific IgE from serum from allergic patients with our bead system. These experiments were in part conducted in parallel to the c-myc experiments described above, hence here too, different secondary antibodies and fluorophores were used.

We employed serum from peanut-allergic patients which had been shown beforehand to have a high specific IgE reactivity against the major peanut allergen Ara h 2 (**Table 2**). Applying a microarray-based epitope mapping technique (27, 28) we could identify several linear epitopes on Ara h 2 which are recognized by these IgE (data not shown). Based on these mapping experiments, we chose the 8mer R-D-P-Y-S-P-S-P as typical Ara h 2 epitope, which also encompasses two of the three immunodominant Ara h 2 epitopes previously described (37) and which reacted with all sera used by us (three different peanut-allergic donors, **Table 2**). This 8mer epitope was synthesized on polystyrene beads and "irrelevant" matrix beads again carried a scrambled version of the peptide (P-P-D-R-S-Y-P-S).

Both bead species were mixed in defined ratios and binding of IgE from human serum from Ara h 2-sensitized peanut-allergic donors was investigated. Even with 1:2, 1:4 and 1:8 diluted serum from one anti-Ara h 2 IgE-positive peanut-allergic donor, Ara h 2 peptide beads could be detected with a green- or red-labeled anti-IgE antibody (**Figures 3A,B**), and their identity could be verified by manual isolation of the beads and subsequent peptide sequencing.

Improved Binding of IgE to Its Target Epitope With IgG-Depleted Serum From Allergic Donors

When comparing the sera from different Ara h 2-sensitized peanut-allergic donors in our bead screening assay, we found considerable differences in the fluorescence intensities of the corona-positive Ara h 2 peptide-bearing beads. This is certainly due to the different Ara h 2-specific IgE content of these samples (varying from 50 to $\geq$240 ng/ml), and to different reactivities against our chosen epitope peptide. A further reason for low IgE detectability may be the presence of IgG with identical epitope recognition in the sample. Along that line, we had realized already from our epitope mapping experiments that we could improve the IgE signals by depleting the serum samples of IgG. Hence, we wanted to port these findings to our bead-based IgE detection, in order to further improve the system.

We therefore compared the bead-based IgE detection of untreated serum with serum which had been depleted of IgG by a protein G-sepharose matrix. We used a serum containing approximately 50 ng/ml of Ara h 2-specific IgE (patient 2), which had shown a specific, albeit not very strong, epitope reactivity in our previous mapping experiments. Yet, the total, untreated serum could not detect any signals above background in our artificial library of Ara h 2/scrambled Ara h 2 peptide beads in combination with a monoclonal, phycoerythrin-labeled anti-IgE antibody (**Figures 3C,D**). Fluorescence intensities of randomly chosen areas, documented with the MORE life cell imaging microscope and analyzed with ImageJ software, were not different between serum-incubated beads and control beads incubated with only the phycoerythrin-labeled anti-IgE antibody. However, after removal of IgG from this serum, IgE binding

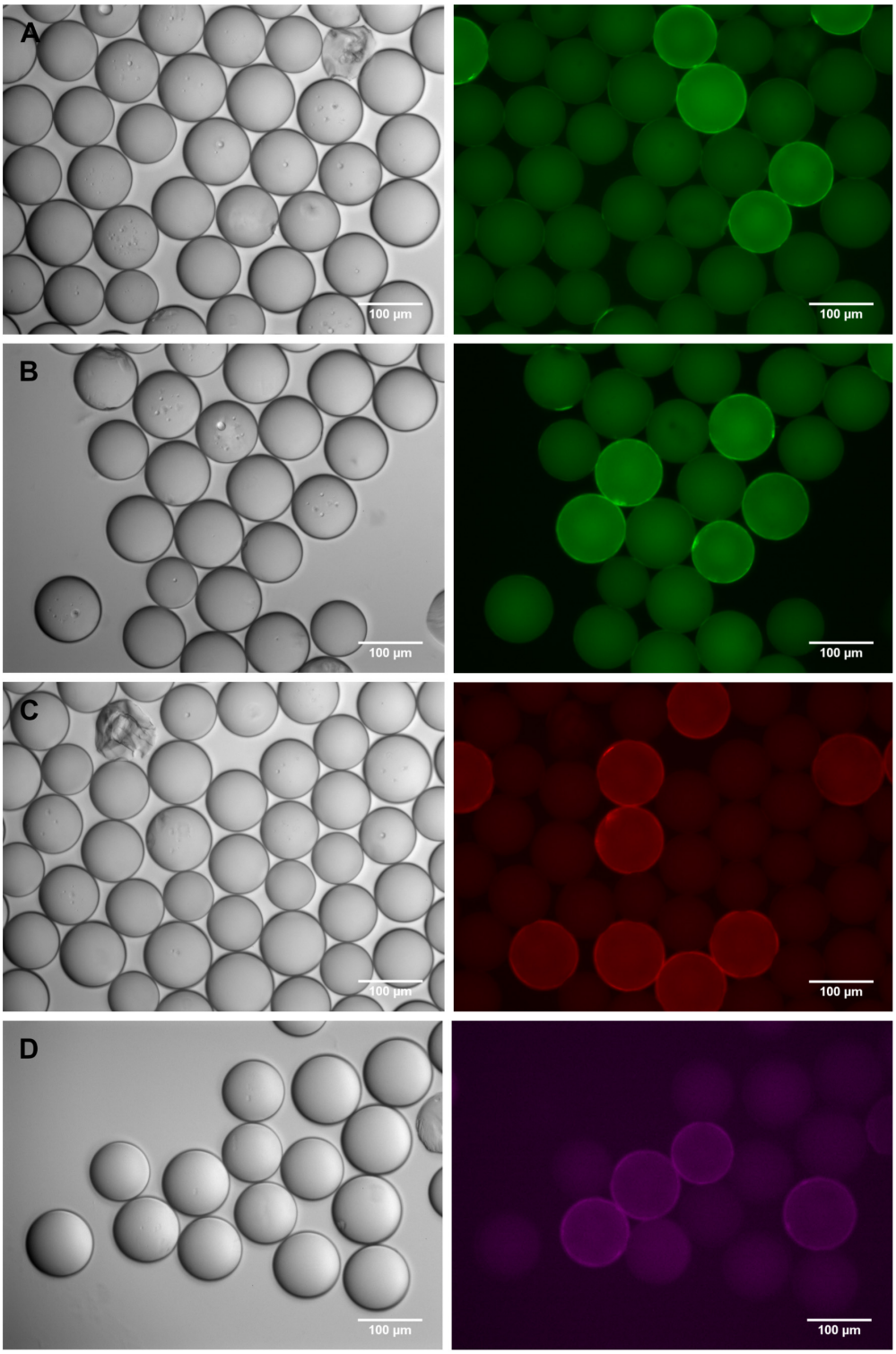

FIGURE 2 | Detection of c-myc-beads, diluted 1:10, in an artificial peptide library of scrambled c-myc beads. Different concentrations of anti-c-myc IgE were used and IgE-binding was detected with various fluorophore-labeled anti-IgE antibodies. Phase contrast images are shown on the left, corresponding fluorescence images of the appropriate channel on the right side of the panel. **(A)** 100 ng/ml anti-c-myc IgE detected with FITC-labeled monoclonal anti-human IgE (FITC-channel); **(B)** 20 ng/ml anti-c-myc IgE detected with polyclonal anti-human IgE labeled with DyLight488 (FITC-channel); **(C)** 20 ng/ml anti c-myc IgE detected with polyclonal anti-human IgE labeled with DyLight550 (rhodamine-channel); **(D)** 2 ng/ml anti c-myc and polyclonal anti-human IgE labeled with DyLight650, (Cy5-channel). High autofluorescence background is predominant in the green channel **(B)**, but true positives can be distinguished by presence of a more brilliant "corona." Autofluorescence decreases at higher wavelength **(C,D)**. Quantitation of fluorescence intensities can be found in the **Supplementary Figure 2**.

TABLE 2 | Characteristics of patient sera.

Patient ID	Total IgE		Anti-Ara h 2 IgE	
	ng/ml	U/ml	ng/ml	U/ml
P1	3,040	1,267	182	75.8
P2	624	260	53	22
P3	3,880	1,616	>240	>100

to the Ara h 2 peptide positive beads could clearly be detected with this anti-IgE antibody (**Figure 3E**). This also resulted in a more than threefold increase in the fluorescence intensity measured (see **Supplementary Figure 3**). Even a serum with a very high anti-Ara h 2 IgE concentration (≥240 ng/ml; patient 3), whose binding to the Ara h 2 peptide beads can be detected by the phycoerythrin-labeled anti-IgE antibody without serum pretreatment, profits from prior removal of the IgG. Here, too, a strong increase in the signal and in the total fluorescence intensity values is observed when the IgE-only fraction is used (data not shown). For both donors the red corona fluorescence-positive beads were verified to be the relevant Ara h 2 peptide beads, via isolation and peptide sequencing.

In addition to enhancing the specific IgE binding, removal of IgG from the serum enabled us to test the anti-IgE antibodies for potential cross-reactivity with IgG. We used the IgG fraction separated from serum of a donor where IgG reactivity with the Ara h 2 epitope had been demonstrated before in our epitope mapping experiments. We incubated these IgG with the artificial Ara h 2 bead library, followed by the monoclonal, phycoerythrin-labeled anti-IgE antibody. Although one can presume, due to our previous data, that IgG-binding to the Ara h 2 beads occurred, no corona-positive red fluorescence was detected, and total fluorescence values were not different from background values. This demonstrates that IgG-cross-reactivity of the monoclonal anti-IgE antibody was negligible.

In total, these experiments show that serum depleted of IgG may improve the detection of target-specific IgE, especially when IgE with only low/medium abundance are analyzed.

Detection of Specific IgE-Epitopes in One-Bead-One-Compound Combinatorial Peptide Libraries

Having established the IgE screening assay with artificial peptide libraries consisting of mixtures of defined peptides, we next moved to the detection of IgE epitopes within a true combinatorial one-bead-one-compound library. An OBOC-library of 8mer peptides was synthesized using the split-and-mix procedure (**Figure 1**). Portions of the library were spiked with specific c-myc or Ara h 2 beads in defined ratios and screened with anti-c-myc IgE or serum from a peanut-allergic donor. Using a rather small library of several thousands of OBOC beads and a comparatively high number of specific beads (3–5%), we could identify the respective target beads and verify the c-myc respectively the Ara h 2 identity by peptide sequencing (data not shown). However, when increasing the library size and reducing the fraction of specific beads (1:1,000), beads visually identified as being "positive" due to a corona-positive fluorescence were by majority not bearing the specific IgE target peptide when analyzed *via* peptide sequencing. We suppose that this is due to the presence of peptide sequences within the library that directly cross-react with the secondary (anti-IgE) antibody, even without an IgE bound to the beads. In fact, when we incubated the OBOC beads with anti-IgE antibodies alone, without prior addition of IgE or serum, corona-positive fluorescent beads were detectable. This effect was seen – albeit to a varying degree – with all the different anti-IgE antibodies we tested, polyclonal as well as monoclonal, and irrespective of the fluorophore used (**Figure 4**).

We attempted to suppress this cross-reactivity by variations in the blocking conditions as well as in the secondary anti-IgE antibody concentrations that were used during the screening procedure. Neither of these approaches was successful.

We then tried to block the cross-reactivity by incubating the OBOC library – spiked with specific c-myc or Ara h 2 beads at a ratio of 1:1,000 – with a FITC-labeled anti-IgE antibody at 10-fold the usual concentration. This pre-adsorbed library was then submitted to the standard screening procedure, using anti-c-myc IgE or anti-Ara h 2 IgE-containing serum as primary antibody and a phycoerythrin-labeled anti-IgE antibody for detection. To warrant maximum blocking of cross-reactivity, both secondary antibodies used were derived from the same monoclonal antibody (clone BE5), differing only in their fluorophore label.

This strategy proved successful in essence, but the parallel use of two fluorophores, and the inherent bead auto-fluorescence problem associated with the green channel, brought about difficulties in the visual inspection and identification of true positive beads. As exemplified in **Figure 5**, a variety of staining patterns was observed which required interpretation and verification. Basically, fluorescent beads fell into three categories: (1) cross-reactive beads of bright green fluorescence, carrying a corona, which were negative in the red channel (open arrowheads in **Figure 5**), (2) true positive beads with distinct fluorescence and corona in the red channel and no or only weak (auto-) fluorescence in the green channel (arrows in **Figure 5**), and (3) questionable beads, where the fluorescence and/or the corona was equally strong in both, the red and green channel (filled arrowheads in **Figure 5**). The discrimination between the three categories became more difficult if the beads were diluted for manual bead selection. Nevertheless, we were able to isolate single beads from each category and, after sequencing, could verify that beads which had been classified as true positives by their bright red fluorescence were carrying the specific peptides as expected. Questionable beads, on the other hand, which were either less bright in the red channel or also showed up strongly in the green channel, turned out to be of irrelevant sequence. These results demonstrate that the strategy of pre-adsorbing the anti-IgE-cross-reactivities in the OBOC-library before the actual screening process is a feasible approach given the final selection of positive beads is restricted to candidates of strong red and negligible green fluorescence.

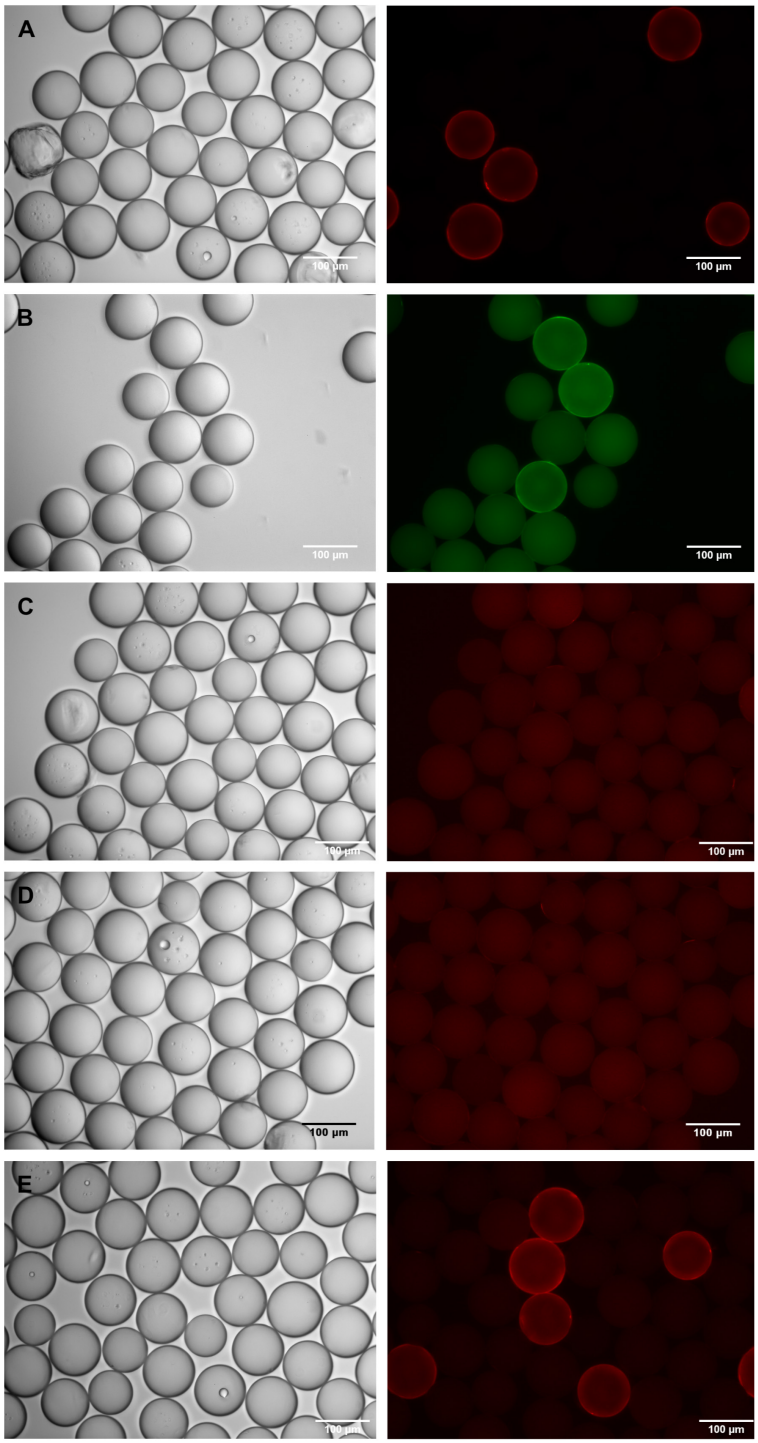

FIGURE 3 | Detection of Ara h 2-beads, diluted 1:10, in an artificial peptide library of scrambled Ara h 2 beads. Beads were incubated with serum from two different peanut-allergic patients, and IgE-binding was detected with fluorophore-labeled anti-IgE antibodies. Phase contrast images are shown on the left, corresponding fluorescence images of the appropriate channel on the right side of the panel. **(A)** Beads incubated with serum from patient 1, diluted to approximately 90 ng Ara h 2-specific IgE/ml; IgE-binding detected with phycoerythrin-labeled monoclonal anti-IgE antibody; **(B)** Beads incubated with serum from patient 1, diluted to approximately 23 ng Ara h 2-specific IgE/ml; IgE-binding detected with DyLight488-labeled polyclonal anti-IgE antibody; **(C)** Beads incubated with total serum from patient 2, diluted to approximately 33 ng Ara h 2-specific IgE/ml; IgE-binding detected with phycoerythrin-labeled monoclonal anti-IgE antibody; **(D)** Beads incubated only with phycoerythrin-labeled monoclonal anti-IgE antibody; **(E)** Beads incubated with IgG-depleted serum from patient 2, diluted to approximately 33 ng Ara h 2-specific IgE/ml; IgE-binding detected with phycoerythrin-labeled monoclonal anti-IgE antibody. Quantitation of fluorescence intensities can be found in the **Supplementary Figure 3**.

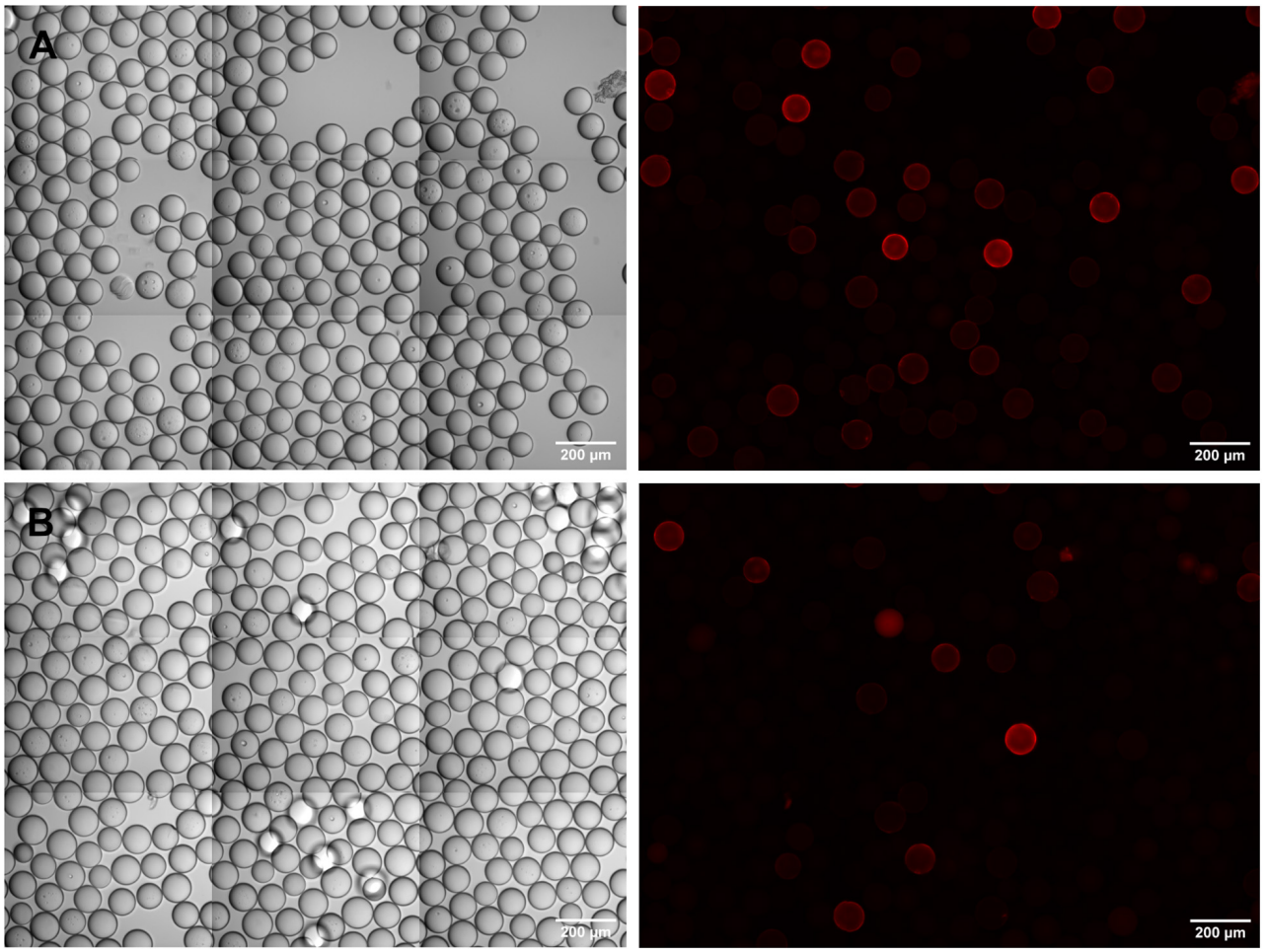

FIGURE 4 | Sporadic cross-reactivity of anti-IgE antibodies with bead-bound random peptides in an OBOC-library. **(A)** DyLight550-labeled, polyclonal anti-IgE antibody; **(B)** phycoerythrin-labeled, monoclonal anti-IgE antibody. Phase contrast images are shown on the left, corresponding fluorescence images on the right side of the panel.

Removal of Anti-IgE Cross-Reactive Beads From an OBOC-Library

Although our approach to pre-adsorb anti-IgE cross-reactive sequences in an OBOC library before the screening process worked reasonably well, we decided to evaluate another, different strategy to solve the cross-reactivity problem. In this approach, rather than leaving the pre-adsorbed beads within the library and having the green (auto) fluorescence interfere with the subsequent bead isolation process, we wanted to remove the cross-reactive beads from the library pool before the actual screening procedure. To do so, beads of the peptide library were again pre-incubated with high concentrations of a fluorophore-labeled secondary, anti-IgE antibody. Afterward the fluorescence-positive, anti-IgE cross-reactive beads were separated from the fluorescence negative beads (with no intrinsic affinity to the secondary antibody) by sorting with a BioSorter (**Figure 6**). This procedure resulted in the loss of a fair part of library beads and their potential IgE target sequences (from 2% up to 20%, depending on the individual OBOC library, the secondary antibody used and the exact gating conditions). As a consequence, however, the pre-cleaned library ought not to produce false positive signals due to anti-IgE cross-reactivity, and it does not necessitate the use of a green fluorophore, thereby avoiding the associated auto-fluorescence problems.

Detection of Specific IgE-Epitopes in OBOC Libraries After Removal of Anti-IgE Cross-Reactive Beads

For our final screening experiments, we used an OBOC peptide library where the anti-IgE cross-reactive beads had been removed via BioSorter separation after treatment with a phycoerythrin-labeled monoclonal anti-IgE antibody. One portion of this pre-cleaned OBOC library was spiked with c-myc beads (ratio c-myc to OBOC = 1:500) and incubated with anti-c-myc IgE (100 ng/ml). A second portion of the pre-cleaned OBOC library was mixed with Ara h 2 beads (ratio Ara h 2 to OBOC = 1:1,000) and incubated with serum from a peanut-allergic donor (specific anti-Ara h 2 IgE = 90 ng/ml). IgE-binding to the beads was

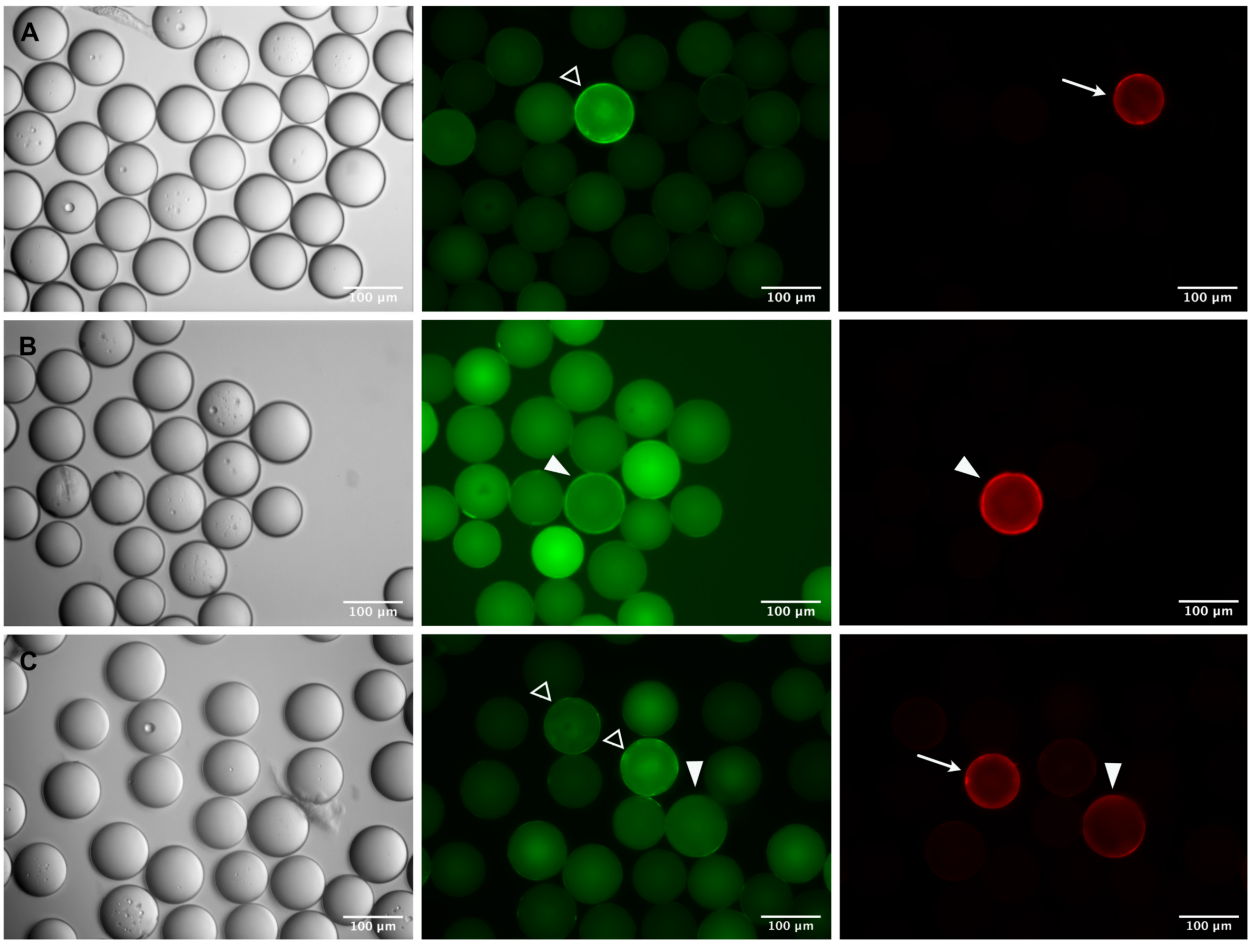

FIGURE 5 | Identification of specific epitope-carrying beads in a pre-adsorbed OBOC library. Specific c-myc- **(A,B)** or Ara h 2- **(C)** beads were mixed 1:1,000 with an OBOC library. Beads were incubated with a FITC-labeled monoclonal anti-IgE antibody to block cross-reactive sequences and subsequently screened with anti-c-myc IgE **(A,B)** or serum from a peanut-allergic patient **(C)** followed by detection of IgE-binding with a phycoerythrin-labeled monoclonal anti-IgE antibody. Phase contrast images are shown on the left, corresponding fluorescence images of the green and the red channel are in the middle and on the right side of the panel. "True positive" beads carrying the specific epitope sequence are recognizable by strong fluorescence only in the red channel (arrows) and only slight autofluorescence in the green channel; pre-adsorbed "cross-reactive" beads show a strong fluorescence with corona in the green channel (open arrowheads), and no signal in the red channel. A number of fluorescent beads cannot be clearly assigned to either "positive" or "cross-reactive," exhibiting similar fluorescence intensities and/or coronas in both, the red and the green channel (closed arrowheads).

detected with the same phycoerythrin-labeled monoclonal anti-IgE antibody that had been used for the pre-cleaning process.

Under microscopic examination, both screening set-ups showed a small number of red fluorescent beads, in line with the low abundance of beads in the library that were carrying specific epitopes. Due to the brightness of the fluorescence, and the low background in the red channel, the individual positive beads could easily be detected and singularized. For each of both set-ups, 10 red-fluorescent beads were isolated and their peptides were sequenced. The correct c-myc sequence could be confirmed for 9 out of the 10 c-myc candidates, and for the Ara h 2 screening set-up, also 9 out of 10 isolated beads could be confirmed to carry the Ara h 2 peptide. Moreover, after repeating the Ara h 2 screening experiment in an identical set-up, but using a different batch of the same monoclonal anti-IgE antibody for detection, 10 of 10 isolated beads carried the Ara h 2 epitope sequence.

In conclusion, we demonstrate here that we have established a method of pre-cleaning and screening an OBOC library that can be used to identify specific IgE-epitope bearing beads within a library of $\geq$100,000 different peptides.

DISCUSSION

A considerable number of asthmatics display high total serum IgE levels along with the respective airway pathology but do not react with the typical aeroallergens they are tested for. Although some disorders such as parasite infections or hyper-IgE-syndrome promote the formation of IgE, and atopic predisposition may support class-switch of natural antibodies to class E, it is unlikely that those afflictions account for the high total serum IgE levels that are often associated with asthmatic airway pathology.

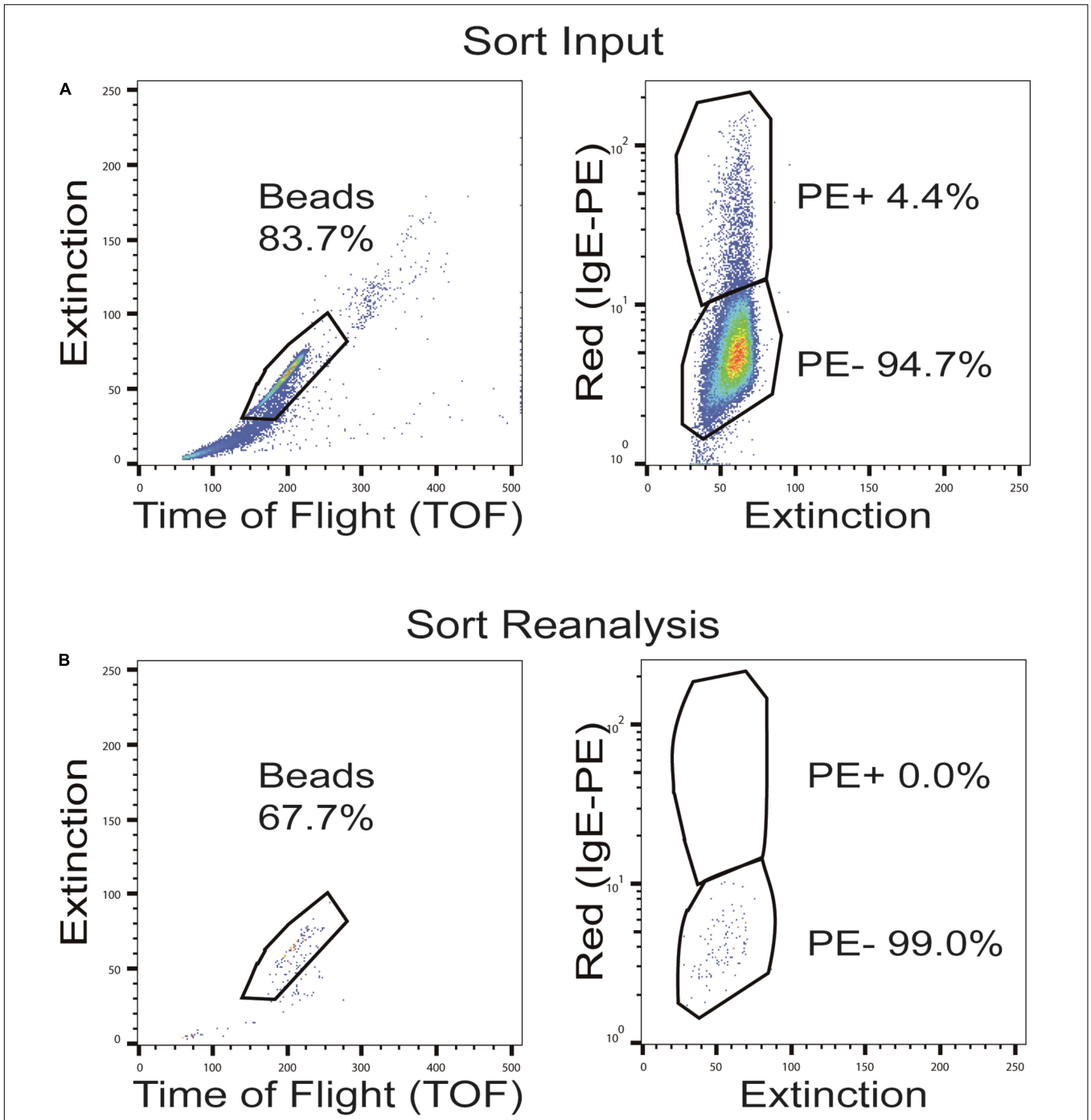

FIGURE 6 | Sorting of OBOC beads after pre-adsorption with the phycoerythrin (PE)-labeled anti-IgE antibody. Representative dot plots with full gating of **(A)** the total pre-adsorbed OBOC library showing the anti-IgE cross-reactive beads in the upper PE+ gate and the non-cross-reactive beads in the lower PE- gate, and **(B)** re-analysis of the pre-cleaned non-cross-reactive bead fraction (PE-) after sorting.

Parasite infections were shown to protect against asthma (38), cases of hyper-IgE-syndrome are extremely rare (39) and natural antibodies are usually of low affinity (40, 41). However, high affinity seems to be necessary for the pathology that accompanies high IgE levels in allergy and asthma (42). It is thus probable that the high total serum IgE levels in asthmatics are largely composed of IgE which underwent affinity maturation against some specific antigen even if they are negative for the typical aeroallergens. The shortfall of proper allergy diagnosis in such cases is mostly due to the fact that *in vitro* routine allergy diagnostic tests include only a limited number of clinically relevant allergen sources and often lack relevant single allergenic

components. *In-vivo* allergy diagnostic tests (skin prick tests) represent the main approach to confirm clinical suspicion of allergic sensitization, but they are mainly based on the application of crude allergen extracts, and standardization remains difficult (43). In this respect, the Global Allergy and Asthma European Network (GA^2LEN) has established a protocol for a standard prick test panel with a total of just 18 inhalant allergens (43), which they propose to be sufficient for general respiratory allergy testing in Europe. However, considering the more than 3,200 different allergenic molecules identified to date (44) and the often unsatisfactory outcome of allergy testing in high-IgE asthmatics, this test panel appears to be all too limited for a personalized diagnostic approach.

Anyhow, as soon as an allergy could be proven to be the initiator of asthma, and the corresponding allergen could be identified, causative treatment strategies (like allergen-specific immunotherapy) can be introduced to the respective patients. Thereby a specific anti-inflammatory treatment can be provided and – if necessary – can be combined with treatment of disease-relevant target molecules (target treatments), like the application of biologicals against certain cytokines (45). In any case, the patients will profit considerably from an improved molecular allergy diagnostic test based on specifically recognized epitopes.

In principle, the specificity of high affinity IgE can be deduced from their selective recognition of cognate allergen or allergen epitope upon its being offered for binding. Unfortunately, most bioinformatical approaches to predict an epitope on the basis of the paratope/CDR sequence (46, 47) did not meet up to the expectations as yet. Hence, a procedure where an abundance of potential epitopes can be offered to an IgE population with unknown specificities appears to be more promising. Although phage display libraries may be an apparent choice in this context, the presentation of an epitope in a permissive scaffold phage protein may be futile. An epitope's presentation in the original topology of its parent allergen is usually indispensable for recognition, and restricted epitope flexibility may prevent induced-fit-binding. On the other hand, the structural constraints and rigid presentation of the peptide insert as part of the scaffold protein render phage display libraries more suited to identify mimotopes that imitate the structure of a conformational epitope (48). In such a case, the amino acid sequence of the peptide insert does not necessarily have any resemblance to that of the natural IgE epitope, and deductions as to the respective antigen/allergen recognized are not feasible. Thus, a display system such as chemically synthesized peptides flexibly linked to the surface of microparticles, where an epitope is free to adopt any conformation, may be more advantageous to identify a linear epitope or the linear core motifs of conformational epitopes. We therefore decided to adapt the OBOC technology for our scientific research question.

Any library, OBOC as well as phage display, is limited by the number of peptides which can be presented. A comprehensive 8mer motif library will contain 1.7×10^{10} different permutations when 19 amino acids are used. This translates into about 2 times 10 billion different phages or 240 kg of OBOC beads. Such huge numbers of phages and amounts of beads are impossible to handle and to screen. Yet, typical epitopes contain a core region of 4–7 amino acids (mean 5.5) (49, 50) which translates into roughly 10 million possible permutations. In view of this, in our OBOC-library of 10 million beads all relevant core motifs should theoretically be contained in the carboxy terminal part (that is the first 5–6 amino acids) of all peptides as a whole. The closer to the amino terminus we get, the more permutations are not represented. We nevertheless decided to extend the OBOC library toward being "non-representative" by using 8mer peptides in order to further increase our chances to identify epitopes which require more than the minimal core region for recognition. We consider this length a good compromise between being representative with the number of beads that can be handled, and the chances of displaying epitopes sufficiently long to be specifically recognized.

In order to establish a robust screening procedure that uses OBOC-libraries for epitope discovery, we decided for a stepwise, systematic approach to solve any intrinsic and extrinsic problems. The main problem to be overcome turned out to be the necessity for high sensitivity of the assay system in order to enable us to detect a specific reactivity with IgE at physiological concentrations. In healthy people the total serum IgE level is usually below 100 IU per ml. A total serum IgE value $\geq$100 IU/ml (corresponding to 240 ng/ml) is considered indicative of allergy/atopy, and in some allergic patients serum IgE against one specific target may reach 1,000 IU/ml (2,400 ng/ml) and above. Still, these immunoglobulin concentrations are in a range where detecting their specific reactivities on a one-bead-level may become a problem. Addressing the sensitivity issue, we tested a variety of different reporter systems attached to anti-IgE antibodies to visualize binding of IgE to the beads. Initial attempts with enzymatic reporters that produce insoluble dyes failed to provide the desired sensitivity and/or specificity, even when tyramide signal amplification (51) or fluorescent substrates such as Amplex Red (52) were used. We therefore switched to fluorophore labels directly conjugated to the secondary antibody, additionally providing the possibility to detect, (pre-) sort and isolate specific beads automatically via fluorescence properties with cell sorter techniques. While this system proved more sensitive, we encountered another difficulty associated specifically with fluorescence detection. The polystyrene-based OBOC bead matrix, in particular when covered with peptide, is not optically inert (33, 34). It tends to display a considerable auto-fluorescence at green emission wavelength (535 ± 50 nm), which obscures the specific signal when e.g., FITC is used as label. We therefore decided to move to reporter fluorophores with higher emission wavelength. Here bead auto-fluorescence was considerably lower and detection sensitivity higher. The sensitivity benefit of migrating toward near infrared was, however, limited by the capacity of the human eye to detect far red light since the beads are being picked manually under visual inspection. For the future, we envisage that the whole screening and bead-isolation process might be automated in a microfluidic system. If this is equipped with an infrared camera the use of near infrared dyes such as DyLight755 or DyLight800 becomes feasible and detection limits can be extended beyond the limits reported in this study.

A further problem which we had not initially anticipated was the capacity of certain beads in the OBOC library to directly acquire secondary antibody. Although the extent of direct binding to the beads varied with the secondary antibody(-conjugate) used, it appears likely that the main determining factor for this cross-reactivity resides in the peptide sequence motifs presented on specific beads. This includes peptides which by chance resemble part of the IgE heavy chain or motifs that have affinity to some region of the secondary antibody or of the fluorophore. Analysis of such undesired binders revealed that they mostly contain aromatic amino acids and proline. Similar results were reported in another study, where OBOC beads were screened with a probe equipped with the fluorophores ATTO590 and TexasRed. Here, the amino acids leucine/isoleucine, histidine, phenylalanine and tyrosine were enriched in false positives reacting with the dye (32). These data are intriguing. Yet, when considering that in an OBOC library where all amino acids are used in equal amounts during synthesis, the three aromatic amino acids as well as histidine are overrepresented as compared to their natural frequency observed in proteins (53), the undesired binders may at least be explainable. Unfortunately, little information is available about amino acid distributions in IgE binding sequences. So far only one study (54) addressed this question and reported a preference for Ala, Ser, Asn, Gly and especially Lys in IgE epitopes. In light of this, it may be advisable to create "natural amino acid frequency-representing" OBOC libraries where the occurrence of certain amino acids reflects the natural occurrence of those building blocks. Further restrictions, on the other hand, such as overweighing the amino acids preferentially found in IgE epitopes (54) may limit the diversity of the OBOC library offered.

In order to reduce undesired direct secondary antibody labeling of beads without tampering with library composition we followed two avenues of resolution for the problem, both of which proved practicable. In the first approach, the complete OBOC-library was pre-incubated with an excess of secondary antibody equipped with a green fluorescent dye, then incubated with the IgE pool and finally exposed to the same secondary antibody labeled with a red fluorophore. In this case the decision between false and true positives has to be made by comparing green and red fluorescence of individual beads upon microscopic inspection. In practice, this turned out to be a rather time-consuming process with a certain operator variability. Focusing on beads with a strong corona in the red fluorescence channel and negligible green fluorescence yielded an excellent true-positive rate – as verified by sequencing – in our experimental set-up. However, it must be surmised that the same strategy decreases the sensitivity of the procedure and reduces the probability to positively identify epitopes of rare IgE species. It therefore seemed reasonable to remove any cross-reactive beads from the OBOC library before the actual screening process. This was achieved by sorting the fluorescently pre-adsorbed OBOC library with a large particle sorter (BioSorter). While this approach led to satisfactory results in our set-up, it must be conceded that the limited availability of suitable sorting devices renders this procedure not highly practicable for general use. Here certainly remains room to improve the protocol, either by adaption of more accessible sorting devices (e.g., FACS) to the specific particle size used in the libraries, or by refining the back-up protocol employing two differently labeled anti-IgE antibodies with the use of fluorophores less sensitive to the interfering bead auto-fluorescence.

The final step to validate our OBOC library screening procedure was the verification of the true positives after bead isolation by peptide sequencing. First attempts using mass spectrometric analysis (MALDI-TOF-MS) of fragments after ammonolysis with aqueous or neat ammonia, however, were not successful. Although some studies report mass spectrometrical sequencing of peptides released from beads upon ammonolysis (32, 55–57), in our hands OBOC beads did not liberate equal amounts of cleavage product upon ammonia exposure which caused difficulties to reconstruct the synthesized motif *in silico*. Even known peptide sequences on defined c-myc or Ara h 2 beads could not be resolved by MALDI-TOF-MS. We therefore switched to Edman degradation-based peptide sequencing on an automated protein sequencer. Single isolated beads were directly subjected to the sequencing process, without the necessity of first removing bound antibodies or cleaving the peptide off the bead. Although the low amount of peptide present for sequencing (maximum theoretical capacity per bead 50 pmoles) is close to the sensitivity limit of the sequencer, the peptide sequence on the isolated beads could be successfully resolved in the great majority of cases. Furthermore, beads with higher peptide capacity (e.g., 100 pmoles) are available. All procedures described here could be adapted without much effort to those beads, in case of ambiguous results in terms of peptide sequencing during OBOC library screening.

Taken together, we here present a detection system to identify unknown IgE reactivities by using chemically synthesized one-bead-one-compound libraries. We are confident that this technology will aid in the identification of novel allergens for asthmatic individuals with high total serum IgE and with no specific allergic reaction detectable to date.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the University of Lübeck. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

TK designed the study, designed and performed the experiments, analyzed and interpreted the data, and wrote the manuscript. NR and BM designed and performed the experiments, analyzed the

data, and wrote the manuscript. HS, CS, and SM performed the experiments, analyzed the data. UJ recruited and characterized peanut-allergic patients, collected the samples, and discussed the data. AF conceived and supervised the study, discussed the data, and wrote the manuscript. All authors read, revised and approved the manuscript for submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.565243/full#supplementary-material

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Mimicking Antigen-Driven Asthma in Rodent Models—How Close Can We Get?

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Asthma is a heterogeneous disease with increasing prevalence worldwide characterized by chronic airway inflammation, increased mucus secretion and bronchial hyperresponsiveness. The phenotypic heterogeneity among asthmatic patients is accompanied by different endotypes, mainly Type 2 or non-Type 2. To investigate the pathomechanism of this complex disease many animal models have been developed, each trying to mimic specific aspects of the human disease. Rodents have classically been employed in animal models of asthma. The present review provides an overview of currently used Type 2 vs. non-Type 2 rodent asthma models, both acute and chronic. It further assesses the methods used to simulate disease development and exacerbations as well as to quantify allergic airway inflammation, including lung physiologic, cellular and molecular immunologic responses. Furthermore, the employment of genetically modified animals, which provide an in-depth understanding of the role of a variety of molecules, signaling pathways and receptors implicated in the development of this disease as well as humanized models of allergic inflammation, which have been recently developed to overcome differences between the rodent and human immune systems, are discussed. Nevertheless, differences between mice and humans should be carefully considered and limits of extrapolation should be wisely taken into account when translating experimental results into clinical use.

Keywords: mouse model, asthma, T2 airway inflammation, non-T2 airway inflammation, endotypes

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INTRODUCTION

Asthma is a heterogeneous disease which affects around 300 million individuals of all age groups and its prevalence is increasing worldwide. Its impact is considered similar to other major chronic diseases such as diabetes or Alzheimer disease (1, 2). Asthma is defined by a history of respiratory symptoms such a wheeze, shortness of breath, chest tightness, cough and variable expiratory airflow limitation (3). A chronic airway inflammation leads to airway remodeling with hyperplasia of goblet cells and mucus hypersecretion, hypertrophy and hyperplasia of smooth muscle cells and lung fibrosis. Different asthma phenotypes have been described, which drove the development of the concept of asthma endotypes, where each endotype is a subtype of a disease condition and defined by a distinct pathophysiological mechanism, in contrast to the disease phenotype, which comprises the observable characteristics of the disease (4, 5). Generally, asthma can be separated in two

main endotypes, a so-called Type 2 endotype, characterized by T-helper Type 2-high inflammatory response and a non-Type 2 endotype, whereby also mixed endotypes are not rare (6, 7). While asthmatic reactions can also be induced without exogenous triggers (8, 9), only antigen-driven asthma models will be discussed here.

Airway Type 2 immune responses are mainly mediated by eosinophils, mast cells, basophils, T2 cells, group 2 innate lymphoid cells (ILC2s) and IgE-producing B cells (10, 11). The whole inflammatory process starts with the activation of epithelial cells and release of cytokines such as IL-25, IL-31, IL-33, and TSLP which contribute to downstream T cells- and innate lymphoid cells-mediated T2 immune responses. These are characterized by the release of cytokines such as IL-4, IL-5, IL-9, and IL-13, consequent production of allergen-specific IgE, recruitment of eosinophils and other inflammatory cells, production of mucus and smooth muscle hyperreactivity (12). Non-Type 2 asthma, instead, is characterized by airway inflammation in the absence of eosinophils and is often associated with environmental and/or host hazards, such as cigarette smoke, pollution, work-related agents, infections, and obesity. These risk factors, alone or in conjunction, can activate specific cellular and molecular pathways leading to non-type 2 pulmonary inflammation (13). Growing evidence supports two major characteristic features of non-Type 2 asthma, namely a neutrophilic-driven inflammation and an IL-6-driven activation of the IL-17-dependent pathway (14, 15). To allow for a detailed investigation of molecular pathways critical for this complex disease or for a specific endotype in a functioning immune and respiratory system, many animal models of asthma have been developed, each of them trying to reproduce specific aspects of the human disease. Because of their low cost, high breeding efficiency and the large availability of transgenic models, rodents, and especially mice have classically been employed in asthma research, although considerations have been made regarding their limitations in mimicking human asthma (16, 17). In this review we will focus on antigen-driven asthma models and methods used for the elicitation and quantification of allergic airway inflammation, including lung physiologic, cellular and molecular immunologic responses. Furthermore, approaches to study exacerbations, chronicity and non-allergic airway inflammation as well as the value of humanized models will be discussed. Nevertheless, differences between mice and humans should be carefully considered and limits of extrapolation should be wisely taken into account when translating experimental results into clinical use.

ELICITING ALLERGIC AIRWAY INFLAMMATION

Historically, experimental asthma research was performed sensitizing rodents intraperitoneally with chicken ovalbumin (OVA) in combination with the pro-T2 adjuvant aluminum hydroxide (alum), followed by repetitive OVA exposures via the airways in order to elicit a Th-2 skewed adaptive immune response leading to eosinophilia, goblet cell hyperplasia and airway hyperresposiveness (18–20). Alum plays an important role in boosting the adaptive immune system via the inflammasome (21). The benefits of OVA lie on the fact that this substance is efficient, inexpensive and has well-characterized MHCI and MHCII epitopes and moreover OT1 and OT2 T-cell receptor transgenic mice are available, which allow monitoring of OVA-specific immune responses in the airways (22, 23), making OVA a very good option for unraveling underlying mechanisms of the disease. However, OVA is not allergenic upon inhalation, therefore it has been more and more replaced by naturally occurring allergens which possess higher clinical relevance.

Allergens frequently used in sensitization protocols include the house dust mites (HDM) *Dermatopagoides pteronyssinus* and *farinae*, the fungus *Alternaria alternata*, cockroach and pollen extracts. The principle of sensitization and challenge remained the same as it was for OVA, but here the use of the adjuvant became dispensable. Adjuvant-free models have been established using several intranasal instillations of these allergens, mimicking the natural exposure to airborne allergens via the nasal mucosa and airway tract (24–27). Some of these allergen complexes like HDM are characterized by an intrinsic protease activity which favors the initiation of the allergic response, stimulating the production of interleukin-25 (IL-25), IL-33, and thymic stromal lymphopoietin (TSLP) from airway epithelial cells, which in turn activate both dendritic cells, promoting T2 responses (28), and local ILC2, leading to the increased release of IL-5, IL-9, IL-13, and amphiregulin (26). Some others, like birch, grass or ragweed pollen grains, do not only release allergens, but also proinflammatory and immunomodulatory lipids and adenosine, which act as critical co-factors in the development of lung allergic inflammation (24, 29).

Whereas models using allergen sensitization/provocation via the airways is reminiscent of the standard route of sensitization in asthma and hay fever, there is also compelling data on the relevance of cutaneous exposure in the development of pulmonary allergy along the lines of the so-called "atopic march" in which eczema precedes food allergies, asthma or hay fever (30). Mouse models have confirmed that repeated epicutaneous sensitization to protein (aero)-allergens leads to phenotypes of atopic dermatitis and to increased risk of allergic rhinitis, lung inflammation and airway hyperresponsiveness, where skin barrier dysfunction and TSLP expression from keratinocytes play essential roles (31–35).

Besides the pulmonary inflammation upon allergen exposure, exacerbations induced by other factors like viral and bacterial infections are a characteristic feature in the course of disease (36, 37). Here murine models of asthma have been especially useful to identify possible effects of infections with the development of the pathology. Particularly, influenza (38–40), rhinovirus (41, 42) and respiratory syncytial virus (38, 43) are important pathogens in the childhood that have been associated with exacerbations in asthma.

Haptens are also broadly used in rodent models to investigate exacerbation in airway inflammation. Studies with toluene diisocyanate (TDI), trimellitic anhydride (TMA), dinitrofluorobenzene (DNFB), and picryl chloride (PCL), allowed dissecting the hapten-induced allergy as well as the

similarities and differences between the different compounds (44–47). Rodent models of DNFB, a powerful sensitizer of non-atopic asthma (47), have recently shown increased numbers of macrophages in bronchoalveolar lavage fluid (BALF), tracheal hyper-reactivity and a strong neutrophilic-based lung inflammation that could reflect characteristic features of non-atopic asthma in humans (46, 48).

QUANTIFYING ALLERGIC AIRWAY INFLAMMATION

The physiological characteristics of asthma are mediated by a complex interaction between multiple effector cells and mediators.

The increased infiltration of inflammatory cells is determined by total and differential cell counts as well as measurement of inflammatory mediator content in the BALF or lung tissue (24, 49, 50). Upon allergen provocation, especially the role of eosinophils is shown to be indispensable for the development of allergic airway inflammation by mediating influx of T cell subsets [reviewed in (51)] into the lung (52, 53). For their release of pro-inflammatory mediators these cells are important contributors to pathophysiological changes, including airway epithelial cell damage, mucus hypersecretion and goblet cell hyperplasia which can be observed and quantified in histological staining of lung tissue (20, 54). In this context, eosinophils can be quantified by cell surface markers and by direct counting of stained cells in histological specimen (55).

Regarding the measurement of inflammatory mediators, tissue-based *ex vivo* cultures are another way to examine which cytokines are regulated in the development of airway inflammation and asthma and which cell type plays a decisive role in the concerned organs [reviewed in (56)]. As an alternative to the determination of cytokines in the supernatant of lung homogenates, stimulation of cells isolated from lung tissue or draining lymph nodes, by adding e.g., the allergen is used to evaluate the distinct cytokine patterns and to examine cell type specific responses more precisely (57, 58), allowing initial mechanistic conclusions about the observed phenotype.

As a hallmark of T2-driven allergic asthma, allergen-specific IgE responses are quantified in murine sera e.g., by means of ELISA (enzyme-linked immunosorbent assay) or functional cellular assays (59). Another factor to be taken into account in this context is IgG (and its subclasses), which are known to modulate inflammation via its receptors (FcγR) (60, 61). For example, antigen-specific IgG has been shown to improve allergic airway inflammation when signaling via FcγRIIB on DCs (62) and triggering different FcgR via certain IgG subclasses engage different pathways in murine IgE-independent anaphylaxis (63). Interestingly, similar mechanisms are discussed to take place in humans as well (64).

Airway hyperresponsiveness (AHR), defined as the predisposition of the airways to react excessively to bronchoconstrictor agents or to noxious stimuli, is an essential component of the asthma phenotype. The degree of AHR usually correlates with disease severity (65), and can be employed clinically for therapy management (66). AHR may not replace measurements of lung function such as FEV1, however it has been proposed to be included with other indices of lung function for asthma control (67). Similarly to spirometry in cooperative humans, lung function testing has been developed for rodents. Analysis of AHR in animal models is usually performed using one cholinergic agonist (methacholine, carbachol, histamine, serotonine), which act on the muscarinic receptor transduction pathway coupling to airway smooth muscle contraction (68). Measurement of AHR is usually performed shortly (24–48 h) after allergen challenge either in whole body chambers in conscious animals (body plethysmography) or in tracheostomized animals, using systems such as the Buxco® or the Flexivent®, with the agonist being either injected or aerosolized (24, 50, 69). Whilst the measurement of Penh (enhanced pause) using body plethysmography has lost acceptance in the scientific community (70), measurement of respiratory system resistance (RL) and dynamic compliance (DC) together with other physiologic parameters under mechanical ventilation in tracheostomized animals is often employed in asthma research (50, 71, 72). An increase in RL reflects both narrowing of the conducting airways and alterations in the lung periphery (distal airways and parenchyma). On the contrary, decreases in DC reflect only events occurring in the lung periphery. Therefore, if the response to an intervention is limited largely to RL, then a relatively proximal location is implicated for the effect, whereas a distinctive effect on DC is indicative of a more distal site of action. (73).

The limitation of this technique is based on the fact that it is only applicable in terminal experiments. This has been overcome by the use of oro-tracheal intubation technique, allowing for repetitive measurements in the same animals, which can be of advantage in longitudinal studies (74, 75).

NON-ALLERGIC ASTHMA MODELS

Since the non-allergic asthmatic phenotype occurs also in patients with severe, steroid resistant asthma and management of asthma evolves into precision medicine with therapies directed toward specific phenotypes/endotypes (76–78), proper models of these conditions are needed to facilitate research on adequate therapeutic options (79). In this regard, it was shown that a Th17-driven non-eosinophilic lung inflammation is insensitive to several treatment options including steroids, by using adoptive transfer of *in vitro* polarized antigen specific Th17 cells with subsequent pulmonary allergen application (80, 81). Manni et al. could create a mixed phenotype by adoptive transfer of T2 and Th17 cells enabling them to dissect contributions of the different cytokine pathways to distinct features of airway disease like mucus metaplasia or tissue inflammation (82). Microbial components like bacterial lipopolysaccharides (LPS) used as adjuvants in airway application of allergen have been proven to elicit a non-eosinophilic airway inflammation by triggering pathogen recognition receptors (PRR). Kim et al. could demonstrate that in such models the dose of LPS during

sensitization plays a decisive role in shaping the resulting lung inflammation either toward eosinophilic (low dose LPS) or neutrophilic (high dose LPS) inflammation (83). Comparing this airway sensitization model to intraperitoneal allergen application (with alum) Wilson et al. could illustrate how different sensitization regimes lead to different molecular and phenotypical pattern in the resulting airway disease identifying a prominent role for Th17 in neutrophilic airway inflammation and AHR (84). Hadebe et al. demonstrated the importance of microbial triggers in airway immune responses via initiation of a non-allergic steroid-refractory airway inflammation by combining two different agents (LPS and beta-glucan) (85). A more sophisticated approach uses biolistic transfection of a plasmid containing the genetic information of the allergen via gene-gun, with targeted expression in dendritic cells ensured by a specific promotor, leading to a Th1/Tc1 driven inflammation depending on IFNγ that is sensitive to steroid treatment (86). Application of Poly I/C, a dsRNA analog mimicking a viral infection, in combination with an allergen results in a Th1-driven airway inflammation as well, offering the possibility to study the pathomechanism underlying virus-induced airway inflammation (87). Taking advantage of the possibility to shape the resulting airway inflammation by means of different sensitization regimes (using the same allergen: house dust mite), Tan et al. were able to directly compare transcriptomic lung profiles of eosinophilic, neutrophilic and mixed phenotypes enabling identification of molecular pattern that are linked to distinct inflammatory phenotypes (88).

Aspirin-exacerbated respiratory disease (AERD) is a common, severe variant of asthma, which affects 7–10% of all asthmatics and is associated with overproduction of cysteinyl leukotrienes (cysLTs) and respiratory reactions to drugs that block cyclooxygenase 1 (89). The pathophysiology has not been fully solved yet, but in order to model this disease deficiency or overexpression genetic animal models have been used presenting severe eosinophilic respiratory mucosal inflammation (90, 91).

CHRONICITY AND REMODELING

Most of the above-mentioned models focus on the development of symptoms after a short period of antigen exposure. While this has provided a broad range of information on causal and mechanistic effects on asthma, it usually cannot mimic characteristics like chronic inflammation of the airway wall, mucus production and remodeling (92–95).

To compensate that limitation, several methods applying allergen for a longer period of time have been established. This causes a protracted experimental window up to several months and in some cases, due to the continuous exposure to the allergen, leads to tolerance in the mice (96–101). The transgenic technology allowed the generation of mice with characteristics of chronic asthma and airway remodeling (102, 103). Furthermore, transgenic models allowed the identification of an important migration factor of DCs to the lung (104) and the role of IL-33 receptor suppressor of tumorigenicity 2 (ST2) in development of chronic asthma in mice by regulating ILC2s, mast cells, IL9 and IL-13 in the lungs (105). In addition, recent gene modification in mice allowed to identify for example the role of the potassium channels Kca3.1 in airway remodeling (106), and the regulatory role of semaphorin 3E (Sema3E) in inflammatory and remodeling responses in chronic asthma (107).

Recently, CRISPR/Cas 9, a gene disruption technology, allowed to knock-out/down several genes in associated with exacerbation, inflammation and remodeling in asthmatic diseases, identifying roles for these molecules in some pathophysiological features of asthma. For example, using the CRISPR/Cas 9 technology the transient receptor potential (TRP) 1, an ion channel was successfully knocked-out by Reese et al. They could demonstrate its role in the protection from airway inflammation in rats as well as in mice, suggesting TRP1A as a therapeutic target in asthma (108). In another study depletion of long non-coding RNAs (lncRNAs), particularly AK085865, led to reduction of the inflammatory response in a murine model of asthma, by modulating differentiation of innate lymphoid cells progenitor (ILCP) into ILC2s (109). CRISPR/Cas 9, because of its high target specificity, is a tool that could be of high importance in the understanding of the pathomechanisms of asthma and identification of novel therapeutic targets.

HUMANIZED MOUSE MODELS

Despite the widespread use of mouse models for the evaluation of asthmatic diseases, there are restrictions when comparing components of the murine biology (e.g., the immune system) with those of the human biology (110). Humanized mouse models, that are immunodeficient mice engrafted with functional human (immune) cells, help to overcome some of these discrepancies. They have become an important pre-clinical tool for biomedical research, but to date only a small number of humanized mouse models are available in the research field of asthma.

Currently immunodeficient mouse strains for this purpose are often based on IL2rg^{null} mice, which lack a functional common gamma chain (γc) of the IL-2 receptor. This chain is not only part of the receptor complex for IL-2, but assembles with other chains to form receptors for IL-4, IL-7, IL-9, IL-15, and IL-21 as well, which are expressed on several cells of the immune system and signaling via these receptors is essential for homeostasis of these immune cells [reviewed in (111)]. Thus, the lack of γc results in absence of functional T, B, and NK cells.

The three most commonly used strains in humanized models are: the NSG mouse, the NOG mouse and the BRG or BALB/c-Rag2^{null} IL2rg^{null} mouse. BRG and NSG mice have no gamma chain while NOG mice have a truncated cytoplasmic domain of the gamma chain, preventing signal transmission (112, 113). All three models allow for efficient engraftment with human immune cells, due to a severe impairment in development of T and B as well as NK cells. These new models have enabled a multitude of new findings in the field of asthma research such as the interaction of allergen immunotherapy, clinical tolerance and cellular response, as well as new therapeutic options through the induction of peripheral

TABLE 1 | Advantages and disadvantages of T2-driven asthma mouse models.

Mouse model	Advantages	Disadvantages
OVA models	High efficiency, reproducibility, low cost Well-characterized MHCI and MHCII epitopes OT1 and OT2 T-cell receptor transgenic mice can be used to study OVA-specific immune responses in the airways	Adjuvants are needed for sensitization Provides good mechanistic insights, but no clinical relevance
Aeroallergen models	Do not need adjuvants Mimic natural exposure to airborne allergens via nasal mucosa and airway tract	Need several consecutive applications of allergens Amount of allergen exposure might not reflect natural exposure of patients
Epicutaneous sensitization models	Allow studies on atopic march Mimics physiologic condition of repeated skin exposures to allergens	Needs intradermal applications of allergen or damaged skin barrier
Chronic models of asthma	Allows the study of a chronic phenotype as frequently observed in asthma patients Allows to investigate lung tissue remodeling	Longer duration of experiments with frequent allergen applications Risk of tolerance induction
Transgenic models	Allows evaluating the role of particular cells, receptors or mediators in asthma pathophysiology Helps evaluating disease development/progression	The genetic modification can affect other phenotypes in the model Challenges in translating murine biology in human biology
Humanized models	Help to mitigate the inherent differences between mouse and humans that limit translation of the findings	Paucity of humanized mouse models for asthma research Anatomical discrepancies between mice and humans (e.g., lung anatomy, cell composition in the airways)

tolerance by sGARP (glycoprotein A repetitions predominant) (114, 115). New mechanistic relationships were also clarified, such as the influence of the IL-33/IL-13 axis on the asthmatic airway inflammation or the anti-inflammatory effect of IL-35 in asthmatic diseases (116). Based on the immunodeficient IL2rg^{null} mouse, further mouse models emerged, including the Hu-SRC-SCID mouse and the BLT mouse as well as the Hu-PBL-SCID mouse providing further insight into our understanding of the development of AHR as a characteristic feature of allergic asthma (117) and discovery of new therapeutics, such as the use of TIM-1 antagonists as a possible treatment strategy for asthma (118).

LIMITS OF EXTRAPOLATION

Taken together, recent developments in asthma research led to a shift from solely applying allergic T2-driven eosinophilic airway inflammation models to a broader variety of airway inflammation models following the demand for precision medicine based on phenotype/endotypes in asthma management. However, it is important to be aware that, while the main hallmarks of asthmatic airway inflammation can be mimicked in such models, there are certain differences between mice and men which are reviewed in detail elsewhere (119, 120), that might limit translational impact of results obtained in mouse models. Some of these differences include immunological features (121, 122), which might be overcome by using humanized models, whereas others like anatomical discrepancies [e.g., lung anatomy, cell composition in the airways (123, 124)] will still differ in humanized mice. Moreover, the course of disease and treatment can often not be mimicked: asthma often begins in childhood when the lung is not fully developed yet, whereas experiments are mostly done in mice which do not spontaneously develop asthma, using adult animals with fully developed lung structure. Since the immunological response is shaped not only by the route, but also the amount and frequency of allergen exposure (23, 125, 126), a model that efficiently results in allergic airway inflammation might not necessarily mimic allergen exposure as it is experienced by the patients. Direct extrapolation of efficacy for therapeutic interventions obtained in mouse models is hampered by the fact that mouse models are conducted under highly controlled conditions (e.g., under specific pathogen-free conditions) which substantially affects the diversity of intrinsic and acquired immune responsiveness and may cause substantial immunological differences between these models and human (127, 128). Moreover, experiments are usually performed in genetically similar animals, which do not reflect the heterogeneity of asthmatic patients. To sum this up there is not the "one asthma model" mimicking human disease, but there is a huge variety of different approaches that allow to closely reproduce certain aspects of this complex syndrome with certain advantages and disadvantages (**Table 1**), enabling researchers to examine a scientific question from several different angels in order to contribute mosaic pieces for better understanding asthma.

AUTHOR CONTRIBUTIONS

FA and MA conceived topic and structure of this mini review. FA, SM, ES, FB-P, and MA wrote the review. FA, FB-P, and MA reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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The History of Carbohydrates in Type I Allergy

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Although first described decades ago, the relevance of carbohydrate specific antibodies as mediators of type I allergy had not been recognized until recently. Previously, allergen specific IgE antibodies binding to carbohydrate epitopes were considered to demonstrate a clinically irrelevant cross-reactivity. However, this changed following the discovery of type I allergies specifically mediated by oligosaccharide structures. Especially the emerging understanding of red meat allergy characterized by IgE directed to the oligosaccharide alpha-gal showed that carbohydrate-mediated reactions can result in life threatening systemic anaphylaxis which in contrast to former assumptions proves a high clinical relevance of some carbohydrate allergens. Within the scope of this review article, we illustrate the historical development of carbohydrate-allergen-research, reaching from only diagnostically relevant crossreactive-carbohydrate-determinants to clinically important antigens mediating type I allergy. Focusing on clinical and immunological features of the alpha-gal syndrome, we highlight the discovery of oligosaccharides as potentially highly immunogenic antigens and mediators of type I allergy, report what is known about the route of sensitization and the immunological mechanisms involved in sensitization and elicitation phase of allergic responses as well as currently available diagnostic and therapeutic tools. Finally, we briefly report on carbohydrates being involved in type I allergies different from alpha-gal.

Keywords: alpha-gal, carbohydrate, allergen, crossreactive carbohydrate determinants, glycolipid, glycoprotein, IgE, type I allergy

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INTRODUCTION

A key function of the immune system is to distinguish self from altered-self and non-self in order to subsequently induce tolerance or a specific immune response, respectively. Environmental factors like pollen or food are potential allergic substances which are normally tolerated by the immune system. However, in some individuals, the immune system mounts a type-2 biased reaction in response to such factors. Type 2 immune responses comprise, among others, Th2 cells, type 2 innate lymphoid cells (ILC2) and basophils. As hallmark type 2 cytokine, IL-4 drives the switch in B cells to the production of allergen-specific IgE antibodies which are bound by the high affinity FcεRI on mast cells and basophils allowing the elicitation of immediate allergic reactions that are called type I in contrast to e.g., directly cell-mediated type IV allergic reactions. Subsequent exposure to the allergen results in cross-linking of these FcεRI-bound IgE followed by the release

of vasoactive substances such as histamine by the mast cells and basophils which in turn mediate typical type I allergy-associated local and systemic symptoms, at worst, anaphylaxis. While, in the past, mainly proteins have been described as allergy-eliciting components within pollen, venom or food, carbohydrates have been considered as non-immunogenic and thus negligible in the promotion of allergic responses. However, recent observations clearly show that carbohydrates as well as glycolipids are involved in sensitisation as well as elicitation of hypersensitivity reactions. Especially the identification of alpha-gal as the epitope responsible for triggering anaphylaxis in response to red meat, innards and therapeutical monoclonal antibodies such as cetuximab, drastically changed the accepted view of carbohydrates as allergens [for an overview see also (1–4)]. How the role of glycans as allergens changed over time and the immunological mechanisms involved in sensitisation to as well as elicitation of allergic responses by carbohydrate allergens will be discussed in this review.

CARBOHYDRATE FUNCTION AND STRUCTURE

Carbohydrates are organic biomolecules consisting of one or more simple sugars. These so-called monosaccharides like glucose, fructose, mannose or galactose are built according to the very basic formula $C_nH_{2n}O_n$. By N-, C-, or O-glycosidic linkage, monosaccharides can be coupled to form disaccharides, oligo- and polysaccharides or complex biomolecules with non-sugar constituents (5). At a first glance the most important function of carbohydrates seems to be the storage of energy and assembly of structural components like cellulose in the cell walls of plants (6). However, as independent molecules but even more as side chains of peptides, proteins or lipids, so called glycopeptides, glycoproteins and glycolipids, carbohydrates have crucial functions in e.g., development, immune regulation, blood clotting and many other vital physiologic processes. Accordingly, changes in glycosylation patterns have severe and systemic consequences resulting in disease (7). Peptides and proteins exhibit so-called glycosylation sites provided by, if accessible in the final tertiary structure, certain amino acids. In principle, carbohydrates can be bound N-linked to a nitrogen of the amino acids arginine or asparagine, O-linked to a hydroxyl group of tyrosine, serine, threonine or hydroxyproline or, much less common, C-linked to a carbon of tryptophan (8). Therefore, most larger proteins potentially qualifying as allergens possess one or more glycosylations. In addition, it is well-known that especially carbohydrate determinants are potent immunogens with a broad clinical relevance e.g., as vaccination antigens like bacterial polysaccharides being part of the Haemophilus influenza type b vaccine or as blood group antigens (9). Strikingly, it could also be shown that especially carbohydrate antigens might serve as triggers of Th2 immunity (10, 11). Consequently, a glycan-related IgE-reactivity has been demonstrated in most allergen sources (12). Likewise, carbohydrates and their possible role in allergy became an early focus of allergy research (for an overview, see **Figure 1**).

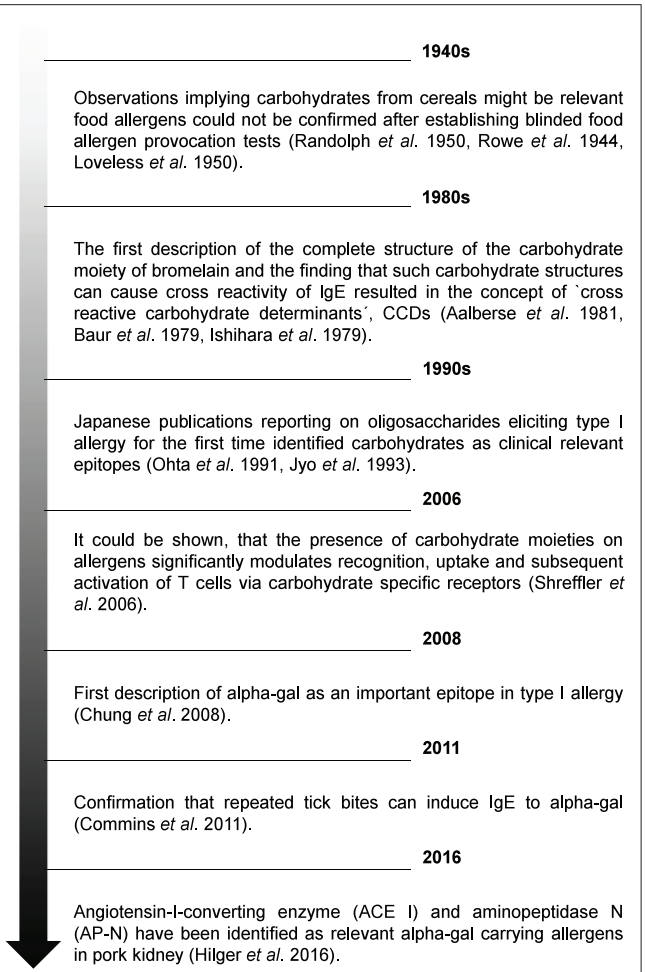

FIGURE 1 | Timeline showing relevant milestones in understanding the role of carbohydrates in allergology.

THE HISTORIC VIEW OF CARBOHYDRATE ALLERGENS

In the 1940's a series of articles reported an important role of corn starch and sugars from cereals as food allergens (13, 14). Because of hearings at the US Food and Drug Administration in 1949, where the advisability of labeling foods was discussed from the perspective of allergy research, these publications received a lot of attention. However, some of these reports referred to quite unspecific symptoms for diagnosing allergy to starches, syrups and sugar from cereals and could not be confirmed by most contemporary specialists. Interestingly, the controversy about the relevance of the reported observations resulted in the development of the most objective diagnostic procedure for food allergy, the blinded provocation test. This even led to the appeal to introduce such controlled, objective methods to study food allergy. Using the new established method, by the way, an important role of corn starches or sugars as allergens could not be confirmed (15). At the same time Coulson et al. reported

evidence that carbohydrates associated with allergenic proteins (they investigated cottonseed allergens) do a) not determine the antigenic specificity and b) not influence the "shocking capacity" of the allergenic protein (16). Both findings together indicated that carbohydrates do not play a major role as allergens.

CROSS REACTIVE CARBOHYDRATE DETERMINANTS

In the 1960's, scientists increasingly hypothesized that allergens must be characterized by a common feature defining an allergen as an allergen (17). Several groups systematically investigated the clinical reactivity of allergic patients to different allergen extracts in cutaneous tests and, according to the calculated correlation coefficients, proposed that allergens can be grouped into clusters or families of closely related allergenic potential (18). Based on these results, others hypothesized that the crucial chemical properties of allergens are N-glycosidically linked sugars (19). Although this did not turn out to be the key to understand the allergic potential of certain antigens in general, it proved to be correct: Many allergens, especially those from the plant kingdom, possess common N-glycosidically linked immunogenic carbohydrate determinants with IgE binding properties. In 1979, a Japanese group described the complete structure of the carbohydrate moiety of stem bromelain, which is generally seen as the starting point of identifying cross reactive carbohydrate determinants and their relevance (20). In the same year, Baur et al. described a mutual inhibition of Radio-Allergo-Sorbent-Test (RAST) to papain, bromelain, wheat flour, rye flour, grass pollen, and birch pollen (21). However, the relevant cross-reactive structures were difficult to identify with radioimmunoelectrophoresis, which was the standard method at the time. In the early 1980's, Aalberse and his colleagues identified IgE antibodies that crossreact with vegetable foods, pollen, and Hymenoptera venom with the new and preferred procedure "immunoblotting" (22). Especially two findings allowed to establish the concept of 'cross reactive carbohydrate determinants', shortly CCDs: Their results showing that the cross-reactive inhibitory effect of grass pollen in RAST could be destroyed by periodate pretreatment, a procedure resulting in breakdown of carbohydrates, and observations, that lectin containing gums like tragacanth gum - lectins very specifically bind certain carbohydrates - can inhibit RAST levels to buckwheat or potato. The findings of Aalberse et al. were supported by following publications showing that IgE from patients allergic to honeybee venom binding to phospholipase A 2 (PLA 2; Api m 1) reacts with the same CCD which is also present on plant glycoproteins (23, 24). Later, these findings were proven by showing that glycopeptides made from pineapple stem Bromelain can inhibit IgE binding to Api m 1. Immunogenicity of CCDs is caused by differences in the cascade of synthetisation. The initial steps of protein N-glycosylation are essentially conserved in all eukaryotic organisms (9, 25), however, the following steps differ between 'higher animals', i.e., the deuterostomia on the one hand and the protostomia (e.g., insects), the acoelomata (e.g., many parasitic worms) and the plants on the other hand (9). Therefore, the prototypic N-glycan CCD structures MMXF 3 and MUXF 3 (**Figures 2A,B**) exhibited by horse radish peroxidase (HRP) or bromelain, respectively, show non-human linkage: Fucose alpha 1,3 is linked to the core region of glycoprotein N-glycans. In humans, the N-glycan core fucose is linked to position 6 of the first GlcNAc unit. The non-human monosaccharide β-(1,2) xylose is bound to the first mannose in the N-glycan core region (26, 27). These immunogenic fucose and xylose moieties have been identified responsible for IgE binding and IgE cross-reactivity. It is expected that about 15–30% of atopic patients mounting IgE responses have anti-CCD IgE (24, 28–30). In principle, binding to anti-CCD IgE is able to cross-link FceRI and activate human mast cells and basophils (29, 31, 32). However, in comparison to protein antigens usually much higher concentrations, up to 10-fold, are necessary demonstrating low potency (31, 33, 34). Thus, there is broad consensus that anti-CCD IgE generally does not cause clinical symptoms when crosslinked by CCDs even though single cases remain, in which a clinical relevant anaphylactic potential is still debated. Interestingly, recent publications also discuss the possibility that CCDs might have a clinical relevance by rather being disease protective (35, 36). Nkurunungi et al. could show, that in ugandan schoolchildren the presence of IgE to a subset of core α-1,3-fucose substituted N-glycans was lower in the asthmatic population. However, those are initial observations which at first should be corroborated by more mechanistic studies.

CARBOHYDRATES AS IMPORTANT EPITOPES IN TYPE I ALLERGY

Understanding the role of IgE recognizing CCDs and its structures allowed to crucially improve allergy diagnostics. However, it might have delayed the understanding of a possible role of carbohydrates as clinically relevant allergens (9). This has changed drastically since in 2009 Commins et al. reported that the carbohydrate allergen galactose-α-1,3-galactose (**Figure 2C**) can induce severe anaphylaxis. Specifically, IgE antibodies to the carbohydrate galactose-α-1,3-galactose (alpha-gal) were found to elicit serious, even fatal, reactions to the monoclonal antibody cetuximab (37). Alpha-gal is a carbohydrate broadly expressed, even by bacteria, and can be found in high density in red meat and especially in innards like kidney and patients with alpha-gal allergy were increasingly recognized (38–41). However, alpha-gal has been recognized as a relevant antigen much earlier. By investigating the cross reactivity between human sera and animal blood, Landsteiner and Miller (42) discovered that 'in twelve species of seven genera of Platyrrhina (New World monkeys) [. . .] a factor similar to the human isoagglutinogen B was present; in 18n species of four genera of Cercopithecidae (Old World monkeys) it was absent, although the latter are more closely related to man than the former' (42). Indeed, the alpha-gal epitope is highly similar to the human blood group B antigen (**Figure 2D**). Roughly at the same time as the role of carbohydrate antigens as CCDs in 1983. Galili et al.

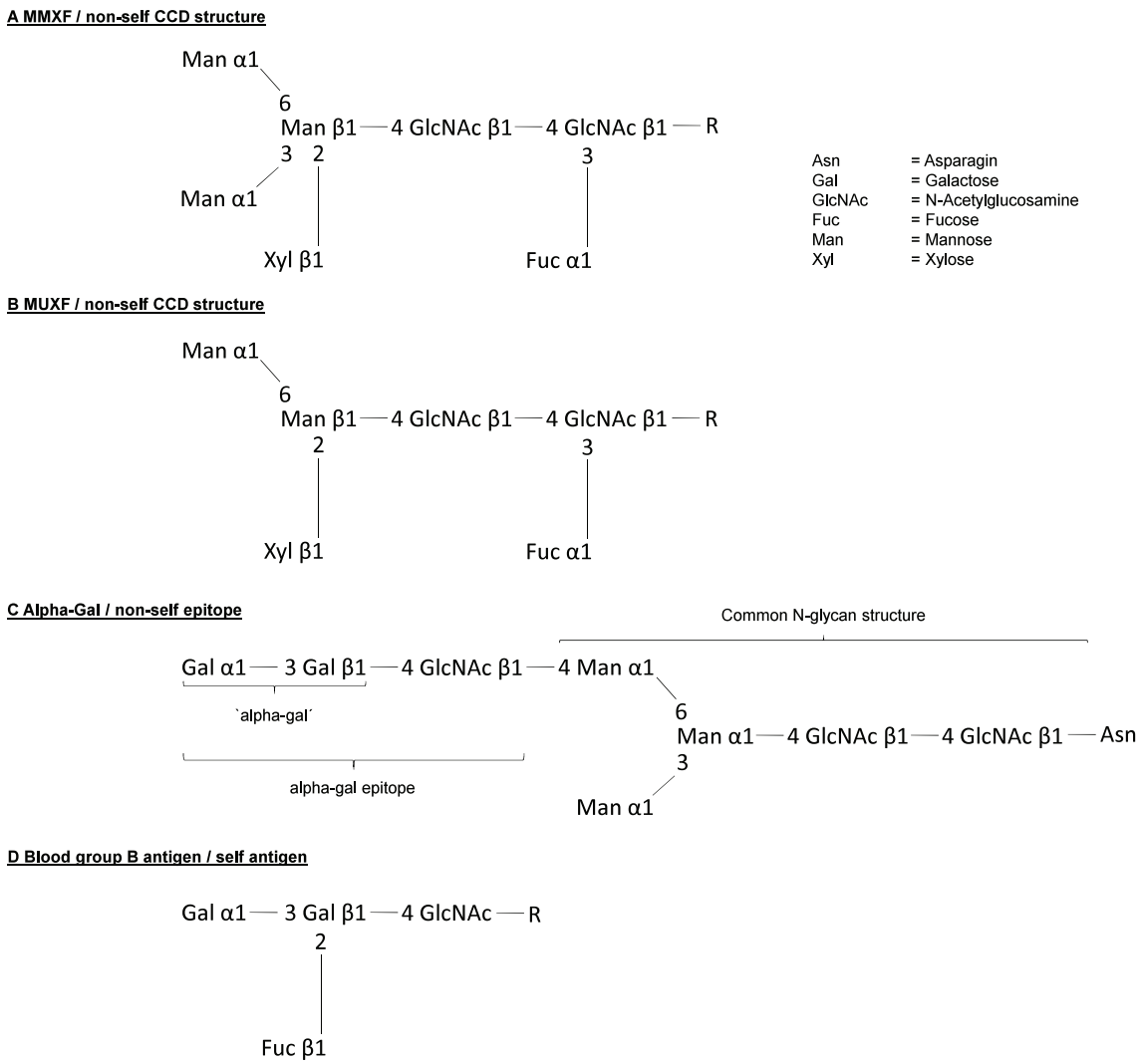

FIGURE 2 | Structure of the most relevant carbohydrate allergenic structures. **(A,B)** Structure of the CCDs MMXF and MUXF. **(C)** Structure of the allergen alpha-gal and **(D)** shows in comparison the difference to the blood group B antigen.

observed a high number of IgG antibodies directed to alpha-gal, initially in thalassemia patients, representing up to 1% of all IgG (43). Further investigations showed that these antibodies very specifically recognize only the alpha-gal epitope showing no cross reactivity with most similar carbohydrate epitopes (44, 45). The development of anti-alpha-gal antibodies is the result of an evolutionary loss of alpha-gal epitopes due to frameshift mutations in the α-1,3-galactosyltransferase gene in humans and old world apes between 25 and 40 million years ago (46). As a consequence, alpha-gal is recognized by the human immune system as a non-self and potentially harmful molecule capable of triggering an immune reaction. It is not totally understood why this mutation could prevail, but it must have provided an evolutionary survival advantage. Indeed, anti-alpha-gal IgG antibodies significantly improve the immune defense against pathogens expressing the epitope like *Plasmodium species* and *Trypanosoma cruzi* (47–49). Accordingly, human anti-alpha-gal IgG were also shown to bind to a number of Gram-positive and Gram-negative bacterial pathogens (41). After its discovery, potential clinical implications of the alpha-gal epitope remained elusive for many years. While a role in immune reaction to cancer cells has been suspected early and is still discussed, alpha-gal initially became most prominent as a relevant xenoantigen impairing efforts to establish xenotransplanation from pig to human (50). Thanks to the extensive research in this field, precious knowledge about the biology of alpha-gal, especially organ and species specific expression patterns and specificity of antibodies, had been gathered even before the discovery of alpha-gal allergy. Xenotransplantation research also led to the generation of α-1,3-galactosyltransferase and consequently also alpha-gal deficient mice and pigs (51–53). It was a surprise when in 2008, Chung et al. observed that in some patients

who developed a severe anaphylaxis following even the very first administration of the new epidermal growth factor receptor (EGFR)-antibody cetuximab, the relevant epitope turned out to be alpha-gal and that affected patients seemed to have preformed IgE directed to it (37). Further research, inspired by the observation that 'the geographical distribution of cases matched the reported distribution of a tick-borne disease called Rocky Mountain spotted fever' which is transmitted by bites of *Amblyomma americanum* (the lone star tick), revealed that indeed tick bites seem to have the potential to induce IgE production to alpha-gal. In 2011, Commins et al. could show that the serum concentration of IgE directed to alpha-gal and IgE reacting with extract of the lone star tick significantly correlate (54). In addition, it could be shown that alpha-gal is present in the gut and in the salivary glands of the ticks *Amblyomma americanum* and *Ixodes ricinus,* the latter being endemic in Europe (55–58). Finally, repeated tick bites can booster the immune reaction to alpha-gal and vice versa subjects living in areas void of ticks do not develop IgE to alpha-gal. Because allergy to alpha-gal means not just red meat allergy but also allergy to other alpha-gal residue carrying substances like gelatin or recombinant pharmaceuticals from mammalian cells and sometimes also comprises allergic reactions to tick bites, it is also called "alpha-gal syndrome" (59, 60) (summarized in **Figure 3**). Next to the induction by repeated tick bites, clinical hallmarks are a typically relative high age of about 50–60 years confirmed in most cohorts and an often delayed onset following meat consumption of at least 2 h. Interestingly, after consumption of pork kidney allergic reactions can occur within < 1 h (61–63). These differences in latency might be caused by the required digestion, absorption and conversion of alpha-gal containing glycoproteins and / or -lipids crucially preceding their recognition by FcεRI-bound IgE on mast cells and basophils. While most ingested proteins are transported to the bloodstream within 1–2 h after ingestion, the majority of lipids enters the blood as part of chylomicrons around 4 h postmeal (64). Indeed, using proteins and lipids extracted from grilled beef, Roman-Carrasco et al. demonstrated that alpha-gal containing glycolipids but not -proteins were able to cross a monolayer of intestinal cells as part of chylomicrons (65). As relevant alpha-gal carrying allergens in pork kidney, angiotensin-I-converting enzyme (ACE I) and aminopeptidase N (AP-N) have been identified (66). The clinical diagnosis is based on a case history with occurrence of systemic symptoms of type I allergy after consumption of red meat or offal and the presence of specific IgE to alpha-gal. The most commonly used commercially available reagent for detecting alpha-gal specific IgE is native bovine thyroglobulin. Prick test solutions authorized and available to confirm meat allergy show a low sensitivity in alpha-gal allergic patients and are usually not suitable to prove allergy to alpha-gal (61). In contrast, prick-to-prick tests, especially those using fresh pork or beef kidney lysate or red meat, as well as intradermal test with Cetuximab or 4 % gelatin polysuccinate have shown to provide a higher sensitivity and can be helpful to diagnose alpha-gal syndrome (61). Importantly, a relevant portion of individuals with IgE to alpha-gal remains clinically tolerant with no symptoms of alpha-gal syndrome upon exposure. In a cohort of German forest workers, the prevalence of IgE to alpha-gal was 35% but only 8.6% of the participants with alpha-gal-sIgE levels $\geq$0.35 kU_A /L had a manifest allergy to alpha-gal (67). Therefore, as established in the early 1950's, oral provocation tests are the tool to establish the clinical relevance of IgE sensitization to alpha-gal. As elicitation of clinical symptoms depends on the presence of additional augmentation or cofactors like exercise, alcohol consumption or intake of non-steroidal anti-inflammatory drugs in some alpha-gal allergic patients, titrated exposure to such cofactors should be included in the test procedure (61, 68, 69). Interestingly, it might be possible to distinguish patients with a relevant sensitization from those with IgE to alpha-gal but persistent tolerance by performing basophil activation test (BAT). Mehlich et al. reported that alpha-gal allergic patients showed a significantly higher%CD63/anti-FcεRI ratio using either alpha-gal-HSA, pork kidney extract or bovine thyroglobulin than alpha-gal tolerant patients also having IgE to alpha-gal (70).

Regarding basic research, data on allergy to alpha-gal in animal models allowing a better understanding of underlying immune mechanisms is still sparse. However, as a proof of concept Araujo et al. could show that effective sensitization of α-1,3-galactosyltransferase deficient mice to alpha-gal can be achieved by either subcutaneous injection of alpha-gal carrying virus like particles, each displaying 540 copies of alpha-gal on its surface or by injection of tick saliva or by feeding ticks on the back of a mouse for 9 days using a feeding chamber. An Elisa specific for anti-alpha-gal antibodies showed a strong induction of alpha-gal specific IgE in mouse serum after tick feeding but a clearly less effective induction following subcutaneous sensitization using virus like particles or tick saliva (71). Our own unpublished results confirm that a percutaneous sensitization to alpha-gal in mice is effective and induces clinically relevant allergy in mice, however, further research is necessary to understand how allergy to alpha-gal is induced and how it might be prevented or more efficiently treated.

Interestingly, clinically relevant type I allergy to carbohydrates other than alpha-gal has also been reported but this is much less well-understood than the alpha-gal syndrome (72). Initially, in the 1980's in workers on Japanese oyster farms, a noticeable increase of occupational asthma has been observed. Analyzing these cases, Ohta et al. could identify a number of different oligosaccharides isolated from the H-antigen of the sea squirt as the relevant allergens (73, 74). Some of these oyster farm workers later on also suffered from anaphylactic reactions after drinking a lactic acid beverage especially popular in Japan. In these patients 1–3 or 1–6 linked so called galacto-oligosaccharides (GOS) consisting of four saccharides elicited positive results if used in skin-scratch tests and in a histamine release assay. GOS consist of 2-6 mostly galactose molecules and a terminal glucose but can significantly vary in length and type of linkage between the monomers (75). Most interestingly, IgE antibodies directed to these GOS also cross-react to the sea squirt antigens (76). In addition, allergic reactions to other GOS used as probiotic supplements in beverages, infant milk products or commercially available milk drinks even outside of Japan have been reported (77, 78). Taken together, these observations demonstrate that

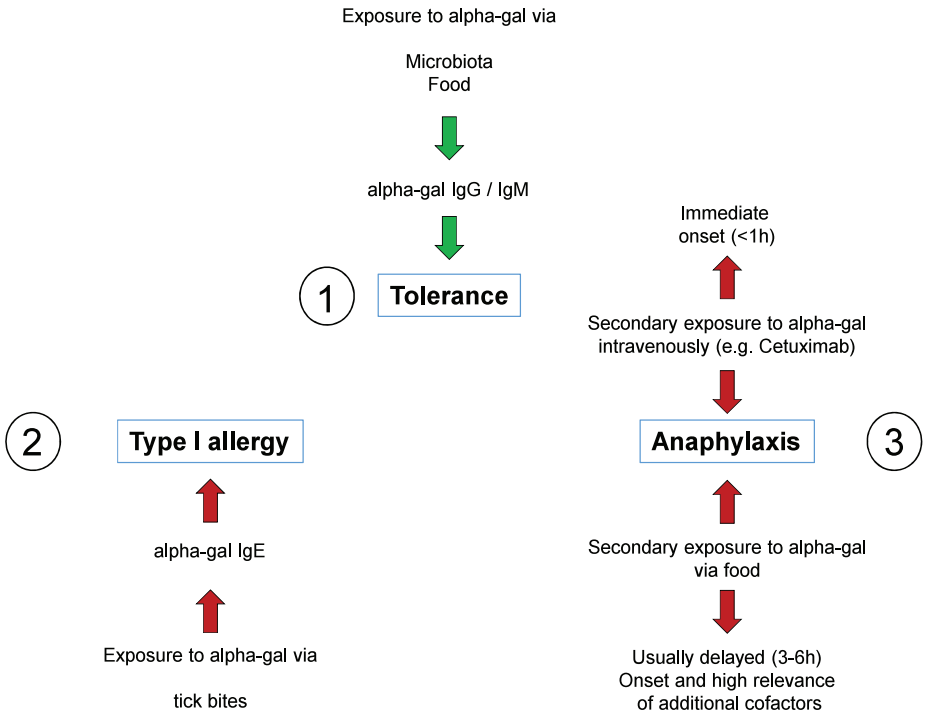

FIGURE 3 | The alpha-gal syndrome. All individuals initially develop tolerance to alpha-gal mediated by constant exposure through bacterial colonization of the intestine and potentially also to food, associated with alpha-gal specific IgG and IgM antibodies (1). However, in some individuals, repetitive tick bites can result in a break of tolerance by induction of alpha-gal specific IgE (2). Upon consumption of red meat and innards as well as administration of alpha-gal containing drugs such as the therapeutic monoclonal antibody cetuximab, these individuals experience symptoms up to fatal anaphylaxis. While symptoms in response to cetuximab occur immediately after administration, anaphylaxis in response to red meat or innards occurs in a delayed fashion 3–6 h after consumption (3).

other type I allergies to carbohydrates than that to alpha-gal exist. Indeed, it seems likely that the increasing understanding about the immune mechanisms underlying allergy to carbohydrates results in identification of additional and clinically relevant carbohydrate allergens.

TODAY'S VIEW OF CARBOHYDRATE ALLERGENS

Immune responses associated with allergy are in most cases elicited by binding of the allergenic substance to specific IgE antibodies coupled to the high affinity IgE receptor FcεRI on mast cells and basophils, leading to the release of mediators such as histamine. Thus, an induction of a type 2-dominated immune response and the associated polarization of CD4+ T cells to become Th2 cells and the production of type 2 cytokines, among them IL-4, can be anticipated to be underlying the class switching of allergen-specific B cells to produce IgE antibodies. To allow the Th2 cell development, IL-4 as dominant education factor is crucial. One possible initial cellular source of IL-4 are the basophils which are well-known for their substantial role in allergic inflammation as well as in parasitic infections (79–82). Basophils have even been described to function as non-professional antigen presenting cells able to take up, process and present allergen on MHCII and induce Th2 responses in mouse models of papain immunization and ovalbumin-induced food allergy, however, their role in "real life" Th2 responses is still a matter of debate (83, 84). Of note, most allergens are proteins, however, the majority of allergens from sources such as pollen, food and insect venom also carry carbohydrates and (glyco-)lipids. While the cascade of events leading to adaptive immune responses to protein antigens are increasingly well-understood (85), the mechanisms underlying carbohydrate-specific humoral and cellular immune reactions are less well-defined (summarized in **Figure 4**). Innate immune cells recognize so-called pathogen associated molecular patterns (PAMPs) on pathogens leading to their activation and subsequent initiation of the adaptive immune response. Pathogens are taken-up and processed by activated antigen presenting cells (APCs), of which the dendritic cells are the most specialized, which in turn present pathogen-derived peptides on MHCII molecules. Dendritic cells subsequently migrate to draining lymph nodes where they initiate the adaptive immune response by activating antigen-specific naïve T cells which specifically recognize the presented peptide-MHCII complex. T cells in turn differentiate into specialized effector T cell subtypes, depending on the cytokine milieu in the cellular environment. Antigen-specific B cells in the lymph node are subsequently activated by a specialized subset of T helper cells,

the follicular helper cells, which provide both cytokines and co-stimulatory molecules leading to B cell activation, proliferation, plasma cell differentiation, antibody secretion and memory formation (85).

CARBOHYDRATE SPECIFIC ANTIBODY RESPONSES – REQUIREMENT FOR T CELLS?

While efficient antibody responses to protein antigens in general require T cell help and thus presentation of antigenic peptides via MHCII, B cell activation mediated by carbohydrate antigens, according to traditional views, has been suggested to occur independent of T cells due to extensive cross-linking of B cell receptors by repetitive glycan epitopes (**Figure 4C**) (85, 86). Consequently, antibody responses to carbohydrate antigens differ strongly from those to protein antigens. First, carbohydrate-specific antibodies use a restricted panel of variable gene pairs as well as confined antibody isotypes, with an overrepresentation of IgM and defined IgG isotypes, namely IgG2 (human) and IgG3 (mouse) (87–89). Due to their capability to form antibody multimers, the overrepresentation of IgM and IgG2 or IgG3 isotypes provides carbohydrate-specific antibodies with the capability to efficiently bind to multivalent glycan structures on the surface of microbes, cells and glycoproteins. Second, mainly due to the seemingly limited contribution of carbohydrate antigens to memory responses and the resulting limited affinity maturation, carbohydrate-specific antibodies primarily exhibit only low affinity compared to antibodies recognizing protein antigens. These characteristics are the main reason for the usage of thymus-dependent forms of vaccines to capsular bacteria such as *Haemophilus influenzae* type b, where carbohydrate-protein conjugates are used to facilitate higher antibody affinities as well as memory responses (90, 91). However, more recent studies described a similar affinity of carbohydrate-specific antibodies of IgE and IgG isotypes for the CCDs α1,3-fucose and xylose as well as high affinity binding of anti-alpha-gal and anti-LPS antibodies (92–94). T cell-independent natural antibodies are primarily produced by B1 cells in response to damage-associated molecular patterns (DAMPs). Interestingly, glycoproteins and glycolipids in vertebrates primarily terminate with sialylic acid residues, which are recognized as normal self by various receptors such as Siglecs (95) while asialylated glycoproteins e.g., with terminal galactose are recognized as altered self or DAMPs. Indeed, anti-alpha-gal IgG and IgM are highly abundant antibodies, originally sorted into the pool of natural antibodies, and are present in all humans with up to 1% of B lymphocytes specific for alpha-gal (44, 96). Of note, it has been described that B1 cells can enter germinal center reactions under certain circumstances, particularly in autoimmune conditions like systemic lupus erythematosus (SLE), allowing class switch recombination and somatic hypermutation, leading to high affinity IgG or IgA responses (97). Thus, B1 cells are likely involved in antibody responses to carbohydrate allergens, especially in those to alpha-gal. In contrast to these observations, using Ggta1 Tcrβ double deficient mice, Cretin et al. showed that antibody responses to alpha-gal are depending on T cells. They observed an increase in alpha-gal -specific IgM titers with age in T cell carrying but not T cell deficient Ggta1 ko mice. Additionally, immunization with pig cells boosted the alpha-gal-specific antibody response only in mice carrying T cells and blocking CD40-dependend co-stimulation abolished the increase in IgM titers in Ggta1 ko mice, indicating that antibody responses to alpha-gal require T cell help (98). Indeed, some carbohydrate antigens, especially zwitterionic polysaccharides from the capsules of some bacteria, have been shown to be presented by MHCII molecules to activate CD4 T cells (99–101), indicating that at least some carbohydrates can be presented by MHCII molecules in certain conditions. Cobb et al. showed that polysaccharide A from *B. fragilis*, a zwitterionic polysaccharide, can be taken up, processed and presented on MHCII by human as well as murine B cells. Confocal microscopy of splenocytes from PS-A treated mice confirmed the formation of an immunological synapse between polysaccharide-MHCII complexes on professional antigen-presenting cells (APCs) and the corresponding T cell receptors (αβTCRs). Specific glycoproteins and glycoconjugates can also be presented via the classical MHCII pathway and in turn activate specific T cells, whereby the glycan group can either be preserved, converted or removed from the presented peptide fragment (102–105). Most interestingly, carbohydrate-dependent proliferation of T cells from bee venom allergic patients in response to the allergen phospholipase A2 derived of bee venom has been demonstrated (102). Thus, carbohydrates with certain molecular characteristics as well as glycopeptides can indeed be presented by the classical MHCII pathway to activate CD4 T cells. A need for T cells for the production of carbohydrate allergen-specific IgE antibodies is also in line with the T cell dependency of the majority of high affinity antibody responses. During the course of the antibody response, both the effector functions of an antibody and the affinity for its cognate antigen are specifically adapted in so-called germinal center reactions, which are crucially depending on co-stimulatory signals as well as cytokines expressed by T helper cells. The affinity of the antibody is increased by somatic hypermutation of the variable regions and the subsequent selection of B cells with high affinity for their cognate antigen, a process called affinity maturation. The effector function of an antibody is mediated by its constant region, which determines the isotype of the antibody. All mature B cells initially express antibodies of IgM isotype, which can be modified by the process of class switch recombination initiated by co-stimulation as well as a defined cytokine milieu. In the case of high affinity IgE antibody responses, a possible sequential class switching of IgG1 to IgE has been proposed, in accordance with the limited and transient participation of IgE positive B cells in germinal center reactions and memory responses (85, 106–108).

GLYCOLIPID ALLERGEN SENSING – NKT CELLS DO THE JOB

While glycoproteins can be presented via the classical MHCII pathway, glycolipids are uniquely presented by the MHC Class I

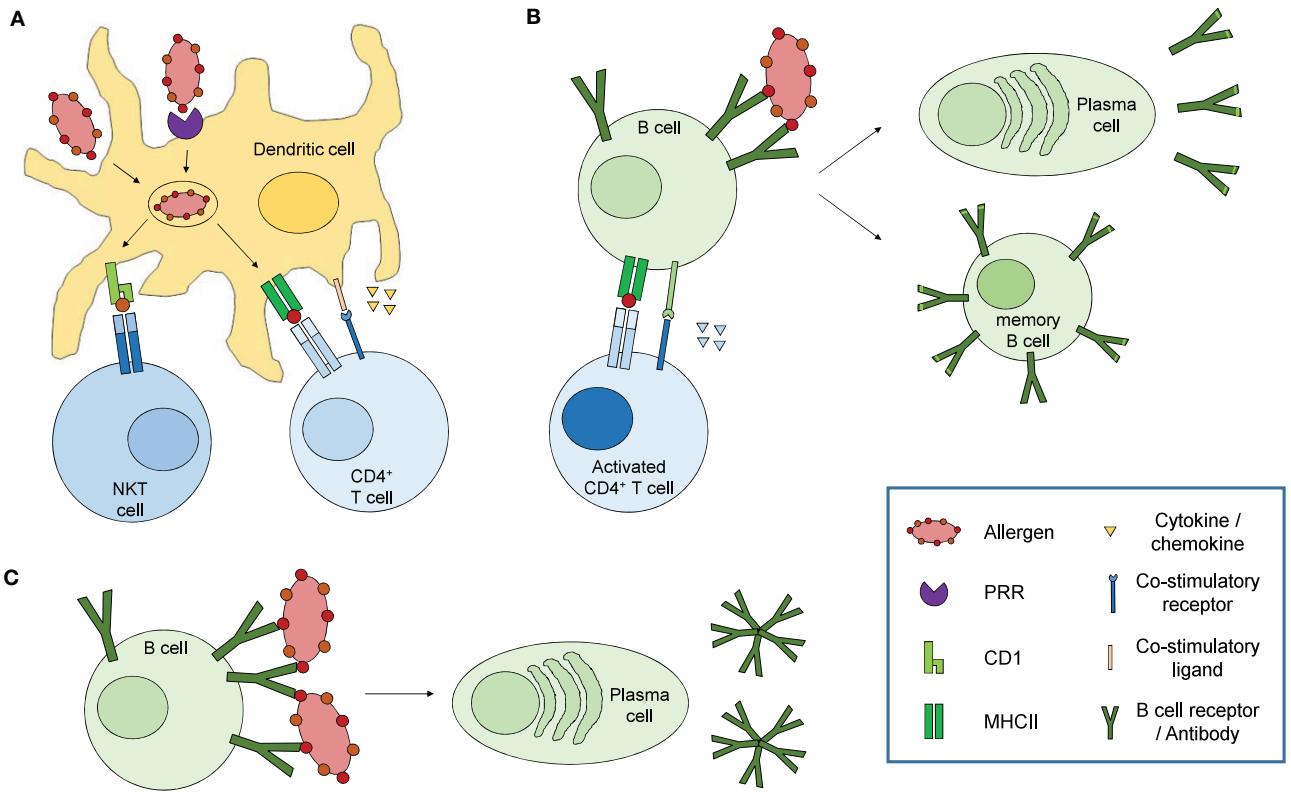

FIGURE 4 | Immune response to carbohydrate antigens. **(A)** Dendritic cells sense carbohydrate constituents on allergens via pathogen recognition receptors (PRR) such as C-type lectin receptors, leading to their activation. Allergens are taken up by receptor mediated endocytosis or phagocytosis, processed and presented on MHCII (glycoproteins, defined polysaccharides) or CD1 (glycolipids) to specifically activate $CD4^+$ T cells or NKT cells, respectively. Activated dendritic cells additionally secrete different cytokines and chemokines and express co-stimulatory molecules which initiate and modulate the adaptive immune response. **(B)** Activated $CD4^+$ T cells in turn activate naïve B cells recognizing and presenting the same allergen on MHCII via co-stimulatory signals and secretion of specific cytokines resulting in affinity maturation, class switch recombination, memory B cell and plasma cell differentiation and antibody secretion. **(C)** Specific B cell subsets, mainly B1 cells, can be activated in a T cell independent fashion via extensive cross-linking of the B cell receptors by repetitive epitopes, resulting in the secretion of so-called natural antibodies characterized by only low antigen affinity and mainly IgM isotype.

like molecule CD1 mainly expressed by B cells, dendritic cells and thymic T cells (109). Self and non-self lipids are in turn sensed by a separate T cell lineage, the NKT cells. NKT cells co-expressing natural killer (NK) cell markers and carry semi-invariant CD1-restricted αβTCRs on their surface (110). While humans express five different CD1 molecules which are organized into two classes based on sequence homologies (class I: CD1a, b, c; class II: CD1d), mice lack expression of the class I genes and thus solely express CD1d (111, 112). Upon ligand-CD1d recognition, NKT cells exhibit cytotoxicity mediated by Fas-Fas ligand interaction and rapidly secrete large amounts of cytokines including IFNγ, GM-CSF and the Type-2 cytokines IL-4, IL-5 and IL-13 (113, 114). The glycolipid alpha-galactosylceramide (α-GalCer), first identified in a marine sponge, is a well-known high affinity NKT antigen (115). Indeed, CD1d-α-GalCer tetramers stained up to 95% of human and mouse NKT cell clones (116). Additional CD1 ligands include microbial lipids such as glycosphingolipids and α-galactosyldiacylglycerols from the Gram-negative, LPS-negative bacteria *Sphingomonas* and *Borrelia burgdorferi*, respectively (117, 118), as well as self-lipids like the lysosomal glycosphingolipid isoglobotrihexosylceramide (iGb3) (119). Interestingly, while mice showed an increase in IFNγ as well as IL-4 and IL-10 after a single immunization with α-GalCer, repetitive immunisations resulted in a Th2 polarization of the immune response, with a dramatic reduction in IFNγ and an increase in IL-4 and IL-10 secretion, accompanied by an increase in serum IgE levels (120). Indeed, lipids from pollen, insects and food allergens have been described to play a role in allergic sensitization by direct recognition of these lipids via NKT cells. Agea et al. showed that phospholipids isolated from cypress pollen grains induced proliferation and secretion of both IFNγ and IL-4 in T cells isolated from allergic patients. Pollen grains directly interacted with dendritic cells and this interaction was blocked by anti-CD1d as well as anti-CD1a antibody treatment. Finally, NKT cell clones from cypress allergic individuals efficiently induced IgE production by autologous B cells *in vitro* and phospholipid-specific IgE antibodies could be detected in the serum of allergic but not control individuals (121). Another work by Abos-Gracia et al. showed that different lipids from olive pollen induce maturation of immature dendritic

cells, accompanied by CD1d upregulation and IFNγ as well as IL-4 production, which in turn activated iNKT cells (122). By investigating PBMCs from food-allergic children, Jyonouchi et al. demonstrated that iNKT cells from allergic individuals produced higher amounts of the Th2 cytokines IL-4 and IL-13 in response to α-GalCer stimulation compared to iNKT cells from non-food allergic individuals. Stimulation of iNKT cells from milk-allergic individuals with milk sphingolipid- as well as α-GalCer-loaded CD1d tetramers specifically induced iNKT cell activation and proliferation, while egg ceramide-loaded CD1d tetramers did not. Interestingly, stimulation with milk sphingolipid- but not α-GalCer-CD1d tetramers resulted in clear IL-4 secretion by iNKT cells. When cultured in presence of milk sphingolipid, iNKT cells from allergic but not non-allergic children showed a clear Th2 response (123). Bourgeois et al. could show that phospholipase A2 from bee and wasp venom indirectly mediated a CD1a-restricted T cell response by cleaving cellular phospholipids which in turn served as neoantigens for CD1a-restricted T cell activation by antigen presenting cells (124). In a follow-up study, an increase in bee and wasp venom-reactive CD1a-restricted T cells with increased IFNγ, GM-CSF and IL-13 production in response to venom or venom-derived phospholipase was observed in venom-allergic individuals (125). Taken together, CD1-mediated presentation of lipids to NKT cells plays a crucial role in sensitization to allergens whereby the allergenic compound is either itself the lipid ligand presented on CD1 or is involved in lipid ligand processing.

SENSING OF CARBOHYDRATE ALLERGENS

In order to present allergens to T cells, APCs have to sense, take-up and process the allergen. Carbohydrate antigens are specifically recognized by a subset of glycan-binding proteins, containing carbohydrate recognition domains with specific binding grooves for certain self and non-self carbohydrate structures, the so-called lectins (126, 127). Among those, C-type lectin receptors (CLRs) essentially contribute to the pattern recognition ability of myeloid cells and mediate dendritic cell activation, antigen uptake and presentation to T cells. Interestingly, the allergy-promoting effects of some allergens such as the major peanut allergen Ara h 1 and the house dust mite allergen Der p 1 have been shown to clearly depend on their glycosylation and interaction with glycan-binding receptors on dendritic cells (128, 129). Shreffler et al. investigated the sensing, uptake and subsequent presentation of glycosylated vs. deglycosylated peanut allergen by dendritic cells and could show that recognition, uptake and subsequent activation of T cells was depending on the glycosylation status of peanut allergen and mediated by interaction of Ara h 1 with the C-type lectin receptor DC-SIGN on dendritic cells (128). Al-Ghouleh et al. investigated the capability of immature dendritic cells to take up Der p 1 and showed a carbohydrate-dependent uptake as well as interaction of Der p 1 with the CLR mannose receptor, which was increased when the allergen was hyperglycosylated but abolished after periodate treatment. Additionally, treatment of human lung epithelial cells with glycosylated but not periodate-treated Der p 1 resulted in increased secretion of the type-2 cytokine TSLP (129). Interestingly, beside their role in antigen recognition and uptake, CLRs can influence the polarization of T cell responses by inducing signaling cascades resulting in the expression of specific cytokines and chemokines as well as the modulation of signaling pathways induced by other (pattern recognition) receptors. Concerning DC-SIGN, it has been shown that distinct signaling circuits are induced depending on the recognized PAMP-associated carbohydrate. Thus, in response to fucose-expressing *Schistosoma mansoni* and *Heliobacter pylori*, DC-SIGN signaling via LSP1 induces a Th2-dominated response while inhibiting TLR4-induced pro-inflammatory cytokine responses such as IL-6 and IL-12. In contrast, sensing of mannose-expressing *Mycobacterium tuberculosis* or *HIV-1* resulted in an enhanced pro-inflammatory response (130, 131). Moreover, major peanut allergen Ara h 1-mediated T cell activation by dendritic cells resulted in a Th2-skewed response in a carbohydrate depending manner (128). Taken together, CLRs function as self and non-self carbohydrate-specific pattern recognition receptors involved in antigen uptake and presentation, activation of dendritic cells as well as the orchestration of individual immune responses, including the skewing of type 2 responses in the context of parasitic infections and allergies.

TOLERANCE AND ALLERGY TO CARBOHYDRATES – THE ALPHA-GAL STORY

While eliciting Th2 immune responses in a few individuals, potential allergenic substances such as from pollen, food and mites are in general tolerated by the immune system. In the case of alpha-gal, clinical tolerance is the main finding in humans and exposure to alpha-gal from the environment is manifold through food and bacterial colonization of the surface organs, resulting in abundant levels of natural IgM and IgG antibodies directed against alpha-gal in human serum (38). These so-called natural alpha-gal specific antibodies are beneficial in the protection against pathogens carrying alpha-gal on their surface, suggesting that the frameshift mutation in the *Ggta1* gene and the associated loss in α-galactosyltransferase function in apes and humans provided an evolutionary advantage with respect to infections with alpha-gal carrying pathogens (132–134). Indeed, Yilmaz et al. showed that colonization of germfree mice with the alpha-gal-expressing *E.coli* strain O86 resulted in induction of alpha-gal-specific IgM and IgG antibodies which provided protection against malaria infection (47). However, in some individuals, repetitive tick bites can break this immune tolerance to alpha-gal by the induction of alpha-gal specific IgE (54, 67, 135). To date, the origin of the tick-transmitted alpha-gal carrying glycoproteins and glycolipids as well as the mechanisms inducing the IgE response remain elusive. Concerning the source of alpha-gal, tick saliva-derived proteins, mammalian proteins and glycolipids ingested by the tick during a previous blood meal as well as alpha-gal expressing bacteria, viruses or parasites potentially transmitted by the tick have been proposed (56, 57, 71,

136). Beside the transmission of alpha-gal carrying components to the host skin, tick bites induce a variety of host immune mechanisms which likely contribute to the induction of a type 2-dominated response in the case of alpha-gal allergy (137, 138). Tick saliva contains a variety of immunomodulatory molecules such as PGE_2 and evasins which are involved in the suppression of pro-inflammatory immune responses, thereby favoring a Th2 polarization. Indeed, mice infested by ticks showed increased levels of both IL-10 and IL-4 which progressively increased with subsequent tick infestations, while IFNγ and IL-12 levels were reduced (139). Interestingly, PGE_2 has been shown to be involved in the regulation of IgE class switching thereby enhancing IgE production by B cells (140). Thus, in the special case of alpha-gal and potentially other tick bite-mediated allergic responses, the tick bite itself may be critical for the induction of the type 2-dominated immune response resulting in the expression of allergen-specific IgE antibodies. After penetration of the skin barrier, carbohydrate allergens are likely phagocytosed by dendritic cells which in turn activate T cells in skin-draining lymph nodes. Although the exact mechanism of alpha-gal uptake by APCs remains unknown, Ristivojevic et al. showed that bovine serum albumin (BSA) uptake by human immature dendritic cells was significantly higher when the protein was decorated with alpha-gal while protein degradation was reduced, suggesting that the carbohydrate is specifically recognized by the dendritic cell inducing uptake and processing, possibly involving lectin-mediated recognition (141). As described above, T cell help was described to be crucial for the induction of alpha-gal-specific antibody responses (98). Thus, dendritic cells may migrate to the skin-draining lymph node to activate antigen-specific T cells which in turn activate their cognate B cells, resulting in production of allergy-eliciting alpha-gal-specific IgE antibodies. Class switching to IgE as well as differentiation of type 2 helper T cells crucially requires the presence of the cytokine IL-4 and thus an initial cellular source of this Th2-associated cytokine, potentially basophils. Indeed, basophils have been shown to be recruited to the site of tick bites in an IL-3 and CD4 T cell dependent manner (142) and basophil numbers were enriched in the skin of alpha-gal-allergic patients at the site of tick bites (143). Taken together, allergic responses to carbohydrate allergens likely rely, similar to protein allergens, on antigen presentation by APCs, either via the classical MHCII pathway or, in the case of glycolipids, via CD1 molecules to T cells, and the subsequent T cell-dependent induction of B cell responses. However, for a fundamental and more general understanding of the mechanisms and cell types involved in immunity to carbohydrate allergens including alpha-gal, further studies are urgently needed.

CONCLUDING REMARKS

In this review, we described how the role of carbohydrates as mediators of allergic diseases changed over time, from crossreactive carbohydrate determinant to specific allergens eliciting anaphylactic responses. Especially the recent identification of alpha-gal as the allergen responsible for elicitation of allergic responses to red meat, innards and therapeutic monoclonal antibodies such as cetuximab changed researchers' view on carbohydrate allergens dramatically. However, the exact mechanisms involved in the break of tolerance as well as the elicitation of the allergic response to carbohydrate allergens are still elusive. Since the prevalence for allergic diseases is still increasing in developed and developing countries and specific therapies for allergic diseases are limited to the treatment of symptoms and the avoidance of the allergenic substance, new therapeutic tools are urgently needed. The development of such therapies in turn requires in-depth understanding of the immunological mechanisms behind recognition, presentation and initiation of the adaptive immune response to carbohydrate allergens, finally resulting in the production of allergy-eliciting IgE antibodies by allergen-specific B cells.

AUTHOR CONTRIBUTIONS

MH, FW, and TB took the lead in writing the manuscript. MH and FW designed the figures. CH, JF, and NH contributed relevant parts to the manuscript and provided critical feedback. All authors contributed to the article and approved the submitted version.

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Precision Medicine in Hymenoptera Venom Allergy: Diagnostics, Biomarkers, and Therapy of Different Endotypes and Phenotypes

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Allergic reactions to stings of Hymenoptera species may be severe and are potentially fatal deviations of the immunological response observed in healthy individuals. However, venom-specific immunotherapy (VIT) is an immunomodulatory approach able to cure venom allergy in the majority of affected patients. An appropriate therapeutic intervention and the efficacy of VIT not only depend on a conclusive diagnosis, but might also be influenced by the patient-specific manifestation of the disease. As with other diseases, it should be borne in mind that there are different endotypes and phenotypes of venom allergy, each of which require a patient-tailored disease management and treatment scheme. Reviewed here are different endotypes of sting reactions such as IgE-mediated allergy, asymptomatic sensitization or a simultaneous presence of venom allergy and mast cell disorders including particular considerations for diagnosis and therapy. Additionally, phenotypical manifestations of venom allergy, as e.g. differences in age of onset and disease severity, multiple sensitization or patients unsusceptible to therapy, are described. Moreover, biomarkers and diagnostic strategies that might reflect the immunological status of the patient and their value for therapeutic guidance are discussed. Taken together, the increasing knowledge of different disease manifestations in venom hypersensitivity and the growing availability of diagnostic tools open new options for the classification of venom allergy and, hence, for personalized medical approaches and precision medicine in Hymenoptera venom allergy.

Keywords: allergy diagnosis, biomarkers, immune tolerance, molecular allergology, precision medicine, venom allergen, venom-specific immunotherapy, Hymenoptera venom allergy

INTRODUCTION

Hymenoptera venom allergy (HVA) is one of the most serious IgE-mediated hypersensitivity reactions due to the high risk of severe and even fatal anaphylaxis. In the majority of patients, venom allergy can be effectively treated by venom-specific immunotherapy (VIT), the only available immunomodulatory and curative approach. However, a comprehensive diagnostic work-up, including the identification of the allergy-relevant venom, with different biomarkers and diagnostic tools is a prerequisite for proving clinically relevant sensitization and ensuring therapeutic success.

HVA is caused by insects of the order Hymenoptera, which inject the venom as a defense mechanism. In Northern and Central Europe, the most common elicitors of venom allergy are honeybees (*Apis mellifera*) and yellow jackets (*Vespula* spp.). Venom allergy to hornets (*Vespa* spp.) is less common and it has been demonstrated that the vast majority of patients with anaphylactic reactions to hornet venom appear to be primarily sensitized to yellow jacket venom (YJV) (1). As bumblebees (*Bombus* spp.) are increasingly used for pollination in greenhouses, allergy to their venom has become more important but is still considered rare (2). In addition to honeybees and yellow jackets, paper wasp (*Polistes* spp.) venom allergy is of relevance in Southern Europe and Northern America. Allergy to the venom of other Polistinae such as *Polybia paulista* is prevalent in South America (3). Whereas allergies to stinging ants are rare in Europe, they are of great importance in Australia (jumper ant, *Myrmecia pilosula*), Asia (Asian needle ant, *Pachycondyla chinensis*) and America (fire ant, *Solenopsis invicta*). The taxonomy of allergy-relevant Hymenoptera is depicted in **Figure 1**.

The frequency of stings and, thus, of allergic reactions, depends on geographical, environmental, and ecological factors (4) which can rapidly change. For instance, *Polistes dominula*, domestic in Southern Europe, has invaded the United States (5), South Africa (6), and central Europe (7). Therefore, allergy to *Polistes dominula* venom (PDV) will probably become more important in the future. A second highly invasive Hymenoptera species, *Vespa velutina nigrithorax* (yellow-legged or Asian hornet), is gaining ground in Europe, although its natural habitat is tropical areas in Southeast Asia. Starting from France, it has spread rapidly across Europe, facilitated by suitable climatic conditions (8). *Vespa velutina nigrithorax* has become a common cause for Hymenoptera anaphylaxis in areas of Europe where it has become endemic (9).

In adults (> 18 years), 48.2% of cases of severe anaphylaxis are caused by insect stings (20.2% in children) (10). The prevalence of systemic sting reactions (SRs) in the adult population ranges between 0.3% and 8.9% and is lower in children (11). The estimated number of annual mortalities due to insect sting-induced anaphylaxis ranges from 0.03 to 0.45 per one million inhabitants (12). However, this number could be underestimated as many fatal reactions following insect stings may remain undetected (13). Large local reactions (LLRs) at the site of the sting that are characterized by a swelling with a diameter exceeding 10 cm and lasting for more than 24 h, occur in 2.4% to 26.4% of the general population (14).

The classification of allergic reactions to Hymenoptera venoms into different endotypes and phenotypes, which can be assigned through various biomarkers and diagnostic strategies (**Figure 2**), enables individual risk stratification for the patients and personalized therapeutic strategies.

ENDOTYPES AND CLINICAL MANIFESTATIONS OF HYMENOPTERA VENOM ALLERGY

Like other diseases, reactions to Hymenoptera stings can be divided into different endotypes, such as the physiological sting reaction in healthy individuals, IgE- and T cell-mediated allergic reactions, venom allergy in patients with mast cell disorders, asymptomatic sensitization and toxic or unusual reactions (**Figure 2**).

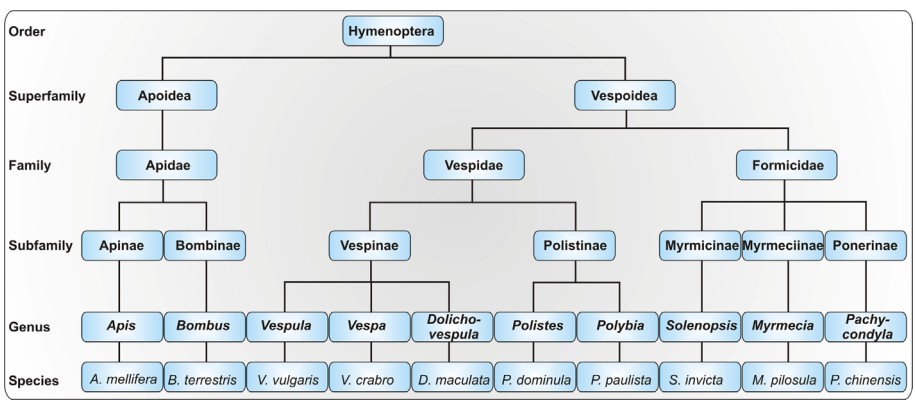

FIGURE 1 | Taxonomy of allergy-relevant Hymenoptera. As the taxonomy of the order Hymenoptera is highly complex, only a selection of allergy-relevant taxa is shown. Displayed are exemplary species with particular relevance for Hymenoptera venom allergy.

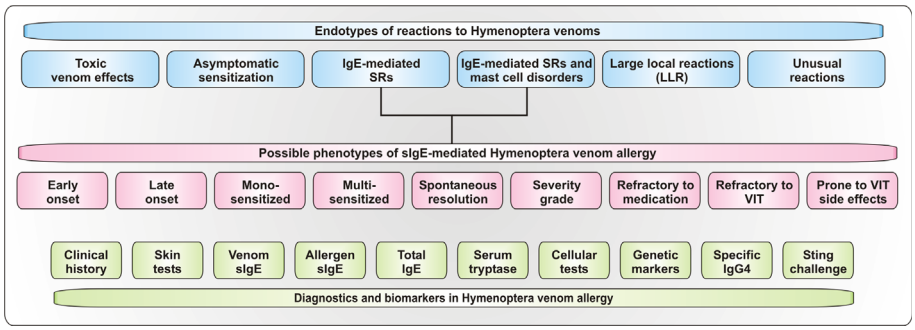

FIGURE 2 | Endotypes und Phenotypes of reactions to Hymenoptera stings. Depicted are proposed endotypes of reactions to Hymenoptera venoms as well as of proposed phenotypes of IgE-mediated systemic allergic reactions. Additionally, available diagnostic tools and biomarkers for the assessment of the reaction are shown. SRs, systemic reactions.

Sting Reaction in Healthy Individuals / Toxic Venom Effects

Between 56.6% and 94.5% of the general population state to be stung by an insect of the order Hymenoptera at least once in their lifetime (12). The normal sting reaction consists of pain and inflammation (swelling, redness and itching) and is not dangerous. However, massive attacks with numerous sting events, for instance by Africanized honeybees, can be life-threatening to humans due to the toxicity of the venom (15–17). On rare occasions, single oropharyngeal stings can induce critical airway obstruction in non-allergic individuals by local swelling (18). If this reaction can be considered as LLR in the oropharynx is unknown so far.

IgE-Mediated Systemic Reactions

In allergic individuals, already a single sting can lead to severe and fatal reactions (19). These IgE mediated reactions depend on an initial step of sensitization. During the encounter with a venom via stings, venom allergens enter the body. Antigen-presenting cells, such as dendritic cells, B cells or macrophages, incorporate, process and subsequently present the allergens to naive $CD4^+$ T cells. These T cells either differentiate into Th1, Th2, or Th17 effector cells or take on a regulatory function as regulatory T cells (Treg). B cell class-switch and differentiation into IgE-producing plasma cells is induced by the cytokines IL-4 and IL-13 which are secreted by mature Th2 cells. After sensitization, the actual allergic reaction can occur if Fc epsilon receptors I (FcεRI) on the surface of mast cells and basophils loaded with allergen-specific IgE antibodies are cross-linked during a further encounter with the allergens. This in turn leads to the degranulation of mast cells and basophils and the secretion of pro-inflammatory compounds such as histamine, proteases, cytokines, and lipid mediators. This mix of immunological active compounds leads to the induction of allergic symptoms in susceptible patients [for further information see Rindsjö and Scheynius 2010 (20)].

SRs can be mild (generalized skin symptoms such as urticaria or angioedema), moderate (e.g. dyspnea, gastrointestinal symptoms or dizziness) or severe (e.g. unconsciousness, anaphylactic shock, respiratory or cardiac arrest) (21). Of note, there is no necessary correlation between the severity of sting reactions at two different times (22). The worst-case scenario, anaphylaxis, is characterized by the involvement of at least two organ systems (23). The most frequently affected organs are the skin and mucosa, followed by the cardiovascular system. Gastrointestinal symptoms occur in one third of the patients (10). It has been reported that 0.7% to 2% of all cases of anaphylaxis are lethal (24) and anaphylactic deaths to insect stings occur in most cases within 15–20 min after exposure (25). SRs usually begin 10 to 30 min after the sting but can also arise faster (e.g. in individuals suffering from mast cell disorders) or slower (1–4 h), although being less life-threatening in the latter case (4). The severity of symptoms can be boosted by different risk and co-factors such as suffering from mast cell disorders, physical exertion, male sex or older age (26).

IgE-Mediated Reactions in Patients With Concomitant Mast Cell Disorders

Mast cell disorders, such as mastocytosis, are common cofactors for severe allergic reactions to Hymenoptera venoms. Mastocytosis is a clonal, neoplastic and heterogeneous (cutaneous, systemic and rare subtypes) disorder characterized by proliferation and accumulation of mast cells in the skin, bone marrow and other tissues (27). Mastocytosis frequently involves the somatic c-kit D816V mutation and elevated baseline levels of serum tryptase (27). The prevalence of mastocytosis may be as high as 7.9% in patients suffering from HVA which is significantly higher than that of the general population (28). Similarly, HVA causes anaphylaxis in nearly 30% of patients with mastocytosis (29). In addition to higher incidence, there is also convincing evidence of a strong association of mast cell disorders with an increased severity of sting-induced anaphylaxis (30). The anaphylactic reactions in patients with systemic mastocytosis are characterized in the majority of cases by the absence of angioedema and erythema and the predominance of cardiovascular symptoms, such as hypotension, leading to loss

of consciousness (31). The Spanish Network on Mastocytosis (REMA) has built and validated a simple clinical score associated with both a high sensitivity and specificity to predict systemic mastocytosis among patients who present with mast cell activation symptoms in the absence of skin lesions (32). Of note, anaphylactic sting reactions in mastocytosis patients have previously been thought to also occur in the absence of specific IgE (sIgE) (33) due to potential pharmacological mechanisms of mast cell degranulation. However, with the introduction of new methods and parameters of evaluation in the diagnostic work-up, this historic diagnostic gap has been closed and sIgE can be detected in the vast majority of patients (34, 35). Importantly, negative sIgE and negative skin tests have been reported in up to 15% of patients with systemic mastocytosis and history of a systemic reaction to insect stings (36), thus, restricting them from VIT. A recent study demonstrated that in mastocytosis patients suffering from YJV-allergy diagnostic sensitivity can be improved by lowering the cut-off for positive sIgE detection without marked changes in specificity (34). Here, a cutoff of 0.17 kU_A/l gave an acceptable sensitivity and specificity (83.6 and 85.0%, respectively). Indeed, sIgE levels between 0.1 and 0.35 kU^A/l should be considered relevant in patients with a clear clinical history, irrespective of the presence of mast cell diseases (35, 37, 38). VIT may be less protective in patients with severe initial SRs and mastocytosis and/or elevated serum tryptase (>11.4 ng/ml). Therefore, for safety reasons, it should be prolonged in those patients; it remains unclear whether it should be given lifelong or after which duration of treatment it should be stopped (21).

Asymptomatic Sensitization

Interestingly, the presence of sIgE does not necessarily imply clinically relevant venom allergy. Between 9% and 29% of the population are sensitized to Hymenoptera venoms without previous clinical history of a sting reaction (39, 40). For most of these patients it is likely that the sensitization is asymptomatic and, thus, of no clinical relevance (41). However, the possibility of a reaction to a future sting cannot be fully excluded. To date, no indications are available on how to effectively manage these cases (42).

Large Local Reactions

LLRs are defined by edema, erythema and pruritus and supposed to be an IgE-dependent late-phase allergic reaction that follows the local recruitment and activation of Th2 cells, eosinophils, basophils and other leukocytes (43, 44). Most studies find positive skin tests for venoms or venom-sIgE in 70%–80% of patients with LLRs (45). It was demonstrated that only very few patients suffering from LLRs develop more severe reactions when re-stung by the same insect (46). However, a recent study showed that SRs occur more frequently after a previous LLR than reported by previous literature (47). Here, 24% out of 225 patients with a previous LLR developed a SR after the first field re-sting. Among the 35 patients clearly re-stung by the same insect, according to their history, 11% reported a SR. A conclusive statement on the connection of LLRs and SRs is challenging.

Unusual Reactions

In addition to the well described allergic reactions to Hymenoptera stings, a variety of extremely rare and unusual reactions may occur. Examples are serum sickness-like manifestations, thrombocytopenic purpura, hemolytic anemia, Schönlein-Henoch purpura, Guillain-Barré syndrome, vasculitis, glomerulonephritis and demyelinization-related neurological complications (48, 49). The pathogenesis of most of these unusual reactions remains unclear but might involve toxic, autoimmune and type II and III hypersensitivity reactions.

PHENOTYPES IN HYMENOPTERA VENOM ALLERGY

Different phenotypes of IgE-mediated HVA can be described by the age of onset, the course and severity of the disease, sensitization profiles and the response to therapy (**Figure 2**).

Age of Onset

Systemic insect sting reactions seem to be rare in children, ranging between 0.9%–3.4% for mild systemic and 0.5%–0.9% for severe SRs (50, 51). However, according to the European Anaphylaxis Registry, HVA is the second most frequent cause of severe reactions in children (20.2%) after food allergy (10). Most studies on the pediatric group reveal the predominance of skin symptoms (60% of cases) and dyspnea (52, 53) in the course of anaphylaxis in children as compared to adults where cardiovascular symptoms more frequently occur (39, 52, 53). Elderly patients develop severe SRs more often and the fatality rate is higher than in children and young adults (39). This might be due to the fact that the cardiovascular system in children is more efficient compared to adults, hence, even the possibility of self-limitation of anaphylactic reactions exists.

Spontaneous Resolution of the Disease

Despite the high prevalence of asymptomatic sensitization (up to 29%), the prevalence of sting-induced SRs is low (41). Why some sensitized patients do not react to a future sting is still unknown, but it is probably due to loss of sensitization over time and, thus, spontaneous resolution (11). On the other hand, the risk for adults who experienced a first anaphylactic reaction to suffer from a SR to a further sting is not 100% but between 40% and 60%. In the remainder, symptoms may be less severe or even completely absent (54). The natural history of insect sting allergy differs between children and adults. Early studies found that children have a favorable prognosis regarding re-stings, both, in studies based on sting challenge (55) and field stings (56, 57). In particular, children with mild SR outgrow their HVA in the majority of cases (58, 59). However, in children not treated with VIT and who have a history of moderate to severe SRs, the risk of future SRs remains as high as 40% after 1 to 9 years, and as high as 30% in years 10–20 after anaphylaxis (58).

Severity of the Disease

Allergic SRs may involve one or more organ systems (i.e. cutaneous, respiratory, gastrointestinal, neurologic and cardiovascular systems), while the simultaneous involvement of two or more organ systems during an acute allergic event is a prerequisite for the diagnosis of anaphylaxis (23, 60). Several classifications were proposed to assess the degree of severity of anaphylaxis, each of which has limitations (61–64). The reason why some sensitized subjects develop mild systemic symptoms while others experience severe, even fatal SRs is not completely understood, even though several risk factors are known. The combination of several concomitant factors, which include environmental, genetic and individual factors, may account for the occurrence of SRs in individual patients (11). Patient-related risk factors for severe SRs in the adult population are older age, clonal mast cell disorders and/or elevated basal serum tryptase and accompanying respiratory or heart diseases (30, 65, 66). Available data regarding potential effects of beta-blockers and/or angiotensin-converting enzyme (ACE) inhibitors in coexisting venom allergy are inconclusive; further studies are required to assess the impact of specific cardiovascular comorbidities (30). Risk factors and co-factors for severe SRs after Hymenoptera stings in children were identified in atopy (asthma, allergic rhinitis, and atopic eczema) (50, 53, 67, 68) and exercise (69). Moreover, the severity of the reaction was also associated with the severity of asthma (67). However, these findings should be confirmed in larger pediatric populations. Taken together, the aforementioned data hints to the existence of several subgroups of phenotypes in relation to the severity of SRs.

Mono- and Multi-Sensitization

Patients with a history of SRs might show positive test results with one, two, or multiple venoms in the following diagnostic work-up (42, 70, 71). Particularly when the allergy-eliciting insect could not be identified by the patient, these double or multiple sensitizations challenge decisions concerning the proper therapeutic strategy as they might be a result of true primary allergy to more than one venom, cross-reactivity between venoms or asymptomatic sensitization (42). Only in the first case is VIT with all relevant venoms recommended, while for other scenarios VIT with the primary sensitizing venom only is sufficient. Fortunately, diagnostic tools, which in many cases allow the differentiation between primary allergy and cross-reactivity, exist.

Patients Refractory to VIT

Although VIT is an effective curative treatment in the majority of Hymenoptera venom-allergic patients, in some cases it is not able to induce immunologic tolerance. To date, the reasons for treatment failures during VIT remain unclear. Risk factors for VIT failure are HBV allergy, very severe sting reactions, SRs induced by VIT, clonal mast cell disorders and/or elevated baseline levels of serum tryptase and perhaps the use of ACE inhibitors (72). A recent retrospective multicenter study of HBV-allergic patients demonstrated that a dominant sensitization to Api m 10 (>50% of sIgE to HBV) is a relevant risk factor for treatment failure with an odds ratio of 8.44 (73). Furthermore, all patients who showed sIgE to Api m 10 that was higher than 60% of HBV sIgE were therapy non-responder. Nevertheless, in most cases in which standard VIT fails, increasing the dosage successfully induces tolerance (74). Risk factors associated with a loss of protection after discontinuation of VIT include those mentioned above and failure to achieve protection during VIT (72). As longer treatment periods are associated with a lower risk of relapse (75), prolonging treatment or even maintaining it lifelong can be a reasonable option to achieve or retain tolerance, especially for high-risk patients (21).

Patients Refractory to Medication

Refractory anaphylaxis (unresponsive to treatment with at least two doses of minimum 300 μg adrenaline) is a rare form of a life-threatening hypersensitivity reaction with high mortality. Comprehensive data on its definition, prevalence and risk factors is missing. Using the data from the European Anaphylaxis Registry (11,596 cases in total), 42 cases of refractory anaphylaxis of different origin were identified and compared to a control group of severe anaphylaxis cases (n = 4820). Cases elicited by insects were very few (n = 8) and often due to bee stings (76). Specific risk factors were not identified in Hymenoptera venom-allergic patients. Rudders *et al.* reported that among 153 emergency department patients with systemic insect sting reactions who received adrenaline, 16% received a second dose, without evaluating their characteristics (77). Although studies have demonstrated an association between beta-blocker use (or multiple antihypertensive drugs) and increased anaphylaxis severity (regardless of the trigger), as evidenced by increased organ system involvement and hospital admission (78–80), it is not yet established whether taking beta-blockers influences the number of adrenaline doses needed, thus, identifying a particular phenotype unresponsive to adrenaline therapy in case of anaphylaxis is not possible, including venom anaphylaxis. A recent case control study in adults did not find a significant link between beta-blocker use and the need for increased adrenaline dosing among emergency department patients with anaphylaxis (81). This suggests that the effects of beta-blockers may not be as significant in the clinical routine as previously thought. The lack of response to initial adrenaline may be due to insufficient drug delivery secondary to reduced venous return (82). A very recent study advocated for rapid escalation with early intravenous fluid therapy in patients where anaphylaxis is refractory to initial intramuscular adrenaline, even in patients without obvious hemodynamic instability (83). Patients suffering from mast cell disorders and venom allergy may need more doses of adrenaline because of the increased severity of anaphylaxis (84, 85) due to massive mast cell activation. Therefore, they can be identified as a specific patient phenotype, also in regards to the refractoriness to pharmacological treatment.

Patients Prone to Adverse Reactions During VIT

VIT may induce adverse reactions. In large multicenter studies, the frequency of SRs reactions during VIT ranges from 8% to

20% (86–88). A slightly elevated risk for SRs during VIT is observed in vespid venom-allergic patients with elevated baseline serum tryptase levels, while this association was not found for treatment with HBV (88). Nevertheless, the most important risk factor for systemic adverse events with VIT (3.1- to 6-fold increased risk) is treatment with HBV (88–90). Although only shown in small patient populations, Api m 4 sensitization might be a risk factor for SRs during the up-dosing phase of VIT with HBV (91, 92). In a prospective study it was demonstrated that patients who had sIgE to Api m 4 >0.98 kU_A/L show higher rates of SRs during the VIT induction phase (91).

According to the recent guidelines of the European Academy of Allergy and Clinical Immunology (EAACI) (21), in the case of systemic adverse events during the build-up phase of VIT, in addition to initially reducing the dosage, premedication with H1 antihistamines should be established. In case of repeated systemic adverse events during up-dosing, pretreatment with Omalizumab may be recommended (21). Currently, case reports and a case series have documented the usefulness of Omalizumab for the pre-treatment of patients who experienced systemic reactions to VIT, including patients with systemic mastocytosis (93–99). Most of these patients were able to tolerate VIT after Omalizumab pre-treatment. However, treatment regimens varied greatly. In some cases a single or a few injections before initiation of VIT were used (94, 98), while in other cases Omalizumab therapy and VIT were combined for several months (93, 97) or pre-treatment before every maintenance dose was administered (96). This suggests that the optimal treatment schedule with Omalizumab depends on the individual response to VIT.

ALLERGENS OF HYMENOPTERA VENOMS

Hymenoptera venoms are complex mixtures of a variety of substances which mediate the toxic effects. These include numerous proteins that represent potential allergens. In recent years, biochemical and molecular biological methods have made a significant contribution to the identification and characterization of new allergens of Hymenoptera venoms, shifting the focus from the whole venom to individual allergenic molecules (100).

To date, honeybee venom (HBV) is the best characterized Hymenoptera venom. In the last years, proteomic approaches have contributed to the identification of a variety of potential new allergens, including those of very low abundance (101, 102). Moreover, recombinant production strategies together with detailed immunologic analyses have enabled the identification of five major allergens in HBV (103): Api m 1 (phospholipase A2), Api m 2 (hyaluronidase), Api m 3 (acid phosphatase), Api m 5 (dipeptidylpeptidase IV), and Api m 10 (icarapin) with sIgE sensitization rates in HBV-allergic patient populations in the range of 57%–97%, 47.9%–52.2%, 49.6%–50%, 58.3%–61.7%, and 61.8%–72.2%, respectively (73, 103–109). Less information concerning sensitization rates is available for other HBV allergens and most of them appear to be of minor importance, not excluding that they might be of particular relevance for some patients. Bumblebee venom closely resembles HBV and both exhibit extensive cross-reactivity (110).

Similarly, the venoms of Vespoidea species are mostly alike (110). Shared between almost all of them is the highly abundant major allergen of unknown function named antigen 5. Moreover, most of the Vepoidea venoms contain phospholipases A1 as prominent and relevant allergens. The sensitization rates of YJV- and PDV-allergic patients to the phospholipases A1 (Ves v 1 and Pol d 1) and antigens 5 (Ves v 5 and Pol d 5) are 33.3%–54% and 87% and 84.5%–100% and 69%–72%, respectively (105, 108, 111–116).

HBV and Vespoidea venoms contain homologous allergens that can lead to cross-reactivity between the venoms. For instance, in addition to HBV, YJV and PDV contain dipeptitylpeptidases IV (Ves v 3 and Pol d 3) as major allergen (117, 118). Furthermore, hyaluronidases were identified in different Vespoidea venoms. However, in contrast to HBV Api m 2, their relevance as allergens in YJV (Ves v 2.0101 and Ves v 2.0201) seems to be limited (119).

DIAGNOSTICS AND BIOMARKERS IN HYMENOPTERA VENOM ALLERGY

Diagnosis of HVA comprises the clinical history of a systemic sting reaction and the proof of sensitization to the relevant venom by *in vivo* or *in vitro* testing (21, 39, 120). For successful VIT, the correct venom for treatment is of major importance. Due to the pronounced cross-reactivity between venoms, choosing the right venom for therapy is a challenging task if the patient was not able to identify the allergy-eliciting insect. Nevertheless, several advanced diagnostic tools and biomarkers exist (**Figure 2**) that facilitate accurate diagnosis and contribute to personalized risk stratification in HVA. Diagnostic algorithms to discriminate between HBV and vespid venom and YJV and PDV allergy are given in **Figures 3A, B**, respectively.

Clinical History

The verification of a previous SR by clinical history should build the basis for a subsequent diagnostic work-up (**Figure 3**) since asymptomatic sensitization to Hymenoptera venoms is observed frequently (39, 40). A thorough clinical history includes information on number and date of sting reactions, symptoms, severity and time course of the reaction as well as the applied treatment. Additionally, individual risk factors for anaphylaxis such as mast cell disorders, medication, cardiovascular risks and other diseases as well as frequent exposition to relevant insects should be considered.

A special focus during the assessment of the patients' history lies on the identification of the culprit insect. However, as many patients (and even allergy specialists) are not able to discriminate different Hymenoptera species (123, 124), all information has to be used with care and verification of the responsible insect with additional diagnostic tests is necessary.

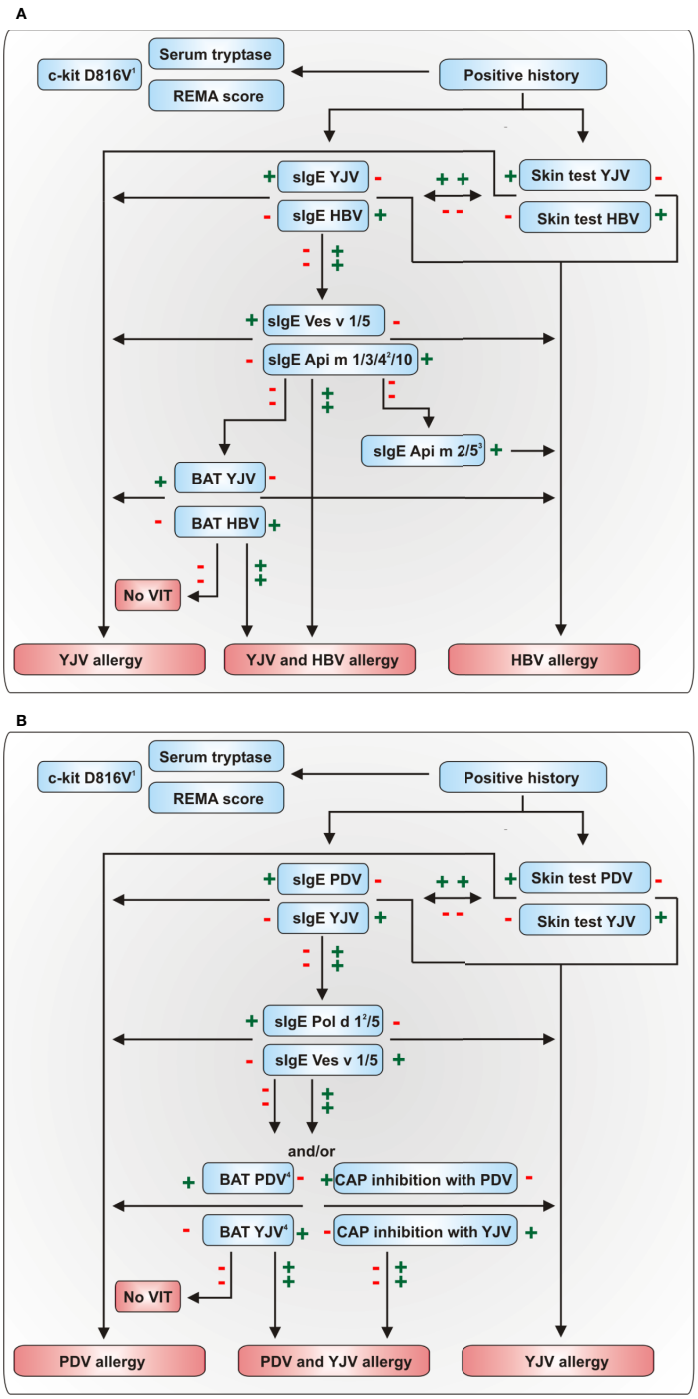

FIGURE 3 | Diagnostic algorithms for the discrimination of **(A)** HBV and YJV allergy and **(B)** YJV and PDV allergy. The diagnostic algorithm presented in **(A)** can also be used to discriminate between HBV and PDV allergy using the *Polistes dominula* homologues of Ves v 1 and Ves v 5, Pol d 1 and Pol d 5, respectively. A plus indicates a positive and a minus a negative test result. [1]In the majority of cases, positive PCR results proving the presence of the c-kit D816V mutation in peripheral blood mononuclear cells can confirm systemic mastocytosis (121). [2]These allergens are only available for selected multiplex sIgE platforms. [3]The HBV allergens Api m 2 and Api m 5 show potential cross-reactivity to not commercially available homologous allergens of YJV and PDV so that a positive test result does not necessarily preclude YJV or PDV allergy. [4]BAT proved to be an effective tool for the assessment of double-positivity in HBV and YJV allergy (122). However, currently, no studies have analyzed its usefulness for the discrimination of PDV and YJV allergy. BAT, basophil activation test; HBV, honeybee venom; PDV, *Polistes dominula* venom; REMA score, score of the Spanish Network on Mastocytosis for predicting mast cell clonality and systemic mastocytosis in patients who experience anaphylaxis without cutaneous mastocytosis; VIT, venom-specific immunotherapy; YJV, yellow jacket venom.

Skin Tests

Both, skin testing with venom extracts and sIgE measurements, should be performed in patients with a history of a SR (72). Skin testing with venom extracts can be done either as skin prick or intradermal testing following different protocols (125). Skin prick tests are performed at a concentration between 1 and 100 µg/ml, while an initial concentration in the range of 0.001–0.01 µg/ml is sufficient for intradermal tests. It should then be increased tenfold per step to a maximum concentration of 1 µg/ml (126). The sensitivity of skin prick test alone is around 64%, while the combination of prick test and intradermal test reaches a sensitivity of 94% (72). Despite a low risk of SRs (127), many institutions recommend to perform a graduated approach for skin testing (126). The simultaneous intradermal testing with different venoms is safe and efficient (127). Since the intradermal test is more sensitive, it should be used to confirm negative skin prick test results. Skin tests should be done at least 2 weeks after the sting reaction to avoid false-negative results during the refractory period (128). In case of negative tests despite a convincing history of a SR, skin tests should be repeated after 1–2 months. Of note, there is no correlation between the severity of a sting reaction and reactivity in skin testing (129).

Baseline Serum Tryptase

It is recommended to determine the baseline tryptase level in the serum of all patients with a history of systemic sting reactions to identify patients at higher risk of developing severe reactions due to undiagnosed clonal mast cell disorders. High baseline levels in repeated measurements (particularly above 25 µg/ml) suggest mast cells disorders which need a further diagnostic work-up (e.g. by testing for somatic c-kit mutation or bone marrow analysis) (72). Adult patients with mast cell disorders and/or elevated baseline tryptase are not only at higher risk of more severe sting reactions but in some studies are also considered a risk population during VIT (28, 66, 130).

Genetic Markers

Due to the increasing implementation of genome-wide association studies since the early 2000s, a multitude of different candidate genes with marker properties have been described. Most of these candidate genes have little or no clinical value and only a small fraction of the initial pool is being further investigated and implemented into the clinical routine. Nevertheless, genetic markers are an up-and-coming field in allergy research. One prominent example is the somatic c-kit D816V mutation which is used as minimally invasive secondary diagnostic criterion to confirm systemic mastocytosis, since >80% of patients with systemic mastocytosis are tested positive for this single nucleotide polymorphism (SNP) (131–133). As described earlier, systemic mastocytosis in combination with sensitization to Hymenoptera venom allergens is considered a risk factor for severe SRs. Therefore, c-kit D816V mutation is no direct genetic marker for venom allergy or increased risk of systemic allergic reactions but can offer added value to a thorough diagnosis and assessment of the individual risk.

A more straightforward marker is the polymorphism in the angiotensinogen AGT p.M235T gene which might be associated with more severe SR in patients with Hymenoptera venom allergy. Patients allergic to insect venoms have a higher prevalence of carrying this mutation and suffer more often, with an odds ratio of 2.5, from grade IV reactions (134).

Furthermore, a variety of studies focusing on the connection of HLA class I and class II genotypes and (venom) allergy have been published. For instance, HLA-B*18 and HLA-Cw*07 were significantly more frequent among Turkish bee- and/or wasp venom-allergic patients (135). Among HLA class II genotypes, DRB1*0101, DRB1*0103, DQA1*0101, and DQB1*0501 were found to be associated with an increased risk of being sensitized to Api m 1 (136). Still, to our knowledge, no conclusive statement regarding HLA class I and II frequencies and venom allergy or risk of SR is possible.

An elevated basal serum tryptase level might be caused by alfa-tryptasemia, a hereditary trait that was reported by Lyon et al. in 2014 (137). Affected persons carry additional copies of TPSAB1, the gene encoding for alpha-tryptase. Alpha-tryptasemia is discussed as one of the main sources for elevated serum tryptase and is associated with a 2–4 fold increased risk of systemic reactions (138, 139). The link between alpha-tryptasemia and mast cell activation disorder is part of ongoing research and not easy to assess (140).

Total IgE

The measurement of the total IgE (tIgE) levels in combination with sIgE test results can be useful to improve and simplify interpretation. This is particularly important in connection with very low sIgE levels, since each sIgE level has a different relevance if produced in an environment with high or low tIgE values (141). Moreover, sIgE to Hymenoptera venoms is frequently observed in asymptomatic individuals with high tIgE (40, 142). Hence, the measurement of tIgE can provide guidance in the context of the ratio sIgE/tIgE, although it is not generally recommended in the guidelines.

Specific IgE to Venom Extracts

Besides skin testing, the detection of sIgE to whole venom extracts is the most established diagnostic method to detect sensitization to Hymenoptera venoms. However, the diagnosis of clinically relevant allergy can only be made in combination with a corresponding clinical history. This also holds true for skin testing and other diagnostic approaches.

Although 0.35 kU_A/L is commonly used as the lower threshold for sIgE detection, sIgE concentrations can be measured with high accuracy on the major singleplex sIgE immunoassay platforms with the lower end threshold of 0.1 kU_A/L. Hence, sIgE levels between 0.1 and 0.35 kU_A/L can be considered in the context of a clear clinical history (37, 143, 144). Ideally, sIgE measurements should be performed one to 6 weeks after the sting event. It should be kept in mind that negative sIgE test results in patients with convincing history of anaphylaxis can be caused by very low levels of sIgE or too long latency between the last sting and the diagnostic measurement (14, 35, 145).

Using the cut-off of 0.35 kU_A/L, 90%–100% of HBV-allergic patients are tested positive for sIgE to HBV. The sensitivity of sIgE detection to YJV for YJV-allergic patients ranges between 83% and 97% (108, 116, 146). Nevertheless, sIgE testing of allergic patients with venom extracts in clinical routine frequently leads to multiple positive test results with different venoms. Intriguingly, for many of these patients only one venom is allergy-relevant. The clinical relevance of positive test results with other venoms with regard to systemic symptoms is limited (41, 147). However, as many patients are not able to identify the allergy-eliciting insect, clinically relevant allergy cannot be excluded. In addition to primary allergy to more than one venom, multiple positive test results with limited or no clinical relevance can be caused by: i) IgE antibodies directed to protein epitopes on homologous allergens present in the venoms, ii) sIgE to clinically irrelevant cross-reactive carbohydrate determinants (CCDs), and iii) asymptomatic sensitization (42). Hence, this often leads to unnecessary VIT with more than one venom, resulting in higher costs, potentially increased risk of side-effects and the possibility of *de novo* sensitization.

Overall, venom extract-based diagnostics has some pitfalls that complicate the differentiation of true primary allergy and cross-reactivity and, thus, the identification of the allergy-relevant venom and selection of the optimal therapeutic strategy.

IgE-inhibition tests with whole venom extracts can be used in particular cases to detect the primary sensitizing venom in patients double-positive to venoms without marker allergens, e.g. YJV and PDV (**Figure 3B**) (148–150). However, IgE-inhibition tests are costly, time-consuming and results occasionally difficult to interpret (149).

After an initial rise during the first months of treatment, sIgE levels to the respective venom decrease during VIT and usually remain low after discontinuation of VIT (151, 152). However, there is no evidence that they can be used as biomarker to predict success of therapy (21).

Specific IgE to Individual Venom Allergens

In the recent past, the identification of relevant venom allergens has led to the development of molecular or component-resolved diagnosis (CRD) in HVA (42, 70, 153, 154). In CRD, sIgE against single allergens of venoms is determined. Thus, CRD not only provides information on whether a patient has sIgE against the whole venom, but also on exactly which allergens are relevant for a patient.

Due to the number of commercially available allergens, CRD has particularly increased diagnostic accuracy for the discrimination between HBV and YJV allergy. Diagnostic sensitivity of a combination of the two commercially available YJV allergens Ves v 1 and Ves v 5 ranges between 92% and 100% (35, 112, 113, 116, 155–158). CRD of HBV allergy is more complex in terms of diagnostic sensitivity. The first commercially available HBV allergen Api m 1 yielded a diagnostic sensitivity of 58% to 97% depending on the inclusion criteria of the patient population, geographical differences and sensitivity of the immunoassay platform used (103, 105–109). Hence, missing sensitization to Api m 1 does not exclude a genuine allergy to HBV. After the relevance of additional HBV allergens was demonstrated, these became available for routine diagnosis and it was shown that a combination of the allergens Api m 1, Api m 2, Api m 3, Api m 4, Api m 5, and Api m 10 leads to a diagnostic sensitivity of 94.4% in a population of HBV-allergic patients (103). However, this sensitivity might be lower in patients with sensitization to HBV only compared to those sensitized to both, HBV and YJV (159). Nevertheless, the extension of the panel of commercially available HBV allergens added clinical benefit as two-thirds of patients with negative sIgE to Api m 1 can be diagnosed using Api m 3 and Api m 10. In patients double-sensitized to HBV and YJV who were not able to identify the allergy-relevant insect, the combination of Api m 1, Api m 3, and Api m 10 increased the sensitivity of HBV allergy verification to 78.6% compared to 54% using Api m 1 only (104).

Recombinant allergens can be produced with the full protein epitope spectrum of the native allergens but without CCDs (160). Hence, positive sIgE test results indicate sensitization to protein epitopes only and not to CCDs, thereby excluding many clinically irrelevant sensitizations (106).

In addition to CCDs, cross-reactivity between different venoms can be caused by homologous allergens that share common IgE epitopes. The potential of CRD is evident from the fact that HBV and vespid venoms contain species-specific marker allergens (Api m 1, Api m 3, Api m 4, and Api m 10 for HBV and phopholipases A1 and antigens 5 for vespid venoms) in addition to homologous allergens. For many patients, the measurement of sIgE directed against these marker allergens allows the identification of the allergy-relevant venom and the discrimination between cross-reactivity and primary allergy (**Figure 3A**) (104). However, a clear limitation of the currently available CRD is the unavailability of potentially cross-reactive allergens of vespid venoms such as hyaluronidases and dipeptidylpeptidases IV as marketed allergens for sIgE detection, as they might be of relevance for particular patients.

While CRD is able to adequately distinguish allergies to HBV and vespid venom (particularly YJV), this is not the case when a differentiation between allergies to various vespid venoms is required. For instance, in Southern Europe, double-sensitization to YJV and PDV is much more frequent than to YJV/PDV and HBV (161–163). Although *Polistes* venom is devoid of CCDs (164), a definite discrimination is rarely possible due to the high degree of cross-reactivity between the major allergens of these venoms (110, 165). Moreover, only the PDV allergen Pol d 5 is available for CRD on the most common sIgE assay platform. Nevertheless, a previous study demonstrated that the measurement of relative levels of sIgE to the phospholipases A1 (Ves v 1 and Pol d 1) and antigens 5 (Ves v 5 and Pol d 5) of YJV and PDV allows the identification of the primary sensitizing venom in many cases (115). Therefore, the additional availability of these and other (e.g. dipeptidylpeptidases IV) cross-reactive allergens from vespid venoms for CRD would represent an added value for advanced precision diagnostics in HVA.

Additionally, some allergens may act as biomarkers for personalized risk stratification in patients undergoing VIT. As

discussed in section *Patients Refractory to VIT*, dominant Api m 10 sensitization is a relevant risk factor for honeybee VIT (73). Thus, the knowledge of patient sensitization profiles allows choosing a therapeutic venom preparation for VIT that contains the highest amount of Api m 10 in a patient-tailored manner (73, 166, 167). Moreover, Api m 4 sensitization might be a marker to identify HBV-allergic patients with increased risk of SRs during the up-dosing phase of VIT (section *Patients Prone to Adverse Reactions During VIT*) (91, 92).

sIgG4

With less than 5% of total IgG, IgG4 is the least abundant IgG subclass in human serum. However, IgG4 levels increase with chronic antigen exposure and are believed to induce immune tolerance and weaken inflammatory responses (168–171). IgE mediated hypersensitivity reactions are dampened by IgG4 by inhibiting IgE activity (172–174). Two different mechanisms have been proposed: i) IgG4 scavenges immunogenic epitopes on antigens and acts as a blocking antibody that prevents the downstream crosslinking of FcεRI (175, 176). ii) IgG4 co-stimulates the inhibitory FcγRIIb. This IgG receptor regulates signal transduction and inhibits the activation of effector cells (177).

VIT is associated with a significant increase in sIgG4 antibodies (178). However, after stopping VIT, sIgG4 levels start to decrease (179). In grass pollen allergy it was demonstrated that IgE-blocking capacity persisted for several years and correlated with clinical efficacy, although IgG4 levels rapidly decreased after stopping allergen-specific immunotherapy (172). This suggests that not the levels of sIgG4 but rather their functional activity might correlate with clinical efficacy and long term protection (180). Therefore, no evidence for the use of levels of venom-sIgG4 as biomarker for prediction of therapy success in VIT is given (21). Nevertheless, although IgG4 induction *per se* is no marker for therapeutic success, lack of IgG4 induction might be a marker for immunological unresponsiveness.

Basophil Activation Test

The basophil activation test (BAT) mimics the activation of effector cells (basophils) responsible for IgE mediated allergic reactions *ex vivo*. Basophils in fresh patient blood are stimulated with allergens and the (up-)regulation of basophil specific markers, such as CD63 or CD203c, is observed.

Although BAT is not part of the routine diagnostics of venom allergy in all patients, it is well established and can be used in cases of unclear or negative skin and sIgE test results or when clinical history and diagnosis are contradictory. Studies demonstrated that BAT is able to detect sensitization in 81% of venom-allergic patients with negative sIgE and in 60% of patients that additionally exhibit negative intradermal skin tests (181, 182). Moreover, BAT is useful to correctly diagnose double-positive patients with inconclusive skin test or sIgE test results, particularly when the patient reacted only to one insect (122). Perhaps, the basophil response can also be used as biomarker for successful tolerance induction after VIT. It was demonstrated that, although unchanged after the first year of treatment, a significant and approximately fourfold decrease of basophil activation was observed in all tolerant subjects in response to submaximal allergen concentration after VIT (183).

BAT can further be used as biomarker to monitor ongoing VIT and to assess the success. Here, discrimination between BAT sensitivity and reactivity is needed. While the reactivity of basophils corresponds to the quantity of allergen needed to induce CD63 on the cell surface, the sensitivity is linked to the change of cell marker (e.g. CD63) amount (184). A successful VIT, which necessarily induces long term tolerance, decreases BAT sensitivity without changing the reactivity (183, 185, 186). Furthermore, a high sensitivity in BAT during the initial VIT phase is also associated with a higher risk of side-effects (186, 187).

Sting Challenge Test

Due to the risk of severe reactions or *de novo* sensitization, sting challenge tests using living insects should not be used as diagnostic tool in untreated patients (188). However, apart from a well-documented field sting, the sting challenge test is the only recommended diagnostic method for the prediction of success of VIT (21). Moreover, a patient's quality of life can be significantly improved by experiencing a tolerated sting challenge (189).

THERAPY OF HYMENOPTERA VENOM ALLERGY

Due to the high risk of very severe and even fatal reactions in venom-allergic patients, a careful patient management and proper therapeutic intervention is of major importance. Although some behavioral rules that might contribute to minimize the risk exist, avoiding stings completely is challenging. Therefore, patients with venom allergy should carry an emergency kit for self-administration including an adrenaline autoinjector as well as orally administered H1-antihistamine and corticosteroids. It is still a matter of debate, if the emergency kit should be carried during and after VIT as most patients are protected after reaching the maintenance dose (190).

VIT is the only disease-modifying and curative treatment of venom allergy that is able to efficiently protect patients against future severe sting reactions. VIT is recommended in adults and children with detectable sensitization and SRs exceeding generalized skin symptoms as well as in adults with generalized skin symptoms if quality of life is impaired (21). Although VIT is one of the most effective treatments in the field of clinical allergology, choosing the correct venom based on a comprehensive diagnostic work-up represents a crucial prerequisite for effective protection. Nevertheless, different biomarkers and diagnostic strategies are available that allow the classification into endotypes and phenotypes in HVA. Hence, they facilitate the correct implementation of VIT, the identification of patients at high risk for severe sting reactions and the adjustment of treatment protocols and times.

The detailed mechanisms of tolerance induction during VIT are not completely understood. Nevertheless, several immunological changes, which are associated with the success of therapy, are well described. Venom-specific regulatory T cells (Treg) and Th1 cells are thought to be induced during VIT and

are able to suppress pro-allergic Th2 cells. Further, the Th2 suppression leads to reduction of the levels of cytokines such as IL-4, IL-5, IL-9, and IL-13 resulting in a desensitization of mast cells and basophils (191). Moreover, an induction of specific IgG4 antibodies might be of relevance as blocking antibodies are supposed to have a protective anti-inflammatory role (192). Additionally, the induction of B cells with a regulatory phenotype (Breg) was shown to be an important event during VIT (193). Bregs are able to suppress venom-specific T cell proliferation (194) and to induce Tregs (195, 196), thus, boosting the shift towards a tolerogenic phenotype.

VIT is performed by subcutaneous injections of whole venom extracts. The suggested maintenance dose of 100 μg can be reached using different protocols. In conventional protocols, maintenance dose is reached in several weeks to month, whereas in rush and ultra-rush protocols that use several injections per day on consecutive days, maintenance dose is reached within a few days or hours, respectively. In cluster protocols, patients receive several injections per day in intervals of 1–2 weeks. Intervals between maintenance injections can be gradually increased from 4 weeks (first year) to 6 (second year) and 8 weeks (in case of a 5-year treatment from year 3–5) without loss of clinical protection (21, 197).

Several studies showed that in most patients clinical protection is achieved as soon as the maintenance dose is reached (190, 198). Most of the patients who are still reacting to a sting while receiving the conventional maintenance dose of 100 μg will be protected by increased venom dosages during VIT (74, 199). VIT is reportedly effective in preventing future SRs in 77%–84% of patients treated with HBV, 91%–96% of patients receiving vespid venom (200, 201), and 97%–98% of patients treated with ant venom (202, 203). The reasons for the lower efficacy of VIT with HBV are still unclear. Potential explanations might be the much larger and consistent venom amount delivered by a honeybee sting (204) or the broad sensitization profiles of HBV-allergic patient with different major HBV allergens (103), including those that might be underrepresented in certain therapeutic venom preparations (73, 166).

VIT should be performed for 3–5 years, whereby most experts recommend 5 years (120). Of note, stopping VIT after 3 years might only be feasible for patients with mild to moderate reactions and should not be done when sting challenge during therapy cannot be performed (205, 206). VIT with a minimum duration of 5 years is superior for long-term effectiveness and protects the majority of patients (207, 208). A recent study on the outcome of re-stings on a long follow-up period after VIT discontinuation (up to 26 years) showed a very low risk of relapse (3.4%) in patients treated on average for about 10 years (209).

According to some studies, risk factors that are associated with a loss of protection after discontinuation of VIT include very severe initial SRs, systemic adverse events during VIT (injection or sting), treatment of less than 5 years, elevated basal serum tryptase and/or mastocytosis, HBV allergy, cardiovascular disease and others (21, 197). However, all patients continue to have a 10% chance of having a reaction to a future sting (210) and the only way to keep the risk down to 2% is to remain on maintenance VIT (205). Lifelong therapy should be particularly considered in high-risk patients such as those suffering from mastocytosis as well as in patients at high risk for future stings such as beekeepers. A recent study on the outcome of re-stings on a long follow-up period after VIT discontinuation (up to 26 years) showed a very low risk of relapse (3.4%) in patients treated on average for about 10 years (209).

CONCLUSIONS

In the first placebo-controlled trial in 1978, allergen-specific immunotherapy with insect venoms has proven to be superior over therapy with whole body extracts of the insects (211) and since then demonstrated to be a highly effective curative treatment of venom allergy. Nevertheless, the growing knowledge of different disease manifestations of HVA and of disease-influencing comorbidities has increasingly improved adequate diagnostics and patient management. For instance, the availability of CRD has facilitated the differentiation of primary allergy and cross-reactivity and, thus, therapeutic decisions in multiple-sensitized patients. Moreover, biomarkers such as the c-kit D816V mutation or elevated baseline tryptase levels that allow to identify patients at risk for very severe sting reactions were identified and allow a personalized patient management. Nevertheless, there is a need for additional biomarkers which reliably allow therapy monitoring, the identification of potential VIT non-responders and patients at risk for severe side effects as well as to monitor immunological tolerance after discontinuation of VIT. There is some evidence that the analysis of patients' sensitization profile might help to predict the outcome of VIT in the future, to better adjust treatment strategies and to select the most suitable venom preparation in a personalized manner (73, 167).

To further classify endotypes and phenotypes in HVA might be a promising approach to better understand the disease, to strengthen personalized treatment strategies and, thus, precision medicine in HVA. Moreover, detailed molecular analyzes of the immunological processes occurring during VIT might contribute to a deeper understanding of immune tolerance to allergens. This in turn can support the development of novel immunomodulatory strategies that might enhance tolerance induction as well as the identification of new biomarkers that indicate therapeutic success in an early state of treatment.

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All authors contributed to the article and approved the submitted version.

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REVIEW
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Shaping Modern Vaccines: Adjuvant Systems Using MicroCrystalline Tyrosine (MCT®)

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The concept of adjuvants or adjuvant systems, used in vaccines, exploit evolutionary relationships associated with how the immune system may initially respond to a foreign antigen or pathogen, thus mimicking natural exposure. This is particularly relevant during the non-specific innate stage of the immune response; as such, the quality of this response may dictate specific adaptive responses and conferred memory/protection to that specific antigen or pathogen. Therefore, adjuvants may optimise this response in the most appropriate way for a specific disease. The most commonly used traditional adjuvants are aluminium salts; however, a biodegradable adjuvant, MCT®, was developed for application in the niche area of allergy immunotherapy (AIT), also in combination with a TLR-4 adjuvant—Monophosphoryl Lipid A (MPL®)—producing the first adjuvant system approach for AIT in the clinic. In the last decade, the use and effectiveness of MCT® across a variety of disease models in the preclinical setting highlight it as a promising platform for adjuvant systems, to help overcome the challenges of modern vaccines. A consequence of bringing together, for the first time, a unified view of MCT® mode-of-action from multiple experiments and adjuvant systems will help facilitate future rational design of vaccines while shaping their success.

Keywords: adjuvants, virus-like particles, MicroCrystalline Tyrosine (MCT®), allergy, disease, immunization, Monophosphoryl Lipid A (MPL®), vaccines

INTRODUCTION

The Evolution of Vaccines and Adjuvants

The concept of variolation (human inoculation/insertion of pathogens) dates back to the 10th century in China, here, immunization against small pox used the live virus itself. Edward Jenner practised variolation in the UK and moved the field to the next level in the last decade of the 18th century by using a cowpox virus for immunization which eventually led to the first vaccine (derived

from vaccinia virus from vacca, the Latin word for cow), and the eradication of small pox in the 20th century (1–3). Again, during the early 1900's the pioneering work of Louis Pasteur, Alexandre Yersin, and others was associated with the development of attenuated and inactivated vaccines which progressed for a variety of pathogens such as cholera, tetanus, polio, tuberculosis, and a severe pneumonia-form of plague (*Yersinia pestis*) (4–10).

> "Discoveries [made] by accidents and sagacity, of things [the observers] were not in quest of" (1754, quoted in Merton and Barber 2004, p. 2) (11).

In the 1920's, both Alexander Glenny and Gaston Ramon were working with diphtheria toxins (12, 13). Production of bacterial toxins became very efficient (14). It was not long before Glenny referred to the use of a toxoid in humans for the first time in 1923 (13). Serendipity has led to some of the greatest discoveries and breakthroughs in science and medicine over the past century. Indeed, the story of adjuvants begins with a French veterinarian who unlocked a secret weapon, at an intersection of chance and wisdom. Gaston Ramon's (1886–1963) crucial discovery, whilst at the Pasteur Institute in Paris in the 1920's, made the observation that "local infection" (or abscesses at the injection site) was in some way enhancing antibody (Ab) production (15). As such, a series of experiments were set out and by adding a variety of substances (e.g., agar and starch oil) to an inoculation—substances he referred to as adjuvants (from the Latin adiuvare, meaning to help or aid) - resulted in enhanced tetanus and diphtheria anti-serum production (15, 16). When Gaston Ramon discovered the immune potentiating effect of such adjuvants, the human population was reeling from the aftermath of the Spanish flu and faced burgeoning health risks from pathogens. Moreover, vaccination against viruses, for example, represented more of a challenge than vaccination against bacteria, mostly because it was more difficult to grow them.

As part of Glenny's work dealing with bacterial toxins, metal salts (precipitates thereof) were employed during the purification process, the adsorbed toxoid was subject to the wisdom of Glenny to perform comparative immunological studies, which indicated greatly enhanced immunological effects (14, 17), illuminating the serendipitous points of discovery that have shaped the modern world. Today, optimized versions of "alum" salt precipitates [e.g., aluminium oxyhydroxide; AlO (OH), aluminium phosphate; AlPO4)] have been the mainstay of adjuvants in clinical vaccines for more than 70 years (16).

For most of this time, the scientific community considered the principle or "dogma" of explaining the effectiveness of aluminium adjuvants in the context of the "depot" effect - immune stimulation through prolonged exposure of the antigen (18, 19). However, more research devoted to this question has revealed evidence that better explains adjuvancy in the context of alums physicochemical attributes and biological properties than a depot effect alone (20–26).

Tools to study the genome or cellular systems have developed rapidly. This has inspired new strategies from empirical to rational approaches to vaccine design and antigen carrier (nano)-systems, for targeting both innate and adaptive immune responses in tackling more challenging or emerging diseases or improvements in safety and efficacy of others (27–29). Vaccines are disruptive technologies and one of the most cost-saving medical applications ever developed, and in the last decades, their application in non-infectious diseases such as allergy, cancer, diabetes, and even smoking cessation continue to be developed (30–34).

While recombinant vaccines have generally improved safety profiles compared with live-attenuated and whole-pathogen vaccines, they are also often less immunogenic due to the removal of their inherent pathogenic features and patterns. Modern vaccine development focusses on bridging or substituting this gap in order to improve their effectiveness without compromising safety. As a consequence, the development of new and sophisticated rational technologies such as antigen (nano)-carrier systems [e.g., virus-like particles (VLPs)] or combination of adjuvants (adjuvant systems) are being employed to help overcome these challenges (29, 35).

Adjuvant Systems

Adjuvant Systems may comprise of a variety of classical adjuvants or immunomodulators that are combined and tailored for the specific antigen and target application. The immune system has evolved to recognise repetitive surface features like pathogen-associated molecular patterns (PAMPs), which forms the basic principles in how they are able to activate the innate immune system, which, in turn, leads to orchestration of a specific adaptive response.

The benefits of vaccines and immunization against pathogenic threats demonstrate a convincing positive benefit-risk ratio over many decades, with the scope to eradicate disease. The existing and evolving threats have been brought to light recently with the spread of SARS-Cov-2, which some have described as natures wake-up call to complacent civilisation; threatening our era of peak globalisation, which has grown under a safety net of medical and scientific advances. The consideration of adjuvants in new vaccine development can be the difference to what makes a vaccine effective or not. Particularly, so where pathogens with more complex life cycles with intracellular habits or pathogens with genetic variability exist. Optimizing vaccines for this purpose has been historically slow and cumbersome (e.g., influenza, HIV, and malaria) and often requires a more robust adaptive response. For billions of years, microbes have evolved in this way, and this complexity has only just begun to be better understood by scientists.

Immunology, Immunization, and Immunotherapy

The innate and adaptive responses cover two broad phases of the body's response to a pathogen or vaccine. Pattern recognition receptors (PRRs) on innate and adaptive immune cells [i.e., macrophages, dendritic cells (DCs), monocytes, neutrophils, and B cells] have evolved to recognise conserved features that are typical of pathogenic surface patterns [pathogen-associated molecular patterns (PAMPs)], thus being able to signal an incoming agent as a threat, that is distinguishable from "self" (16, 36, 37). PRRs will trigger intracellular signaling cascades,

resulting in the production of pro-inflammatory cytokines. This early inflammatory response to infection or immunization is diverse and tightly regulated, its early orchestration shaping the quality in adaptive immunity. A key mediator in shaping the quality of this adaptive response are antigen-presenting cells (such as DCs, macrophages, and B cells), particularly where vaccines are concerned (16).

How effectively a pathogen is removed will depend on the interplay between the innate and adaptive response and the quality that sits behind this immune reaction. In essence, the immune response to infection involves innate immune activation and antigen-specific responses of B and T cells, with the ideal vaccine typically able to induce Th1/Th17 immune responses that can direct this toward inactivation and removal of the threat, followed by development of immune memory (**Figure 1**) (16).

Allergy or parasitic infections are somewhat distinct, inducing strong type-2 immune responses. While Th2/IgE responses control parasitic infections, robust response to parasitic infections is also associated with allergic phenomena (38). Type 1 allergy is mediated by specific IgE, which results in an exaggerated immune response against an otherwise harmless substance. However, growing evidence of a negative association between parasitic infections and allergy at an ecological level highlights the complex inter-relationship between the two (39). Allergic disease is considered a new epidemic of the 21st century, a burgeoning disease particularly in urban areas (40). The most effective way to treat IgE-mediated allergies is through allergen-specific immunotherapy (AIT), which entails repeated administration of specific allergens to patients resulting in protection against the allergic and inflammatory reactions (41). Despite its success, subcutaneous immunotherapy is generally slow and cumbersome for the patient. However, the advances in vaccinology may be exploited here too with the advent of new antigen nano-carriers, modified ways in presenting the allergen and next-generation adjuvants that may advance treatment for chronic diseases and emerging/re-emerging diseases into the modern world (42).

Tailoring Adjuvant Systems

The combination of adjuvants (adjuvant systems) have been pioneered for the last few decades and has resulted in significant advancements in vaccine design and treatments. However, only few alternative adjuvants (other than alum) have been approved for human use (**Table 1**).

Antigen carrier systems such as VLPs can be engineered to optimise antigen presentation and harbour intrinsic adjuvanticity, as these can be packaged with immunomodulators/adjuvants or combined with depot adjuvants to further tailor and optimise the immune response appropriately (29, 30, 50). The most commonly used traditional adjuvants are aluminium salts; however, for decades, a biodegradable adjuvant based on the crystalline form of the non-essential amino acid L-Tyrosine, MCT®, has been utilized in the niche area of allergy immunotherapy (43, 45). It is only in the last decade that its use and effectiveness across a variety of challenging disease models in the preclinical setting highlights it as a promising platform for adjuvant systems to help overcome the challenges associated with modern vaccines and challenging diseases (29).

The application of MCT® as an adjuvant has more recently been extended across a broader vaccine scope with and without VLP antigen carrier systems; one such VLP system uses the cucumber mosaic VLP ($CuMV_{TT}$), which includes intrinsic adjuvant features such as an engineered universal T helper cell epitope ($CD4^+$, based on the tetanus toxin) and encapsulated RNA (TLR7/8 agonists) (51). The disease challenge models which have screened MCT® -adjuvanted vaccines consist of

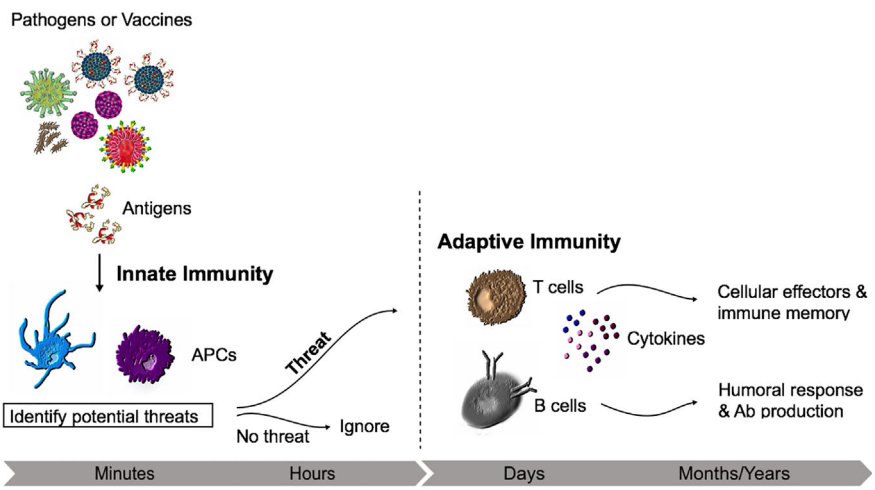

FIGURE 1 | Innate and adaptive immunity time course. The non-specific early inflammatory response is characterized by cells of the innate immune system (e.g., Macrophages) which will recognise conserved repetitive features from bacteria or viruses. If recognized as a threat, the adaptive immune responses develops with the activation of lymphocytes.

TABLE 1 | Adjuvants used in licensed vaccines and immunotherapy [adapted from Di Pasquale et al. (16)].

Adjuvant	Composition	Immunomodulation	Product Indications
Aluminium (alum)	Aluminium salts mixed with antigens aluminium oxyhydroxide; AlO(OH), aluminium phosphate; AlPO4	Th2-biased, prolonged immune exposure (DC uptake), DAMP, Inflammasome activation, potent innate/Ab and inflammatory responses	Diphtheria, tetanus, pertussis, poliomyelitis, hepatitis A, hepatitis B, meningococcal, pneumococcal
Virosomes	Phospholipid membrane (either a mono- or bi-layer) vesicle incorporating virus derived proteins	Target antigen-presenting cells (APCs) and B cells	Hepatitis and influenza
AS03/ Oil-in-water/ MF59	Squalene-based	Increase antigen uptake by APCs, Ab B cell responses,	Influenza pandemic and seasonal.
AS04	• Aluminium salt; AlO(OH) • 3-deacyl-monophosphoryl lipid A	Increase antigen uptake by APCs, TLR-4 agonist, Th1 – biased Ab responses	Hepatitis B and Human Papillomavirus
AS01	Liposome-based • 3-deacyl-monophosphoryl lipid A • Purified saponin; QS-21	Th1-immunity Early innate inflammasome activation, Antigen-specific CD4+ T cells in addition to antigen-specific Abs, robust IFN-γ response.	Recombinant zoster vaccine (Shingrix, RZV). Mosquirix (*Plasmodium falciparum*; RTS, S').
Montanide ISA51	Mineral oil	Increase antigen uptake by APCs, Ab B cell responses	Non-small cell lung cancer
MCT®	Crystalline form of L-Tyrosine (MicroCrystalline Tyrosine); MCT®	Biodegradable depot (43, 44), Th1-biased, Increase antigen uptake by APCs, highly immunogenic B and T cell responses (45).	Pollinex® short-course allergy immunotherapy.
MCT®-MPL®	Crystalline form of L-Tyrosine (MicroCrystalline Tyrosine); MCT® 3-deacyl-monophosphoryl lipid A	Th1-biased, Increase antigen uptake by APCs, highly immunogenic B and T cell responses. TLR-4 agonist, Th1 – biased Ab responses (46, 47),	Pollinex Quattro® short-course allergy immunotherapy (48, 49).

largely murine data (malaria and cancer melanoma models), and one Ferret model (H1N1 Influenza) (50, 52–54). It is important to note that the proof of concept disease models capture biomarker measurements indicative of protection (efficacy) compared to control groups, with performance of the vaccine assessed against groups formulated with alum. Extended pharmacokinetic experiments featured in the Melanoma model (VLP-MCT®)provides unique insights into the importance of the depot effect of MCT® when combined with nanoparticles in orchestrating a robust adaptive cytotoxic T cell response (50).

MCT® AND MONOPHOSPHORYL LIPID-A IN ALLERGY IMMUNOTHERAPY

MCT® is a biodegradable depot adjuvant developed primarily for use in short-course subcutaneous allergy immunotherapy (AIT), in combination with native allergens or modified allergens (allergoids) with or without Monophosphoryl Lipid A® (MPL®, a Toll-like receptor 4 agonist) (48). Allergoid MCT®-MPL® formulations are referred to as Pollinex Quattro®. Clinical evidence for the use of allergoid-MCT®-MPL® adjuvant systems in allergy immunotherapy is well documented (55, 56). Combining an allergoid with an adjuvant system pays tribute to the short-course posology of the vaccine, which is administered in four to six injections within a year pre-seasonally, as opposed to longer-treatment courses (>30 injections) that are commonly applied in AIT and which are typically combined with alum depots (57).

The most recent phase II studies (including the optimal dose levels planned for Phase III) have recently been published for a six-injection presentation of Pollinex Quattro (PQ) Birch and PQ Grass (49, 55). These products are subject to further clinical development, and a phase III trial for both PQ Birch and PQ Grass are currently planned. Furthermore, a combined transcriptomic and proteomic biomarker analysis is pending in a phase III study for PQ Grass, while a smaller preliminary data set is available from an earlier trial, establishing some initial hypotheses related to mode-of-action/predictive efficacy biomarkers (46). Furthermore, Pollinex Quattro is listed in the current European Academy of Allergy and Clinical Immunology (EAACI) AIT guidelines with grade IA recommendation (56).

The PQ products employing the MCT® and MPL® adjuvant system are designed to desensitise allergic individuals by modulating the inherent Th1/Th2 imbalance of atopic disease. The mechanism involved in MCT® -MPL® adjuvancy has not been fully elucidated, but the synergistic attenuation of IgG may prolong protective immunity, which is a further benefited by combining the two adjuvants. The added benefit of MPL® has been demonstrated in the clinic too (58). Several possible mechanisms might account for Toll-like receptor 4 (TLR-4) mediated effects in atopy and asthma. For instance, signaling through the TLRs is generally associated with production of Th1 cytokines by DCs *via* IL-12, leading to increased IFN-γ production (59).

For the PQ product portfolio, in total, 26 Phase I-III clinical trials have been conducted using various allergoids, with different formulations and dosing posologies, including 4695 patients in total (**Table 2**).

The combination of MCT® and MPL® has been shown to be safe and well tolerated in these Phase I-III studies and based on post-marketing data, i.e., >150,000 individuals have received PQ treatment (2004–2019) and an estimated >450,000 treatment courses have been dispensed (Data on file, Allergy Therapeutics plc). Moreover, the safety of MPL® has been demonstrated in several products using MPL® as an adjuvant (60). MPL® is currently used as an adjuvant in the licensed product Cervarix

TABLE 2 | Overview of clinical studies performed with Pollinex Quattro (PQ) products (Data on file, Allergy Therapeutics Plc).

	Phase I	Phase II	Phase III	Total
PQ Ragweed	1	3	1	5
PQ Grass	4	8	1	13
PQ Tree	1	3	1	5
PQ Birch	0	2	1	3

(human papilloma virus vaccine), Fendrix (hepatitis B vaccine), and Shingrix [herpes zoster (shingles)] (60). Since first being licensed in 2006, over 200 million doses of HPV vaccines have been distributed globally, no significant safety issues have been observed (WHO, 2016).

In relation to MCT®, 1575 patients have received MCT® alone as placebo group in placebo controlled GCP studies (including 9 million injections of all MCT® platforms) (Data on file, Allergy Therapeutics plc). MCT® as an adjuvant alone has been shown to be safe and well tolerated, without any treatment related serious adverse events (SAEs) being reported and no relevant effects observed in safety laboratory and vital signs. In a recent position paper, authored by an independent taskforce of EAACI members, a review of adjuvants and formulations currently used in marketed allergy immunotherapies discussed, stating, *"Since its introduction into AIT in 1970, there are no specific safety concerns known for MCT®. It can be anticipated that this fully biodegradable adjuvant will also in future studies not reveal side effects"* (61).

MCT® MODE OF ACTION

In depth comparative adjuvant studies are, in general, limited in number, which may in part be due to the proprietary nature of investigational adjuvants. Since alum is the adjuvant of choice and most broadly studied, it is a useful comparator to use when studying vaccine mode-of-action.

MCT® and alum have been compared head-to-head in a number of preclinical mouse models. In one such study, MCT® combined with Ovalbumin stimulated striking and comparable B cell responses (antigen-specific IgG1, IgG2a, IgG2b, and IgG3) (45). The relevant induction of IgE was of interest, since IgE antibodies (Abs) are the key mediator of the allergic response and an "unwanted" reaction. Here, MCT® triggered less IgE production than alum. This is an observation that has been consistently described in other studies, highlighting a key benefit in using a Th1-biased depot adjuvant in AIT and its reported synergy when combined with MPL® as an adjuvant system (45, 52–54). The specific T cell (CD4+) cytokine response may, in part, explain this since MCT® induced a more Th1-biased response. Of note, IL-4 is required for the Ig switch to IgE, and the lower propensity to induce IL-4, compared to alum, supports this notion (45). Both Alum and MCT® were found to activate the inflammasome but this activation was not essential for the stimulation of B and T cell responses, nor early inflammatory markers (i.e., eosinophils and neutrophils) which were induced by MCT® and alum adjuvants, when assessed by peritoneal lavage (45). Similar results have been reported for alum in mice deficient in IL-1R or NLRP3 (26, 62). Hence, although alum and MCT® may activate the inflammasome *in vitro*, this does not affect the adaptive immune response needed for Ab production in AIT. Furthermore, increased B and T cell responses induced with alum or MCT® -based vaccines did not depend on signaling through toll-like receptors, which is distinct from the TLR agonist MPL® (45).

MCT® 's half-life at the injection site was modelled in preclinical models, with an estimated half-life of 48 hours (44). MCT® has a broad adsorption capacity with model allergens and carriers such as VLPs (63). The depot effect has been characterized with VLP nanoparticles, and this prolonged immune exposure was attributed to play an important role in priming T cells and, in particular, stimulating cytotoxic T cells—a response in which other adjuvants struggle to confer (50).

Shardlow and Exley have further characterized the physicochemical properties of MCT®, which describes needle-like crystalline structures, some of which stack together, to produce a high degree of structural order (64). The resultant crystals combined to form extensive rod-like features the majority of which exceeded 10 μm in length under physiological conditions (median size ca. 21 μm). MCT® also appeared to lack a water decomposition phase by Thermogravimetric analysis, which indicated the lack of physically adsorbed moisture at the surface interface. A decrease in hydroxyl display/surface functionality has been associated with the reduced reactivity of aluminium salts *in vitro* in terms of proinflammatory cytokine production, reactive oxygen species (ROS) generation and inflammasome activation. The size of MCT® may influence its recognition and uptake by THP-1 macrophages *in vitro* (64). In general, adjuvant particles between 1 and 3 μm in size have been considered optimal for recognition and engulfment by macrophages (65). The large hydrodynamic length of MCT® crystals in biological medium (>ca. 10 μm) appeared to partially stymie the scavenging capacity of THP-1 macrophages *in vitro* (64). This may contribute to the safety profile of MCT®, since limited macrophage uptake may prohibit transport *via* barriers such as blood-brain and rapid transport to lymph nodes. The lower propensity to induce IgE/Th2-polarized responses and early inflammatory responses compared to alum, as described in Leuthard et al., 2018, may be partly attributed to the size and distribution of larger and more ordered crystalline structures of L-Tyrosine (45). This is in stark contrast to results obtained using a crystalline aluminium adjuvant where its optimal particle size (median size, 1.4 μm) appeared to more readily facilitate cytoplasmic loading (64).

Both adjuvants were characterized by immediate infiltration of neutrophils and eosinophils (MCT® to a lesser degree) (45). This was the only study, to our knowledge, to characterize such inflammatory responses for MCT®. Although many innate reactions are important for the onset of adaptive immunity, the role of inflammasome activation in immunization and AIT has not been precisely defined. Indeed, MCT® harbors different physicochemical properties, such as particle size, morphology, adsorption characteristics, and local pharmacokinetics compared to alum, which undoubtedly plays a pivotal role in shaping the quality of the Th1/Th2 biological response. MCT®'s roles in the innate and adaptive response are outlined in **Figure 2**.

MCT® -MPL® "Synergy"

The physical association of MPL® for MCT® has been characterized using fluorescently labeled LPS (Lipopolysaccharide) as a substitute for MPL® (**Figure 2**). The LPS was labeled with fluorescein isothiocyanate (FITC). Through confocal microscopy, it was possible to see that the labelled LPS is associated with the MCT® depot. Furthermore, in Bell et al., 2015 allergoid and MPL® adsorption to MCT® in PQ allergy AIT formulations was determined *in vitro* using specific allergen IgE allergenicity and MPL® content methods (63). The predominant mode (i.e., force) of adsorption between MPL® and MCT® was investigated by competition inhibitor binding experiments. This was predominantly inferred as C–H···π interactions between the 2-deoxy-2-aminoglucose backbone on MPL® and aromatic ring of L-tyrosine in MCT® (63) (**Figure 3B**). Furthermore, the physical association of MPL® across the needle-like crystalline structure of 20 mg/ml MCT® has been characterized using fluorescently labeled LPS as a substitute for MPL® *via* confocal microscopy (**Figure 3A**).

Immunological synergy has been documented in allergoid formulations with or without MPL®, highlighting a synergistic relationship in IgG induction (47, 58). Formulation science is often an overlooked or under-appreciated discipline and often adjuvants may be included into formulations without having an extended level of characterisation of their interactions and compatibility with active substances and/or other adjuvants. Indeed, adsorption characteristics of adjuvants may shape bio-availability and in turn vaccine effectiveness (ratio of free versus adjuvants bound antigen may determine antigen draining kinetics) (28). MCT® demonstrates consistent adsorption characteristics, when combined with antigens and allergoids (63). As such, quality attributes may be controlled for over the course of the products shelf life and investigated in preclinical

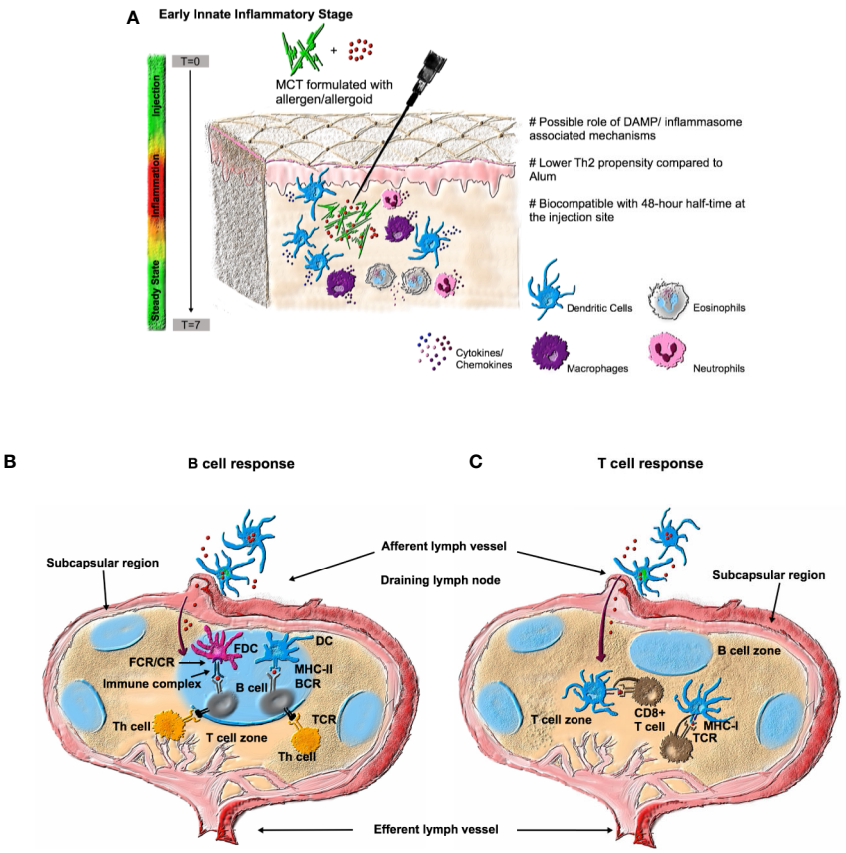

FIGURE 2 | An overview of the immune response after vaccination with an MCT® depot. **(A)** The early innate response is characterized by immediate exudation of neutrophils and eosinophils *in vivo*. The role of inflammasome/DAMP-associated mechanisms have not been precisely defined. The innate response has recorded an increase in dendritic cells (DCs), observed 24 h post-injection (45). MCT® is biodegradable/biocompatible with an estimated half-life of 48 h at the injection site (44). As a result, it is cleared within 7 days with a return to a local steady state. The biodegradable depot properties of MCT® are thought to be key in orchestrating the subsequent adaptive response**. (B)** The infiltrating antigen presenting cells to the draining lymph node, induce sustained and robust B cell response, *via* MHC class II antigen presentation (45, 52–44, 54), with sustained IgG antibody titers. The prolonged immune exposure of antigen is thought to further DC uptake and initiate CD4 T helper cell (Tfh) clonal expansion and differentiation (45). Furthermore, immune complexes may form with follicular dendritic cells (FDCs) *via* Fcγ receptors (Cd16 and CD32) and complement receptors (CD35). **(C)** The depot properties of MCT® have been shown to be key in generating a more robust cytotoxic T cell response, thus the priming of T cells combined with optimal antigen delivery, such as when combined with VLPs, are key drivers in orchestrating this arm of the adaptive response (50).

immunogenicity models of the disease to further tailor and optimise properties of vaccines.

MCT® Combined With Poorly Immunogenic Antigens

It is important to note that the combination of MCT® with poorly immunogenic antigens such as Ovalbumin (45), CSP (53), and H1N1 (54) produce consistent results in generating a robust B cell response and protective efficacy in preclinical models. MCT® was found to possess high protein-binding capacity (adsorption compatibility with the antigens) (54, 63). In the influenza study, a close correlation of haemagglutination inhibition and neutralization titres in groups formulated with MCT® or alum suggests that the two adjuvants were inducing functionally equivalent influenza-specific Abs. Leuthard et al., 2018 using Ovalbumin, highlighted similar findings (45). However, key differences related to MCT®'s physicochemical properties (particulate structure), depot function and biased Th1 specificity highlights some key distinctions of the platform that should be considered when assessing other adjuvants to combined, tailor and optimise the immune response appropriately for specific disease applications.

MCT® IN VIRUS-LIKE PARTICLE FORMULATIONS: HELPING OVERCOME THE CHALLENGES OF MODERN VACCINES

VLPs can be engineered a specific way to modulate the immune response. In pathogen-specific prophylactic applications, they have proven to be well tolerated and highly immunogenic. The 21st century sees further advancements of the technology harnessing state-of-the-art techniques in leveraging the platform to tackle complex diseases. Mohsen et al., 2020 pay tribute to these advancements in the context of the design, delivery and draining dynamics of VLPs (29) and their respective stages of clinical development and success (30). **Table 3** summarizes immunological mechanisms of VLP-based

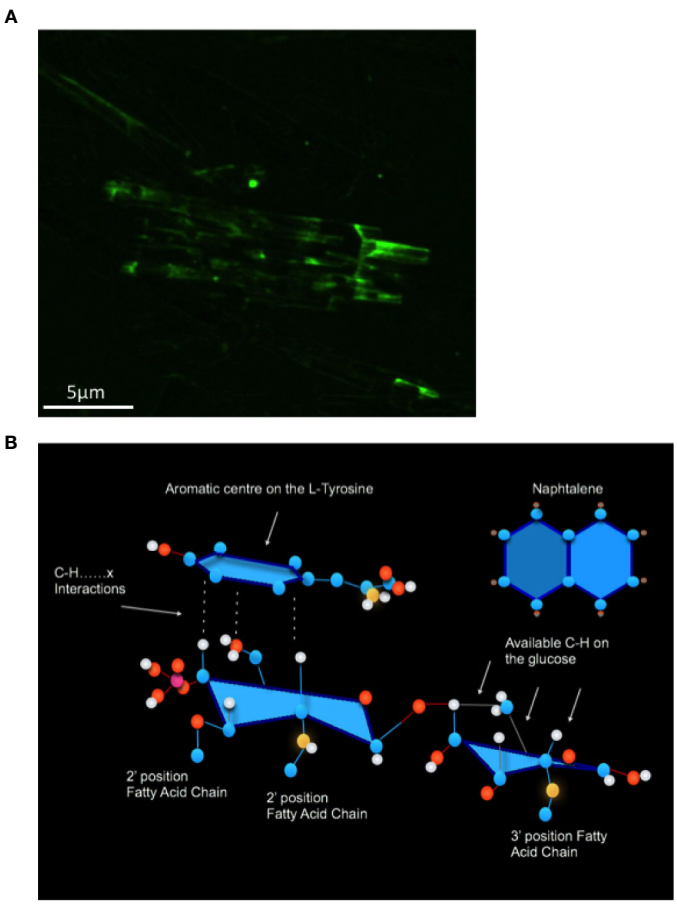

FIGURE 3 | **(A)** The physical association of MPL® across the needle-like crystalline structure of 20 mg/ml MCT® has been characterized using fluorescently labeled LPS (100 µg; Lipopolysaccharide) as a substitute for MPL® *via* confocal microscopy. **(B)** Proposed C–H···π interactions between the 2-deoxy-2-aminoglucose on MPL® and the aromatic ring on L-tyrosine, based on inhibitor studies with Naphthalene (Adapted from Bell et al., 2015).

vaccines in the context of tailoring VLP-platforms with MCT® as an adjuvant system.

In regards to targeting B cells and Abs, a major factor here relates to the size and ability of VLPs to display antigens in optimal fashion [repetitive antigen display, Pathogen Associated Structural Patterns (PASPs)], resulting in very robust induction in Ab responses. In an elegant study by Link and colleagues, 2012 the importance of size and repetitive structure as critical factors for efficient Ag presentation to B cells was demonstrated. In this case, IgM Abs which VLPs are recognized by, recruit the complement component C1q followed by activation of C3, resulting in persistent deposition of antigen on follicular dendritic cells (FDCs) *via* complement receptor CD35 (29, 69). Furthermore, the physical association of a repetitive antigen display distanced by 5–10 nm permit optimal B cell receptor crosslinking. The size of VLPs (20–200 nm) enable efficient fast and transient trafficking of native antigen to the lymph nodes highlighting pharmacokinetic advantages of the platform and their ability to target APCs to orchestrate a robust adaptive response (66).

Vaccines targeting pathogens that are more complex will need to induce both B and effector T cells, which is where adjuvant design may come into play more deeply. If our understanding related to the mode-of-action of depot adjuvants/immunomodulators, continue to grow and become more well established, effective rational approaches in VLP vaccine design may be taken in tailoring *dynamic responses* of *desired specificity*.

Adjuvants physically associated with VLPs (e.g., TLR ligands) may enhance B cell responses. Prokaryotic RNA is known to be more effective and superior in this regard and, most importantly, is the ability of this adjuvant-effect to help differentiate a memory B cell pool into secondary plasma cells, which produce very high levels of Abs. This may allow for more efficient and rapid control of an evolving pathogen (67, 70, 71). The $CuMV_{TT}$ VLP is an example of this, based on an ssRNA plant virus, engineered to harbour a universal T cell epitope derived from the tetanus toxoid, to optimise T cell help for B cells (51). The $CuMV_{TT}$ encapsulates pRNA, which acts as a TLR 7/8 ligand. This particular platform has been remarkably effective in generating proof of concept data in different veterinary vaccines for insect-bite hypersensitivity in horses (IL-5), atopic dermatitis in dogs (IL-31), and preclinical PoC in allergy (peanut and cat), pain in osteoarthritis (NGF), Zika virus infection (ED-III), psoriasis (IL-17a), and malaria (PvTRAP and PvCSP) (51–53, 72–78).

$CuMV_{TT}$ in a Peanut Allergy Model

Where allergic disease is concerned, VLPs have achieved preclinical proof of concept and are subject to further clinical development, notably for peanut allergy (32, 74). Here, targeting B cells using $CuMV_{TT}$ combined with a *single major allergen*, was able to protect against a complex peanut extract in a murine anaphylaxis model (74). In this study mice were immunized with one of three vaccines containing either a mixture of allergens found in whole extract of roasted peanut or with just one single, purified peanut allergen ("Ara h 1" or "Ara h 2"). Regardless of which vaccine was used, immunization strongly reduced systemic and local allergic symptoms in vaccinated subjects and protected against anaphylaxis upon subsequent challenge with a whole peanut allergen mixture. The fact that one injection against a single allergen was sufficient to induce protection against a whole peanut allergen mixture has never been described before and could be applied in different relevant allergies. In addition, the vaccine proved hypoallergenic as previously described (79), which in peanut allergy is a vital characteristic to avoid anaphylactic reactions upon dosing and to improve patient uptake.

$CuMV_{TT}$-MCT® in a Malaria Disease Model

The inclusion of the depot adjuvant MCT® has highlighted the effectiveness of prolonged physical release of VLP nanoparticles, which have been shown to be particularly effective at priming effector T cell responses. In a number of different comparative adjuvant studies in disease challenge models for Malaria (*P. vivax*) and Cancer (Melanoma), a step-wise improvement in biomarkers/disease progression, with the addition of MCT®, has been consistently demonstrated (50, 52, 53). In these studies, the $CuMV_{TT}$ VLPs was screened in a comparative adjuvant study with alum. **Table 4** summarizes the findings from a comparative adjuvant study using $CuMV_{TT}$ in the Malaria disease model, which highlights the effectiveness in combining nanoparticles with MCT® as an optimal way to formulate VLP-vaccines, taking advantage of the physiological properties of the lymphatic system.

In this study, the vaccine efficacy in the malaria survival challenge models were significantly improved if the vaccines were formulated with MCT®, compared to alum. This was explained, in part, due to the high and sustained Ab titres induced in a step-wise improvement by adding MCT® (compared to non-adjuvanted groups) which indicated a more

TABLE 3 | Immunological mechanisms of VLP-based vaccines complement other adjuvants like MCT® and may provide added benefit (29, 43, 45, 47–66–68).

VLP scaffold	MCT®
Repetitive and native antigen display - optimal BCR-crosslinking (PAMP; Pathogen Associated Molecular Pattern)	Local inflammation (early innate responses)
Complement activation	Inflammasome activation
Recognition by natural Abs and other innate humoral factors	DC activation
	Particulate for APC targeting
	B cell activation
	T cell activation
Fast – transient migration to draining lymph nodes	Depot – prolong immune-exposure
Co-delivered adjuvant (e.g., TLR-ligands)	

polarized Th1 biomarker specificity compared to alum as indicated by the IgG subset data (see **Table 4**).

$CuMV_{TT}$-MCT® in a Cancer (melanoma) Model

Combining $CuMVTT_{TT}$- VLPs displaying T cell epitopes with MCT® as an adjuvant has been tested in an aggressive transplanted melanoma murine model B16F10. The results showed improved anti-tumor efficacy when formulating the nano-vaccine with the micro-sized adjuvant MCT® (**Figure 4**). This hybrid system facilitated an optimal delivery of the vaccine to efficiently prime the adaptive immune system. Furthermore, the MCT® adjuvant was as potent as B type CpGs in a direct comparative assessment of efficacy. These findings highlight the translational potential for application for any solid tumor.

CONCLUSIONS

- MCT® is the crystalline formulation of the non-essential amino acid L-Tyrosine, biodegradable, with an estimated half-life of 48 h at the site of injection (44, 80).
- Formulated as a depot for controlled release from injection site - immunomodulation with allergens, antigens, whole cells, polysaccharides, and lipids.
- Characterized adsorption capacity and stability (broad vaccine scope) facilitating Th1-specific immunological augmentation.
- MCT® and Alum [AlO(OH)] are both distinct crystalline depot adjuvant formulations and induced broadly comparable B- and T-cell responses in mice (45).
- MCT® induced less Th2 polarisation than Alum (less IL-4 and IgE). A higher ratio of IgG/IgE (i.e., relatively higher

TABLE 4 | Summary of vaccine efficacy with MCT® and Alum –depot adjuvants. The respective studies conjugated $CuMV_{TT}$ with TRAP or a CSP antigen from *P. vivax* (independent of $CuMV_{TT}$). Formulations were compared against vaccines formulated with Alum.

Protection against *Plasmodium berghei/vivax*				
Formulations screened	**Humoral response**	**Cellular response (CD8+ T cells)**	**Vaccine efficacy in survival challenge**	**Reference**
CMVtt-PvTRAP + MCT®	** (PvTRAP + MCT®) IgG2b > IgG2a > IgG1	** (PvTRAP + MCT®)	*** (PvTRAP + MCT®)	(52)
CSP + MCT®	** (CSP + MCT®) IgG2a > IgG2b > IgG1	N.D	* (CSP + MCT®)	(53)

****p = 0.0001 **p = 0.001; *p = 0.01 (one week after second boost); N.D; not determined.*

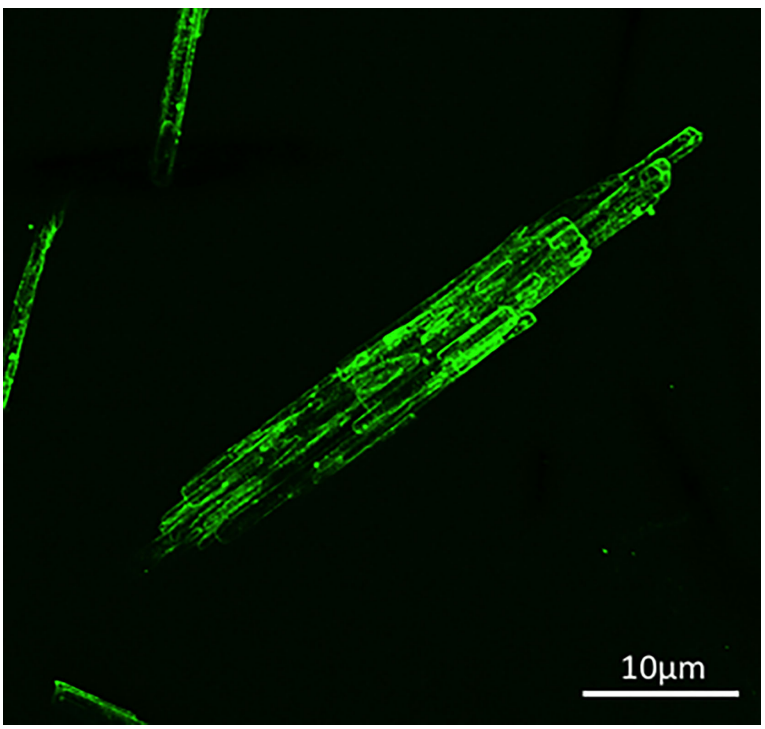

FIGURE 4 | Confocal microscopy imaging of fluorescent dye AF488 $CuMV_{TT}$-p33 nano-vaccine following formulation with the MCT® (20 mg/ml) adjuvant.

IgG to IgE) which has been reported to be a surrogate marker indicative of efficacy of AIT in humans (81).

- MCT® facilitates induction of CD8 T-cell responses (45, 50).
- AIT with MCT® adjuvanted allergens induce protection in a mouse model of anaphylaxis (45) and is formulated (adsorbed) with MPL® as an adjuvant system to provide short-course AIT in humans.
- MCT® induces IL-1β secretion *in vitro*, but inflammasome activation does not affect B- and T-cell responses *in vivo* (45).
- MCT® acts independent of TLR activation (45).
- The combination of MCT® with poorly immunogenic antigens such as Ovalbumin (45), CSP (53) and H1N1 (54) produce consistent results in generating a robust B cell response and protective efficacy in preclinical challenge models.
- The adsorption of MCT with $CuMV_{TT}$ virus-like-particles demonstrates significant added benefit in enhancing immunological (B and T cells) responses in Malaria and Cancer (Melanoma) preclinical disease models (50, 52).

AUTHOR CONTRIBUTIONS

MH conceived and wrote the manuscript. MB, TK, MK, and MS are senior authors and chief or principal investigators of the MCT-based research and contributed to the scope and discussions. SH and MS are inventors of MCT technology and contributed to the scope and discussion. TC and MM designed the figures. P-JK contributed to the clinical development sections. All authors contributed to the article and approved the submitted version.

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IgE-Mediated Peanut Allergy: Current and Novel Predictive Biomarkers for Clinical Phenotypes Using Multi-Omics Approaches

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Food allergy is a collective term for several immune-mediated responses to food. IgE-mediated food allergy is the best-known subtype. The patients present with a marked diversity of clinical profiles including symptomatic manifestations, threshold reactivity and reaction kinetics. In-vitro predictors of these clinical phenotypes are evasive and considered as knowledge gaps in food allergy diagnosis and risk management. Peanut allergy is a relevant disease model where pioneer discoveries were made in diagnosis, immunotherapy and prevention. This review provides an overview on the immune basis for phenotype variations in peanut-allergic individuals, in the light of future patient stratification along emerging omic-areas. Beyond specific IgE-signatures and basophil reactivity profiles with established correlation to clinical outcome, allergenomics, mass spectrometric resolution of peripheral allergen tracing, might be a fundamental approach to understand disease pathophysiology underlying biomarker discovery. Deep immune phenotyping is thought to reveal differential cell responses but also, gene expression and gene methylation profiles (eg, peanut severity genes) are promising areas for biomarker research. Finally, the study of microbiome-host interactions with a focus on the immune system modulation might hold the key to understand tissue-specific responses and symptoms. The immune mechanism underlying acute food-allergic

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events remains elusive until today. Deciphering this immunological response shall enable to identify novel biomarker for stratification of patients into reaction endotypes. The availability of powerful multi-omics technologies, together with integrated data analysis, network-based approaches and unbiased machine learning holds out the prospect of providing clinically useful biomarkers or biomarker signatures being predictive for reaction phenotypes.

Keywords: endotypes, food allergy, peanut allergy, phenotypes, predictive biomarker

TYPE-I HYPERSENSITIVITY TO FOOD

Food allergies (FA) are considered as an important public health concern (1, 2). FA can be classified into IgE-mediated, non-IgE-mediated and mixed types (3). This review focuses on IgE-mediated food allergy which is the best-known type among those food-adverse events.

Epidemiology

There is a general perception that FA prevalence increased during the last twenty years. FA prevalence has been estimated up to 8% in the pediatric and 11% in the adult population based on a number of surveys (2, 4, 5). Beyond the sheer patient numbers, FA entails an important socioeconomic impact, causing fear of accidental exposure in patients and their families, reduced quality of life and relevant healthcare costs nearly double the amount compared to non-allergic individuals (6, 7).

Pathophysiological Basis

IgE-mediated FA is considered as an epithelial barrier disease, resulting from food protein uptake *via* disrupted barriers (gastrointestinal tract, skin, lung) which in turn, leads to an immune dysregulation, and finally, food proteins being recognized as hostile invaders in a T helper type 2 (Th2)-skewed immune response (3, 8). During sensitization, epithelium-derived danger signals and pro-inflammatory cytokines, including interleukin 25 (IL-25) and IL-33, orchestrate the activation and expansion of type 2 innate lymphoid cells (ILC2) and dendritic cells (DCs) (9–11). Those activated DCs promote again the differentiation of naive T cells into a Th2 phenotype cells. Th2 cells and ILC2 foster the recruitment of basophils and eosinophils into the tissue beneath the epithelium (mucosa, lamina propria) through the secretion of pro-inflammatory cytokines (eg, IL-4, IL-5, IL-13) (3). Th9 cells, another effector T helper subset maturating under the influence of IL-4 and transforming growth factor beta (TGF-β), release IL-9, a cytokine which promotes the tissue accumulation of mast cells. B cell class switching to plasma cells producing food antigen-specific IgE is also fostered through IL-4 secretion by Th2 cells. Specific IgE-antibodies bind to the high-affinity IgE receptor (FcεRI) on effector cells, basophil granulocytes and mast cells (11–14). In the elicitation phase, food antigens undergo molecular interactions with cell-bound IgE-antibodies *via* specific epitopes, leading to cell activation and mediator release *via* crosslinking of FcεRI-bound IgE. Subsequently released inflammatory mediators, including histamine, prostaglandins, tryptase, and platelet-activating factor (PAF), contribute to the clinical symptoms.

Clinical Features

Food-allergic patients present with a marked diversity by reactivity profiles (15). Clinical symptoms range from mild to severe (severity score) as does eliciting doses (sensitivity score) and time to reaction onset are highly variable (16, 17). The estimated dose likely to trigger reactions in 10% of a study population (ED_{10}) vary also for specific foods (eg, peanut 11 mg; shrimp 12.8 g protein) (18). The organ involvement may relate to the skin and/or gastrointestinal tract, but also respiratory/cardiovascular symptoms in the case of potentially life-threatening anaphylaxis. Although most patients suffer from stereotypic symptoms, threshold doses depend on multiple factors under real-life conditions (atopic comorbidities; cofactors eg, exercise, alcohol, nonsteroidal anti-inflammatory drugs) (19–21). Disease prognosis and progression may also vary depending on the food allergy, such as in milk or egg allergy, which is commonly outgrown, compared to peanut allergy, which often persists lifelong (22).

Food Allergens

A large variety of foods can cause allergic reactions and constantly, new allergenic foods are reported (23–25). The most allergenic foods include plant (peanuts, tree nuts, wheat, soy) and animal sources (milk, eggs, fish, shellfish) (13). Food allergens, the molecular drivers of allergen-specific Th2-immune responses, share molecular properties and belong to few structural protein superfamilies (26). Commonly, food allergens involved in food anaphylaxis exhibit a higher stability from digestion/processing as compared to low-allergenic homologs (27–29). Intrinsic characteristics contributing to Th2-immune modulation, such as the house dust mite allergen Der p 2 acting with auto-adjuvant properties *via* Toll-like receptor (TLR)4 signaling, are less known for food allergens (30). In-vitro models suggest that matrix effects might contribute to facilitate cross-barrier allergen uptake (eg, peanut lipids inhibiting immune-suppressive IL-10) (31). Class I food allergens are primary food allergens (eg, peanut Ara h 2). The "pollen-fruit syndrome" is mediated by specific IgE to pathogenesis-related protein-10 (PR-10; eg, birch Bet v 1) and/or profilins (eg, birch Bet v 2) as well as antibody cross-recognition of homolog class II food allergens (eg, PR-10: peanut Ara h 8). Those patients experience usually mild FA symptoms (32).

Food Allergy Diagnosis

Usually a detailed anamnesis and IgE-tests are combined (33). In single cases, oral food challenges (OFC) are necessary, time-consuming procedures entailing a significant health risk (34). IgE (skin prick test, SPT; serum specific IgE/sIgE) is an important biomarker. Though, there is a clear trend to overdiagnosing FA as specificity of the testing is low at diagnostic cut-offs (skin wheal size diameter 3 mm, serum sIgE 0.1 kU_A/L). Combining medical data, SPT and sIgE (extract, component-resolved sIgE) increase the diagnosis performance and might approximate OFC outcome (26, 35–37). There is the general notion that multiple IgE epitope recognition patterns correlate to FA severity and unfavorable disease progression (38, 39). Other serological parameters (e.g., total IgE, food-specific IgG4, sIgE/IgG4) are reported as discordant data. Functional assays using living cells, basophils (blood-/cell line-based) or mast cells (cell lines) feature an important added value in FA-diagnosis, although not yet being implemented into routine (29). Serum mediator levels, including histamine, tryptase and prostaglandin D2 metabolite levels, provided less consistent data like in venom- and drug-induced anaphylaxis (40). Overall, usable, reliable and affordable in-vitro predictors of clinical presentation (eg, severity, sensitivity) and risk stratification are evasive and considered still as important knowledge gaps (41). Such predictors may vary depending on the eliciting food, and therefore need to be evaluated for each food allergy.

EMERGING OMIC-AREAS FOR CLINICAL ENDOTYPING

Peanut allergy (PA) is the focus of many research studies, due to its high prevalence, spectrum of clinical phenotypes, severity and lifelong duration, therefore it will be used as an example throughout. Here, we will give an overview of the immune basis for phenotype variations. We span from non-omics to omic-areas, with a focus on studies using cutting edge-technologies and studies based on patient reactivity stratification. A complete overview on biomarker approaches in peanut allergy can be found in **Table 1**.

Molecular Endotyping

The deep analysis of allergens by proteomic technologies (*allergenomics*), based on mass spectrometry (MS), pushed the boarders of knowledge around allergenic peanut proteins including basic aspects of primary structures and post-translational modifications (95–97).

Non-Omics

A total of 18 unique peanut iso/allergen are reported (with Ara h 4 now being considered an isoallergen of Ara h 3) (98). Serum IgE-reactivity to seed storage proteins, including 2S albumins (Ara h 2, Ara h 6) and cupins (Ara h 1, Ara h 3) relates to primary PA. Cross-reactivity markers are the PR-10 protein Ara h 8 (birch allergy) and the non-specific lipid transfer proteins (nsLTP) Ara h 9 (peach-related fruit allergy). The diagnostic relevance of molecular vs extract-based IgE-signatures seems to vary for patient groups from different geographic origins (42, 99–101). However, a recent meta-analysis summarized the overall high diagnostic accuracy of sIgE to Ara h 2 in terms of sensitivity and specificity (95% CI 75.6, 88.9 and 95% CI 77.4, 88.4, respectively) at a cut-off of 0.35 kU_A/L (37). Patients are often IgE-positive for both Ara h 2 and Ara h 6. Recently, Ara h 2 was described as the immunodominant molecule among the two allergens, with higher capacity to activate in-vitro effector cells (basophils, mast cells), pointing to a greater role of Ara h 2 in both disease pathophysiology and as diagnostic severity marker (**Figure 1**) (43). Even allergen peptides can be beneficial. Indeed, systematic peptide-based scanning approaches (epitope mapping) revealed that increasing IgE-epitope diversity correlated with a more severe phenotype (38, 67). In-vitro basophil activation tests (BAT), using basophils from peanut-allergic patients challenged with peanut protein, revealed dose-dependent activation (%-CD63+ basophils). High performance to identify clinical PA (98.7% specificity, 74.7% sensitivity) and high precision to identify individuals with severe outcome (97% specificity, 100% sensitivity) has been recently reported for large UK study populations (**Table 1**) (65). Here, the best prediction of low threshold reactivity was determined in a multivariate statistical tool combining various parameters, SPT, sIgE (Ara h 2, peanut extract), peanut extract-sIgG4/IgE quotient and BAT. In a similar integrated approach, a predictive algorithm based on the CD63 ratio (BAT with peanut protein) and clinical parameters (eg, exercise-induced asthma), had been proposed to predict severe reactions (66).

Allergenomics

As an extension to allergenomics as an analysis of the allergen repertoire of an allergen source, a new research axis applied proteomic approaches to study in-vitro degradation patterns of peanut digests by simulating gastric or small intestine milieus (102, 103). The pronounced digestion stability of Ara h 2, 6, and specific peptides was linked to IgE-recognition and suggested as triggers of the immune response in-vivo. Those peptide structures might be novel candidates for serological assays, be it as antigens in immunoassays or as references for peptide identification in patient blood. In fact, upon ingestion, peanut allergens are degraded, followed by absorption across biological barriers and distribution *via* the bloodstream (20, 60, 104–106). The analysis of allergen residues in human samples after peanut ingestion has been recognized as an important challenge (60, 61). Recent antibody-based studies, combined with removal of interfering endogenous immunoglobulins, succeeded to detect peanut allergens in a reliable fashion (62). Proteomic analyses of such peripheral allergen peptides, together with MS-based analyses of serological metabolomics signatures (68), might be promising avenues toward molecular endotyping of peanut-allergic patients, and toward marker discovery for phenotype prediction (**Figure 1**).

Immunological Endotyping

Non-Omics

Deep immunological endotyping, including aspects of the genome, epigenome, transcriptome and proteome provided

TABLE 1 | Summary of the main approaches (non-/omics) toward phenotypic biomarkers in peanut allergy based on molecular, immunological and commensal endotyping.

Method	Biomarker research area	Interpretation in peanut allergy	C	E*	References**
Molecular endotyping					
Non-omics					
Immunoassays	Serum IgE to Ara h 2, Ara h 6 (less Ara h 1 and Ara h 3)	Primary peanut allergy; often, presence of sIgE and high titers (cut-off titers unequivocal) along with severity		#	(37, 38, 42–47)
	Serum IgE to Ara h 10, Ara h 11, Ara h 14, Ara h 15	Primary peanut allergy; often, presence of sIgE related to severity		#	(48)
	Serum IgE to Ara h 8	Primary sensitization to pollen (eg, birch, alder); mostly, mild reactions to peanut		#	(49–52)
	Serum IgE to Ara h 9	Primary sensitization to nsLTP (eg, peach Pru p 3); severe reactions to peanut in Ara h 1-7 negative patients		#	(53–55)
	Serum IgE to peanut epitopes	High diversity of sequential IgE-epitopes (Ara h 1, Ara h 2, Ara h 3) along with more severity		#	(38, 56–59)
	Serum peanut peptides upon in-vivo ingestion	Digestion-stable Ara h 6-peptides as candidate markers for in-vivo reactivity and serological proteomics			(60–63)
	Serum IgE-bound soluble FcεRI	Soluble FcεRI levels together with correlating IgE-titer as putative markers for in-vivo reactivity and severity			(64)
Integrated algorithms	Serum IgE to Ara h 2 and peanut extract, BAT together with clinical variables (eg, skin test, asthma)	Prediction of risk to experience severe events (symptoms scoring, threshold reactivity)		#	(65, 66)
Allergenomics					
Proteomics	Simulated digest-derived peanut peptides	Digestion-stable Ara h 2/Ara h 6-peptides as candidate markers for in-vivo reactivity and serological assays			(38, 67)
	Serum metabolomic signatures	Metabolites (eg, from dysreguated tryptophan metabolism) as candidate markers for phenotypic severity			(68)
Immunological endotyping					
Non-omics					
Immunoassays	PBMC peanut-stimulated $CD4^+$ T cells	Increased Th2 cytokine expression (IL-4, IL-5, IL-9, IL-13) correlating with elevated peanut-specific IgE-titers and low threshold reactivity		#	(69) ((70))
Mass cytometry	Blood peanut-stimulated $CD45^+$ cells, basophils (CD63, FceRI, CD23)	Basophil-platelet complexes (CD61, CD141, CD42b) with potential to contributing to severity and PAF-related anaphylaxis			(71) ((72, 73))
	PBMC un-/peanut-stimulated $CD45^+$ cells, 11 cell types within CD4 T-cells, CD8 T-cells, B-cells, myeloid cells	Increased prevalence of activated B cells ($CD19^{hi}HLDR^{hi}$) and peanut-specific CD4 T cells ($CD40L^+CD69^+$, memory $CD45RA^-CCR7^{+/-}$) correlating with in-vivo reactivity			(74)
Flow cytometry	Blood peanut-/anti-IgE–induced $CD63^{high}$ basophils (%)	Reduced basophil response and FcεR-expression, together with low sIgE, as putative markers for severity			(52) ((75–77) (78, 79))
	PBMC peanut-stimulated $CD154^+$ T cells	Increased cytokine-positive $CD4^+$ T cell counts ($CD154^+CD4^+IL\text{-}4^+$ or $IL\text{-}13^+$) correlating with increased sIgE-titers and clinical threshold reactivity		#	(69, 70, 80)
		Increase of $CD4^+$ T cell homing populations (CCR4: skin, lung; CCR6: mucosa; CXCR5: B cell follicle) correlating with clinical reactivity		#	(69, 81), ((82, 83))
		Increase of IL-2-dependent $CD154^+$ Treg cells with regulatory ($CD3^+CD4^+CD25^hiCD127^{low}FoxP3^+$) and memory markers (CD45RO) correlating with clinical threshold reactivity		#	(69)
		Increased coefficient of Teff/Treg ($CD25^+CD127^{+/-}$) correlating with clinical threshold reactivity		#	(70) ((83))
	PBMC peanut/peptide-stimulated T cells	Phenotype Th2 shift (expression gut-homing factor Integrin $\beta7^-$, CRTh2) relating to clinical reactivity			(84)
Multimodal omics					
Genomics	PBMC-derived peanut-activated $CD154^+$ Teff/Treg ($CD25^+CD127^{+/-}$)	Increased diversity of the peanut-specific TCRβ repertoire (CDR3 sequences) and enrichment in the Teff compartment, in correlation with low threshold reactivity		#	(70)
	Salivary DNA-based HLA gene SNPs	With the HLA-DQB1 region confirmed as a risk factor for clinical allergy, increased odds ratios for SNPs in HLA (rs17612852, rs9275596, and rs1612904) correlate with low reaction severity		#	(85) ((86) (87, 88))
Transcriptomics	PBMC-derived peanut-activated $CD154^+$ T cells	Differential gene expression patterns according to clinical phenotypes stratified by threshold reactivity, pronounced gene expression associated to Th2 and Th17 cells in individuals with low threshold reactivity		#	(69) ((70))
	Whole blood cells during food challenge	Leucocyte compositional changes (naive B cells/$CD4^+$ T $cells^-$, neutrophils) associated with severity, upregulated "peanut severity genes" (eg, neutrophil-related function, leucocyte function) correlating with severity scores		#	(89) ((90))
Epigenetics	Whole blood $CD4^+$ lymphocytes	"Peanut severity CpG" methylation associated with "peanut severity genes" (eg, immune response, chemotaxis, macroautophagy regulation; moderator genes NFKBIA and ARG1) and clinical reactivity scores		#	(90)

(Continued)

TABLE 1 | Continued

Method	Biomarker research area	Interpretation in peanut allergy	C E*	References**
Commensal endotyping				
Non-omics				
Flow cytometry	Fecal microbiome	Increased IgE-binding to fecal microbes suggesting an anti-commensal Th2 response contributing to the clinical reactivity and phenotype outcome		(91)
Microbiomics and gut issue typing				
Genomics	Fecal microbiome	Decreased microbial richness associated with PA		(92)
		Increased alpha-diversity in low responsive individuals		(93)
		Bacteroidales, especially *Bacteroides fragili* increased in PA, *Clostridium* sp increased in low threshold; high *Oscillosiraceae* sp, *Lachnospiraceae* sp, *Ruminococcaceae* sp, *Frimicutes* sp and *Bacteroides* sp correlating with low threshold reactivity		(92, 93)
		Clostridiales abundance decreased in PA, potentially leading to a decrease in protective ROR-γt+ iTreg cell populations		(91, 92)
Transcriptomics	Gut tissue-derived peanut-specific IgE+ plasma cells	Local class switch resulting in IgE+ B cell counts correlating with serum sIgE-titers; at the gut microbiome interface, local IgE-reservoir for mast cell FcεRI-coating and thus, candidate factors triggering clinical reactivity profiles		(94)

BAT, basophil activation test; CDR3, complementarity-determining region 3; FceRI, high-affinity IgE receptor; HLA, human leucocyte antigen; nsLTP, non-specific lipid transfer protein; PBMC, peripheral blood mononuclear cell; sIgE, specific IgE; SNP, single nucleotide polymorphism; TCR, T cell receptor; Teff, effector T cell; Treg, regulatory T cell.
**Clinical evaluation (CE), as defined here by a patient-based study design with participant severity stratification (eg, symptoms, threshold dose); #, results reported in severity stratification approach. **References in double-brackets, additional background literature based on different study design but supporting the relevance of the respective biomarker/biomarker approach.*

insights in the immune landscape of PA phenotypes, with prospect of future multimodal omics, meaning the integration of heterogeneous data from those sources.

Tordesillas et al. studied the in-vitro activation of granulocytes (basophils, eosinophils, neutrophils), monocytes, dendritic cells, T cells, B cells and NK cells in whole blood using single-cell mass cytometry (71). More B cells and eosinophils but less neutrophils were found in resting $CD45^+$ cells from peanut-allergic vs healthy individuals. After stimulation with peanut protein, the highest response was observed for basophils (CD16, CD23, CD63), but also monocytes, dendritic cells and neutrophils became activated, pointing to an emerging role of these myeloid cells related to clinical PA. Elevated plasma PAF levels had been associated with increased severity of PA earlier (72, 73). Basophils were found to form physical complexes with platelets (CD61, CD141, CD42b) upon peanut activation, suggesting a novel way of PAF-related anaphylaxis (**Figure 1**) (71). Neeland et al. applied mass cytometry to study peripheral immune signatures associated with clinical PA, using peripheral blood mononuclear cells (PBMC) from peanut-allergic and controls (74). Allergic infants distinguished from sensitized infants by increased prevalence of a B cell cluster ($CD19^{hi}HLDR^{hi}$). Upon in-vitro stimulation with peanut protein, increased levels of CD4 T cells ($CD40L^+CD69^+$, memory $CD45RA^-CCR7^{+/-}$) discriminated peanut-allergic from controls.

Other studies focused on analyzing the T cell compartment in PA (81, 107–109). Chiang et al. compared peanut-allergic (OFC positive at <1g cumulative peanut dose), high-threshold (OFC negative at ≤1g cumulative peanut dose but sIgE to peanut and clinical PA history), and healthy individuals (69). In-vitro PBMC stimulation induced a significant increase in peanut-responsive T cells ($CD154^+CD4^+$) and significant cytokine increases (mainly IL-4, IL-13) in peanut-allergic patients only. Cytokine-positive T cell counts ($CD154^+CD4^+IL\text{-}4^+$ or $IL\text{-}13^+$) correlated with sIgE-titers. Peanut-allergic patients had higher shares of peanut-activated Th2 cells with homing markers (CCR4: skin, lung; CCR6: mucosa; CXCR5: B cell follicle) as compared to controls. Peanut-responsive T cells presented with surface marker heterogeneity as well as enrichment for effector memory T cells (CD45RO) and regulatory marker expression ($CD3^+CD4^+CD25^{hi}CD127^{low}FoxP3^+$, delayed IL-2-dependent activation). RNA sequencing of peanut-activated T cells confirmed proinflammatory Th2-polarization with multicytokine expression. This study pointed to the heterogeneous nature of the peanut-specific Th2 response in presence of functional Treg cells. The lack of T cell reactivity (peanut-specific Th2, Treg) in high-threshold individuals discriminated those from peanut-allergic individuals. In a similar approach, Ruiter et al. investigated the T cell response to peanut protein comparing peanut-allergic (OFC positive at <0.5g cumulative peanut dose) and hyporeactive (OFC negative at ≤0.5g cumulative peanut dose but clinical PA history) individuals (70). Compared to hyporeactive patients, stimulated PBMC from peanut-allergic patients showed a higher $CD154^+CD4^+$ T cells response and stimulation index correlating with elevated peanut-specific $CD4^+$ T cell and complementarity determining region 3 (CRD3; T cell receptor domain identified by RNA sequencing) counts. Indeed, CDR3 constitutes the most critical region responsible for recognizing processed antigens (110, 111). Some peanut-specific CRD3 (17%) were found exclusively in $CD154^+CD4^+$ T cells from peanut-allergic individuals (70). CRD3 were also more variable in effector T cells ($CD25^+CD127^+$) than Treg cells ($CD25^{++}CD127^-$), suggesting skewing toward a compartment with expanded effector T cell repertoire in allergic but not in hyporeactive patients. The ratio of peanut-specific effector T cell vs Treg discriminated individuals stratified by threshold doses. The pronounced clinical reactivity of peanut-allergic patients was concluded to correlate to peanut-specific effector T cells characteristics (frequency, proportion, reactivity), rather than a defective Treg response (**Figure 1**).

Multimodal Omics

Gene sequencing studies provided insights into FA immune regulation and epithelial barrier function (112, 113). Genome-

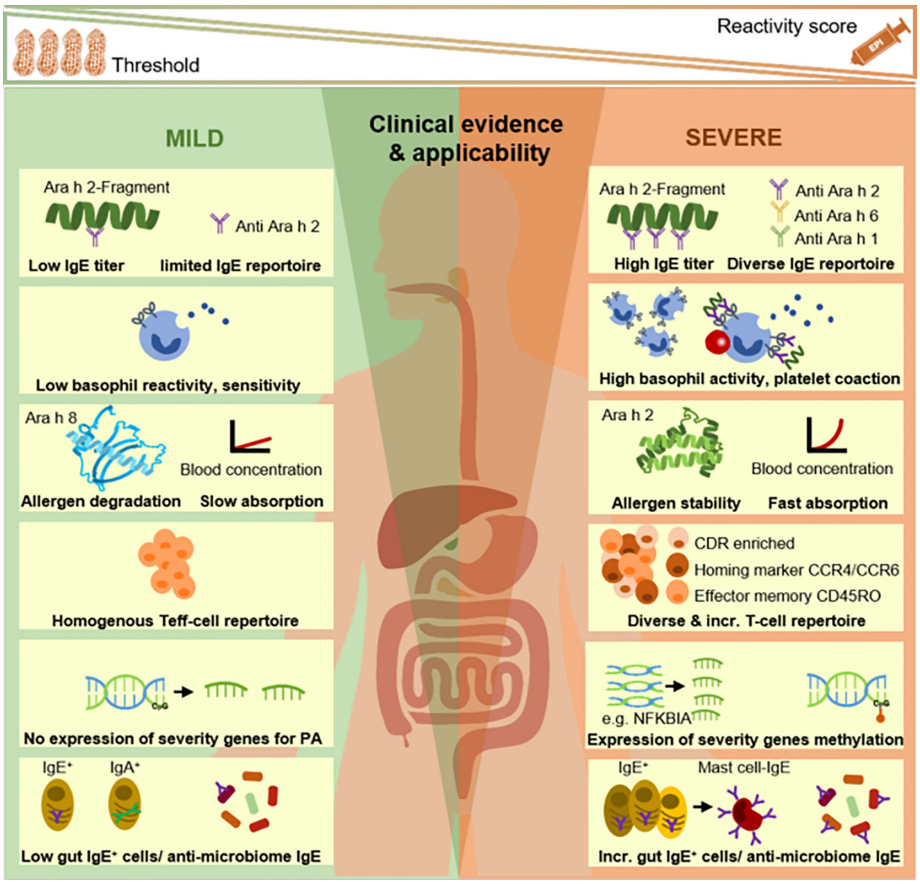

FIGURE 1 | Endotyping of peanut-allergic patients: from selected, established to newly discovered approaches. The association of IgE-signatures (IgG-profiles not shown/reviewed in text) and basophil reactivity profiles with clinical phenotypes is widely established. Research fields on peripheral allergen tracing, deep immune typing (eg, T cells), gene expression/modification as well as local gut immune responses and gut microbiome-host interactions represent putative endotyping axes which require further investigations and finally, systems-level integration in future studies. Incr., increased; PA, peanut allergy.

wide association studies reported on loci correlated to specific FAs (**Table 1**) (85–87, 114). Beyond aspects of disease susceptibility, recent studies focused on genes involved in acute inflammation in PA patients. Watson et al. analyzed the time-resolved transcriptome in peripheral blood sampled from peanut-allergic individuals during OFC (at baseline, 2 h/4 h later), comparing peanut vs placebo (89). Indeed, specific gene expression changes were induced by peanut intake. Gene upregulation was commonly found (1,411/2,168 genes) correlating with evolving peanut-induced inflammation. In leucocyte cell subsets after deconvolution, resting macrophages (M0) and neutrophilic granulocytes increased while naive $CD4^+$ T cells decreased during OFC. Genes associated with peanut-allergic reactions were mostly found in a co-expression module with upregulated genes related to inflammatory processes. Six key driver genes were identified (3/6 with established role in inflammation) as modulators of the peanut-reactive co-expression module. The data-driven approach on genes involved in peanut-allergic reactions was further developed by Do et al. using transcriptome analysis during OFC (at baseline, 2 h/4 h later), in combination with baseline epigenomic profiling (90). The participants' clinical reactivity was stratified by threshold-weighted severity grades. More than 300 genes ("peanut severity genes") had significant expression changes during OFC and were found to be associated with reaction severity. Biological processes related to upregulated peanut severity genes clustered by function, mostly around neutrophils (activation, degranulation, neutrophil-mediated immunity). With pronounced reaction severity, neutrophilic granulocytes increased also in number while naive $CD4^+$ T cells and naive B cells decreased significantly during the course of OFC. Most peanut severity genes clustered together by co-expression. Gene interaction network analysis indicated the central role of two genes, NFKBIA (NF-kappa-B-inhibitor alpha, a regulator protein) and ARG1 (arginase, a catabolic enzyme and immune regulator), on reaction severity. Epigenetic modification correlating with reaction severity, as measured by methylation signatures of CpG dinucleotides in $CD4^+$ lymphocytes, was found for more than 200 CpGs ("peanut severity CpG"). A causal relationship between methylation and peanut severity genes gene expression was established, pointing

further to the relevance of epigenetic modifications in the context reaction severity.

Commensal Endotyping

The gut represents the largest interface for the interaction between the human body and food allergens. There is a constant regulatory interaction between the immune system and the intestinal microbiome (115). The microbiome might promote Th2-immunity to food by regulating eosinophils (frequency, function) in the gut (116).

Non-Omics

More recently, sIgE-binding to commensal bacteria was discovered in food-allergic children, suggesting structural similarities between food allergens and microbial structures (91). Molecular mimicry of the microbiome plays a role in a number of inflammatory diseases, such as celiac disease. Here, structures on P. *fluorescens*, a commensal which is overrepresented in celiac disease, have been found to mimic human leukocyte antigen (HLA) locus HLA-DQ2.5- and activate mucosal T cells, suggesting a pathological dysfunction of the gut barrier (117). First studies comparing amino-acid sequence similarities between known food allergens and microbiome data revealed conserved regions of T-cell immune recognition on commensal bacteria (118). Carrasco Pro et al. further showed similarities between human microbiome sequences and inhalation allergens (119).

Microbiomics and Gut Tissue Typing

The adaptive immune response is influenced by microbial interactions with secreted IgA (120), together with lower richness and lower local species diversity (alpha diversity), accompanied by a dysbiosis of commensal strains (92, 93). A previous study demonstrated that peanut allergy is marked by higher Bacteroidales, especially *Bacteroides fragilis* and reduced Clostridiales abundance (92). Low threshold reactivity to peanut has been connected to an increase in *Clostridium* sp, *Oscillosiraceae* sp, *Lachnospiraceae* sp, *Ruminococcaceae* sp, *Frimicutes* sp, and *Bacteroides* sp (91–93).

At which gastrointestinal sites immune dysregulation and allergic sensitization might develop is unexplored. Recently, large numbers of allergen-specific B cells were described in the gut (stomach, duodenum) of peanut-allergic patients (94). These IgE^+ cells are rarely found in the blood (121, 122). Gut IgE^+B cells counts were found to correlate with serum IgE-titer concentrations. Importantly, inter-individual variations in this local IgE^+B but also variable mast cell IgE-loading by different IgE^+B clones might explain differential reaction phenotypes in peanut-allergic patients' reservoir (**Figure 1**). The high number of IgE^+ B cells in the gut combined with increased intestinal permeability might explain the high sIgE-levels found in fecal samples of food-allergic patients (123).

These findings give an idea of a new mechanism in which the microbiome may initiate, trigger and influence allergic reactions. This in turn may lead to novel ways to stratify patients, due to their metaproteomic profile, as has been shown for other inflammatory diseases (124, 125).

CONCLUSION: PERSPECTIVE TOWARD NEW INTEGRATIVE APPROACHES

Deciphering the immunological response to food proteins shall enable the stratification of patients into reaction endotypes, for advanced understanding of their phenotypic heterogeneity. The ambitious but ultimate goal will be to identify clinically useful predictors for allergic reactions to food, with emphasis on predicting clinical outcome, severity and threshold dose, upon allergen exposure in order to adapt avoidance protocols and symptomatic medication (15, 126). Recent PA-studies demonstrated the complexity of the immune mechanism, as investigated during simulated allergen-specific stimulations or during the course of clinical reactions (71, 74, 89, 90). Several studies did even compare immune targets in individuals with variable clinical reactions, based on severity or sensitivity (69, 70, 90). To explain clinical manifestations of reaction phenotypes, various aspects are considered of fundamental relevance, including the molecular IgE-signature/-repertoire, the potency/repertoire of effector cells, the kinetics of allergen degradation/absorption, allergen-specific T cell reactivity profiles, genes/methylations and aspects of the gut microbiome including composition and host interaction (**Figure 1**). The availability of multiple omics technologies, proteomics, high-dimensional mass cytometry, transcriptomics and epigenomics, allowed identifying promising molecular and immunological targets for future human studies. Taken individually, each omics-approach has assets and drawbacks (reviewed by (127, 128) but together they might unfold their full potential. Unbiased machine-learning, integrated data analysis of heterogeneous datasets as well as network-based approaches will be required to establish algorithms for providing insights in disease pathophysiology and for inferring biomarkers or biomarker signatures being predictive for reaction phenotypes (33, 127, 129, 130). Finally, those insights shall advance the stratification of individuals prior to selection for oral immunotherapy or early food introduction for prevention, both pioneer areas research in PA (131, 132).

AUTHOR CONTRIBUTIONS

RC and JK wrote the manuscript. MS, FC-M, and CB-J revised the manuscript as to clinical content. CH, PW, PS, and MO provided critical feedback to the manuscript concept and scientific content. AK developed the review concept together with RC/JK and helped to shape the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Microarray-Based Allergy Diagnosis: Quo Vadis?

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More than 30% of the world population suffers from allergy. Allergic individuals are characterized by the production of immunoglobulin E (IgE) antibodies against innocuous environmental allergens. Upon allergen recognition IgE mediates allergen-specific immediate and late-phase allergic inflammation in different organs. The identification of the disease-causing allergens by demonstrating the presence of allergen-specific IgE is the key to precision medicine in allergy because it allows tailoring different forms of prevention and treatment according to the sensitization profiles of individual allergic patients. More than 30 years ago molecular cloning started to accelerate the identification of the disease-causing allergen molecules and enabled their production as recombinant molecules. Based on recombinant allergen molecules, molecular allergy diagnosis was introduced into clinical practice and allowed dissecting the molecular sensitization profiles of allergic patients. In 2002 it was demonstrated that microarray technology allows assembling large numbers of allergen molecules on chips for the rapid serological testing of IgE sensitizations with small volumes of serum. Since then microarrayed allergens have revolutionized research and diagnosis in allergy, but several unmet needs remain. Here we show that detection of IgE- and IgG-reactivity to a panel of respiratory allergens microarrayed onto silicon elements is more sensitive than glass-based chips. We discuss the advantages of silicon-based allergen microarrays and how this technology will allow addressing hitherto unmet needs in microarray-based allergy diagnosis. Importantly, it described how the assembly of silicon microarray elements may create different microarray formats for suiting different diagnostic applications such as quick testing of single patients, medium scale testing and fully automated large scale testing.

Keywords: allergy, allergen, IgE, molecular diagnosis, microarrayed allergens, allergen chip, precision medicine

BACKGROUND

The major difference between allergic patients and healthy, non-allergic subjects is that allergic patients produce IgE antibodies against certain environmental antigens, termed allergens, whereas non-allergic subjects produce IgG antibodies (1, 2). IgE antibodies bind specifically to high (FcεRI) and low affinity receptors (FcεRII) for IgE present on inflammatory cells which become activated by IgE-allergen immune complexes to release inflammatory mediators, cytokines, and proteases and/or to activate allergen-specific T cells (3, 4). Therefore, allergen contact induces in allergic patients containing specific IgE, allergic inflammation in different tissues and organs leading to a variety of allergic symptoms comprising rhinoconjunctivitis (hay fever), asthma, skin inflammation, gastrointestinal symptoms, and systemic symptoms such as anaphylactic shock (5). By contrast, IgG recognition of allergens does not trigger allergic inflammation because allergen-IgG immune complexes cannot bind to Fcε receptors and thus fail to trigger allergic inflammation. IgE antibodies occur in very small concentrations in the blood and therefore were identified only in 1966 (6). Due to their importance for triggering allergic reactions already in 1967 the first serological test for measuring allergen-specific IgE in the blood of allergic patients was developed and termed radioallergosorbent test (RAST) (7). Before the discovery of IgE antibodies, allergic sensitization was diagnosed by exposing subjects with suspected allergic sensitization to extracts made from the disease-causing allergen sources in order to study if this would induce immediate allergic inflammation. One of the first descriptions of controlled allergen provocation dates back to a study performed by Charles Blackley in 1873 (8). Since the induction of allergic inflammation resulting from the activation of mast cells by IgE-allergen immune complexes occurs within few minutes, IgE-associated allergy was also termed immediate type hypersensitivity in the classical description of the four types of immunological hypersensitivity of the immune system published by Coombs & Gell (9). Accordingly the diagnosis of allergy has been based on three elements, one is the case history trying to relate the occurrence of allergic symptoms in a patient to exposure to certain allergen sources; the second element is trying to induce allergic reactions in the patient by exposing the person to the allergen source and recording of subsequent allergic symptoms; and the third by confirming IgE sensitization by demonstrating the presence of IgE antibodies specific for the allergen source in the blood or tissue fluids of the patient (10). Traditionally, testing is performed exactly in the described order by starting with the anamnesis followed by provocation testing and final confirmation of sensitization by measuring specific IgE antibodies.

Abbreviations: IgE, Immunoglobulin E; IgG, Immunoglobulin G; Ab, antibody; AIT, allergen-specific immunotherapy; Ig, immunoglobulin; RAST, radioallergosorbent test; GP, general practitioner; OAS, oral allergy syndrome; RV, rhinovirus; HDM, house dust mite; Der p, *Dermatophagoides pteronyssinus*; Blo t, *Blomia tropicalis*; Fel d, *Felis domesticus*; PR10, pathogen-related protein 10; Bet v, *Betula verrucosa*; Gly m, *Glycine max*; Ara h, *Arachis hypogaea*; Pru p, *Prunus persica*.

TRADITIONAL FORMS OF ALLERGY DIAGNOSIS

Traditional allergy diagnosis always starts with a detailed anamnesis trying to identify the presence or absence of allergic symptoms. The next step is to try associating the occurrence of symptoms with contact to certain allergen sources and to verify that controlled exposure to allergen extracts prepared from the allergen source will elicit an allergic reaction. For this purpose, allergen extracts are prepared from the natural allergen sources. These allergen extracts represent mixtures of allergenic and non-allergenic, potentially also irritating substances which may elicit an inflammatory reaction without underlying IgE sensitization. Some examples are the presence of histamine in fish or adverse reactions to milk due to lactose intolerance (11). Accordingly the next step for confirming the condition of an IgE-associated allergy is to verify that the patient serum contains IgE antibodies which react specifically with the allergen extract. However, the demonstration of the presence of allergen-specific IgE with allergen extracts is problematic. First of all, the disease-causing allergen molecules cannot be identified with allergen extracts because they represent mixtures of different allergen molecules and non-allergenic materials. Furthermore, the quality of allergen extracts may strongly vary and depend on various factors which are out of the control of the manufacturer. For example, certain allergens may be lacking in certain extracts (12) and there may be contaminations with allergens from other sources just to name a few problems. It also seems that allergen extracts for *in vivo* provocation testing are becoming less available because they do not meet current standards for medical products (13). Accordingly the use of allergen extracts has several disadvantages which are reviewed in (14). In order to address the urgent needs and bottlenecks of diagnostic allergens the European Academy of Allergy and Clinical Immunology (EAACI) has founded a task force which has set up an action plan to maintain the supply of diagnostic allergen extracts (15). This action plan comprises i.) a simplification of authorization, ii.) specific regulations for special types of extracts, iii.) new models beyond the current model of homologous allergens, iv.) simplification of pharmacovigilance reporting, v.) reduction of regulation fees and vi.) reimbursement for diagnostic allergen extracts. Nevertheless, the practice of traditional allergy diagnosis becomes now challenged with the appearance of molecular allergy diagnosis.

MOLECULAR ALLERGY DIAGNOSIS

Shortly after the first allergen-encoding DNAs had been isolated (16–18), the first two studies were published showing that one can replace complex allergen extracts such as birch pollen or grass pollen extract with a few defined recombinant allergen molecules for IgE-based serological diagnosis without losing sensitivity or specificity (19, 20). These results were quite surprising because at that time it was thought that allergen molecules may exist in different isoforms with variable IgE reactivity and that it may be impossible to find one isoform

which would be suitable for the diagnosis in all patients (21, 22). Furthermore, it was not clear if a few allergen molecules would be sufficient to cover the IgE epitopes of whole allergen extracts. The early studies performed with recombinant allergens for diagnosis also indicated that patients who are sensitized to a certain allergen source may react with different molecules in this source and accordingly show different clinical phenotypes that need "patient-tailored treatment concepts" (19). Subsequently, an increasing number of allergen molecules from different sources were produced and recombinant allergens became available for the first time in the most commonly used fully automated *in vitro* allergy diagnosis system, the ImmunoCAP system (23). However, the principle of ImmunoCAP testing was that one test provided only one information so that for each allergen source several allergen molecule-based tests would be needed to cover the spectrum of the allergens of the allergen source. Thus molecular testing with single allergen molecules would have increased the costs for testing considerably. It was therefore, clear that using this test one would always start testing for allergen-specific IgE with allergen extracts and only if deemed necessary and affordable, one would continue with molecular testing.

In order to utilize the increasing numbers of allergen molecules which were developed by time for diagnostic testing, new test platforms were needed. It was a co-incidence that microarray technology became available for printing nucleic acids onto chips leading at that time. The first DNA chips were manufactured by the company Affymetrix which was based in Santa Clara, California, and a similar technology was applied by the Vienna start-up company VBC Genomics headed by Manfred Müller from Vienna, Austria which had the instruments for printing microarrays. In a collaboration between Manfred Müller and Rudolf Valenta, which was the first to develop chips containing microarrayed allergen molecules were then developed (24). which were not only one of the first protein arrays for diagnostic purposes but also represented the first microarrays for *in vitro* allergy diagnosis (24). These microarrays contained more than 90 different allergen molecules from different allergen sources provided by researchers from all over the world. The exciting thing with allergen microarrays was that one could test IgE reactivity simultaneously to a large number of different allergen molecules with a few microliters of serum or other body fluids. Compared to other existing allergy test systems the allergen chip thus represented a breakthrough which may be also considered a "disruptive technology" because it had the potential to change allergy diagnosis completely. One year after the appearance of the study describing the first allergen chip, the concept of microarray-aided allergy diagnosis was considered the first time (25).

Figure 1 provides a comparison of traditional allergy diagnosis, which in principle is an approach driven by the hypothesis developed by the physician based on the anamnesis. According to the information collected by the anamnesis the physician selects certain allergen sources for a first round of *in*

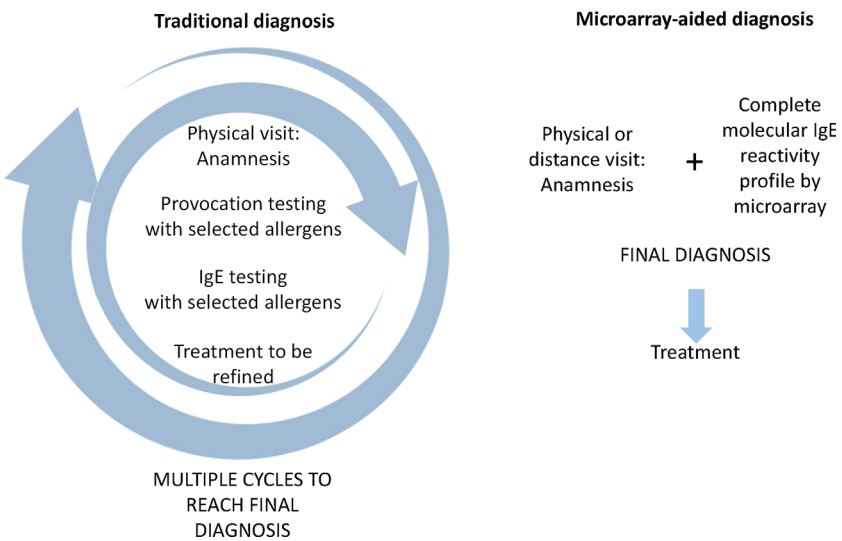

FIGURE 1 | Comparison of traditional allergy diagnosis and microarray-aided allergy diagnosis. In traditional allergy diagnosis (left part) an anamnesis of allergic symptoms is recorded which serves as the basis for targeted provocation testing, usually skin testing with a limited number of allergen extracts selected according to the anamnesis and eventually collection of a serum sample for measuring IgE specific for the suspected allergen sources. In the best case, the patient receives first treatment suggestions according to skin test results. Usually at least one, but often several additional visits are necessary to adjust the treatment to the IgE test results and/or to conduct further targeted testing to determine more precisely the patients sensitization profile and to further adjust treatment. Regarding microarray-aided allergy diagnosis (right part), it can be envisioned that the first visit can be conducted even in a virtual, telemedicine-like form because no provocation testing is required. The anamnesis and complete molecular IgE reactivity profile would be available to the specialist online who could then prescribe precise treatment taking clinical information and the complete sensitization profile into consideration.

vivo provocation testing which is usually conducted by skin testing, and eventually the confirmation of IgE sensitization by serology is performed. According to the *in vivo* provocation results which can be evaluated during the first visit, the physician may already make first preliminary treatment recommendations. Results from serology are usually not available on the same day, and often it may turn out that the original hypothesis, what the culprit allergens might have been, may need refinement through additional anamnesis and repeated testing (**Figure 1**, left). Therefore traditional allergy diagnosis requires usually multiple visits until a more or less complete picture of the sensitization profile of the patient has been obtained. It is needless to say that the traditional allergy testing path may become challenging because it may require repeated consultations and thus long time to reach a final diagnosis, and this may reduce patients' compliance (**Figure 1**).

By contrast, microarray-aided allergy diagnosis has the potential to reduce the time to reach the final diagnosis and to select the correct treatment albeit it may be initially more expensive because it would place the assessment of the complete sensitization profile of the patient already at the beginning of the first consultation. One could imagine that based on validated allergy questionnaires such as the ISAAC questionnaire (26) already the general practitioner (GP) initiates microarray-based allergy screening in subjects with suspected allergy. The patient could then have a first consultation with a specialist presenting already the anamnesis and the complete molecular IgE reactivity profile so that the specialist can immediately determine the best treatment practice. In contrast to the traditional "hypothesis-driven approach", this pathway is reminiscent of a "discovery approach" because the comprehensive IgE test result might stimulate the specialist to refine the anamnesis according to the IgE sensitization profile. Obviously the microarray-aided diagnosis would need *in vivo* provocation testing only in certain cases for confirmatory purposes, if at all and hence the consultation could be performed also in a telemedicine-based format. The advantage of such a telemedicine-based approach is that it reduces the number of visits, avoids eventual long travelling, and remote areas in a country can easily benefit from specialist knowledge (**Figure 1**, right). In this context the possibilities of mobile health (mHealth) technology using mobile communication devices to support and improve health-related services, data flow, patient management, surveillance, and disease management should be mentioned. A task force of EAACI has reviewed all these possibilities, discussed advantages, limitations, and risks such as data protection, and provided recommendations (27). In the context of precision medicine, the potential of changing the practice from clinician- to patient-centered health care is highlighted.

Some recent reports underline the potential of molecular diagnosis in the field of health economics and suggest that it can help to save costs for the diagnosis of respiratory allergies (28) and food allergies (29).

Currently, allergy diagnosis is still dominated by the traditional approach of diagnosis as indicated in **Figure 1**, but more and more specialists in allergy start to use molecular allergy diagnosis, and already quite a few prefer microarray-aided diagnosis as shown also in **Figure 1**. There are many different reasons for the different preferences. For example, traditional allergy diagnosis is often preferred because it is currently reimbursed by the health care system, whereas serological diagnosis and, in particular microarray diagnosis, requires laboratory facilities and a different mode of reimbursement shared between specialists and diagnostic laboratories. Factors limiting the increased use of microarray-guided diagnosis are that practitioners must be skilled in interpreting complex molecular test results and/or have well-trained algorithms for clinical decision making (*i.e.*, clinical decision support systems) available. Information on sensitizations not linked to symptoms require appropriate and time-consuming information of patients, and certain allergen molecules for obtaining complete results may still need to be discovered and included in the microarrays. Finally, it will be necessary to reduce the costs of microarrays, which are often driven not only by costs of goods for manufacturing but also by costs due to quality control, validation, and registration. However, once the latter issues are resolved one may expect that microarray-based allergy diagnosis will become a highly cost-effective diagnostic tool. One must consider that microarray-based allergy diagnostic approaches can provide more than hundred individual test results from one sample and present the comprehensive picture of allergen sensitization, which can be used for precision medicine treatment. In fact, unrecognized and untreated allergy is a major cost factor for the management of allergic diseases. With the adherence to proper treatment and the precise diagnosis and proper management of allergic diseases, it has been estimated that high costs can be saved (30, 31).

MEASUREMENT OF ALLERGEN-SPECIFIC IGE AND IGG RESPONSES WITH MICROARRAYED ALLERGENS

Microarrayed allergens can be used for measuring simultaneously IgE reactivity to a large number of different allergen molecules with very small volumes of serum. In this context, it should be mentioned that it has been shown that allergen-specific IgE can be also measured in plasma and other body fluids such as nasal secretions and in milk samples (32–35). Moreover, it has been shown that dried blood spots on paper can be recovered for specific IgE and IgG measurements which allows sending serum samples as paper dried blood spots in simple envelops without requiring expensive packaging, cooling, and sophisticated transportation (36). Several recent studies have confirmed the importance of allergen-specific IgG antibodies for the protective effects of AIT, and the measurement of allergen-specific IgG antibodies which block allergic patients IgE binding to allergens is therefore considered as an important biomarker for the clinical efficacy of AIT (37). Accordingly, certain commercial allergen arrays allow measuring specific IgE and IgG antibodies (*e.g.* Thermo Fisher ImmunoCAP ISAC Immuno-solid-phase Allergen Chip, which contains 112 allergens from 51 allergen sources) (38); however,

this has not been shown for all available allergen arrays (*e.g.*, MADx Allergen Explorer ALEX; containing 282 allergens: 156 extracts and 126 components) (39). The measurement of allergen-specific IgG antibodies in cohorts has provided new insights in beneficial functions of IgG. For example it has been suggested that allergen-specific IgG transmitted from the mother to the child *via* the placenta during pregnancy may protect the off-spring from allergic sensitization (40). Likewise, evidence has been provided that the production of allergen-specific IgG antibodies follows different pathways and mechanisms than those involved in the production of allergen-specific IgE antibodies (2, 41–43).

EXAMPLES FOR THE USE OF MICROARRAYED ALLERGENS IN ALLERGY RESEARCH

Microarrayed allergens have been used in research to address several questions. For example, it has been shown that adult allergic patients do not change their IgE sensitization profiles for a decade demonstrating that there is no acquisition or loss of IgE sensitizations in adult allergic patients (44). Furthermore, information was obtained about the characteristics of IgE sensitization profiles in different populations. For example, it was found that sensitization to clinically relevant grass pollen allergens is rare in tropical climates and that most of the grass pollen-specific IgE is directed to non-allergenic carbohydrate epitopes (45). In another study it was observed that man-made changes of the environment as for example obtained by replanting of certain plants can alter the allergic sensitization profiles towards plant-derived allergens in populations within two generations (46).

However, without any doubt, the most exciting results were obtained when microarrayed allergen molecules were used to study the development of the allergic sensitization profiles in longitudinal birth cohorts allowing to analyze the evolution of IgE sensitization profiles from birth to early adolescence (47, 48). Importantly it was found that allergy frequently starts with early asymptomatic IgE sensitization and that early assessment of IgE sensitization profiles and IgE-levels allow predicting the development of allergy later in life (49–52). This finding suggests that for allergy similar as for other diseases such as cancer, cardiovascular diseases, and metabolic disease, early screening in the form of a preventive medical examination by determination of IgE sensitization profiles early in life might allow initiating preventive measures (*e.g.*, allergen avoidance, early allergen-specific immunotherapy) (53–55) to prevent the development of allergic symptoms (*i.e.*, secondary prevention) later in life.

PRECISION MEDICINE BY CHIP-BASED ALLERGY DIAGNOSIS

In allergy, like in other important diseases, it has become clear that it is necessary to transform healthcare towards the principles of "P4 Medicine" for predictive, preventive, personalized (precision), and participatory medicine by developing new diagnostic and predictive tests as well as therapies and preventive strategies which affect the course of disease or prevent the development of disease (56). Allergy is ideally suitable for a precision medicine approach because patients are sensitized to different allergens and allergen combinations and suffer from a wide variety of symptoms which may change during the course of disease. Furthermore, there are several different strategies for the treatment of allergy available which require the identification of the disease allergens. It is also clear that early preventive measures should be more effective than late mending of severe disease (5, 57). Accordingly, it has been suggested that molecular allergy diagnosis improves treatment especially in pediatric allergy (58). In this context, examples of how molecular diagnosis helped in the diagnosis of children suffering from complex allergic sensitizations and tailoring the treatment according to the needs of the children should be mentioned (59). Furthermore, evidence accumulates that allergic phenotypes and symptoms are associated with certain patterns and/or levels of allergen-specific IgE in children and adult allergic patients suggesting that serological surrogate parameters for diagnosis can be developed (60–63).

Microarrayed Allergens in Food and Respiratory Allergy

The diagnosis of food allergy is often challenging because the frequency of IgE-associated food allergy is often considered higher than it is in reality. For example, adverse reactions to cow's milk due to lactose intolerance are much more common than IgE-mediated allergy (11). Although many clinicians consider only the double-blind, placebo-controlled food challenge as gold standard for the diagnosis of food allergy, this test is cumbersome, and there may be severe and even life-threatening side effects. Accordingly, alternative diagnostic tests are needed. So far a considerable number of food allergen molecules have been identified which are associated with severe, mild, or even no reactions allowing for serological testing of food allergy also by microarray-based IgE testing (64).

In this context a study demonstrating different IgE sensitization profiles in children suffering from severe peanut allergy and in peanut-sensitized but asymptomatic children should be mentioned (65). Screening for IgE sensitizations using a large panel of food allergen molecules is useful for several reasons. First, it allows testing simultaneously for IgE sensitization to a large panel of allergen molecules with high anaphylactic capacity to predict the risk of food allergy, and thus it may help to reduce hazardous food challenge testing (29, 66, 67). Second, and importantly, negative test results to a large panel of food allergen molecules are helpful in searching for other reasons of food intolerance beyond IgE-mediated allergy. Besides IgE testing to food allergen molecules, it has turned out that measuring IgE sensitizations to food allergen-derived peptides may be useful to discriminate patients with no or mild symptoms from those suffering from severe symptoms (68, 69). Microarrayed peanut allergen molecules were also used to

investigate the course of peanut sensitization in childhood and to predict symptomatic peanut allergy in a birth cohort (70). Very recently it was found that oral allergy syndrome (OAS) to Bet v 1-related food allergen molecules of the pathogen-related protein 10 (PR10) family was associated with the levels of Bet v 1-specific IgE and the numbers of recognized PR10 molecules (61).

Microarrayed allergen molecules are not only useful for the diagnosis of IgE-mediated food allergies but are also considered for the diagnosis of asthma triggered by allergens in sensitized allergic patients (71). The two major trigger factors for asthma are allergens for patients with IgE sensitizations and infections with respiratory viruses, in particular with rhinoviruses (RVs) (72). For example, it has been shown for house dust mite allergy that children suffering from allergic asthma differ regarding their IgE reactivity profiles and ability to produce allergen-specific IgG antibodies (60). Children with asthma showed higher IgE levels to certain allergens, reacted with a larger number of molecules, and produced less allergen-specific IgG as compared to children suffering only from rhinitis (60). Furthermore, machine learning approaches have been suggested to identify pairwise interactions of IgE antibodies and their association with asthma based on microarray results (73). Allergen molecules from cat and dog, which are important for the development of respiratory allergy in childhood, have been identified in the Swedish BAMSE birth cohort (74).

For the diagnosis of RV-induced asthma, a chip containing peptides derived from the N-terminus of VP1 proteins from a representative number of RV strains covering RV-A, RV-B and RV-C species has been produced (75). This chip allowed measuring species-specific increases of RV-specific IgG antibodies in children who had experienced RV-induced asthma exacerbations, and cumulative IgG responses were higher in children with RV-induced exacerbations of respiratory illnesses (76, 77). Accordingly, it has been proposed to use microarrayed allergens and respiratory virus-derived peptides for diagnosis of allergen and/or RV-induced asthma and personalized treatment according to the test results (77).

Microarrayed Allergens for Prescription and Monitoring of Allergen-Specific AIT

AIT is an allergen-specific form of therapeutic vaccination which is based on the administration of the disease-causing allergens or modifications thereof with the goal to induce allergen-specific protective IgG antibodies and alterations of the cellular immune response to reduce symptoms of allergy upon allergen contact. Accordingly, the accurate prescription of AIT requires that the culprit allergens are identified. Since allergen sources often contain cross-reactive allergens the identification of the culprit allergen source can be challenging. It has therefore been suggested to use marker allergen molecules which are specific for given allergen sources as diagnostic marker allergens for improving the prescription of AIT (78). The marker allergen concept can be applied to almost all allergen sources and accordingly has been suggested for several common respiratory allergen sources (79–81). The use of marker allergen molecules was suggested not only for refining the prescription of AIT but also for the monitoring of treatment response by measuring the development of allergen-specific IgG antibodies which are considered as biomarkers for the success of AIT (37, 82). In this context an interesting discovery was made which suggested that the ImmunoCAP ISAC microarray platform which utilizes small amounts of immobilized allergens is very useful for AIT monitoring. In fact, when small amounts of allergens are immobilized on the solid phase of immunological tests for detecting allergen-specific IgE, IgG antibodies can compete with IgE for allergen binding when they block epitopes recognized by patients IgE (83, 84).

When patients develop such blocking IgG antibodies in the course of AIT, these IgG antibodies will then compete with IgE antibodies which then causes a reduction of IgE binding similar as it is observed by skin testing when allergen-specific IgG antibodies block IgE-mediated mast cell activation thus leading to a reduction of skin responses. The reduction of allergen-specific IgE binding by blocking IgG antibodies can only be measured with IgE binding assays containing small amounts of immobilized allergens such as the ImmunoCAP ISAC chip but not by the traditional ImmunoCAP test which contains larger amounts of immobilized allergens (83–85). No other diagnostic platform with similar properties has been identified so far. In fact, two independent studies have shown the reduction of IgE binding to allergens by AIT-induced IgG antibodies on the ImmunoCAP ISAC platform and suggested it as a possible biomarker for AIT (86, 87).

Several studies support the concept of using molecular testing for the refined prescription of AIT (88), and the cost-effectiveness of molecular diagnosis as compared to traditional allergy diagnosis has been shown (89). Two more recent studies should be mentioned which have shown that it may be possible to enhance the success of AIT by selection of patients whose IgE reactivity profiles match the immunogenic components present in the AIT vaccines (90, 91). Accordingly, microarray-based molecular diagnosis seems to be well suited as companion diagnostic tool for the selection of patients for AIT and for the monitoring of treatment success.

UNMET NEEDS IN MICROARRAY TECHNOLOGY

Since the first description of the use of microarrayed allergens in 2002 for allergy diagnosis the technology has become available world-wide and has been used extensively in research. However, several unmet needs remain which are summarized in **Figure 2** and discussed below.

The Library of Allergen Molecules and Peptides

The allergen molecules and allergen derived peptides and their quality can be considered the heart of any allergen microarray because it determines what application can be addressed with the allergen chip. For example, it is important to cover the most common respiratory, food, venom, and other allergen sources by

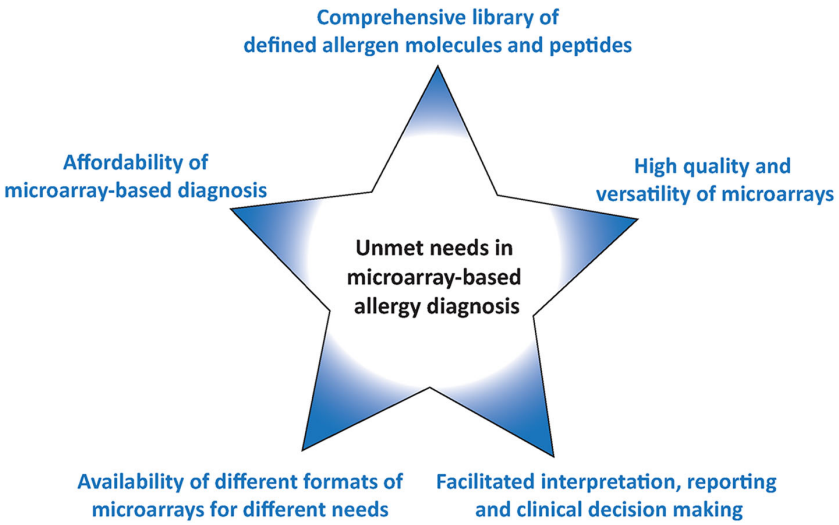

FIGURE 2 | Unmet needs in microarray-based allergy diagnosis.

a representative collection of allergen molecules. In this context it should be noted that there are different opinions regarding the inclusion of certain allergen molecules in screening assays. For example, some argue that the inclusion of venom allergens may create ethical and legal issues because in case of a positive or negative test result one cannot predict or exclude a sting reaction. However, this is in principle applicable for every IgE test result which needs to be considered in the context of clinical information and/or results from provocation testing. Therefore others think that the inclusion of venom allergens in screening tests is not a problem provided the patients are adequately informed about the relevance of the IgE test results.

The addition of allergen-derived peptides may be interesting for example in the diagnosis of food allergy where sequential IgE epitopes play a role and for the monitoring of AIT-induced IgG responses. Since only small amounts of allergen are immobilized on microarrays it is important to use highly pure allergens of high quality to detect also low specific IgE responses. Natural allergen preparation containing impurities in terms of unrelated allergens or cross-reactive carbohydrates may give rise to unclear, false-positive test results. Although carbohydrates are highly cross-reactive, it seems that patients mount quite specific and selective IgE to certain carbohydrates which cannot be completely inhibited by pre-incubation of sera with a single carbohydrate (92) so that unclear background reactivity may remain. This is of particular relevance because IgE-reactive carbohydrates have been shown to have little or no clinical relevance. Accordingly it is important to establish a library of high quality allergen molecules and peptides which can be reproduced according to defined protocols. The allergen molecules should be preferentially made as recombinant, non-glycosylated allergens to avoid unclear results due to carbohydrate-specific IgE.

This allergen library should be as complete as possible to pick up every relevant IgE sensitization in a given population. For example it has been shown, that the MeDALL allergen chip, a customized allergen array based on the ImmunoCAP ISAC platform containing more than 170 allergen molecules (83), was more sensitive in picking up IgE sensitizations than traditional allergy tests based on comprehensive panels of allergen extracts for skin testing or IgE serology (93). In order to refine the panel of allergen molecules on a chip it will be necessary to investigate molecular IgE sensitization profiles in different populations in different countries and continents to define the allergen repertoire of a microarray suitable for allergy diagnosis in the whole world. Such a complete representation of allergen molecules seems very important because due to the high mobility of the world population allergen arrays representing only a local allergen repertoire will not be sufficient for diagnosis. The production of microarrays containing subsets of allergen molecules does not seem to have any advantages because the microarray technology does not set limitations regarding the numbers of molecules which can be immobilized, and the costs of goods for production are low due to the low amounts of allergen needed. However, one must consider that costs for quality control, validation, and registration of complex assays may increase costs considerably.

New Materials May Increase the Quality and Versatility of Microarrays

There are basically two types of multiplex diagnostic platforms available. One contains allergen molecules adsorbed to microbeads, whereas the other platform is based on allergens which are immobilized on chips by micro-spotting. Microbead-based multiplex assay usually can accommodate only a limited number of less than 50 different allergen molecules in a single test and require quite expensive instruments such as Luminex readers or FACS-based technology for read out (94, 95). By contrast, microarrays allow measuring specific antibody reactivity to more than hundred different allergen molecules at

the same time. There are currently two major types of allergen arrays available, the ImmunoCAP ISAC platform, using allergen molecules immobilized onto glass (Thermo Fisher ImmunoCAP ISAC Immuno-solid-phase Allergen Chip which contains 112 allergens from 51 allergen sources) (38) and an allergen macro-array prepared on the basis of a nitrocellulose membrane (*e.g.*, MADx Allergen Explorer ALEX; containing 282 allergens: 156 extracts and 126 components) (39). Both systems allow reasonable detection of allergen-specific IgE but one may consider increasing the quality of the arrays by selecting different materials for allergen immobilization. Already in the original patent application made for the ImmunoCAP ISAC technology, silicon-based surfaces have been considered as alternative to glass (96).

In fact, the high sensitivity of protein assays on microarray silicon slides has then been demonstrated (97, 98). The latter studies demonstrated optimized layers of thermally grown silicon oxide with highly reproducible thickness, low roughness, and fluorescence background which yielded fluorescence intensification due to the constructive interference between the incident and reflected waves of the fluorescence radiation. Furthermore, the studies suggested that by combining an optimized reflective substrate with a high performance surface chemistry may strongly improve the quality of diagnostic protein array by obtaining a 5–10 fold enhancement of the fluorescence signals when compared to glass surfaces. The favorable features of silicon slides have been further demonstrated for peptide arrays (99) and for the field of food allergy diagnosis (100).

Below, we have evaluated allergen microarrays based on silicon oxide surfaces and compared them with glass slides currently used in the ImmunoCAP ISAC platform confirming the higher sensitivity of the silicon technology. We are then also discussing advantages of this technology for the versatile production of different formats of allergen microarrays.

Affordability of Microarray-Based Diagnosis

Currently available microarray-based allergy tests are relatively expensive although one has to consider that a single microarray test result provides more than hundred individual test results. For example costs for one array may range between 60 and 100 Euro depending on prices requested by different manufacturers in different countries, and additional costs for the processing of one sample may vary considerably 50–300 Euro depending on costs of laboratory facilities and personnel. Therefore, it will be necessary to decrease the costs of production for the micro-arrays and the costs for the test procedure which is currently performed by hand pipetting.

Different Formats of Microarrays for Different Needs

As mentioned above currently available microarrays are made for manual operation and thus individual testing requires wet laboratory facilities and relatively expensive scanning equipment for the analysis of results. The available formats thus can be used for manual analysis of relatively limited numbers of samples and require trained personnel. Unfortunately, no automatization for the processing of the available allergen arrays is available which would allow large scale and fully automated analysis of large numbers of patients. Therefore, there is still an unmet need for different formats of allergen arrays allowing different applications such as the occasional analysis of few or single serum samples yielding fast results with a minimum of equipment, the medium scale analysis of several serum samples and the fully automated analysis of large numbers of sera (**Figure 2**).

Furthermore, allergen arrays should allow the analysis not only of allergen-specific IgE but also of other immunoglobulin isotypes as well as the visualization of the competitive activity of allergen-specific IgG on IgE binding for the monitoring of AIT.

Interpretation, Reporting and Clinical Decision Making

Since allergen microarrays deliver test results for more than 100 different allergen molecules it is important that doctors who see allergic patients and wish to correctly interpret the sometimes complex test results keep themselves updated by continuous medical education. The transition of allergy diagnosis from the use of allergen extracts to allergen molecules requires knowledge regarding the characteristics of the individual allergen molecules. Thus molecular allergy diagnosis may be compared a bit with the switching from previous old telephones to mobile phones which offer many different additional applications that need to be explored by the user. The challenges of interpreting allergen microarray results may be met by machine learning approaches and other diagnostic algorithms in addition to continuous medical education (10, 101).

A COMPARISON OF DIFFERENT SURFACES FOR MICROARRAYS: GLASS *VERSUS* SILICON

In order to compare the glass surface which currently is used for ImmunoCAP ISAC with silicon slides (97–100) a panel of important respiratory allergens was spotted on the two surfaces and allergic patients' IgE and IgG reactivity was assessed. A set of 24 allergens containing mite allergens (Der p 1, Der p 2, Der p 4, Der p 5, Der p 7, Der p 10, Der p 15, Der p 18, Der p 20, Der p 21, Der p 23, Der p 37, Blo t 5, Blo t 12 and Blot 21), cat allergens (Fel d 1, Fel d 2, Fel d 3, Fel d 4 and Fel d 6), and PR10 allergens (Bet v 1, Gly m 4, Ara h 8 and Pru p 4) were spotted in triplicates on glass and silicon wafers in the order described (**Figure 3**) (*Supplementary Materials and Methods*). In the first experiment we determined the sensitivity of IgE reactivity to Bet v 1 immobilized to glass and silicon chips using a human monoclonal chimeric IgE antibody (IgEmoAb) (102) (**Figure 4**). A two-fold serial dilution of IgEmoAb corresponding to 208–0.025 ISU/ml was used to detect Bet v 1. Silicon microarrays showed a five-fold higher fluorescence intensity of IgE reactivity of IgEmoAb in the range of 52–0.025 ISU/ml to Bet v 1 than the glass surface (**Figure 4**). The silicon surface allowed measuring

FIGURE 3 | Outlay of prototype allergen microarrays made by printing on glass slides and assembled silicon elements. Order of house dust mite, mite, cat, and PR10 allergens microarrayed in triplicates on glass slides (left) and precut and assembled silicon chips-derived elements.

the Bet v 1-specific IgE down to 0.025 ISU/ml which is much lower than the cut-off used in currently available microarray tests (0.3 ISU/ml) and the detection limit 0.1 ISU/ml used for certain research purposes.

Figure 5 shows the comparison of IgE and IgG reactivity to allergens spotted on glass and silicon microarrayed chips determined with a serum pool from allergic patients with IgE reactivity against the tested allergen panel that was diluted 3- to 729-fold for IgE detection (**Figure 5A**) and 27- to 19,683-fold for IgG detection (**Figure 5B**). **Figure 5A** shows that the silicon surface yielded an approximately 10-fold higher IgE binding according to fluorescence intensity to all but one (*i.e.*, Der p 20) allergen compared to the glass surface. **Figure 5B** demonstrates that silicon was superior to glass also regarding IgG detection showing approximately five-fold higher IgG signals, and specific binding was detectable even at a dilution of 1:19,683 of the serum pool on silicon microarrays.

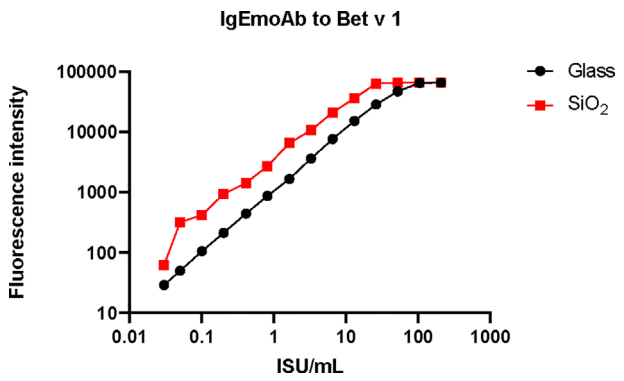

FIGURE 4 | Sensitivity of the reactivity of a human monoclonal Bet v 1-specific IgE antibody to Bet v 1 printed on glass *versus* silicon. Shown are the fluorescence light intensities (y-axis) corresponding to different concentrations (x-axis) of the monoclonal human Bet v 1-specific IgE antibody (IgEmoAb).

Next, we tested sera from HDM and mite- (**Figure 6A**), cat- (**Figure 6B**), birch pollen allergic patients (**Figure 6C**) and non-allergic subjects (**Figure 6D**) for IgE and IgG reactivity to allergens microarrayed on silicon and glass. This experiment confirmed for almost all tested sera and allergens that allergens immobilized on silicon have higher IgE and IgG detection signals. Importantly, IgE detection was highly specific for glass and silicon because none of the non-allergic subjects showed detectable allergen-specific IgE reactivity (**Figure 6D**). Our results thus indicate that allergen microarrays based on silicon are superior to glass for IgE and IgG detection of allergens. It should be also noted that another advantage of silicon arrays is that spotting can be performed on small, precut silicon elements which can be assembled in different formats (**Figure 7**, right upper part).

MANUFACTURING OF MICROARRAY ELEMENTS AND SUBSEQUENT ASSEMBLY MEETS DIFFERENT NEEDS IN ALLERGY DIAGNOSIS

In **Figure 7** we try to provide an overview of how microarrays based on silicon elements may contribute to innovation in allergy testing as compared to currently available chips and arrays used for IgE serology. Currently available allergen arrays are manufactured in one predetermined format which is a chip, containing one or several identically prepared allergen arrays (**Figure 7**, left part) which then need to be processed in the laboratory by hand pipetting.

Disadvantages of chips containing more than one array are that the spotting of the microarrays is performed directly on chips which are relatively large in comparison to a single silicon element. However, the single silicon elements can be arranged in much shorter distance close to each other for the spotting than preformed chips. Accordingly the spotting machine (microarray printer)

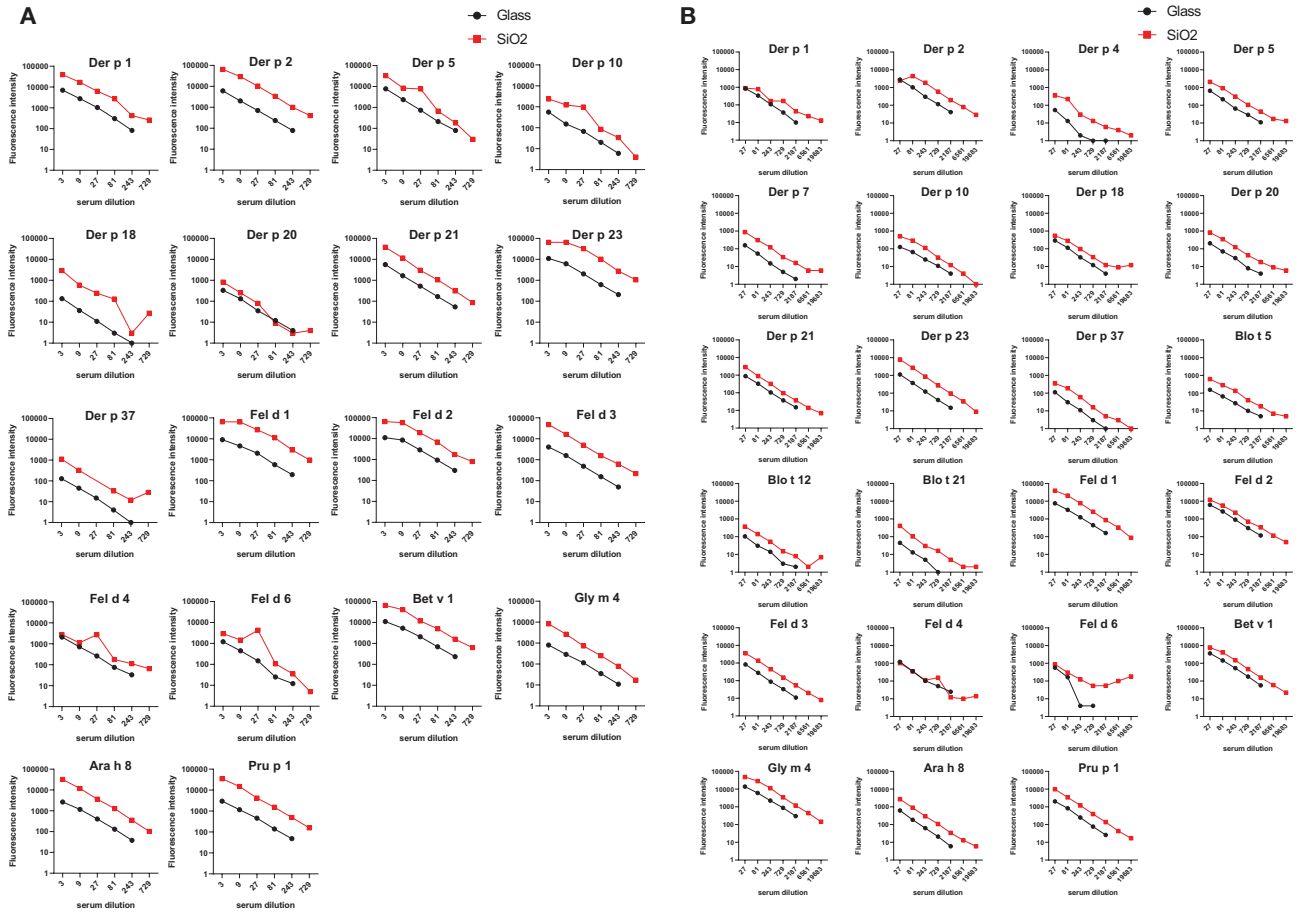

FIGURE 5 | Comparison of IgE and IgG reactivity to HDM/mite, cat, and PR10 allergens which had been microarrayed on glass and silicon. A serum pool containing IgE and IgG antibodies against each of the tested allergens was tested for **(A)** IgE and **(B)** IgG reactivity to the individual allergens in different dilutions (x-axes). Fluorescence intensities corresponding to bound antibodies are shown on the y-axes.

makes much shorter movements when spotting closely assembled silicon elements as compared to premade chips which should speed up the production by shortening the production time. Another disadvantage of chips containing several microarrays is that one array of bad quality will lead to the discarding of a complete chip although the other arrays may meet the quality criteria. By contrast, when single silicon elements are used only the few poor quality elements will be discarded keeping the loss of material low. However, the most important advantage of microarrays based on single silicon elements is that after spotting, the single elements can be assembled in different formats for different uses. This allows producing chips containing only one microarray for fast testing of single serum samples. Furthermore, chips containing several silicon elements for testing of several sera can be assembled. Importantly, silicon elements can be also assembled in plates (*e.g.*, ELISA plate format) for automated processing of samples which can be incubated with samples, washed, developed, and read in machines without requiring hand pipetting. Thus microarrays printed on silicon elements allow assembling of different devices for testing based on one standardized element. Furthermore, silicon surfaces give 5–10-fold higher sensitivity as compared to glass which should allow detecting also low allergen-specific IgE levels with high precision, and the measurement can be done with very simple and inexpensive detection devices.

CONCLUSION

Since the first description of allergen microarrays for allergy diagnosis almost 20 years ago, these multi-allergen tests have been successfully used to answer many research questions and have proved highly valuable for allergy diagnosis in multiple applications. However, several needs for improvement have remained unmet until today limiting the broad application of microarray-aided allergy diagnosis. We introduce here a novel concept for improving allergen microarray technology by showing that microarrays prepared on silicon offer higher sensitivity for the detection of specific IgE than the currently used glass surfaces and other surfaces with similar sensitivity as

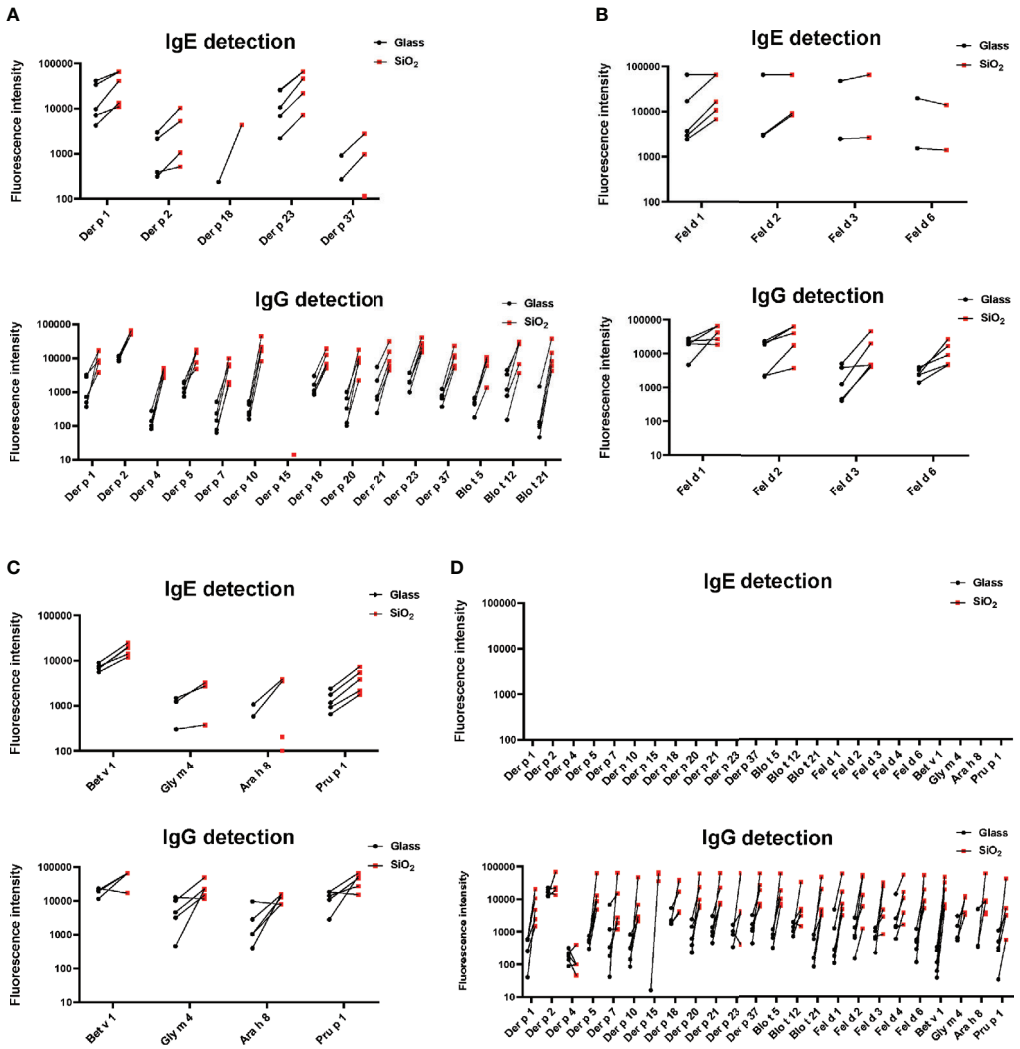

FIGURE 6 | IgE- and IgG-reactivity (y-axes: fluorescence intensities corresponding to bound antibodies) of allergic patients to **(A)** microarrayed HDM/mite allergens, **(B)** cat allergens and **(C)** PR10 allergens (x-axes) and of non-allergic subjects to all tested allergens **(D)**. Glass: black dots; Silicon: red dots.

glass. Instead of spotting allergen arrays on preformed, inflexible devices we propose manufacturing of flexible silicon elements containing microarrays which then can be assembled in different formats. This allows addressing the different needs of allergy diagnosis ranging from manual testing of single or few sera to fully automated processing of large numbers of sera. Microarrays based on silicon elements are versatile arrays that can be easily produced. Using this technology it should be possible to decrease the costs of microarray testing to make the technology affordable to the health care systems. The heart of the allergen array is a library of high-quality allergen molecules. Microarrays utilizing low amounts of immobilized allergens allowing visualizing the interplay of allergen-specific IgE and IgG mimicking the *in vivo* situation and thus should deliver serological test results resembling the clinical sensitivity. Our vision for microarray-aided allergy diagnosis is to make available to the doctor the complete IgE reactivity profile of the patient already at the initial visit or during teleconsultation to achieve a complete diagnosis and personalized treatment without need for multiple time-consuming visits for the benefit of the patient and to reduce the costs for health care in allergy.

AUTHOR CONTRIBUTIONS

RV and HJH wrote the manuscript. RV, HJH and TS designed the figures and tables. HJH, RC, OA, MC, KR, AnK, OE, EF, AL, KN, ESa, and TS performed experiments and/or contributed materials. RC, OA, MC, ESa, AnK, KR, AlK, OE, EF, AL, ESm, MK, KN, SV, TS, HJH and RV critically read and revised the

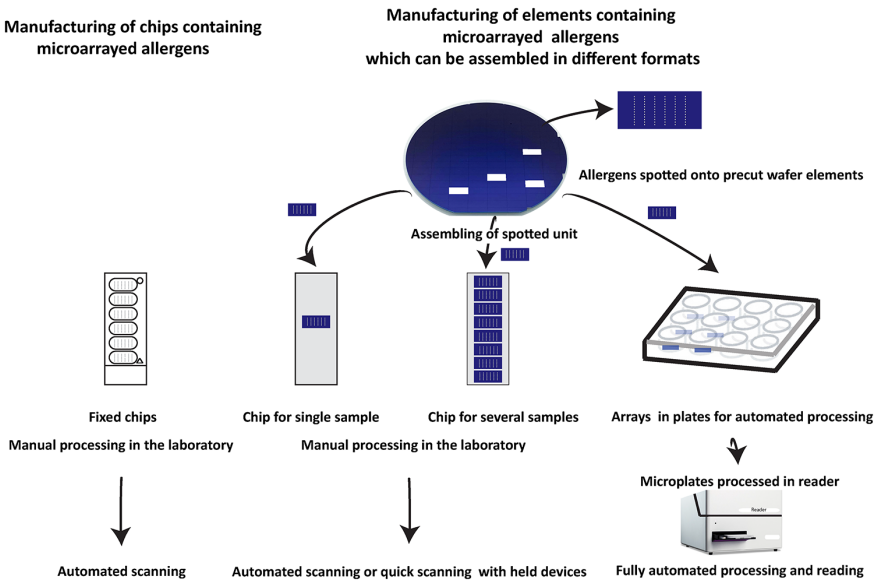

FIGURE 7 | Different assembly of silicon-based microarray elements generates different microarray formats for different diagnostic needs. Currently available allergen chips represent a single format which cannot adapt to different needs as shown for ImmunoCAP ISAC. By contrast, silicon-based microarray elements can be assembled in different formats. For example, chips containing only one array for individualized rapid testing can be produced which due to the high sensitivity can be read in simple scanners or by using mobile phone-based cameras. Alternatively, chips containing up to eight microarrays can be assembled which allow manual testing of medium scale numbers of sera and subsequent analysis by inexpensive automated scanners. For large scale automated testing of large numbers of sera, silicon elements can be mounted in ELISA-type plates and subjected to automated testing in a closed ELISA-based instrument containing an incubation, washing and detection unit followed by a scanning unit.

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SUPPLEMENTARY MATERIAL

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REVIEW
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CpG Adjuvant in Allergen-Specific Immunotherapy: Finding the Sweet Spot for the Induction of Immune Tolerance

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Prevalence and incidence of IgE-mediated allergic diseases have increased over the past years in developed and developing countries. Allergen-specific immunotherapy (AIT) is currently the only curative treatment available for allergic diseases that has long-term efficacy. Although AIT has been proven successful as an immunomodulatory therapy since its beginnings, it still faces several unmet needs and challenges today. For instance, some patients can experience severe side effects, others are non-responders, and prolonged treatment schedules can lead to lack of patient adherence and therapy discontinuation. A common strategy to improve AIT relies on the use of adjuvants and immune modulators to boost its effects and improve its safety. Among the adjuvants tested for their clinical efficacy, CpG oligodeoxynucleotide (CpG-ODN) was investigated with limited success and without reaching phase III trials for clinical allergy treatment. However, recently discovered immune tolerance-promoting properties of CpG-ODN place this adjuvant again in a prominent position as an immune modulator for the treatment of allergic diseases. Indeed, it has been shown that the CpG-ODN dose and concentration are crucial in promoting immune regulation through the recruitment of pDCs. While low doses induce an inflammatory response, high doses of CpG-ODN trigger a tolerogenic response that can reverse a pre-established allergic milieu. Consistently, CpG-ODN has also been found to stimulate IL-10 producing B cells, so-called B regulatory cells (Bregs). Accordingly, CpG-ODN has shown its capacity to prevent and revert allergic reactions in several animal models showing its potential as both preventive and active treatment for IgE-mediated allergy. In this review, we describe how CpG-ODN-based therapies for allergic diseases, despite having shown limited success in the past, can still be exploited further as an adjuvant or immune modulator in the context of AIT and deserves additional attention. Here, we discuss the past and current knowledge, which highlights CpG-ODN as a potential adjuvant to be reevaluated for the enhancement of AIT when used in appropriate conditions and formulations.

Keywords: allergen-specific immunotherapy, CpG-oligonucleotides, allergy, tolerance induction, immune tolerance

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INTRODUCTION

Allergy as a Global Health Issue

The main role of the immune system is to protect the host against external pathogens such as bacteria, viruses, fungi, or parasites. Immune cells are able to discriminate pathogens from self- and harmless external antigens, thus inducing protective immunity to pathogens and tolerance toward self- and harmless non-self-antigens (1–3). Dysregulation of immune tolerance may lead to immune-mediated diseases such as autoimmunity, cancer and various immediate-type allergic disorders (4, 5). Allergic diseases are characterized by diverse clinical disease phenotypes and symptoms, ranging from airway disorders such as allergic rhinitis and asthma (6) to systemic anaphylaxis as in the case of food and insect venom allergy (7, 8), or skin eczema/atopic dermatitis (9). Although some allergic diseases are mild and can be controlled with symptomatic medication, a considerable number of patients are at risk of life-threatening episodes (10). For instance, allergic asthma can become chronic and dramatically dampen respiratory function for life. Despite the disparity of allergic manifestations and symptoms, allergic diseases share underlying molecular and cellular mechanisms characterized by a T helper cell type 2 (Th2) response and the production of allergen-specific immunoglobulin E (IgE) by plasma cells (11). In recent decades, allergy has become a major health issue (12). Prevalence and incidence of IgE-mediated allergic diseases have been increasing over the past years in both developed and developing countries (13), thus impacting on the well-being of millions of patients worldwide and causing high socioeconomic costs (14, 15).

Treatment of Allergic Diseases

Allergic diseases have been treated with a wide range of drugs for systemic or topical application. The main strategy used for many years has been the reduction of allergic symptoms by antagonizing the activity of main allergic mediators through inhibition of their receptors, such as the histamine type-1 (H1) receptor or the cysteinyl-leukotriene receptor type-1 (CysLTR1), by H1-antihistamines or CysLTR1 antagonists. Particularly for allergic rhinitis, nasal sprays containing antihistamines are a preferable option for patients to achieve quick and fast symptom relief (16). When allergic rhinitis becomes chronic and unresponsive to systemic or topical antihistamines, intranasal glucocorticoids are added in addition to antihistamines to target T cells and thus reduce the Th2-mediated allergic airway inflammation. Inhaled corticosteroids are also the most common therapeutic option recommended for the treatment of chronic and persistent allergic asthma. However, when the disease is refractory to this treatment, more targeted approaches are recommended such as the use of biologics. Indeed, anti-IgE monoclonal antibodies like omalizumab have shown beneficial effects in uncontrolled allergic asthma (16). Other disease-modifying biologics in asthma treatment target Th2 cytokines or their receptors (17), showing good results in the clinic (18). Although symptomatic therapies are the first line of action for patients with allergic diseases, their benefit comes with several downsides. Patients have to be treated continuously and upon treatment discontinuation, symptoms commonly relapse (19). Some of the symptomatic anti-allergic drugs have undesirable side effects such as somnolence in the case of first generation antihistamines (20). In the past years, a variety of new symptomatic medications, which show less side effects and a more targeted activity, have been developed. Among them are new generations of H1-antihistamines (21) and disease-modifying biologic monoclonal antibody therapies that interfere with the mediators and effectors of the Th2 response (17, 18, 22). However, the higher costs of such novel therapeutic options are quite often prohibitive for many patients as healthcare systems are restrictive regarding the reimbursement of advanced but more expensive treatments (23).

Allergen-Specific Immunotherapy

AIT consists in delivering increasing doses of the allergen over time with the aim of reaching immune tolerance and clinical non-reactivity to the allergen. By activating long lasting immunomodulatory mechanisms, AIT has long-term effects such as providing sustained relief of symptoms and reducing the need for symptomatic medication (24, 25). Although AIT is a successful therapeutic strategy with dozens of licensed products worldwide, it has some unmet needs to overcome (26), since not all patients experience a significant symptom relief after therapy (27) and AIT imposes a long course with multiple doses (up to 3-5 years), which can lead to lack of patient adherence and thus treatment discontinuation (28). In addition, some patients may experience side effects during the course of AIT, ranging from mild rashes to severe anaphylaxis (29, 30). For these central reasons, AIT needs to be optimized, to make it safer, shorter in time and more successful for a maximum number of allergic patients. Another significant advantage that argues in favor of AIT is its long-term cost-effectiveness compared to symptomatic treatments.

Mechanisms of Allergen Specific Immunotherapy

The establishment of immune tolerance by AIT implies modifications of the immune response such as induction

Abbreviation: AIT, allergen-specific immunotherapy; $Al(OH)_3$, aluminum hydroxide; AP1, activator protein 1; APC, antigen presenting cell; Breg, B regulatory cell; cDC2a, type 2a classical dendritic cell; cDCs, classical dendritic cell; CpG-ODN, CpG oligodeoxynucleotide; CTLA4, cytotoxic T-lymphocyte-associated protein 4; DC, dendritic cells; dsDNA, double stranded DNA; EPI, epicutaneous; FoxP3, forkhead box P3; Gata3, GATA Binding Protein 3; GM-CSF, granulocyte-macrophage colony-stimulating factor; HBsAg, hepatitis B major surface antigen; IBD, intestinal bowel disease; ICOS-L, inducible costimulatory ligand; ID, intradermal; IDO, indoleamine 2,3-dioxygenase; IFN, interferon; Ig, immunoglobulin; IKK, IκB kinases; IL, interleukin; IM, intramuscular; IP, intraperitoneal; IRAKs, IL-1R-ssociated kinase; IRF, interferon regulatory factor; LAG3, lymphocyte-activation gene 3; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; mDC, myeloid dendritic cell; MLP, monophosphoryl lipid A; MyD88, myeloid differentiation factor 88; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NI, nasal instillation; OG, oral gavage; PAMP, pathogen associated molecules pattern; pDC, plasmacytoid dendritic cell; PD-L1, programmed death ligand 1; PD-L2, programmed death ligand 2; PLGA, poly-(lactic-co-glycolic acid); PRR, pattern recognition receptor; SC, subcutaneous; SLE, systemic lupus erythematosus; SLIT, sublingual immunotherapy; TGF-β, transforming growth factor beta; Th1 cells, T helper cell type 1; Th2 cells, T helper cell type 2; TIR, toll/IL-1 receptor domain; TLR, toll-like receptor; TNFR2, tumor necrosis factor receptor 2; TNF-α, tumor necrosis factor alpha; TRAF, tumor necrosis factor receptor-associated factor 6; Treg, T regulatory cell; TRIF, TIR-domain-containing adapter-inducing interferon-β.

of Tregs and B regulatory cells (Bregs), strengthened by immunological memory (31). The induction of Tregs and their essential role in the regression of the disease have been observed in humans and in mice (32). Tregs exert their immune modulatory properties through several synergistic mechanisms such as IL-10, IL-35 and TGF-β secretion (33, 34), as well as through cell surface receptors such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), lymphocyte-activation gene 3 (LAG3), programmed cell death protein 1 (PD-1) and T cell immunoglobulin and ITIM domain (TIGIT) (35–37). Tregs might not be sufficient to reduce allergic inflammation under some circumstances (4). Indeed, Tregs have been shown to act in synergy with Bregs to promote immune tolerance. Bregs compose a specific B cell subtype that produces the anti-inflammatory cytokines IL-10 and/or IL-35 (38–41). They have been found to synthesize allergen-specific IgG4 in AIT treated patients (42, 43). Blocking antibodies that prevent specific IgE binding to the allergen are also crucial for mediating clinical non-responsiveness as it has been shown in a recent clinical trial. Blocking the major cat allergen Fel d 1 through passive immunotherapy by injecting a single dose of two monoclonal IgG4 antibodies successfully mitigated acute symptoms in cat-allergic patients (44). IgG4 is absent in mice, but IgG1, IgG2a and IgG3 have been associated with protective effects in murine AIT models (45). IgA has also been shown to be protective by neutralizing the allergen on mucosal surfaces in the context of AIT (46). The innate immune system is mainly engaged at early stages of AIT. Dendritic cells (DCs) and especially pDCs have been described as the main cellular mediators in tolerance induction during AIT by leading to the generation of Tregs (32). DCs utilize several factors to promote Treg differentiation, which include soluble factors such as IL-10, TGF-β, or IDO (47–49), as well as cellular ligands like the inducible costimulatory ligand (ICOS-L) or the programmed death ligands 1 (PD-L1) and 2 (PD-L2) (50–52).

Improving Allergen-Specific Immunotherapy

Several strategies have been investigated to improve AIT since its first application back in 1911 by Leonard Noon (53), among which are bypassing IgE binding, use of modified allergens or chimeric proteins, delivery of recombinant hypoallergenic proteins, new routes of administration, allergen-derived peptide immunotherapy, combination with biologics and adjuvants/immune modulators to enhance tolerance effects (4, 27). Immune adjuvanticity in the context of AIT relates to the addition of one or more compound(s) to an allergen formulation in order to improve its tolerance-inducing immunogenicity, thereby overcoming some of the unsolved needs described above. Several adjuvants have been tested over the years in the context of AIT (**Table 1**). Historically, aluminum hydroxide ($Al[OH]_3$) has been the adjuvant used in many AIT formulations for its properties to form a depot (27). More recently, new adjuvants have been studied and tested for their potential to improve AIT effects and reduce its potential pitfalls. These new adjuvants are biological or synthetic compounds with a broad range of effects on the immune system such as induction of T helper cell type 1 (Th1) or regulatory T cell (Treg) responses, recruitment of dendritic cells (DCs) and other antigen presenting cells (APCs), improvement of APC uptake and signaling, or protection of the active compounds from rapid degradation (92). Adjuvants can interact with the host immune system in many ways. In the case of aluminum hydroxide, it mainly activates the inflammasome, leading to high production of the active forms of IL-1β and IL-18 (54, 55). Interestingly, aluminum hydroxide is well known to trigger a Th2 response in mice (93). Besides aluminum derivatives, toll-like receptor (TLR) ligands such as monophosphoryl lipid A (MPL), a lipopolysaccharide (LPS) derivative with TLR4 ligand properties (68), LP40, a TLR2 ligand (61), imidazoquinolines, TLR7/8 ligands (73) and CpG oligodeoxynucleotides (CpG-ODN), a TLR-9 agonist (94), have been used in AIT (**Table 1**). MPL was tested as AIT adjuvant in phase II and III clinical trials and was approved for subcutaneous immunotherapy (SCIT) of allergic rhinitis (Pollinex Quattro®) (95). Regarding CpG-ODN, a series of pre-clinical studies (86, 96, 97) (**Table 2**), as well as clinical trials (92, 110) (**Table 3**) were performed using this adjuvant for AIT. In 2006, data were published on a 2001 completed clinical trial using a CpG-ODN-based formulation for AIT in a randomized, double-blind, placebo-controlled phase II clinical trial for the treatment of allergic rhinitis (111). Although this novel AIT treatment appeared to have long-term clinical efficacy in patients with allergic rhinitis due to ragweed allergy (111), the formulation failed later in phase III clinical studies (26). A very low ragweed pollen exposure in the first pollen season of the phase III trial, which made it impossible to see any measurable disease in any study participant, was announced as the main reason for discontinuing the development of this novel AIT approach (122). Other possible reasons for the failure of the drug were not further discussed in the scientific literature, such as the possible presence of LPS in the purified allergen (Amb a 1), which could have interfered with TLR9 signaling (**Figure 1**), or the low concentration of CpG-ODN used. A decade later, CpG-ODN was tested as immune modulator without allergen to treat allergic rhinitis in a randomized placebo-controlled phase IIb clinical trial (113). Although the drug was proven to be successful in previous clinical trials (92, 110, 112) (**Table 3**), it did not show clinical improvement compared to placebo control (113).

Thus, despite the fact that CpG-ODN-based therapies for allergic diseases have shown early clinical promise, they were not pursued further for the treatment of allergic diseases after experiencing a lack of success in later phases of controlled clinical trials (**Table 3**). Nevertheless, experimental and clinical research on CpG-ODN continued in other biomedical areas, which led to the successful FDA approval of CpG-ODN as immune adjuvant in hepatitis B vaccination in 2018 (120) and to multiple recent clinical studies, where CpG-ODN has been used as immune modulator in cancer immunotherapy (**Table 3**). In this review, we describe and discuss the current knowledge and latest results on CpG-ODN as immune adjuvant for AIT and asthma treatment. Indeed, although other adjuvants, especially TLR ligands, have shown immune-regulatory properties (123), CpG-ODN has been predominantly described for its capacity to induce immune tolerance in comparison to other adjuvants that are also used in the context of AIT (**Table 1**). Our review of the literature, which is based on searches in PubMed "(CpG ODN OR CpG oligodeoxynucleotides)

TABLE 1 | Comparison of adjuvants in the context of AIT.

Adjuvant name	Receptor or mechanism of action	Drug development phase	Effect on the immune system
Aluminum hydroxide ($Al[OH]_3$)	Depot effect and inflammasome activation through NLRP3.	Approved for SCIT in many formulations, e.g. Alutard®.	**Immune tolerance induction**: No. **Inflammatory response**: Yes, general activation of immune cells and production of IL-1β and IL-18 (54, 55)[§#]. Induction of Th1 responses in PBMCs (56)[§]. **Antibody response**: yes, IgG isotypes (57)[§#].
Tyrosine crystals	Depot effect, recognition by APC due to specific crystal size.	Approved for SCIT (MCT®, Acarovac Plus™) (58, 59).	**Immune tolerance induction**: Yes, increased IL-10 production by re-stimulated splenocytes (60)[#]. Higher levels of IL-10 after 1 year patient follow up (59)[§]. **Inflammatory response**: Yes, increase production of IFN-γ by both $CD4^+$ and $CD8^+$ antigen specific T cells (60)[#]. **Antibody response**: yes, IgG1, IgG2a, IgG2b, IgG3 isotypes (60)[#].
LP40	TLR2	Pre-clinical	**Immune tolerance induction**: Yes, increased IL-10 production by PBMCs (61)[§]. **Inflammatory response**: Yes, increased IFN-γ, IL-6 and IL-12 production by stimulated PBMCs and splenocytes (61)[§#]. **Antibody response**: yes, IgG2a (4)[#].
Poly I:C	TLR3	Pre-clinical	**Immune tolerance induction**: Yes, induction of IL-10 by re-stimulated splenocytes (62)[#]. **Inflammatory response**: Yes, increased production of IL-12 from DCs (63)[#]. Increased production of INF-γ by $CD8^+$ antigen-specific cells (62, 63)[#]. **Antibody response**: yes, IgG1 and IgE (64)[#].
Flagellin	TLR5	Pre-clinical	**Immune tolerance induction**: Yes, induction of IL-10 by DCs, and generation of Tregs (65)[§#]. Increased production of IL-10 by bone marrow-derived DCs (66)[#]. **Inflammatory response**: Yes, increased production of IL-6, IL-1β by bone marrow derived DCs (66)[#]. Induction of IL-4, IL-5, IL-13 and IL-17 in the lungs, as well as Th2 cell induction after airway challenge (67)[#]. **Antibody response**: yes, IgG2a (66) and IgE (67)[#].
Monophosphoryl lipid A (MPL)	TLR4	Approved for SCIT to treat allergic rhinitis (Pollinex Quattro®)	**Immune tolerance induction**: No. **Inflammatory response**: Yes, increased production of IFN-γ (68)[§]. Induction of Th1 responses (69, 70)[#]. **Antibody response**: yes, IgG1 and IgG4 (71)[§]. IgG2a (70)[#].
Imidazoquinolines	TLR7/8	Phase II (AZD8848) to treat allergic asthma (72).	**Immune tolerance induction**: Yes, increased IL-10 production by monocytes (73)[§]. Induction of tolerogenic DCs and Treg promotion (74)[#]. **Inflammatory response**: Yes, increased production of IFN-γ by allergen-specific T cells from allergic donors. Increased production of IL-12, IL-18, TNF-α, and IL-15 by monocytes derived (73)[§]. **Antibody response**: lack of IgG induction *in vitro* (75)[#].
CpG-ODN	TLR9	Phase IIb to treat allergic rhinitis (low dose) Pre-clinical (high dose)	**Immune tolerance induction**: Yes (in high doses), increased production of IL-10 by alveolar macrophages (76)[#], production of TGF-β and IDO by pDCs (76)[#§], induction of Treg cells (77–81)[§#], induction of Bregs (82)[§] (**Table 4**). **Inflammatory response**: Yes (at low doses), increased IL-12, IFN-γ, IL-6 (83, 84)[§]. Induction of Th1 responses (85)[§]. (**Table 4**). **Antibody response**: yes, IgG1, IgG2a, IgG2c IgG3, and IgA (79, 84, 86–91)[#§].

NLRP3, NLR family pyrin domain containing 3; BALF, bronchoalveolar lavage fluids. [#]In mouse, [§]In human.

AND (allergy OR allergic disease)", "(CpG ODN OR CpG oligodeoxynucleotides) AND (immune tolerance OR tolerance induction)" and "(CpG ODN OR CpG oligodeoxynucleotides) AND (safety)" points to CpG-ODN as an adjuvant with previously unrecognized potential for AIT that is able to effectively induce immune tolerance to allergens when used at appropriate higher concentrations, which are still safe in humans. This review delineates that, based on more recent evidence, a reevaluation of CpG-ODN as immune adjuvant for AIT seems to be warranted.

CPG OLIGODEOXYNUCLEOTIDES (CPG-ODN)

CpG-ODN as a TLR9 Ligand

To distinguish pathogen structures from self-antigens the immune system has tailored specific systems detecting danger signals or pathogen associated molecules patterns (PAMPs). PAMPs comprise a variety of pathogen structures such as LPS, proteins like flagellin, glycan structures, genetic material (DNA and RNA) among others (124). Immune cells can specifically identify PAMPs through pattern recognition receptors (PRRs). Bacterial genetic material, unlike vertebrate DNA, is enriched of unmethylated deoxycytidyl-deoxyguanosine (CpG) dinucleotides (125). These PAMPs of unmethylated bacterial CG motifs are recognized by the Toll-like receptor 9 (TLR9) (126). Experimentally, short synthetic oligodeoxynucleotide (ODN) sequences containing unmethylated CpG dinucleotides (CpG-ODN), mimicking bacterial DNA signatures, are used to agonize TLR9, thereby avoiding any other TLR co-activation by bacterial contaminants (127).

Different Classes of CpG-ODN

So far, at least four types of CpG-ODN have been identified, which induce different effects on the immune system (**Table 4**). Thus, it is crucial to know the immunomodulatory properties of

TABLE 2 | Summary of pre-clinical studies using CpG oligodeoxynucleotides (CpG-ODN) for allergic diseases.

Model/disease	Drug features	Drug design	CpG-ODN dose*	Administration scheme	Outcome	Reference
Mouse model of asthma to schistosome eggs.	CpG-ODN, B-class. LPS level: undetectable	CpG-ODN + allergen	1 dose: 30 µg per injection. (1.5 mg/kg)	1 IP injection 7 days before challenge (preventing treatment). 1 IP injection together with allergen (co-administration treatment).	Reduction of allergy burden in co-administration and preventive treatment. Eosinophilia, Th2 cytokines reduction and IgE reduction. IFN-γ and IL-12 increase.	Kline et al. (97)
Mouse model of asthma to rBet v 1.	CpG-ODN, B-class. LPS level: <0.1 EU/ 6 µg of DNA	CpG-ODN + allergen	1 dose 61.23 µg (10nmol) per injection. (3 mg/kg)	3 IP, SC or NI administrations 2 weeks before sensitization (preventive treatment). 3 IP, SC or NI administrations 2 weeks after challenge (active treatment). 3 IP injections (co-administration with allergen and $Al[OH]_3$).	Reduction of allergy burden in co-administration, preventive and active treatment. Eosinophilia, Th2 cytokines, and IgE reduction. IgG2a and IgA increase.	Jahn-Schmid et al. (86)
Mouse model of asthma to ragweed extract.	CpG-ODN, B-class. LPS level: <0.02 U/kg	CpG-ODN + extract	1 dose: 35 µg per administration. (1.75 mg/kg)	1 IT administration 48h before challenge.	Reduction of allergy burden in preventive treatment. Eosinophilia, IgE and IL-4 reduction. IFN-γ, IFN-γ/IL-4 ratio and IgG2a increase IFN-γ dependent.	Sur et al. (98)
Mouse model of immune response to mosquito salivary antigen (rAed a 2).	CpG-ODN, B-class. LPS level: undetectable	CpG-ODN + allergen	10 µg, 30 µg and 90 µg per injection in the preventive treatment. (0.5 mg/kg, 1.5 mg/kg and 4.5 mg/kg respectively) 30 µg per injection in the active treatment. (1.5 mg/kg)	1 ID injection 24h before first sensitization (preventive treatment). 2 ID injections during sensitization (co-administration treatment), 1 injection every 4 weeks.	Reduction of Th2 reaction in co-administration and preventive treatment. IgE and Th2 cytokines reduction. IL-12 and IgG2a increase. Delayed-type hypersensitivity reactions in CpG-ODN treated mice.	Peng et al. (99)
Mouse model of immune response to hepatitis B major surface antigen (HBsAg)	CpG-ODN 1826, B-class. LPS level: no mention	CpG-ODN + antigen	10 µg per injection. (0.5 mg/kg)	1 IM injection 2 or 4 weeks before or after sensitization.	Reduction of Th2 reaction in preventive and active treatment. IgG1 reduction. IgG2a and CTL activity increase.	Weeratna et al. (88)
Mouse model of asthma to OVA	CpG-ODN, B-class. LPS level: undetectable	CpG-ODN + allergen	1 µg per administration. (0.05 mg/kg)	3 NI administrations: 2 weeks after sensitization, 1 administration every 2 weeks.	Reduction of allergy burden response in active treatment. Airway hyperresponsiveness, eosinophilia, IgE and Th2 cytokines reduction. IgG2 and IL-10 increase.	Jain et al. (100)
Rhesus monkey model ofexperimentally induced allergic asthma to HDM.	CpG-ODN, A-class. LPS level: ND	CpG-ODN solely	12.5 mg per administration. (1.9 mg/kg)	3 NI administrations: 24h after sensitization, 1 administration every 2 weeks.	Reduction of allergy burden in active treatment. Airway hyperresponsiveness, eosinophilia, mast cell number and tissue remodeling reduced.	Fanucchi et al. (101)
Mouse model of asthma to OVA	CpG-ODN 1826, B-class. LPS level: undetectable	CpG-ODN solely and CpG-ODN + allergen	100 µg per administration. (5 mg/kg)	3 OG administrations: 3 days before, same day of and 7 days after sensitization (preventive treatment). 6 OG administrations: 2 weeks after sensitization, 1 administration every week (active treatment).	Reduction of allergy burden in active and preventive treatment. Eosinophilia, IgG1, IgE and Th2 cytokines reduction. IgG2c increase.	Kitagaki et al. (91)
Mouse model of immune response to OVA	CpG-ODN BL07S from *Bifidobacterium longum*. B-class. LPS level: undetectable	CpG-ODN + antigen	10 µg per injection. (0.5 mg/kg)	2 SC injections: 2 weeks interval co-injected with sensitization.	Reduction of Th2 reaction in co-administration treatment. IgE and Th2 cytokines reduction. IgG2a increase.	Takahashi et al. (102)

(Continued)

TABLE 2 | Continued

Model/disease	Drug features	Drug design	CpG-ODN dose*	Administration scheme	Outcome	Reference
Murine model of asthma to OVA	CpG-ODN B-class. LPS level: ND	CpG-ODN + allergen	50 µg per injection. (2.5 mg/kg)	1 SC injection: 24h after Th2 cell adaptive transfer.	Reduction of allergy burden in active treatment. Eosinophilia, mucus hyper-production, Th2 cytokines and cell migration to the lung reduction. Type I IFN dependent.	Ashino et al. (103)
Mouse model of asthma to OVA	CpG-ODN B-class. LPS level: no mention	CpG-ODN solely	100 µg per injection. (5 mg/kg)	4 IP injections: 6 days after sensitization, 1 injection every 24h.	Reduction of allergy burden in active treatment. Airway hyperresponsiveness, eosinophilia, Th2 cytokines, cell migration to the lung reduction. IgG2a and IFN-γ increased.	Chang et al. (104)
Mouse model of asthma to OVA	CpG-ODN (sensitized using custom primers). LPS level: undetectable	CpG-ODN solely	50 µg/per injection. (2.5 mg/kg)	3 SC injections: 3 days after sensitization, 1 injection every week.	Reduction of allergy burden in active treatment. Airway hyperresponsiveness, eosinophilia, IgE, IgG1 and Th2 cytokines reduction. IgG2a increase.	Fonseca et al. (105)
Mouse model of asthma to the Japanese cedar pollen allergen (Cry j 1).	CpG-ODN 1018, B-class. LPS level: <0.03 endotoxin unit/µg	CpG-ODN coupled to Cry j 1	5 µg per injection. (0.25 mg/kg)	3 SC injections: 3 days before sensitization, 1 injection every 24h.	Reduction of allergy burden in preventive treatment. IgE and Th2 cytokines reduction. IL-12, IFN-γ and IgG2a increase.	Kaburaki et al. (106)
Mouse model of asthma to *Aspergillus fumigatus* extract.	CpG-ODN 1826, B-class. LPS level: endotoxin free	CpG-ODN solely	30 µg per injection. (1.5 mg/kg)	2 IP injections: 7 days after sensitization, 1 injection every week.	Reduction of allergy burden in active treatment. IgE and Th2 cytokines reduction. IL-12, IFN-γ and IgG2a increase.	Volpi et al. (84)
Mouse model of asthma to ragweed extract	CpG-ODN 1018, B-class. LPS level: <5 endotoxin units/mg	CpG-ODN solely	20 µg per administration. (1 mg/kg)	5 to 12 NI administrations: 14 days after sensitization, 1 administration every week.	Reduction of allergy burden in active treatment. Eosinophilia, IgE and Th2 cytokines reduction. IFN-γ increase. IFN-γ dependent.	Cambell et al. (107)
Mouse model of asthma to HDM	CpG-ODN 1826, B-class. LPS level: <0.1 EU per dose	Nanoparticle-conjugated CpG-ODN	2 µg per administration. (0.1 mg/kg)	4 NI administrations: 3 days before sensitization, 1 administration every 2 days (preventive therapy). 4 NI administrations: 3 days after sensitization, 1 administration every 2 days (active therapy).	Reduction of allergy burden in preventive and active treatment. Eosinophilia, IgE, mucus production and Th2 cytokines reduction. INF-γ increase.	Ballester et al. (108)
Mouse model of asthma to cockroach extract	CpG-ODN 1826, B-class. LPS level: ND. HLPC purified	CpG-ODN solely	3 µg per administration. (0.15 mg/kg)	1 NI administration: 3 days before sensitization (preventive therapy). 1 NI administration: 3 days after sensitization (active therapy).	Reduction of allergy burden in preventive and active treatment. Airway hyperresponsiveness, eosinophilia, IgE, goblet cell hyperplasia and Th2 cytokines reduction. IL-10 and $CD4^+$ $Foxp3^+$ regulatory T cells increase.	Kim et al. (80)
Mouse model of food allergy to peanut extract	CpG-ODN 1826, B-class. LPS level: ND	Nanoparticle-conjugated CpG-ODN	1.8 µg per administration. (0.09 mg/kg)	4 OG administrations: 3 weeks after sensitization, 1 administration every week.	Reduction of allergy burden in active treatment. Anaphylaxis, histamine levels, IgE/IgG1 levels, and Th2 cytokines reduction. IgG2a and INF-γ increase.	Srivastava et al. (109)
Mouse model of asthma to HDM	CpG-ODN, A-class. LPS level: <24 EU/mg	CpG-ODN solely	50 µg per administration. (2.5 mg/kg)	1 NI administration: 7 days before sensitization (preventive therapy). 1 NI administration: 7 days after sensitization (active therapy).	Reduction of allergy burden in preventive and active treatment. Airway hyperresponsiveness, eosinophilia, goblet cell hyperplasia and Th2 cytokines reduction. IL-10 increase. IL-10 dependent.	Sabatel et al. (76)

(Continued)

TABLE 2 | Continued

Model/disease	Drug features	Drug design	CpG-ODN dose*	Administration scheme	Outcome	Reference
Mouse model of asthma and active cutaneous anaphylaxis to OVA	CpG-ODN 2395, C-class. LPS level: endotoxin free	CpG-ODN + allergen	10 ug per injection. (0.5 mg/kg)	2 SC injections: co-administrated with the sensitization, 1 injection every week.	Reduction of allergy burden in co-administration treatment. Eosinophilia, IgE and anaphylaxis score reduction. IgG2c and IgG2a increase.	Alberca Custodio et al. (90)
Mouse model of asthma to Fel d 1	CpG-ODN 1668, B-class. LPS level: endotoxin free	CpG-ODN + allergen	21 µg per injection. (1.05 mg/kg)	3 IP injection: 14 days after sensitization, 1 injection every 2 weeks.	Reduction of allergy burden in active treatment. Airway hyperresponsiveness, eosinophilia, mast cell ratio, B cell activation and Th2 cytokines reduction. Gata3^{+} Treg increase.	Leonard et al. (79)

*CTL, cytotoxic T-lymphocyte; OVA, ovalbumin; HDM, house dust mite; IP, Intraperitoneal; NI, Nasal instillation; IT, Intratracheal instillation; ID, intradermal; IM, intramuscular; OG, oral gavage; ND, not determined. *20 g mean weight for a mouse and 6.5 kg mean weight for a rhesus monkey were considered to calculate the dose.*

each CpG-ODN type when formulating CpG-ODN-based treatments. A-class CpG-ODN has a phosphodiester/phosphorothioate backbone containing a single CpG motif which is flanked by palindromic sequences, plus a poly-G string located at the 3' end. B-class CpG-ODN harbors multiple CG doublets within a phosphorothioate backbone (136), the latter providing resistance to nuclease digestion increasing its lifetime by 6-fold compared to the phosphodiester backbone of A-class (83). C-class was reported more recently as a new CpG-ODN. C-class is a combination of both afore-mentioned CpG-ODN classes. As B-class, it is built entirely on phosphorothioate nucleotides but it resembles the A-class by its palindromic CpG motifs (133). The last and more recent CpG-ODN group that was described is the P-class, which, unlike the other CpG-ODN types, contains two palindromic sequences that facilitate the formation of higher-ordered structures (135).

TLR9 Downstream Signaling and Its Modulation by CpG-ODN Concentration

TLR9 is located intracellularly, with the binding site for CpG-ODN pointing to the endosomal compartment. This specific orientation requires the internalization and endosomal uptake of CpG-ODN to agonize TLR9 and trigger its signaling cascade (129) (**Figure 1**). Once TLR9 is activated by CpG-ODN binding, its cytosolic domains, known as Toll/IL-1 receptor (TIR) domains, are stimulated and signaling is transmitted further. TIR domains interact with the common TLR adaptor protein myeloid differentiation factor 88 (MyD88) (137), the classical adaptor protein attributed to many TLRs. Subsequently, IL-1R-associated kinases 1, 2 and 4 (IRAKs) and tumor necrosis factor receptor-associated factor 6 (TRAF6) are phosphorylated (124). From this point, several mitogen-activated protein kinases (MAPK) and other kinases such as IκB kinases (IKK) are sequentially recruited. Those kinases facilitate the translocation to the nucleus of various transcription factors such as the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), the activator protein-1 (AP-1) or the interferon regulatory factor 7 (IRF7) (138–140). These transcription factors activate the transcription of pro-inflammatory genes such as *TNF, IL-1B, IL-1A* and interferon type 1 (*IFNA* and *IFNB*) genes (129, 141). Indeed, type 1 IFN production by plasmacytoid dendritic cells (pDC) in response to TLR9 activation has been demonstrated to be dependent on the MyD88-IRF7 signaling cascade (129). The kinetics of the gene expression signature induced by CpG-ODN are complex. It has been shown that only 30 min after injection of CpG-ODN *in vivo* a panel of genes is upregulated, with a peak of expression around 3 h after administration. Interestingly, a second delayed peak is observed 5 days post administration (141), demonstrating that CpG-ODN can have short-, mid- and long-term effects. This fact is not to be neglected when using CpG-ODN for therapeutic purposes. A few years ago, the TIR domain containing adaptor inducing IFN-β (TRIF) was demonstrated to signal upon TLR3 and TLR4 activation (142–144). More recently, Volpi et al (84). showed that TLR9 can also signal through TRIF besides the classic MyD88 pathway (**Figure 1**). In fact, this group

TABLE 3 | Summary of CpG oligodeoxynucleotides (CpG-ODN) clinical studies for allergy and other diseases.

Disease	Study phase	Identifier	Drug information	CpG-ODN dose*	Administration scheme	Outcome and safety	Completion date	Reference
IgE-MEDIATED ALLERGY								
Allergic rhinitis to ragweed-pollen	Randomized double-blind placebo-controlled phase 2	NCT00346086	CpG-ODN 1018 conjugated to Amb a 1	6 increasing doses: 0.06µg, 0.3 µg, 1.2 µg, 3 µg, 6 µg and 12 µg. (0.00004 mg/kg, 0.00008 mg/kg, 0.000016 mg/kg, 0.0002 mg/kg, 0.0004 mg/kg, 0.0008 mg/kg and 0.00016 mg/kg respectively)	6 SC injections, 1 injection every week.	Long-term clinical efficacy. Drug was well tolerated with mild local injection-site reactions.	Aug 2001	Creticos et al. (111)
Perennial allergic rhinoconjunctivitis with asthma to HDM	Single center open-label phase 1/2a	NCT00652223	CYT005-AllQbG10 (combination of HDM allergen extract with CYT003-QbG10)	§1 dose: 60 µg per injection. (0.0008 mg/kg)	6 SC injections, 1 injection every week.	Patients achieved practically complete alleviation of allergy symptoms. Drug was well tolerated with mild local injection-site reactions.	Nov 2005	Senti et al. (110)
Perennial allergic rhinoconjunctivitis to HDM	Randomized double-blind placebo-controlled phase 2	NCT00574704	CYT005-AllQbG10 (combination of HDM allergen extract with CYT003-QbG10)	Not identified	6 SC injections, no schedule found.	No results accompanying the study	Mar 2008	Not published
Perennial allergic rhinoconjunctivitis to HDM	Randomized double-blind placebo-controlled phase 2	NCT00574223	CYT005-AllQbG10 (combination of HDM allergen extract with CYT003-QbG10)	Not identified	8 SC injections no schedule found.	No results accompanying the study	Mar 2009	Not published
Perennial allergy to HDM and/or Cat	Randomized double-blind placebo-controlled phase 2	NCT00575003	CYT003-QbG10 (VLP filled with an CpG-ODN G10)	Not identified	6 SC injections no schedule found.	No results accompanying the study	Mar 2009	Not published
Perennial allergic rhinoconjunctivitis to HDM	Randomized double-blind placebo-controlled dose-finding phase 2b	NCT00800332	CYT003-QbG10 (VLP filled with an CpG-ODN G10)	§2 doses: 0.1 mg and 0.2 mg per injection. (0.00134 mg/kg and 0.0026 mg/kg respectively)	6 SC injections, 1 injection every week.	Higher dose of the drug improved disease symptoms and reduced medication use in allergic individuals. Drug was well tolerated with mild local injection-site reactions.	Nov 2010	Klimek et al. (92)
Allergic asthma requiring long-term treatment with inhaled corticosteroids	Randomized double-blind placebo-controlled phase 2	NCT00890734	CYT003-QbG10 (VLP filled with an CpG-ODN G10)	§1 dose: 0.18 mg of per injection. (0.0024 mg/kg)	7 SC injections. 3 first injections every week, and 4 next injections every 2 weeks.	Drug may contribute to asthma control during steroid reduction in patients. Drug was well tolerated with mild local injection-site reactions.	Nov 2010	Beeh et al. (112)
Uncontrolled moderate to severe allergic asthma on standard inhaled corticosteroids	Randomized double-blind placebo-controlled dose-finding phase 2b	NCT01673672	CYT003-QbG10 (VLP filled with an CpG-ODN G10)	§3 doses: 0.06 mg, 0.2 mg and 0.4 mg per injection. (0.0008 mg/kg, 0.00266 mg/kg and 0.0054 mg/kg respectively)	7 SC injections, 1 injection every 1 or 2 weeks.	Drug showed no additional benefit in patients. Drug was well tolerated with mild local injection-site reactions.	May 2014	Casale et al. (113)
CANCER								
Chronic lymphocytic leukemia	Randomized open label phase 1	NCT00233506	CpG-ODN 7909	2 doses: (0.15 mg/kg 1.05 mg/kg)	4 to 8 IV and SC injections, 1 injection every week.	Drug was well tolerated with mild side-effects such myalgia, malaise, and fevers.	Jun 2011	Zent et al. (114)
Refractory solid tumors	Randomized open label phase 1b	NCT03052205	CpG-ODN IMO-2125 (Tilsotolimod)	Maximum dose: 32 mg/injection. (0.43 mg/kg)	6 IT injections on weeks 1, 2, 3, 5, 8, and 11.	Drug was well tolerated and no related adverse events were observed.	Oct 2019	Babiker et al. (115)

(Continued)

TABLE 3 | Continued

Disease	Study phase	Identifier	Drug information	CpG-ODN dose*	Administration scheme	Outcome and safety	Completion date	Reference
Metastatic melanoma or recurrent or metastatic head and neck squamous cell carcinoma	Randomized open labelPhase 1b/2	NCT02521870	CpG-ODN SD-101 in combination with Pembrolizumab (anti-PD1)	4 doses: 1 mg, 2 mg, 4 mg, 8 mg per injection. (0.013 mg/kg, 0.027 mg/kg, 0.053 mg/kg and 0.11 mg/kg respectively)	11 IT injections in escalating doses. 4 first injections every week, and 7 next injections every 3 weeks.	Drug was well tolerated with injection-site reactions and transient, mild to moderate "flu like" symptoms.	Feb 2020	Ribas et al. (116)
Malignant melanoma	Non-randomized open label phase 1	NCT03084640	CMP-001b (CYT003-QbG10) in combination with Pembrolizumab (anti-PD1)	§7 escalating doses: 0.2 mg, 1.5 mg. 2 mg, 2.5 mg, 3 mg, 3.5 mg and 4 mg per injection. (0.0134 mg/kg, 0.02 mg/kg, 0.026 mg/kg, 0.034 mg/kg, 0.04 mg/kg, 0.046 mg/kg and 0.054 mg/kg respectively)	7 SC injections into accessible lesion(s), 1 injection every week.	Drug in combination with Pembrolizumab was well tolerated with mild toxicities such as fever and headache.	May 2021	Milhem et al. (117)
INFECTIOUS DISEASES								
Plasmodium falciparum (Malaria) infection	Randomized phase 1	NCT00344539	CpG-ODN 7909 in combination with AMA1-C1/Alhydrogel® (experimental malaria vaccine)	1 dose: 564 µg per injection. (0.0075 mg/kg)	3 IM injections, 1 injection every 4 weeks.	Drug was well tolerated with mild reactions such as local and systemic adverse events.	Jan 2007	Mullen et al. (118)
Bacillus anthracis (Anthrax) Infection	Randomized Double-blind placebo-controlled phase 1	NCT01263691	CpG-ODN 7909 in combination with BioThrax (FDA-licensed anthrax vaccine)	1 dose: 0.5 mg per injection. (0.0067 mg/kg)	2 IM injections, 1 injection every 2 weeks.	Drug was well tolerated with mild injection-site reactions.	Jun 2012	Hopkins et al. (119)
Hepatitis B Virus (HBV) infection	Randomizedobserver–blindedactive–controlledphase 3	NCT02117934	HBsAg-1018 (CpG-ODN 1018 in combination with epatitis B surface antigen (HBsAg))	1 dose: 3 mg per injection. (0.04 mg/kg)	2 IM injections, 1 injection every 4 weeks.	Drug was well tolerated with non-reported side-effects.	Oct 2015	Jackson et al. (120)
Mycobacterium tuberculosis (Tuberculosis) infection	Randomized placebo-controlled phase 1	NCT03255278	GamTBvac: CpG-ODN 2216 in combination with Ag85a and ESAT6-CFP10 *M. tuberculosis* antigens	3 doses: 37.5 µg, 75 µg and 150 µg per injection. (0.0005 mg/kg, 0.001 mg/kg and 0.002 mg/kg respectively)	2 SC injections, 1 injection every 2 months.	Drug was well tolerated with mild injection-site reactions.	Dec 2017	Vasina et al. (121)

*VPL, virus-like particles; HDM, house dust mite; SC, subcutaneous; IV, intravenous; IM, intramuscular; IT, intratumoral; §CpG-ODN G10 represents 20% of the CYT003-QbG10. *75kg mean body weight was considered to calculate the dose.*

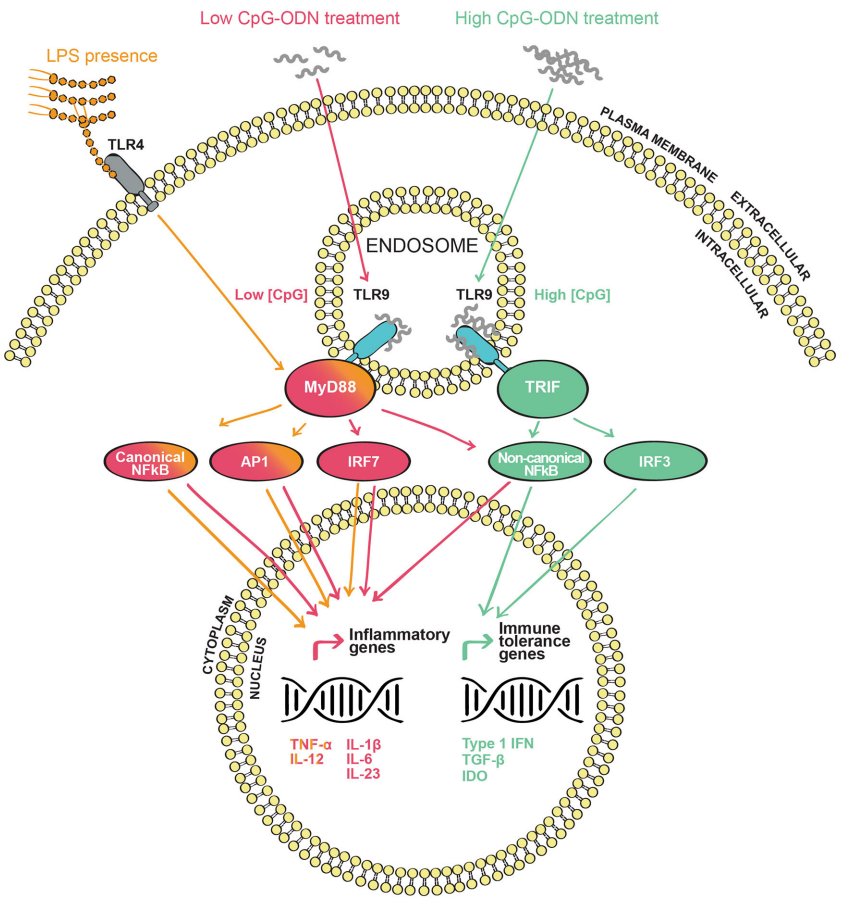

FIGURE 1 | Toll-like receptor 9 (TLR9) signaling pathway(s) upon CpG oligodeoxynucleotides (CpG-ODN) stimulation: CpG-ODN is internalized through endosomes where it interacts with the endosomic domain of TLR9. In the subsequent signaling cascade, the cytosolic domain of TLR9 interacts with the adaptor proteins MyD88 or TRIF in the cytosol. Depending on the adapter engaged, a different signaling cascade occurs. MyD88 signaling (red) is triggered when TLR9 is agonized by a low CpG-ODN concentration. This cascade leads to the induction of the canonical and non-canonical NF-κB pathways, AP1 and IRF7 transcription factors, inducing inflammatory mediators such as TNF-α, IL-12, IL-1β, IL-6, and IL-23. TRIF signaling (green) is triggered when TLR9 is agonized by a high concentration of CpG-ODN. In contrast to the MyD88 signaling pathway, the TRIF cascade leads to the activation of the non-canonical NF-κB and IRF3. These transcription factors will induce the production of type 1 IFN and anti-inflammatory molecules such as TGF-β and IDO. When LPS is present (orange), TLR4 and TLR9 signals are combined to engage MyD88 leading to an enhanced inflammatory reaction by the increased production of IL-12 and TNF-α.

demonstrated that TLR9 signals through TRIF when a high concentration of CpG-ODN is applied to pDCs, while the classical MyD88 adaptor is engaged by TLR9 when a lower concentration of CpG-ODN is used (84) (**Figure 1**). Interestingly, the divergence in TLR9 downstream signaling implies a differential gene expression signature. On the one hand, the MyD88 transduction pathway triggered both the canonical NF-κB (IKKβ-dependent) and non-canonical NF-κB (IKKα-dependent) pathways, leading to the expression of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), IL-1β, IL-12, IL-6 and IL-23. On the other hand, TRIF downstream signaling activated exclusively the non-canonical NF-κB pathway and IRF3, leading to the expression of anti-inflammatory mediators such as transforming growth factor beta (TGF-β) and indoleamine 2,3-dioxygenase (IDO) (145) (**Figure 1**). This research reveals a new concept, in which TLR9 can be differentially activated depending on the ligand concentration. The distribution of TLRs on human peripheral blood mononuclear cells is variable. While TLR9 is not expressed in some human immune cells such as neutrophils and monocytes, it has a high expression in pDCs and B cells, and it is found to a lesser extent in NK cells and T cells (146). In the mouse, TLR9 exhibits similar expression and activation patterns as in humans (146–148) with subtle differences. For instance, and according with their lack of TLR9 expression, monocytes from peripheral human blood are not activated by CpG-ODN (149). However, mouse monocytes express TLR9 and respond to CpG-ODN stimulation by engaging the NF-κB pathway and the subsequent production of cytokines such as TNF-α (148, 150). With regard to the organs relevant for allergic airway disease, human and mouse alveolar macrophages express TLR9 (151).

TABLE 4 | CpG classes: sequences, structure, interaction with Toll-like receptor 9 (TLR9), and response induced in immune cells.

CpG-ODN type	Sequence example	Structure	Effect on Immune cells
A-class (D-type)	GGTGCPuPy**CG**PuPyGCAGGGGGG	Single CpG motif flanked by a palindromic sequence, plus a 3' Poly-G end. Mixed phosphodiester/phosphorothioate backbone.	• IFNγ production by NK cells (83)[§] • Lytic activity by NK cells (128)[§#] • Type 1 IFN production by pDC (85, 129, 130)[§#]
B-class (K-type)	AT**CG**ACTCTCGAG**CG**TTCTC	Multiple CpG motifs. No palindromic sequences. Phosphorothioate only backbone.	• IgM, IL-6 production by B cells and B cell proliferation (83, 131)[§] • Type 1 IFN production by pDC (85)[§] • pDC maturation (130)[§] • Th1 response induction (85)[§] • NK cells activation (85)[§] • Production of TGF-β and IDO by pDC (84, 132)[§#] • Tolerance induction by pDC (77, 84, 132)[§#]
C-class	T**CG**T**CG**TT**CG**AA**CG**A**CG**TTGAT	Multiple CpG motifs and one palindromic sequence. Phosphorothioate only backbone	• IL-6 production by B cells (131, 133)[§] • B cell activation and proliferation (134)[§] • Type I IFN in pDC (130)[§] • pDC maturation (130)[§]
P-class	T**CG**T**CG**A**CG**AT-**CG**G**CG**C**GCG**C**CG**	Multiple CpG motifs flanked and two palindromic sequences. Phosphorothioate only backbone.	• Type I IFN by PBMCs (135)[§] • Increased plasma levels of type 1 IFN and IL-6 *in vivo* (135)[§]

Pu, purine nucleotide; Py, pyrimidine nucleotide; Bold, CpG motifs; Underlined, palindrome sequences; PBMCs, peripheral blood mononuclear cells. #In mouse, §In human.

However, only human but not mouse resident lung DCs show TLR9 expression (152, 153).

Given all these indications, CpG-ODN may induce slightly different immune responses between mice and humans (151, 154). Moreover, optimal CpG-ODN sequences that agonize TLR9 differ among species (127, 131, 155). However, the effect of TLR9 activation remains essentially unchanged between species since the afore-mentioned downstream transduction elements are well conserved. Nonetheless, inter-species differences should be considered when applying CpG-ODN-based treatments in animal models, aiming at translation to humans (**Tables 4** and **5**).

CpG-ODN Effect on Immune Cells

CpG-ODN influences many immune cell types, especially APCs such as DCs and macrophages. Dendritic cell precursors express various TLRs, among which is TLR9 (149), thus enabling their activation by CpG-ODN (159). As pDCs express high levels of TLR9, their response to CpG-ODN has been studied in detail. CpG-ODN enhances pDC migration to lymph nodes and stimulates their production of cytokines such as IL-6, TNF-α, type 1 interferons and granulocyte-macrophage colony-stimulating factor (GM-CSF) (160). Moreover, CpG-ODN induces pDCs to express several co-stimulatory surface molecules such as CD40, CD54, CD80, CD86 (85, 161–163). Very interestingly, unmethylated CG motifs have also been shown to promote an anti-inflammatory phenotype in pDCs. CpG-ODN-treated pDCs can induce $CD8^+$ Tregs that express immune inhibitory molecules such as LAG3 and CTLA4 (164). Similarly, pDCs stimulated with CpG-ODN can prime naïve T cells to differentiate into $CD4^+$ $CD25^+$ Tregs that produce IL-10 and TGF-β (77) (145). Langerhans cells express TLR9 and migrate upon CpG-ODN intradermal injection in mice (165). Finally, a subpopulation of splenic DCs was capable of producing a potent IDO signal upon CpG-ODN IV injection, thereby acquiring a suppressive capacity similar to Tregs (166). Interestingly, it has also been shown that pDCs stimulated with C class CpG-ODN, can directly license human B cells into plasma cell differentiation and antibody production in a T cell independent way (167). B cells are also greatly influenced by CpG-ODN. Indeed, CpG-ODN is a strong B cell mitogen, induces them to produce IL-6 and IgM (127, 168), and boosts their activation and differentiation during Ig production (169). Along the same line, B cells stimulated with CpG-ODN change their Ig profile and produce different antibodies, an effect being dependent on TRL9 and MyD88 signaling (87). NK cells can react through direct or indirect signals upon CpG-ODN stimulation. Certain studies showed that NK cells cannot respond to CpG-ODN directly, but are rather triggered indirectly through soluble factors produced by CpG-ODN-activated pDCs (146). By contrast, other studies support the fact that CpG-ODN can directly induce NK cells to produce IFN-γ and increase their activity (128, 170, 171). Since T cells express very low levels of TLR9, CpG-ODN is assumed not to stimulate these cells directly. Similarly to NK cells, T cells are rather engaged *via* signals transmitted by CpG-ODN-activated pDCs (146). However, although it seems rather evident that there is no direct effect of CpG-ODN on T cells, some research points out that CpG-ODN could stimulate T cells without any intermediate, but in a TLR9 and MyD88 independent fashion (172). This was supported by another study, showing that purified human T and NK cells produce IFN-γ upon direct CpG stimulation (173). In addition, one study has shown MyD88 to be essential for direct CpG-ODN stimulation in $CD4^+$ T cells (174).

In summary, each CpG-ODN type has a wide range of effects on various immune cells, among which pDCs and B cells are the most relevant ones. With regard to the use of CpG-ODN as an adjuvant for AIT, B-class CpG-ODN stands out for its capacity to induce a tolerogenic phenotype in pDCs that can further educate Tregs and protective antibody responses (**Table 4**).

TABLE 5 | Sequence and type of CpG oligodeoxynucleotides (CpG-ODN) used in pre-clinical studies (**Table 2**) and clinical trials (**Table 3**).

CpG-ODN name or study	Species used	Sequence	CpG-ODN type/structure
A-Class			
G10 (156)	Human	5′-GGGGGGGGGGGGACGATCGTCGGGGGGGGGG-3′	A-class
CpG-ODN 2216	Human	5′-GGGGGACGATCGTCGGGGGG-3′	A-class
Fanucchi et al. (101)	Mouse	5′TGACTGTGAACGTTCGAGATGA-3′	A-class
Sabatel et al., 2017 (76)	Mouse	5′-TCCATGACGTTCCTGATGCT-3′	A-class
B-Class			
1018 ISS (CpG-ODN 1018)	Human and mouse	5′-TGACTGTGAACGTTCGAGATGA-3′	B-class
CpG-ODN 7909 = CpG-ODN 2006	Human	5′- TCGTCGTTTTGTCGTTTTGTCGTT -3′	B-class
Kline et al. (97), Jain et al. (100)	Mouse	5′-TCCATGACGTTCCTGACGTT-3′	B-class
Jahn-Schmid et al. (86)	Mouse	5′-ATCGACTCTCGAGCGTTCTC-3′	B-class
Sur et al., 1999 (98)	Mouse	5′-GCTAGACGTTAGCGT-3′	B-class
Peng et al. (99)	Mouse	5′-TCCATGACGTTCCTGACGTT-3′	B-class
CpG-ODN 1826	Mouse	5′-TCCATGACGTTCCTGACGTT-3′	B-class
CpG-ODN BL07S	Mouse	5′-GCGTCGGTTTCGGTGCTCAC-3′	B-class
CpG-ODN 1668 (79)	Mouse	5′-TCCATGACGTTCCTGATGCT-3′	B-class
Chang et al. (104)	Mouse	5′-TCCATGACGTTCCTGACGTT-3′	B-class
C-Class			
IMO-2125 (Tilsotolimod)	Human	5′-TCG*AACG*TTCG*-X-G*CTTG*CAAG*CT-3′	C-class
SD-101	Human	Not described.	C-class
CpG-ODN 2395	Mouse	5′-TCGTCGTTTTCGGCGCGCGCCG-3′	C-class
Others			
MGN1703 (Lefitolimod)	Human	CTAGGGGTTACCACCTTCATTGGAAAACGTTCTTCGGGGC GTTCTTAGGTGGTAACCC by dimer-circularization	Double-stem loop immunomodulators (dSLIM) (157, 158)
Fonseca et al. (105)	Mouse	Not described.	Not described.

CPG-ODN AS A TOLERANCE INDUCER IN ALLERGEN-SPECIFIC IMMUNOTHERAPY

CpG-ODN Induces Allergen-Specific Immune Regulation Through DCs

As discussed above, DCs and especially pDCs, produce IL-10, TGF-β and IDO upon CpG-ODN stimulation (47, 77), which makes them extremely efficient Treg inducers (**Figure 2**). As innate immunity is crucial to induce a tolerance response of the adaptive immune system, the modulation of APCs such as pDCs and of early-activated adaptive immune cells, such as B cells, is consequently a promising tool for improving AIT. Indeed, Volpi and collaborators (84) showed that CpG-ODN has a dual impact on the immune system. While low CpG-ODN concentrations induced inflammation, high concentrations induced immune regulation and tolerance. Their work suggests the new concept that the same TLR ligand can instruct two distinctive measurable effects on the immune system depending on its concentration/dose (**Figure 1**). Indeed, when CpG-ODN has been used as an adjuvant in vaccination, where a cytotoxic or inflammatory response is needed, the doses used have been low (**Figure 3** and **Table 3**). However, when CpG-ODN has been proven successful in inducing tolerance and treating allergy in animal models, the doses employed have been high (**Figure 3** and **Table 2**). The pivotal immune cells in the research of Volpi et al. were pDCs. The classical role attributed to pDCs in the immune system is the promotion of viral defense through the production of type 1 IFN as a result of TLR danger signal activation by viral genetic material (160). As aforementioned, pDCs have been shown to promote tolerance through several soluble factors and membrane signaling proteins. Although it might seem contradictory at first, several lines of research clearly showed that the stimulation of pDC through CpG-ODN leads to the induction of tolerance (**Figure 2** and **Table 4**). This could be the result of a differential type 1 IFN response triggered by a full-fledged viral infection versus the sole activation of TLR9 through high doses of CpG-ODN. To make a further distinction between pDC-derived type 1 IFN effects from these differential stimulation sources would require additional research. Further evidence in line with their potential for tolerance induction, human pDCs show minimal *NLRP1* and *NLRP3* expression, demonstrating their low capacity for inflammasome activation (175). Accordingly, they can hardly induce adverse inflammatory responses, but are rather programmed to induce tolerance. Indeed, a $CD5^+$ $CD81^+$ pDC subset was shown to produce no type 1-IFN upon CpG-ODN stimulation, but to induce a strong Treg differentiation (78). Similarly, it has been shown that pDCs primed with CpG-ODN require direct cell contact to induce $CD4^+$ $CD25^+$ Treg cells (77). Classical dendritic cells (cDCs) have also been shown to promote tolerance through Treg induction in both mice and humans (**Figure 2**). In more detail, a subset of type 2 classical dendritic cells (cDC2a) displays a higher expression of TLR9 and has been shown to induce Treg cell differentiation upon CpG-ODN stimulation (176). Indeed, DCs have been proposed and used to reverse immune-based diseases toward immune homeostasis through tolerance induction (177, 178). Similar to allergy, inflammatory bowel disease (IBD) lacks appropriate immune regulation (179). Studies have shown that CpG-ODN ameliorates IBD hallmarks by inducing tolerance through DCs (180, 181). In accordance with Volpi et al (84)., it was recently shown that AIT using the major cat allergen Fel d 1 together with high doses of CpG-ODN

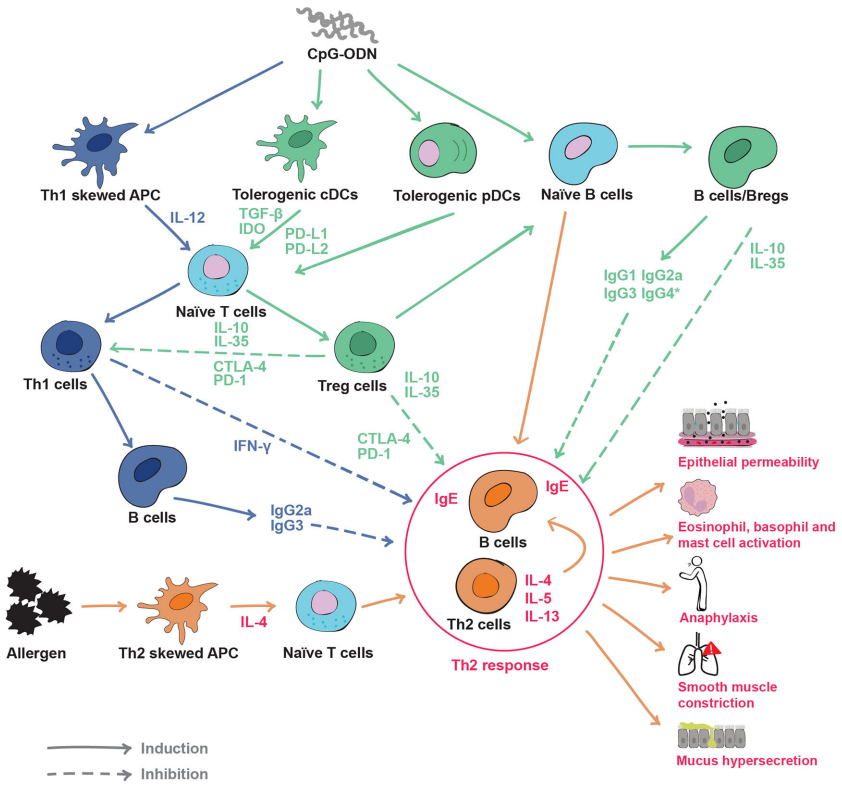

FIGURE 2 | Effects of CpG oligodeoxynucleotides (CpG-ODN) on the immune system and inhibition mechanisms of the Th2 response: In an allergic immune environment (orange and red), allergens skew APCs toward the production of cytokines such as IL-4, prompting naïve T cells into Th2 cells. Th2 cells produce Th2 cytokines (IL-4, IL-5, IL-13) and induce B cells to produce immunoglobulin E (IgE), causing the hallmarks of the allergic disease such as increased epithelial permeability, effector cell activation, anaphylaxis, smooth muscle contraction, mucus hypersecretion etc. CpG-ODN can have different effects on APCs. On one side, it can promote a Th1-like response (blue) by skewing APC to produce IL-12 and then derive naïve T cells to IFN-γ producing Th1 cells. Th1 cells induce B cells to produce neutralizing antibodies such as IgG2a and IgG3. On the other side, CpG-ODN can skew APCs classical dendritic cells (cDCs) and plasmacytoid dendritic cells (pDCs) toward a tolerogenic phenotype (green). Tolerogenic APCs, cDCs and pDCs produce immune regulatory soluble factors such as TGF-β and indoleamine 2,3-dioxygenase (IDO), and express immune modulatory surface molecules like programmed death ligands 1 (PD-L1) and PD-L2. Tolerogenic APCscDCs and pDCs derive naïve T cells to Tregs, initiating an immune regulatory response. CpG-ODN can also engage directly B cells and skew them into a regulatory phenotype (Bregs). Bregs secrete the regulatory cytokines IL-10 and IL-35, as well as neutralizing IgG isotypes. The Th1 response can inhibit Th2 and allergy responses through the production of interferon-γ (IFN-γ) and neutralizing antibodies. However, Treg and Breg cells utilize a wide range of regulatory molecules from soluble IL-10 and IL-35, to the immune checkpoints CTLA-4 and PD-1 to suppress both Th2 and Th1 responses. Solid lines indicate induction, dotted lines indicate inhibition. *IgG4 refers to humans only.

reduces all disease hallmarks in a mouse model of allergic asthma. This was accompanied by an expansion of pDCs and their migration from the injection site to the periphery at early stages of the treatment (79).

CpG-ODN Induces T and B Regulatory Cells

With regard to immune tolerance induction, CpG-ODN was shown to reduce the Th2 response triggered by aluminum hydroxide when these two adjuvants were co-injected, an effect that was dependent on the activity of MyD88 and IL-10 (182). In agreement with this study, several other studies have pointed out that CpG-ODN is able to induce immune regulatory responses. Already in 1997, bacterial DNA containing unmethylated CG motifs was shown to induce IL-10 production in adherent mouse splenocytes (183). CpG-ODN has also been shown to promote other regulatory cytokines such as TGF-β 3 days after *in vivo* injection in a vertebrate species phylogenetically distant from humans, the fish gilthead seabream (184). TGF-β is crucial for immune regulation and it is one of the three key signals that induce Treg cell differentiation. Furthermore, it is known for its role in tissue repair after inflammation (185). Several research groups have tested different administration routes for CpG-ODN. Kim et al. (80) and Sabatel et al. (76) investigated the nasal installation (NI) route for CpG-ODN application in mice, a proxy for bronchial inhalation in humans. In the first study, the authors showed how pre-treatment with CpG-ODN (medium-high dose: 0.15 mg/kg, **Figure 3**) prevented cockroach-induced allergic asthma in a mouse model (169). The authors found increased levels of IL-10 in lung lysates which was attributed to an increase in the $CD4^+$ $FoxP3^+$ Treg population. In the second study, Sabatel and colleagues showed as well that NI of CpG-ODN (high dose: 2.5 mg/kg, **Figure 3**) drastically reduced allergic airway inflammation

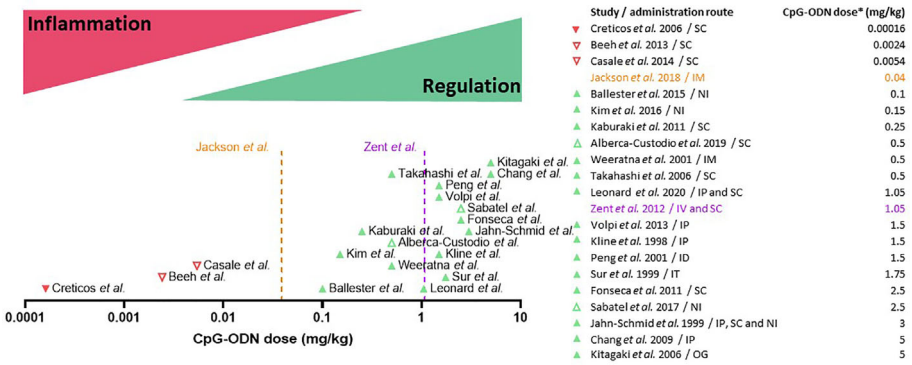

FIGURE 3 | Dual dose effect of CpG oligodeoxynucleotides (CpG-ODN) in AIT: CpG-ODN at low doses triggers immune activation and inflammation, while at high doses it induces immune regulation and tolerance. Clinical (downward-pointing triangles) and pre-clinical studies (upward-pointing triangles) are placed on the X axis (CpG dose in mg/kg body weight, Log10 scale) according to the CpG-ODN dose equivalent used in each study. Unsuccessful or suspended clinical trials and drugs (red triangles) fall under the immune activation/inflammation range of CpG-ODN dosage, whereas most of the successful (green triangles) pre-clinical research studies fall under the regulation/tolerance range. The orange dotted line (Jackson et al., 2018) indicates the dose of B-class CpG-ODN employed to boost the immune response in an FDA-approved vaccine for hepatitis B virus infection. The purple dotted line (Zent et al., 2012) indicates the maximum dose of B-class CpG-ODN that was well-tolerated in humans. Open triangles indicate non-type B CpG-ODN. The table on the right indicates the approximate dose equivalent of CpG-ODN used in each study. *For clinical trials, an average body weight of 75 kg was considered to calculate the CpG-ODN dose. For pre-clinical murine studies, an average body weight of 20 g was considered. IP, Intraperitoneal; NI, Nasal instillation; IT, Intratracheal instillation; ID, intradermal; IM, intramuscular; OG, oral gavage.

through the secretion of IL-10 (76). In this case, Tregs were not the source of IL-10, but the anti-inflammatory cytokine was secreted by regulatory macrophages recruited after CpG-ODN NI treatment. Both results are not mutually exclusive and could be explained by an initial IL-10 production by regulatory macrophages followed by a successive differentiation of Tregs and production of IL-10. Moreover, after completion of CpG-ODN-based AIT, differentially regulated and de-novo-expressed Tregs were found in the spleen of CpG/AIT-treated mice (79), including Treg subtypes which are known to specifically suppress Th2 responses (186, 187). In addition to Tregs, Bregs are also of crucial importance in the success of AIT (40). As B cells express higher levels of TLR9 compared to other immune cells, they are strongly activated by CpG-ODN. Adoptive transfer of splenocytes from mice treated with CpG-ODN conferred resistance to allergic inflammation in a mouse model (188). The authors found that the reduction of the allergic response was dependent on IL-10 producing follicular B cells ($B220^+$ $CD19^+$ $CD23^+$ IgM^+ $CD40^+$ $MHCII^{hi}$), indicating that CpG-ODN-stimulated B cells have a role in promoting immune tolerance in the context of allergy. Furthermore, it has been shown by Ticha et al. that IL-10 producing B cells induced by CpG-ODN have a distinct expression of tumor necrosis factor receptor 2 (TNFR2) (82), which could also be confirmed in the murine CpG-ODN-based AIT model where B cells expressed higher levels of TNFR2 at the injection site (79).

CpG-ODN Induces Protective Immunoglobulins and Improves Epithelial Integrity

As explained above, one of the important mechanisms of AIT in reducing allergic symptoms is the production of protective immunoglobulins by B cells such as IgGs and IgA (46). Indeed, B cell stimulation with CpG-ODN induces the production of neutralizing immunoglobulins such as IgG2a, IgG2b, IgG3 and IgA in mice (87–89, 102). Furthermore, CpG-ODN inhibits IgE production *in vivo* (89, 90). CpG-ODN has also been shown to enhance protection from allergic diseases through co-activation of non-immune cells. For instance, CpG-ODN improves tight junctions in airway epithelial cells, a cell type playing a crucial role in the homeostasis of lungs and the prevention of airway sensitization by allergens (189–191).

CpG-ODN Induces Th1 Responses

As introduced in the previous section, CpG-ODN has been used as an adjuvant in AIT formulations to target the innate immune system and therefore increase therapeutic effects of AIT. Among others, CpG-ODN has been described to induce a Th1 immune response. Indeed, the first assays where CpG-ODN was used *in vitro* reported production of IFN-γ and IL-12, the canonical cytokines produced in Th1 environments (170, 173, 192). Interestingly, *in vivo* preventive allergy treatment with CpG-ODN has been shown to induce an immutable IFN-γ-dependent Th1 response, which prevented the establishment of a subsequent Th2 allergic response (98). Comparably, when mice are primed with the hepatitis B major surface antigen (HBsAg) using aluminum hydroxide as adjuvant, a Th2 response is induced, but when the same mice are subsequently treated with CpG-ODN and HBsAg, the CpG-ODN boost is able to induce a Th1 response, overwriting the pre-established Th2 response (88). Accordingly, the induction of Th1 responses has been proposed in the past as a possible cellular immune mechanism to treat allergic diseases (193, 194).

CpG-ODN-Based Allergen Specific Immunotherapy Considerations

As depicted above, different routes of administration of CpG-ODN such as intraperitoneal (IP), subcutaneous (SC),

epicutaneous (EPI) or NI have been applied to ameliorate allergic disease phenotypes in murine models and humans (**Tables 2** and **3**). Because of this disparity in the approaches with varying outcomes, additional studies are needed to find the optimal route of administration to be used in combination with the most effective dose of CpG-ODN and the most advantageous AIT treatment regimen. In common clinical practice, the subcutaneous route of injection is preferred. However, non-invasive delivery routes are not to be neglected, since AIT has been proven to be successful using a sublingual approach *via* sublingual immunotherapy (SLIT) (195). SLIT has been shown to engage potential tolerance inducing immune cells such as DCs and pDCs or Tregs in the oral mucosa (196, 197). Hence, the usage of a CpG-ODN-based AIT *via* other administration routes such as SLIT would increase its therapeutic value. Although both, Th1 and Tregs are known to suppress allergy (186) (**Figure 2**), it is undoubtedly preferable to induce a Treg/Breg reaction than a Th1 response since Th1 cells have been associated with undesirable side effects such as inflammation or even with autoimmunity (198). Because of these considerations, immune tolerance induction should be preferred to treat allergic diseases over induction of Th1 cells to replace the existing Th2 response (**Figure 2**). Some of the CpG-ODN-based treatment strategies presented in this review use CpG-ODN as an immunomodulator in an allergen-free formulation. This creates an unspecific tolerance induction which is usually not long-lasting due to the lack of immune memory. On the contrary, when CpG-ODN is used together with the allergen, the resulting tolerance induction is allergen-specific and long-lasting, as it is supported by immune memory (31, 40). Accordingly, although treatments for allergic diseases using only CpG-ODN seem to confer a certain level of short-term immune tolerance (76, 104, 105) (**Table 2**), CpG-ODN-based AIT formulations should be preferred in order to induce specific and durable tolerance to the allergen(s). An overview of the literature would also be strongly in favor of using CpG-ODN with allergen as a prophylactic vaccination-like treatment for allergic diseases by inducing either a Th1 or a Treg response (199), thus preventing the subsequent development of a pro-allergic Th2 environment. However, this does not reflect the situation in the clinic, where allergic patients, who are already sensitized and present with a pre-established symptomatic disease, consult the clinicians. Therefore, a prophylactic treatment for allergic diseases represents a future scenario for those in the general population, who are at high risk of developing allergic disease. Based on all current data, CpG-ODN-based therapies for allergy have the potential to be successful as active treatment in AIT when used in a novel formulation. Therefore, this should be the immediate next step to follow when designing novel therapeutic strategies for allergic diseases using CpG-ODN. Interestingly, it has been shown that when pDCs and B cells are stimulated with CpG-ODN, their TLR9 expression is downregulated within 12 h (167), probably due to negative feedback. Consequently, repeated CpG-ODN injections in a short period of time would trigger a much lower effect. Similarly, key cells such as B cells, DCs and macrophages change their TLR9 expression according to the circadian rhythm in mice, showing a higher expression coinciding with the mouse active phase (200). According to this knowledge, a timely spaced injection schedule during the active human phase would be preferable to maximize TLR9 signaling in critical immune cells, thus enabling the desired effect of the adjuvant.

The analysis of the literature discussed above, which is graphically summarized in **Figures 2** and **3**, encouraged us to propose that CpG-ODN used in the adequate conditions and concentrations might be a strong inducer of a tolerogenic immune response for allergy and other inflammatory disorders, acting by modulation of various layers of the immune system (**Figure 2**). Other licensed adjuvants such as $Al(OH)_3$ or MPL have been used and tested in the context of AIT. However, they induce pro-inflammatory responses such as inflammasome activation and Th1 responses (**Table 1**) that can revert the underlying Th2 immune phenotype but lack the tolerance promoting properties of CpG-ODN.

CPG-ODN AS AN ADJUVANT FOR ALLERGEN SPECIFIC IMMUNOTHERAPY

Reconsidering CpG-ODN for Allergen-Specific Immunotherapy

According to the studies discussed in the previous sections, CpG-ODN used in the appropriate conditions induces tolerance in the immune system through various cells and molecular mechanisms. With this systematic review, we intend to support the reconsideration of CpG-ODN as an adjuvant for AIT formulations with the purpose to enhance its therapeutic effects and overcome the aforementioned challenges of AIT such as side effects and burdensome treatment schedules. Indeed, CpG-ODN has shown potential to alleviate the burden of allergic diseases through various immune processes. Multiple lines of evidence have shown that CpG-ODN exerts its immune modulating effects mainly through two complementary mechanisms in many pre-clinical studies (**Table 2**). On the one hand, the induction of an immune regulatory response in the form of tolerogenic pDCs, Breg and Treg cells. On the other hand, the generation of allergen-specific neutralizing antibodies that block allergen binding by specific IgE (sIgE) and thus inhibit allergy effector cells such as eosinophils, basophils or mast cells (**Figure 2**). These immune-modulatory properties of CpG-ODN would ultimately promote immunological and clinical tolerance to the allergen.

TLR-Ligand Interference as a Consideration in the Design of CpG-ODN-Based Allergen-Specific Immunotherapy

A crucial aspect in the design of CpG-ODN-based AIT formulations is the possible interference with other immunostimulatory PAMPs, especially with other TLR ligands such as LPS. Indeed, the immune system is a complex network of receptor-ligand interactions and connected intracellular signaling pathways. Many TLR downstream signaling molecules, particularly kinases, are

shared between the TLR family members (125, 143). Consequently, a secondary TLR-stimulation, in parallel to TLR9 activation by CpG-ODN, could alter the outcome and quality of TLR9 signaling. In the case of CpG-ODN employment as an adjuvant for AIT, the desired result is the induction of immune tolerance without inducing an inflammatory response. The presence of residual LPS would activate the TLR4 signaling pathway, which competes with concurrent TLR9 signaling for downstream signaling molecules, thus biasing and deviating the tolerogenic pathway induced by CpG-ODN. Indeed, it has been shown that co-activation of TLR9 and TLR4 induces a strong inflammatory signal in form of TNF-α and IL-12 secretion (201) (**Figure 1**). Among other reasons, such an interference by low amounts of residual LPS (**Figure 1**) in the natural ragweed allergen Amb a 1 could explain why previous AIT clinical trials using CpG-ODN were unsuccessful (26, 111). However, with the appropriate knowledge from preclinical studies, one has the ability to influence and activate only specific immune cells and pathways to achieve the desired immunotherapeutic precision yield.

High Doses of CpG-ODN Induce Immune Regulation

Another essential factor in CpG-ODN formulations is the dose. As outlined before, to achieve tolerance induction, high CpG-ODN concentrations and doses are needed (84). Indeed, a principal pattern can be extracted when systematically analyzing the numerous clinical trials (**Table 3**) and pre-clinical studies (**Table 2**) in which CpG-ODN has been used in the context of allergic diseases (**Figure 3**). On the clinical side, the maximum CpG-ODN dose used for allergy treatment in humans equals to approximately 0.0054 mg/kg per injection, repeated over 7 injections (113). However, this clinical trial showed no benefit in patients, and was prematurely terminated with no further follow-up. Other clinical trials in humans used even lower dose equivalents of CpG-ODN adjuvant (111, 112). In contrast to human studies in the allergy field, CpG-ODN doses used in preclinical allergy studies were markedly higher and more successful. Indeed, many of the animal research studies with positive findings used at least a 10-fold higher CpG-ODN dose per injection compared to the less successful clinical trials (**Table 3** and **Figure 3**). The fact that lower CpG-ODN doses were employed in human clinical trials could explain the lack of CpG-ODN activity as AIT adjuvant. The only marketed pharmaceutical product currently using CpG-ODN is a subunit vaccine for HBV that has recently received approval by the FDA (120). Vaccines necessitate firm and sustained immune activation, sometimes also referred to as inflammation, to induce protection against pathogens. Interestingly, the new HBV vaccine utilizes a low dose of CpG-ODN to induce an enhanced immune response against HBV. However, this relatively low dose of 0.04 mg/kg CpG-ODN per injection, which was shown by Jackson et al. (120) to display superior vaccination adjuvant activity over aluminum hydroxide for HBV prevention, is still higher than the CpG-ODN dose equivalents previously applied in clinical trials to treat allergy (**Figure 3**). In contrast, CpG-ODN has been successful in reducing allergy burden in animal models when used at higher doses (**Table 2**). Based on the analysis presented (**Figure 3**), we suggest to reconsider CpG-ODN as an AIT adjuvant, but at higher doses than previously applied in humans, to treat Th2-/IgE-mediated allergic diseases. Intriguingly, the maximum dose of well-tolerated B-type CpG-ODN after intravenous injections in humans as shown by Zent et al. is in the range of tolerance-inducing CpG concentrations reported by others (79, 84, 114) (**Figure 3**).

CpG-ODN to Treat Other Non–IgE-Mediated Immune Diseases

Furthermore, one could think of applying the immune regulatory properties of CpG-ODN not only in allergy therapy, but also to treat other diseases in which the immune system is dysregulated such as multiple sclerosis (MS), where the induction of antigen-specific immune tolerance would be needed. As similar or even identical immune tolerance checkpoint mechanisms have been identified across immune-mediated diseases (202, 203), the co-administration of the self-antigen myelin oligodendrocyte glycoprotein (MOG) together with high doses of CpG-ODN could potentially reduce the autoimmune reaction observed in the experimental autoimmune encephalomyelitis (EAE) mouse model, setting the basis for an antigen-specific tolerance induction therapy in MS and other autoimmune diseases.

Route of Administration for CpG-ODN-Based AIT

As aforementioned, CpG-ODN-based AIT has been applied to animal disease models using a wide variety of routes of administration, such as IP, NI, subcutaneous (SC), intradermal (ID), intramuscular (IM) and oral gavage (OG) routes (**Table 2**). While the dosage appears to be key in order to achieve immune tolerance in the context of CpG-ODN-based AIT, the role of the administration procedure is less clear. The application of the therapy *via* a particular method of delivery to treat a tissue-specific allergy phenotypes, for example intranasal or intrabronchial administration to treat airborne allergies, is an interesting point to consider. Indeed, some animal studies successfully treat airway allergy using intranasal application of CpG-ODN-based AIT (76, 80, 108) (**Table 2**). However, a comprehensive analysis of the pre-clinical studies (**Table 2**) suggests that the route of administration does not always have to target the tissue or organ affected by the allergy in order to achieve its therapeutic effect. Indeed, many of the animal studies described in **Table 2** treat respiratory allergies through an IP, SC or ID administration (79, 103–106). Furthermore, in clinical practice the SC route has proven its efficacy to treat airway allergies, indicating that the administration method can be independent of the allergy phenotype to be cured. Nevertheless, novel delivery methods such as sublingual or intrabronchial therapies can result in improved efficacy and ease of application in clinical studies (23, 26, 91, 96, 196), suggesting that an optimized route of application could also improve CpG-ODN-based AIT. However, since the

dose of CpG-ODN is the primary determinant for treatment efficacy, studies would need to be fine-tuned regarding the dosage for each novel route of administration without compromising safety.

CPG-ODN – SAFETY CONSIDERATIONS AND OTHER APPLICATIONS

CpG-ODN as an Adjuvant for Pathogen and Cancer Vaccination

Besides its role as immunomodulatory agent in the treatment of asthma or allergic rhinitis and as adjuvant in AIT, CpG-ODN has been applied in other sectors of pharmaceutical research, mainly as adjuvant for vaccines against pathogens, and as immune-enhancing drug for cancer treatment (204). CpG-ODN has been used to confer protection through vaccination against a wide variety of pathogens (205), including bacteria (199). Indeed, CpG-ODN can induce pathogen-specific IgM enhancing phagocytic activity against *S. aureus* (206). Furthermore, it has been tested with success in a phase I clinical trial of a BCG multi-subunit vaccine against *M. tuberculosis* (121) (**Table 3**), where relatively low CpG-ODN dose equivalents of 0.001 mg/kg (75 μg per injection) and 0.02 mg/kg (150 μg per injection) induced robust IFN-γ and IgG responses. As mentioned above, CpG-ODN can activate and enhance NK cell function, a characteristic that some authors have associated with enhanced anti-viral properties. For instance, CpG-ODN has been shown to effectively fight alphavirus encephalitis in neonatal mice (162). Moreover, CpG-ODN has also been shown to be efficacious as an adjuvant in vaccines against viruses, for example, by generating a humoral protective response to the HBV surface antigen HBsAg (88), which subsequently lead to the development of an FDA-approved vaccine against HBV that was superior compared to an established aluminum hydroxide-based HBV vaccine (120). Similarly, pre-clinical models of vaccination against viruses have also used CpG-ODN as adjuvant in their formulations, such as in vaccination studies targeting the influenza virus strain H1N1 (140). With regard to cancer treatment, CpG-ODN has been extensively used for its immune enhancing properties (136). The first reported case where bacteria were used against cancer was published in 1893 by Dr. William Coley using live bacteria injected directly into tumor tissue (207). Later, Dr. Coley utilized heat-killed bacteria with similar effects (208). These observations sparked the research on bacterial compounds such as CpG-ODN for cancer treatment (209). It has been shown that CpG-ODN potentiates both innate and adaptive anti-tumor immunity, mainly by enhancing NK cell and CD8 T cell cytotoxic activity (81, 210). Among other successful applications in the cancer field (**Table 3**), CpG-ODN was used as prophylactic treatment for reducing brain metastasis through microglia activation (211), or in combination with M362, a TLR6 ligand, with which it potentiates CD8 T cell response against breast cancer cells (212).

Safety Considerations Regarding CpG-ODN

Although CpG-ODN showed a good safety profile, there have been some concerns regarding the potential triggering of autoimmune diseases by this TLR9 agonist (136, 213). For instance, CpG-ODN has been associated with the induction of arthritis *in vivo* when injected into animal joints (214, 215). Similarly, when bacterial DNA containing CG motifs is injected interstitially into the meninges of rats, signs of meningeal inflammation appear on the histopathologic level (216). However, the injection of CpG-ODN directly into articulations or meninges is by no means foreseen in a CpG-ODN-based AIT. Furthermore, neither of these effects have been observed in clinical trials using CpG-ODN (**Table 3**) nor in the individuals that received the approved CpG-ODN-adjuvanted HBV vaccine. Another possible safety concern is that CpG-ODN could activate autoreactive B cells to produce double stranded DNA (dsDNA) autoantibodies and cause autoimmune diseases similar to systemic lupus erythematosus (SLE). However, only very few cases of anti-dsDNA antibodies or other signs of autoimmune disease have been reported in subjects receiving CpG-ODN (136) with no clear link to the adjuvant administration. Similarly, and although CpG-ODN was suspected to exacerbate and maintain dextran sulfate-induced colitis in a mouse model of IBD (217, 218), a later publication found CpG-ODN to be protective for IBD (180).

Safety Considerations Regarding High Dose CpG-ODN

Like many other drugs and compounds, CpG-ODN used in very high doses (>2.4mg/kg) and repetitive injection schemes (daily injections) showed toxicity in mice (219). However, in lower and more conventional dosages, CpG-ODN has been recognized to be a safe compound in humans with relatively benign effects in terms of toxicity (**Table 3**). Consequently, it has been recently approved by the FDA as adjuvant for a HBV vaccine (120). Some of the CpG-ODN-derived side effects described in clinical trials (**Table 3**) are probably caused by its rapid systemic distribution upon injection. Furthermore, all the clinical trials using CpG-ODN for allergy treatment rely on subcutaneous administration (**Table 3**), a route known for its systemic distribution of the compounds through capillaries and lymphatic vessels (220). This presents a challenge regarding possible mild, but tolerable side-effects, which needs to be addressed, since the tolerogenic effects of CpG-ODN are only observed at relatively high doses (**Table 2**, **Figure 3**). However, a high concentration of CpG-ODN does not necessarily have to be systemic and can also act locally to promote immune tolerance. According to *in vitro* experiments (84, 132), a local but high concentration of CpG-ODN should have the same effect on key immune cells such as pDCs and B cells, without causing systemic toxicity. Several pharmacological strategies can be employed to achieve a high local concentration, thus preventing a rapid systemic distribution of compounds. One solution is the usage of drug delivery matrices forming a depot and assuring a slow substance release, like hydrogels, nanoparticles or liposomes (221, 222).

When injected subcutaneously, these substances form a depot that encloses all the compounds of the AIT formulation. In case of a CpG-ODN-based AIT, such an approach would allow for a high local concentration of CpG-ODN, avoiding its rapid systemic release, thereby minimizing side effects, while allergens are also progressively released. Moreover, these drug delivery systems are composed of biocompatible and biodegradable compounds, which makes them safe and suitable as part of pharmaceutical products (223, 224).

Altogether, these data indicate that CpG-ODN can be used as an adjuvant in a variety of therapeutic strategies. Based on current knowledge, CpG-ODN-based AIT using high doses of CpG-ODN to induce tolerance would be a safe and potentially beneficial therapy for patients suffering from moderate or severe allergic diseases.

CONCLUSION

AIT has been used for almost 110 years and remains to this date the only disease-modifying treatment for allergic diseases that can provide allergy cure. It presents several advantages over symptomatic therapies such as its cost-effectiveness and long term tolerogenic effects. Despite being a successful therapy in many aspects, AIT still has unmet needs to be faced and solved. One of the most affordable, suitable, practical and promising strategies relies on the use of optimized adjuvants to boost the therapeutic effects of AIT. CpG-ODN has been used in the past as an adjuvant for AIT with limited success that could be explained by the use of rather low doses of CpG-ODN at that time, probably due to caution and safety concerns when applying it in humans. Based on newer evidence, CpG-ODN induces tolerance in high doses. Therefore, we argue for its reevaluation as a potentially beneficial adjuvant in AIT. We propose that doses between 0.5 and 1.5mg/kg, dependent on the administration route, should induce the desired immune tolerance toward the allergen with a minimal risk of adverse effects. Specifically, B-class CpG-ODN is capable to induce tolerance with very low unwanted reactogenicity, making it a strong candidate for further translation into the clinic. Indeed, by combining CpG-AIT and novel drug delivery systems, even higher doses of CpG-ODN could be tailored to be well tolerated, thus aiding to further improve the safety of CpG-ODN-based AIT. The route of administration is another important factor to be considered. Pairing the route of administration with the organ-specific allergy phenotype could be a promising strategy to enhance the tolerance-promoting capacity of CpG-AIT. Further research will help to unveil the regulatory immune mechanisms of CpG-ODN-based AIT and design better strategies to specifically target key immune cells such as pDCs and other APCs. Overall, CpG-ODN used in AIT has the potential to greatly benefit allergic patients as it represents a safe, effective and possibly curative approach for allergic diseases.

AUTHOR CONTRIBUTIONS

All authors contributed to the conception and writing of the manuscript as well as read and approved the final manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: MO reports personal fees from Allergy Therapeutics/Bencard, Great Britain/Germany; Thermo Fisher Scientific, Sweden; Siemens Healthcare Diagnostics, Germany; Hitachi Chemical Diagnostics, USA; and Hycor Diagnostics, USA outside the submitted work; and is Scientific co-founder of the academic biotech spin-offs PLS-Design GmbH, Hamburg, Germany and Tolerogenics SARL, Luxembourg. CL and MO report to be co-inventors on two patents pending on hydrogel-embedded oligodeoxynucleotides as tolerogenic adjuvant for subcutaneous immunotherapy and induction of allergen-specific Tregs prior to oral or sublingual immunotherapy of food allergy. LK reports receiving research grants from Allergy Therapeutics/Bencard, Great Britain/Germany; ALK-Abelló, Denmark; Allergopharma, Germany; ASIT Biotech, Belgium; AstraZeneca, Sweden, Biomay, Austria, Boehringer Ingelheim, Germany, Circassia, USA; Stallergenes, France; Cytos, Switzerland; Curalogic, Denmark; HAL, Netherlands; Lofarma, Italy; Mylan, USA; Novartis, Switzerland, Leti, Spain; ROXALL, Germany; GlaxoSmithKline (GSK), Great Britain; Sanofi, France and/or has served on the speaker's bureau or was consulting for the above mentioned pharmaceutical companies. LK is the current President of AeDA (German Society of Applied Allergology), Vice-President of the German Academy for Allergy and Environmental Diseases and Chair of EAACI ENT section.

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