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Novel strategies in immunotherapy for allergic diseases

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ABSTRACT

Conventional immunotherapy (IT) for optimal control of respiratory and food allergies has been fraught with concerns of efficacy, safety, and tolerability. The development of adjuvants to conventional IT has potentially increased the effectiveness and safety of allergen IT, which may translate into improved clinical outcomes and sustained unresponsiveness even after cessation of therapy. Novel strategies incorporating the successful use of adjuvants such as allergoids, immunostimulatory DNA sequences, monoclonal antibodies, carriers, recombinant proteins, and probiotics have now been described in clinical and murine studies. Future approaches may include fungal compounds, parasitic molecules, vitamin D, and traditional Chinese herbs. More robust comparative clinical trials are needed to evaluate the safety, clinical efficacy, and cost effectiveness of various adjuvants in order to determine ideal candidates in disease-specific and allergen-specific models. Other suggested approaches to further optimize outcomes of IT include early introduction of IT during an optimal window period. Alternative routes of administration of IT to optimize delivery and yet minimize potential side effects require further evaluation for safety and efficacy before they can be recommended.

Keywords: Immunotherapy; Allergy immunology; Allergoid; Immunologic stimulation; Monoclonal antibody; Probiotics

INTRODUCTION

Atopic diseases, such as asthma, allergic rhinitis, eczema, and food allergies, are increasingly prevalent and are common causes of morbidity, affecting the patients' quality of life [1] and causing significant socioeconomic burden [2].

Symptom control has traditionally been the mainstay of management of atopic diseases as definitive cures are still elusive. Allergen immunotherapy (IT) is the frontier of a more definitive treatment for atopic diseases. IT is the practice of administering increasing quantities of an allergen extract to an allergic subject to ameliorate allergic symptoms during subsequent exposure to the allergen. IT induces allergen specific regulatory T and B cells that downregulate the allergen-specific Th2 response, via interleukin (IL)-10 and transforming growth factor (TGF)-β [3, 4]. In particular, regulatory B cells produce IL-10 and allergen-

specific IgG4 antibodies [5], which capture allergens before reaching effector cells, thereby preventing mast cell and basophil activation [6].

Conventional IT techniques have, however, been beleaguered by significant rates of adverse reactions, raising concerns over safety and limiting its widespread use in the general population. A recent study by Tophof et al. [7] showed a high rate of side effects experienced by patients undergoing subcutaneous IT to a range of aeroallergens such as grass pollen, tree pollen and house dust mites (HDMs). Seventy-five point six percent (75.6%) of patients experienced local side effects while 9.3% developed systemic side effects including cutaneous and respiratory symptoms. Of these, 0.6% experienced hypotension and shock. Rates of adverse reactions are also high in food oral IT. Pajno et al. [8] described 9% of patients receiving oral IT to cow's milk and hen's egg needed to discontinue therapy due to significant side effects. Depending on the food, reaction rates could be higher. In a study by Kulmala et al. [9] looking at wheat oral IT 94% of subjects experienced reactions 64% of which were classified as moderate to severe reactions.

Over the last decade, there have been increasing interests in developing and modifying IT to strengthen its efficacy while maintaining or improving its safety profile. This review summarizes existing literature on the use of novel adjuvants, challenges faced in developing such adjuvants for clinical use and discusses areas for future research and development.

OVERVIEW OF NOVEL STRATEGIES

An ideal adjuvant should be able to accomplish 2 goals. First, to modify the nature of the immune response by inducing a robust Th1 response and/or suppressing Th2 responses arising from the allergic state. Second, to amplify the primary immune response to IT, thus requiring lower doses of allergen to achieve therapeutic effect [10]. Some of the adjuvants described here function as a depot for the antigen, presenting the antigen over a longer period and thus maximizing the body's immune response or may act as irritants, which induce the recruitment of additional immunomodulatory pathways which amplify the primary immune response [11]. **Table 1** provides a summary of available IT strategies which are further described in detail in this paper.

Allergoids

Allergoid vaccines are allergen extracts which have been modified chemically by substances such as glutaraldehyde or formaldehyde. The chemical modification causes irreversible intra- or intermolecular polymerization of the protein, disrupting the conformational IgE epitopes of the allergen. These higher molecular weight complexes result in reduced allergenicity while preserving or improving immunogenicity. This would facilitate improved safety and efficacy compared to conventional IT compounds, and allow a faster up-dosing regimen.

Depigmentation is an additional step involving acid treatment of the extract prior to polymerization with glutaraldehyde. This step reduces the allergenicity of the extract without compromising immunogenicity [12]. Gallego et al. [13] looked at the effect of a mixture of depigmented and polymerized extracts of *Dermatophagoides pteronyssinus* and *Dematophagoides farinae* in asthma patients (Depigoid). Patients treated with alum-adsorbed Depigoid for 54 weeks demonstrated clinical efficacy with significant reductions in median posttreatment bronchial (3.3 to 1.7), nasal (8.6 to 4.7), and ocular (0.7 to 0.6) symptom scores and reduced

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Table 1. Summary table of novel strategies in immunotherapy (IT)

Novel strategies	Mode of action	Results and recommendations
Allergoids	Extracts which have been modified chemically by substances such as glutaraldehyde or formaldehyde.	Extract dependent with one study showing clinical efficacy [13], but mostly demonstrate both reduced allergenicity and immunogenicity [14, 15, 17].
Immunostimulatory sequences	Induce strong Th1 response.	Small scale clinical studies show improved rhinitis symptom scores [23, 24]. *In vitro* studies using food sequences also show suppression of allergen-specific IgE levels [25, 26].
Epitope modification	Modification of IgE-binding epitopes to reduce allergenicity.	Positive studies *in vivo* and mouse models studies [39-41].
Peptide-based immunotherapy	Use of short sequence tolerogenic epitopes which prevent cross-linking of IgE and hence reduce allergenicity.	Protein oligomerization [55-57] and hybrid molecules [59, 63] increase immunogenicity.
Monoclonal antibodies	(1) Anti-IL 4: Suppression of inhibition of FOXP3+ T regulatory cells. (2) Anti-IgE monoclonal antibody: Prevents binding of free IgE to high affinity FcεR1 IgE receptor.	(1) No additional benefit conferred when used together with SCIT [30]. (2) Improved efficacy with grass and pollen SCIT [32] and cow's milk and peanut OIT [41-43] with improved safety profile.
Carriers	Aluminium hydroxide that induce strong Th2 responses by stimulating antigen-presenting cells. Newer lipid based carriers improve stability and drug delivery, also act as immunomodulators.	Greater immunogenicity and reduced allergenicity in mouse models [21, 48, 49].
Probiotics	Tolerogenic effect via dendritic cell and T-cell responses.	Improved clinical efficacy in grass pollen SLIT [69]. Overall lack of studies.
Earlier timing of introduction	Early and controlled introduction of allergens at an optimally defined timing may induce long-term tolerance starting from an early age in predisposed individuals (87).	The optimal timing for introduction of IT is yet to be determined.
Alternative routes of IT	Administration of allergens via tissues which have a high density of antigen-presenting cells such as via the skin and lymphatics may improve efficacy.	Outcomes of efficacy using the intralymphatic route vary between studies. Some report improved efficacy requiring a shorter duration of treatment [88, 89] while others do not report therapeutic efficacy [90]. Some studies have reported increased adverse effects and recommend dose reductions [91, 92]. Treatment via the epicutaneous route has been shown to be effective but only with high doses of IT [94, 95].

IL, interleukin; SCIT, subcutaneous immunotherapy; SLIT, sublingual immunotherapy; FcεR1, high-affinity IgE receptor; OIT, oral immunotherapy.

rescue medication use (15.6 to 7.1). In contrast, there was an increase in median symptom and medication scores in the placebo group. Although there was also a statistically significant increase in specific IgG4 responses in the treatment group, the increase was not large, ranging between 1.4 to 2.8 fold from the baseline.

To date, there has been only a single study directly comparing the outcomes between standard allergen extracts and allergoids. Henmar et al. [14] compared the *in vitro* allergenicity and immunogenicity of 4 commercially available grass pollen allergoids with 3 intact grass pollen allergen vaccines used in subcutaneous IT. In this study, basophil activation was performed using blood from 20 grass pollen allergic patients. Basophil activation was comparable between the allergoids and allergen vaccines and did not show reduced allergenicity. The allergoids demonstrated significantly lower T-cell activation in 29 human cell lines. Moreover, 2 of the 4 allergoids, which demonstrated the lowest allergenicity ($p < 0.001$), unfortunately, also had significantly lower immunogenicity ($p < 0.001$) when compared to the intact allergens. Similarly, studies by Würtzen et al. [15] and Lund et al. [16] also demonstrated the same issues with reduced immunogenicity where the allergoids were not appropriately recognized by the allergen-specific T-cell lines, leading to reduced T-cell activation and diminished specific IgG responses.

Chemical preparation processes also determine the allergenicity and immunogenicity of the extract. Heydenreich et al. [17] demonstrated that glutaraldehyde and formaldehyde preparations had different effects on pollen-derived allergoids. The glutaraldehyde-modified allergoids showed reduced IgE-binding, suggesting that the IgE-binding epitopes of modified allergoids were more efficiently destroyed by glutaraldehyde. However, the glutaraldehyde treatment process also modified T-cell binding epitopes, which diminished T-cell proliferative responses, resulting in reduced immunogenicity.

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Allergoid grass pollen vaccines are now commercially available and studies evaluating the economic benefits show that they might be more cost-effective and affordable than other IT options such as SLIT [18, 19]. However, the efficacy and reproducibility of these vaccines remain questionable. This is likely due to a lack of standardization of chemical modification methods that have consistently shown to preserve important T-cell epitopes and immunogenicity of the extracts.

Routine antibody-based assays also cannot be used to reliably assess the allergen content in these extracts since the process of polymerization disables antibody binding and reduces IgE binding [13]. Alternative methods including tandem mass spectrometry have thus been used to ensure the preservation of important allergens [20]. In addition, indirect assessments of allergenicity can be performed through basophil activation tests and flow cytometric measurements of uptake by immature dendritic cells [21].

Immunostimulatory sequences

Immunostimulatory sequences (ISS) are oligodeoxynucleotide DNA sequences containing unmethylated CpG motifs, which are recognized by Toll-like receptor 9 (TLR 9), an important member of the TLR family of transmembrane signaling molecules that play an important role in the initiation of innate immune responses. ISS induce interferon (IFN)-α and convert a Th2 immune response to a Th1 response [22].

Amb a 1 immunostimulatory phosphorothioate oligonucleotide conjugate (AIC) is a novel adjuvant created by conjugating Amb a 1, the major allergen in ragweed, to an ISS. Its use as an adjunct to ragweed subcutaneous immunotherapy (SCIT) has shown promising long-term results. Creticos et al. [23] demonstrated that the AIC group had lower mean peak-season rhinitis scores (13.2 vs. 40.8, $p = 0.006$), mean peak-season daily nasal symptom diary scores (1.8 vs. 4.0, $p = 0.02$), and improved midseason overall quality of life scores ($p = 0.05$) compared to the placebo group. AIC induced a transient increase in Amb a 1-specific IgG antibodies and suppressed the seasonal increase in Amb a 1-specific IgE antibodies. A reduction in the number of IL-4-positive basophils in AIC-treated patients also correlated with lower rhinitis visual-analogue scores ($r = 0.49$, $p = 0.03$). This response was sustained during the subsequent ragweed season.

Tulic et al. [24] demonstrated both significant attenuation of the increase in eosinophils and IL-4 mRNA-positive cells ($p = 0.008$) and an increased number of IFN-γ mRNA positive cells ($p = 0.002$) in the AIC group compared to placebo-treated patients upon nasal challenge to ragweed 4–5 months post IT, and a postragweed season pollen challenge showed that this effect was sustained long term.

ISS have also been investigated in food IT using mice models. A murine model of peanut IT demonstrated increased peanut IgG2a and decreased IgE, reflecting a shift from Th2 to Th1-type response, following administration of a synthetic TLR 9 agonist consisting of modified oligodeoxynucleotides (CpR) [25]. Another study used CpG-coated poly(lactic-co-glycolic-acid) nanoparticles containing peanut extract (CpG/PN-NPs) in a murine model of peanut IT, resulting in significantly decreased peanut-specific IgE/IgG1 and Th2 cytokine levels as well as increased IgG2a and IFN-γ levels, which were consistent with reduced allergic responses [26].

The use of other TLR agonists such as the TLR4 agonist monophosphoryl lipid A (MPL-A), a bacterial lipopolysaccharide lipid derivative, have also been successful. Rosewich et al. [27]

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recently demonstrated successful induction of tolerance upon bronchial allergen provocation after a single ultrashort course of MPL-A-adjuvant - grass pollen SCIT in pollen-sensitized asthmatics, achieving approximately 50% reduction in bronchial hyper-reactivity after treatment. A small follow-up study [28] of subjects on the MPL-A *Parietaria* grass pollen SCIT showed that the improvement in rhinitis, conjunctivitis and asthma symptoms was sustained up to 5 years after discontinuation of treatment.

Monoclonal antibodies

A number of studies have examined the use of monoclonal antibodies against immunological targets in Th1 and Th2 pathways as adjuvants to IT. Two well-described therapies include anti-IL-4 and anti-IgE antibodies.

Anti-IL-4 therapy

IL-4 plays a pivotal role in the inhibition of FOXP3+ T regulatory cell induction [29]. It sustains Th2 responses by inducing IgE isotype switching, high affinity IgE receptor ($Fc_{\varepsilon}R1$) and major histocompatibility complex class II upregulation and also drives other proallergic cytokines, such as IL-5, IL-9, and IL-31 in allergic disease [6].

Chaker et al. [30] administered anti-IL-4 antibodies as an adjuvant to SCIT with grass pollen in individuals with seasonal allergic rhinitis. Combined treatment with anti-IL-4 antibodies, however, did not improve response to SCIT, as measured through early and late phase cellular responses as well as subjective symptom reduction, compared to the control group.

Anti-IgE monoclonal antibody

Omalizumab is a recombinant humanized monoclonal antibody which binds to free IgE, preventing it from binding to the $Fc_{\varepsilon}R1$ and decreasing the number of FcεRI receptors on basophils. Its administration also decreases inflammatory mediators and down-regulates dendritic cell FcεRI expression [31]. It was first licensed for use in severe persistent asthma and chronic urticaria.

Omalizumab increases efficacy of SCIT when used in combination, compared to SCIT alone. Omalizumab-SCIT combination therapy in grass pollen allergic children with allergic rhinoconjunctivitis showed a 38% reduction in days with nasal symptoms, 76% reduction in days with ocular symptoms and 28% reduction in the use of any rescue medication after treatment [32]. In contrast, the SCIT-only arm did not show a significant reduction in any of the above parameters. Patients with severe asthma who remain at high risk of reactions with aeroallergen IT have also been shown to benefit with concomitant omalizumab administration [33, 34]. Case reports [35-37] also described patients who developed anaphylactic reactions to hymenoptera IT but subsequently successfully completed treatment with concomitant administration of omalizumab.

The first application of anti-IgE therapy for food allergy was in a small, uncontrolled study of 22 food allergic patients who were already on omalizumab for persistent asthma and the authors observed a concomitant reduction in their concomitant IgE-related food allergy symptoms including cutaneous symptoms, respiratory symptoms, and even anaphylaxis on subsequent re-exposure to the sensitized foods [38].

Subsequent pilot studies looking at omalizumab-combined oral IT in severe milk and peanut allergies found that they facilitated rapid oral desensitization with a reduction in the time

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required to achieve maximum doses and tolerability [39-41]. A phase 2 trial of omalizumab in peanut oral IT also demonstrated good treatment efficacy [42]. A recent randomized controlled trial of omalizumab combined with oral IT in cow's milk allergic subjects showed a significantly improved safety profile while maintaining efficacy [43]. However, there were no differences in the rates of sustained unresponsiveness between the placebo and omalizumab groups upon rechallenge, 16 weeks after cessation of omalizumab and 8 weeks post milk oral IT (OIT) (48.1% vs. 35.7%, respectively, $p = 0.42$). Omalizumab might, thus, be preferred in high-risk patients to achieve desensitization more safely and quickly, but long-term efficacy remains in question.

More recently, Brandström et al. [44] individualized dosing of omalizumab to patients with peanut allergy based on basophil allergen threshold sensitivities. A much higher dose was required in these patients as compared to the dose recommended for asthma treatment. Frischmeyer-Guerrerio et al. [45] looked at identifying biological markers to predict the added value of omalizumab as an adjuvant in milk OIT. Patients with high baseline basophil reactivity and higher milk IgE/total IgE ratios who were given omalizumab with milk OIT were less likely to react and more likely to achieve sustained unresponsiveness compared to high risk subjects who received milk-only OIT. A phase I clinical trial [46] also demonstrated successful rapid and safe desensitization to multiple food allergens simultaneously with concomitant administration of omalizumab.

Carriers

Aluminium hydroxide has been present as an adjuvant in vaccines for decades and is now also being used as a safe carrier adjuvant in SCIT. It induces a strong Th2 response by stimulating the activation of antigen presenting cells, independent of TLR signaling. Alum-adsorbed allergens and allergoids showed greater reductions in histamine release and leukotriene release by human basophils, indicating superior allergenic and immunogenic properties, compared to nonadsorbed allergens and allergoids [21].

There are some concerns that the cumulative aluminium dose exposure in 3- to 4-year duration of IT exceeds safe dietary oral intake levels recommended by the World Health Organization [47]. However, no causal relationship has thus far been established between ingested or injected aluminium compounds and short-term adverse neurocognitive effects or longer-term outcomes such as neurotoxicity-induced Alzheimer disease or other cognitive impairments.

Other novel carriers under investigation include liposomes, niosomes (nonionic surfactant vesicles with increased stability), and transferosomes (which possess a fluid membrane, rendering them elastic and able to squeeze through the stratum corneum, allowing for transdermal delivery). These molecules encapsulate hydrophilic antigens [11] which are otherwise unable to permeate hydrophobic cellular barriers, enabling them to reach intracellular targets and function as immunomodulators.

There have been several successful studies in murine models using liposomes as carrier molecules. Arora and Gangal [48] showed that liposome-entrapped pollen allergen elicited down-regulation of IgE responses and allergenicity in mouse models primed and boosted with alum-adsorbed pollen allergen. This appeared to be through a direct effect on inducing T suppressor/cytotoxic cells. Mice primed and boosted with liposome-entrapped allergen had higher levels of specific IgG, suggesting greater immunogenicity of the modified allergen. Similar findings were reported in intranasal liposomal cat allergen [49] and cockroach

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allergen [50] vaccine studies. Sehra et al. [51] also demonstrated that liposomal-entrapped allergen conferred an added protection against anaphylaxis in mice by inducing a high IFN-γ: IL-4 ratio and reduced synthesis of IgE and histamine release.

Recombinant proteins

Advances in molecular cell biology have allowed for the development of standardized and effective recombinant allergen preparations for both SLIT and SCIT [52]. This is vastly different from the crude preparation of allergen extracts of different mixtures and from a multitude of sources in the past, which could vary significantly in terms of molecular composition between batches [53].

The use of hypoallergenic recombinant protein derivatives is a strategy adapted from vaccine therapy, in which 2 general approaches have been proposed: the first, involves modification of IgE-binding epitopes on protein molecules to reduce immunogenicity; and the second, uses synthetic short amino acid segments containing tolerogenic epitopes as peptide-based IT. The latter approach retains antigenicity and allows interaction with antigen-presenting cells but prevents cross-linking of IgE, thus promoting tolerance. Although these approaches are technically not adjuvants, their properties significantly potentiate the effects of conventional IT.

The major advantage of hypoallergenic proteins is the capability to produce an effective immune response with reduced risks of systemic adverse reactions. This has been demonstrated in birch pollen allergy. Vrtala et al. [54] showed reduced IgE-binding capacity and allergenic activity in the basophils of birch pollen allergic individuals treated with recombinant trimeric rBet v1 aa 1–74 compared to those treated with wild type r Bet v1. The rBet v 1 aa 1–74 trimer induced significantly higher levels of Bet v 1-specific IgG1 antibodies than the monomer, and these antibodies also inhibited patients' IgE-binding to rBet v 1 wild type more than 10 fold more efficiently than antibodies induced by the monomer.

A *Fagales* pollen hybrid protein comprising stretches of birch, hazel, elder, oak, and hornbeam linear T-cell epitopes induced greater T-cell proliferation and a more significant reduction in lung eosinophilia and suppression of IL-5 in bronchoalveolar lavage fluid in sensitized mice, compared to a combination extract containing all 5 native pollen allergens [55].

These hybrid molecules have the advantage of better sustained long term immunologic memory. A study by Spertini et al. [56] found that the administration of 3 contiguous overlapping Bet v1 peptides (AllerT) in five injections to patients with allergic rhinoconjunctivitis over 2 months elicited high anti-Bet v 1-IgG_4 levels which remained sustained after more than three years of completion of treatment.

A clinical trial by Zieglmayer et al. [57] administered grass pollen allergy vaccine BM32 to individuals with grass pollen-induced allergic rhinoconjunctivitis. The vaccine contained four recombinant fusion proteins consisting of nonallergenic peptides derived from the IgE-binding sites of the major grass pollen allergens (Phl p 1, Phl p 2, Phl p 5, and Phl p 6) fused to hepatitis B virus-derived PreS which were adsorbed to aluminum hydroxide. Three monthly injections were administered to subjects who were stratified into 3 treatment groups with varying doses. These groups were compared with a fourth placebo group receiving only aluminium hydroxide. The recombinant vaccine induced significant grass pollen-specific IgG without increasing allergen-specific IgE responses. Subjects were able to tolerate high doses

of up to 160 μg of the recombinant protein without requiring an up-dosing phase. There was a significant reduction in total nasal symptoms score (TNSS) in the 20 mg and 40 mg groups with a mean reduction of 1.41 and 1.34, respectively. These groups also showed lower TNSS score after a 6-hour pollen chamber challenge. A dose-dependent reduction of between 11%-24% in the total ocular symptom score was also observed.

On the converse, a small phase II randomized controlled trial comparing a hypoallergenic recombinant birch pollen allergen to standardized extracts in 51 patients with allergic rhino-conjunctivitis did not find any statistically significant differences in outcomes including symptom medication scores, allergen tolerance by nasal provocation tests and increases in birch-pollen specific IgG1 and IgG4 between the groups [58]. The variability in outcomes is possibly related to the variations in extract preparations, extract dosing and dosing intervals, etc.

Recombinant proteins have also recently been used as modifiers in food IT. A phase 1 trial by Wood et al. [59] modified IgE-binding epitopes on three peanut allergens, Ara h1, h2, and h3 through amino acid substitutions and encapsulated these in inactivated *Escherichia coli* (EMP-123) as rectally administered suspensions for peanut IT. Despite promising *in vivo* studies, unexpected significant adverse events occurring in 5 out of 10 peanut-allergic adults required discontinuation of therapy.

In a novel peptide IT approach, 6 major T-cell epitopes from Met e 1, a recombinant shrimp tropomyosin protein, were administered orally to shrimp-allergic mice over 4 weeks. This resulted in increased IgG2a ($p < 0.001$), lower IL-4 and IL-5 levels compared to control mice ($p < 0.001$), shifting the allergenic Th2 cytokine milieu back towards tolerogenic Th1-type responses [60]. Similar studies by Rupa and Mine [61] and Yang et al. [62] using T-cell epitopes in ovalbumin (OVA), a major egg allergen, have also shown promising results in murine models of egg allergy.

Liu et al. [63] used a plant-human fusion protein comprising Ara h 2, the major peanut allergen, and human IgG Fcγ1 (the high affinity IgE receptor) - termed AHG2. This construct was able to inhibit whole peanut extract-induced, peanut-specific IgE-mediated histamine release in human basophils as well as allergic responses in transgenic mice, through indirectly cross-linking FcγRIIb.

Similarly, a hypoallergenic variant of the major fish allergen, parvalbumin, inhibited IgE binding, basophil degranulation, and allergic symptoms in sensitized mice in a mouse model of fish allergy by Freidl et al. [64].

In one of the few trials looking at human subjects, an edible β-lactoglobulin hydrolysate (βLg) with reduced antigenicity, generated through chymotrypsin-assisted hydrolysis, was able to completely suppress IgE-binding capacity in the sera of milk-allergic subjects compared to unhydrolysed βLg in a study by Ueno et al. [65]. This approach could potentially be utilized for the creation of peptide-based vaccines against milk allergy.

Overall, data on the use of recombinant proteins in both animal studies are promising and several peptides that are strong candidates for IT have been identified. There is, however, still a paucity of robust clinical data in humans and large-scale randomized controlled trials comparing recombinant proteins to current standard extracts are needed before definitive conclusions can be made about the efficacy of these products.

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Probiotics

A handful of studies on the successful use of probiotics as adjuvants in IT have been reported. Liu et al. [66] randomized house dust mite-sensitized asthmatic patients to 4 groups receiving standard allergen extract (*D. pteronyssinus*), standard extract with *Clostridium butyricum* as an adjuvant, *Clostridium butyricum* alone and a placebo. Subjects who received the standard extract with probiotic had statistically better and more sustained improvement in asthma symptoms and suppression of serum specific IgE and Th2 cytokine levels ($p < 0.01$) after the discontinuation of IT compared to those who received only the standard allergen alone. No therapeutic effects were observed in the probiotic-only or placebo groups.

The use of probiotics as adjuvants in IT is likely to be strain-specific, given that different strains of bacteria have varying effects on dendritic and T cells in *in vitro* studies [67, 68]. This is demonstrated by Berni Canani et al. [69] who looked at the use of *Lactobacillus rhamnosus* with extensively hydrolyzed casein formula in infants with cow's milk protein allergy. The rate of tolerance acquisition in infants at both 6 months and 12 months as evaluated by double-blinded placebo-controlled food challenge was significantly higher in the probiotic group. However, this effect was not reproducible when using a different combination of probiotics (*Lactobacillus casei* and *Bifidobacterium casei*) [70], suggesting that the effect was strain-specific.

Jerzynska et al. [71] compared the effect of both *L. rhamnosus GG* and vitamin D as adjuvants and found better clinical and immunologic responses with the use of probiotics with SLIT in grass pollen sensitized children with allergic rhinitis. The SLIT-probiotic group showed significantly higher $CD4^+CD25^+Foxp3^+$ induction compared to the SLIT-only group and higher reduction in the percentage of TLR-positive cell group compared to the SLIT-vitamin D group. These were also correlated with greater reduction in symptom and medication scores and improvements in lung function in the children in the SLIT-probiotic group.

Other novel adjuvants

Fungal compounds

Mushroom extracts, specifically fungal immunomodulatory proteins (FIPs), have been shown to have immunomodulatory properties such as the ability to inactivate the innate immune system, such as dendritic cells, NK cells, monocytes/macrophages, as well as induce cytokine/chemokine production which in turn activate the adaptive immune system by polarizing the Th1 or Th2 effector cells and stimulating the differentiation of B cells for antibody production [72]. The FIPs consist of various immunomodulatory proteins derived from the Basidiomycetes.

In an OVA-induced food allergy animal model, mice receiving oral administration of FIP-*fve*, a FIP isolated from the edible oriental mushroom *Flammulina velutipes*, during OVA sensitization showed significant reduction in the anaphylaxis symptoms, lower plasma histamine levels as well as milder mucosal oedema and epithelial damage of intestinal lamina propria compared to OVA-only sensitized mice [73]. These fungal compounds may be promising adjuvant carriers in future allergen IT.

Parasitic molecules

Helminths use several immunomodulatory strategies to evade or modify the host immune response in order to survive in the host. These including suppression or inactivation of host antigen-specific immune response. The modulation of the immune system has been considered symbiotic since helminths can avoid being eradicated and the host is also

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protected from inflammatory responses which may damage the host's tissues and organs. Two helminthic proteins that have been used to evade host's immune response are Brugia malayi TGF-β homologue-1 (TGH-1) and *Brugia malayi* TGF-β homologue-2 (TGH-2). They are 2 TGF-β homologues that have shown to be differentially regulated during the filarial life cycle: TGH-1 in Brugia malayi and B. pahangi [74] and TGH-2 in Brugia malayi [75]. These proteins bind to the human TGF-β receptor [76], mimicking human TGF-β and stimulating regulatory responses in the host. These parasitic molecules could potentially be harnessed as adjuvant carriers for allergen IT.

Vitamin D

Vitamin D may act as an adjuvant by activating specific regulatory immune cells to prevent atopic disease. An immunoregulatory effect of vitamin D3 was reported on dendritic cell migration, development of Fox p 3+-Tregs as well as activation of T cells [11]. Urry et al. [77] found that a vitamin D3 adjuvant to HDM allergoid-grass pollen IT resulted in an increased ratio of regulatory to effector T cells in cell cultures ($p = 0.015$ for HDM; $p = 0.031$ for grass). Grundström et al. [78] demonstrated improved airway hyper-responsiveness in a murine model of asthma receiving subcutaneous IT with the cat allergen *Fel d 1* coupled with vitamin D3.

However, clinical trials using vitamin D as an adjunct to SCIT show conflicting results. Although Baris et al. [79] reported a significantly reduced usage of inhaled corticosteroids ($p = 0.02$) in asthmatic children receiving vitamin D as an adjunct to HDM SCIT, there was no significant difference in several other parameters such as total asthma symptom score, total symptom scores and medication scores compared to the control group.

Traditional Chinese herbs

A traditional Chinese herbal medication (food allergy herbal formula-2) was able to suppress IL-4, IL-5, and IL-13 production and anaphylactic responses in a murine model of peanut allergy for at least 6 months after treatment [80, 81]. Although phase 1 studies in humans showed it to be well tolerated [82], a recent phase 2 trial failed to demonstrate efficacy at the selected dosing regimen and suffered from high rates of drug nonadherence [83]. More studies are required to assess if the positive results in animal models will translate to efficacy in human trials.

Early introduction of IT

In addition to finding an optimal adjuvant, the discovery of an optimal window period for IT and an optimal mode of delivery may also be important in optimizing the outcomes of IT. It may be desirable to start IT earlier in life or even in the antenatal period, at a time when the immune system is still naïve and before any structural complications of allergy have occurred, such as lung remodelling in asthma, or chronic sinusitis in allergic rhinitis. Recent successful studies have looked at the optimal timing of early introduction of foods to prevent specific food allergies in high-risk children [84-86].

Hamelmann et al. [87] suggest that an early and controlled introduction of allergens at an optimally defined timing to induce long-term tolerance in allergic individuals may be the way forward. This may include exposure to allergens via sublingual IT or the mucosal route in sensitized but nonallergic high-risk individuals. In addition to early IT with aeroallergens and food proteins, they suggested that IT with bacterial or helminthic proteins may help induce tolerance from an early age.

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Novel routes of IT

Novel routes of IT administration now include administration of allergens via tissues which have a high density of antigen-presenting cells such as via the skin and lymphatics. Senti et al. [88, 89] demonstrated reduced treatment duration with intralymphatic grass pollen or cat allergen IT from 3 years to 8 weeks in individuals with rhinoconjunctivitis with comparable long-lasting tolerance and that the intralymphatic route was also safer with less adverse events. However, Witten et al. [90] questioned the therapeutic efficacy of intralymphatic grass pollen IT. Moreover, Lee et al. [91, 92] reported that intralymphatic aqueous allergen IT for house dust mite, dog, and cat allergies could provoke serious hypersensitivity reactions including anaphylaxis although this modality could alleviate symptoms during allergen exposure. They recommended reduction of the allergen dose in patients with high skin reactivity to the allergen. Despite several attempts to elucidate the mechanism, therapeutic efficacy, and adverse effects of intralymphatic IT, much remains to be learned [93].

Treatment efficacy via the epicutaneous route has also been demonstrated in studies but only when using high doses of IT [94, 95]. Epicutaneous delivery also has an added advantage of reduced systemic side effects [96] as the allergen is administered to a nonvascularized tissue which minimizes systemic absorption. Kitaoka et al. [97] described successful transcutaneous administration of modified nanodispersed Japanese cedar pollen in a mouse model with significant reduction of specific IgE levels.

CONCLUSIONS

We have discussed several novel strategies to optimize the efficacy, safety and tolerability of IT for the future. Amongst these, the use of adjuvants, in particular, the use of hypoallergenic recombinant proteins and probiotic-based IT appear to be most promising at present, with strong evidence for improved clinical efficacy and sustained unresponsiveness. These adjuvants are also more cost effective in comparison to other alternatives such as immune-stimulatory sequences or lipid-based carriers. Omalizumab as an adjuvant to IT appears to improve its safety profile in high-risk individuals with previous systemic reactions to IT, or in concomitant multiple allergen desensitizations. However, its prohibitive cost, limited spectrum of indications, and lack of data for sustained unresponsiveness may not justify its widespread use.

Of the adjuvants and other strategies described, the majority of positive findings were found mainly in *in vitro* studies and a handful of small scale early-phase clinical trials. There is a need for more robust, larger-scale randomized clinical trials in humans to thoroughly evaluate the safety, clinical efficacy and cost effectiveness of the use of these various modalities across age and disease spectrums.

Considerations for the optimal timing for initiation of IT within an earlier window period may improve the outcomes of IT. Alternative routes of administration targeting tissues with high density of antigen-presenting cells to optimize target effects while maintaining safety will need further evaluation before they can be recommended for use. Overall, there has been increasing interests in the use of novel approaches to augment the effects of IT for atopic disease. This remains a promising field to be studied with vast potential options.

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