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REVIEW

Next generation immunotherapy for tree pollen allergies

Yan Su, Eliezer Romeu-Bonilla, and Teri Heiland

Department of R&D, Immunomic Therapeutics, Inc. (ITI), Rockville, MD, USA

ABSTRACT

Tree pollen induced allergies are one of the major medical and public health burdens in the industrialized world. Allergen-Specific Immunotherapy (AIT) through subcutaneous injection or sublingual delivery is the only approved therapy with curative potential to pollen induced allergies. AIT often is associated with severe side effects and requires long-term treatment. Safer, more effective and convenient allergen specific immunotherapies remain an unmet need. In this review article, we discuss the current progress in applying protein and peptide-based approaches and DNA vaccines to the clinical challenges posed by tree pollen allergies through the lens of preclinical animal models and clinical trials, with an emphasis on the birch and Japanese red cedar pollen induced allergies.

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1. Introduction

Pollen can induce a range of allergic reactions including seasonal allergic rhinitis (SAR) or pollinosis, rhinoconjunctivitis, or a less common but severe form, allergic asthma. Pollen allergies are a major public health problem affecting 10–30% of the world population. Prevalence has increased over recent decades.[1-3] Allergens derived from tree, grass, and weed pollen cause SAR or rhinoconjunctivitis. Standard treatment options include allergen avoidance, systematic pharmacotherapy, and immunotherapy. Allergen-specific immunotherapy (AIT) is frequently prescribed to patients with moderate to severe pollen allergies, as it is the only available potentially curative treatment. Subcutaneous Immunotherapy (SCIT), a classic form of AIT that entails repeated injections of crude pollen extracts and extract mixes matched to a patient's diagnostic and case history, has been used in clinical practice for over 100 years. To achieve long-term benefits, unique patient SCIT preparations are typically administered over a 3 year period in widely ranging dosing regimens. Traditional SCIT is based on two phases: an initial up-dosing phase and a subsequent maintenance phase. The up-dosing phase is an individual titration, where increasing doses are administered in order to build tolerance and assess the sensitivity of the patient to the specific dose(s). The maximum tolerated dose is then given throughout the maintenance phase. Although sometimes effective, SCIT is often associated with severe, even life-threatening side effects and responders cannot be predicted prior to treatment initiation and during treatment. Alternatively, AIT could be self-administrated sublingually (SLIT) with pharmaceutical extract preparations containing drops or tablets. SLIT is considered safer than SCIT[4,5] and is accepted by physicians and patients in Europe and Japan.[4,6,7] Nevertheless, SLIT and SCIT are both plagued by poor patient adherence to the inconvenient dosing schedule required to achieve a therapeutic effect, which can be acerbated by the occasional negative safety profile of SCIT (and to a lesser degree, SLIT). As a result, only 7% of patients receiving SLIT finish the three-year treatment, while 23% of SCIT patients complete the full treatment course.[8] Finally, the cost of long-term treatment may also impact the broader clinical adoption of AIT globally.[9] Thus, there is an unmet challenge to develop a safe, convenient, and low cost AIT capable of rapidly inducing protection from the negative effects of pollen allergies. In this article, we review novel approaches being tested for treating tree pollen induced allergies.

2. Type I allergy and the MoA of AIT

Pollen induced allergies, a type I hypersensitivity reaction, are mediated by allergen-specific IgE antibodies. In susceptible individuals, initial exposure to pollen allergens resulted in the differentiation and/or activation of T helper 2 cells (Th2).[10,11] Th2 cells are characterized by the production of the pro-inflammatory cytokines IL-4 and IL-13, which promote B cells to produce and secrete allergen-specific IgE antibodies. IgE antibodies bind to the high-affinity IgE receptors (FcεRI) on the surface of allergic effector mast cells and basophils. Re-encounters of the allergen results in IgE receptor crosslinking on mast cells and basophils triggering the degranulation of these cells and the immediate release of inflammatory mediators: vasoactive amines, lipidic mediators, cytokines, and chemokines.[10,11] Besides providing help to B cells, Th2 cells produce cytokines IL-4, IL-5, and IL-9, which induce differentiation and/or activation of mast cells, basophils, and eosinophils.[10,11]

The mechanism of action (MoA) of AIT for treating pollen allergies has not yet been fully elucidated, especially due to the complexity of the formulation of allergens and varying treatment protocols for delivery routes and doses. However, the big picture of AIT's MoA has emerged from extensive clinical and

CONTACT Yan Su, PhD ysu@immunomix.com Department of R&D, Immunomic Therapeutics, Inc. 15010 Broschart Rd Suite 250, Rockville, MD 20850, USA.

preclinical studies and it is generally accepted that SCIT and SLIT have the same MoA.[12-15] A very early event (usually within the first few hours after treatment initiation) is mast cell and basophil desensitization.[16] After a few months, allergen-specific $CD4^+Foxp3^+$ regulatory T cells (Tregs) and IL-10 producing $CD4^+Foxp3^-$ type 1 regulatory T cells (Tr1 cells) are induced,[17,18] as well as IL-10 producing B regulatory cells and monocytes.[19-21] Seasonal activation of allergen-specific Th2 cells in allergic patients is suppressed, possibly due to the earlier induction of Tregs/Tr1 cells and/or a Th1 immune deviation.[22-24] From months to years, allergen-specific IgE levels first increase transiently, then decrease while allergen-specific IgG, particularly Th1-type IgG4 blocking antibody levels increase, which is reflected in the skew to a lower IgE-to-IgG4 ratio.[25-27] Long-term clinical symptom improvement is related to the production and maintenance of the high avidity inhibitory allergen-specific IgG4 antibody.[21,28] Besides outcompeting IgE antibodies for allergen binding sites, allergen-specific IgG antibodies also suppress T cell-mediated late phase allergic reactions by inhibiting IgE-facilitated antigen presentation.[29,30]

Thus, through a detailed mechanistic understanding of successful AIT, the attributes of an ideal pollen allergy treatment can be described as including: 1) induction of high avidity blocking IgG4 antibody, 2) inhibition of allergic Th2 response though the induction of Tregs and/or Tr1 cells or an immune skewing toward to Th1 response, and 3) induction of sustained protective effects (long-term memory) even after the discontinuation of treatment. Beyond these, a next generation allergy treatment should be safe, convenient, and cost effective. Addressing such issues, researchers utilized different strategies to generate novel approaches in the next generation immunotherapies of pollen induced allergies.

3. Tree-pollen allergies and allergens

Two recent review articles summarized the molecular characteristics of currently identified tree pollen allergens and their geographic distribution and prevalence rates.[31,32] Briefly, the majority of clinically relevant tree pollen allergens are produced by four orders Fagales, Lamiales, Proteales, and Pinales. Birch pollen (Fagales) has strong allergenicity and is a major source of SAR in Northern Europe and North America.[31,33,34] Bet v 1 is a major allergen in the birch pollen extract and thus the primary focus of birch pollen AIT.[35,36] Bet v 1 belongs to the pathogenesis-related protein class 10 (PR-10) family. PR-10 family includes Aln g 1(alder), Car b 1 (hornbeam), Ost c 1 (hophornbeam), Cor a 1 (hazelnut), Fag s 1 (beech), Cas s 1 (chestnut), and Que a 1 (oak).[31] In Japan, Japanese red cedar (Pinales; JRC) pollen induced SAR affects up to one third of Japanese and JRC induced SAR heavily affects the quality of life of Japanese individuals.[2] Cry j 1 and Cry j 2 are the two major allergens in JRC pollen.[37-40] Cry j 1, a basic glycoprotein of pectate lyase protein family, is a major component of JRC pollen extract. Cry j 1 has high cross-activity with Cup a 1 (*Cupressus arizonica*), Jun a 1 (mountain cedar), and Cha o 1 (Japanese cypress). Cry j 2, a member of polygalacturonases family, shares sequence identity and cross-reactive IgE epitopes with Jun a 2 (mountain cedar), and Cha o 2 (Japanese cypress).[31] Because of the high prevalence of birch and JRC pollen allergy in these heavily populated and developed regions, the interest from both academic and industry in developing novel immunotherapies for treating birch and JRC pollen allergies is much higher than other tree pollen allergies. Thus, in this article, we focus on the novel immunotherapies of birch and JRC pollen allergies. We first discuss the approaches utilizing allergens from natural source (either pollen extracts or purified component allergens) which are formulated with different Th1 inducing adjuvants (Table 1). Then, we present the component based immunotherapies, which use recombinant hypoallergenic derivatives and synthetic peptides. We next talk the approaches of DNA vaccines in which the allergens are endogenously generated by patients after vaccination. Finally, we discuss the approaches in which the allergens are delivered through alternative routes including oral immunotherapy (OIT), intralymphatic immunotherapy (ILIT), and epicutaneous immunotherapy (EPIT).

4. Pollen extracts and purified natural allergens

The conventional SCIT or SLIT are based on the administration of crude natural pollen extracts, which may induce the IgE mediated acute side effects and T cell mediated late phase side effects in patients. To reduce the IgE mediated side effects, allergens could be modified by chemical denaturation (e.g. aldehydes) to generate hypoallergenic allergen derivatives, in which the conformational structure of the IgE recognizing epitopes in allergens is destroyed. Such chemically modified allergens is named allergoids. In 1970s Marsh et al. showed that chemical modification of rye grass extract reduces the allergenicity by destroying the conformational IgE epitope structure yet retains the immunogenicity (blocking IgG epitopes remained).[41] SCIT is usually administrated with an adjuvant (such as aluminum hydroxide) to enhance the immunogenicity of the allergens and reduce side effects by keeping the allergens in local injection sites by a depot effect. In addition, because the major allergens causing tree pollen allergies have been identified, pollen extract could be replaced with the major component allergen(s) to further reduce the side effects of AIT.

4.1. Allergoids and Monophosphoryl lipid A (MPL)

Monophosphoryl lipid A (MPL), is a bacterially-derived adjuvant that acts as a ligand for toll-like receptor 4 (TLR4), thus helping to favor induction of a Th1 immune response.[42] To improve the safety and efficacy of the natural pollen extract products, a combination of allergoids, which are absorbed by L-tyrosine, and MPL adjuvant has been tested for treating grass or tree pollen allergies.[43-45] Based on patient allergic reaction profiles, allergoids are prepared by allergen extraction, diafiltration to remove low molecular weight molecules, and then glutaraldehyde treatment to destroy the IgE binding epitopes. Allergoids were quantitatively measured by using High Performance Liquid Chromatography. The allergoids are preseasonally administrated in an ultra-short injection regimen consisting of four escalating doses of 300 SU (Standardized Units), 800 SU, 2000 SU and 2000 SU administrated weekly. Besides the reduced allergenicity by denaturation of allergens, the 4 injection regimen could further reduce the side effects.

Table 1. Novel approaches for tree pollen allergies.

Approaches	Allergen	Route	Formulation and treatment	Stage	Proposed MoA	References
Natural pollen extract or purified component based AIT	Chemically modified birch pollen extract	SC	Allergoids + MPL 4 weekly injections	Phase III	Induction of Treg and Tr1 cells, and IgG	46
	Purified natural Bet v 1	SC	Bet v 1+ Al(OH)3 Conventional SCIT build-up and maintenance in 2 years	Phase II	Induction of IgG	58
	Purified natural Cry j 1	ID	Cry j 1 entrapped in OMLs (mice: 2 weekly ID injections)	Preclinical	Th1 deviation	50
	Purified natural Cry j 1	SC	Cry j 1 conjugated with CpG (mice: 3 weekly SC injections)	Preclinical	Th1 deviation	52
Recombinant protein or synthetic peptide based AIT	rBet v 1	SC	Bet v 1 + Al(OH)3 Conventional SCIT build-up and maintenance in 2 years	Phase II	Induction of IgG	58
	rBet v 1	SL	rBet v 1 tablet once daily for 5 months	Phase II	Not determined in the reference 59	59
	Hypoallergenic rBet v 1	SC	Bet v 1 + Al(OH)3 Conventional SCIT build-up and maintenance in 1 year	Phase II	Induction of IgG, reduction of IgE	63,64
	Synthetic Bet v 1 COPs	SC	Synthetic peptides + Al(OH) 3 5 injections in 2 months	Phase III	Induction of IgG4, Th1 deviation, and IL-10	72,73
	Synthetic Cry j 2 T cell epitope	SC	T-cell epitope conjugated with CpG (mice: 1 or 2 weekly SC injections)	Preclinical	Suppression of Th2 response	53
	Bet v 1 B cell epitopes	SC	B-cell epitopes + carrier (KLH, PreS) + Al(OH)3 (mice: 3 SC injections in 3 week intervals; rabbits: 3 monthly injections)	Preclinical	Induction of IgG blocking Ab	81,85,86
	Hypoallergenic rCry j 1/2 fusion protein	SC	Hypoallergenic rCry j 1/2 fusion protein conjugated with PEG (monkeys: 4 weekly SC injections)	Preclinical	Induction of IgG and Th1 deviation	88
DNA vaccines	Cry j 1 Cry j 2	IM	Cry j 1-LAMP and Cry j 2-LAMP MHC II targeting DNA plasmids 4 biweekly injections	Phase II	Th1 deviation and induction of IgG antibody	109,110
	Bet v 1a or Cry j 1	ID or IM	DNA plasmid (mice: 1 ID injection or 4 weekly IM injections)	Preclinical	Induction of IgG2a and Th1 deviation	99-101
	Bet v 1	ID	MHC I or II targeting DNA plasmid (mice: 3 weekly ID injections)	Preclinical	Treg induction	103
	T cell epitope of Cry j 2	IM	MHC II targeting DNA plasmid (mice: 4 or 5 weekly IM injections)	Preclinical	Th1 cellular immunity	102
Alternative routes	Purified natural Cry j 1	Oral	Cry j 1 conjugated to galactomannan, Daily administration for 10 weeks (build-up and maintenance)	Phase II	Induction of IgG4 and IL-10	116-119
	Cry j 1 COPs and hypoallergenic Cry j 2 by Transgenic rice:	Oral	Milled transgenic rice seed powder (mice: freely feed for 7 days or 20 days)	Preclinical	Suppression of Th2 and Th1 responses	122-124,127
	Synthetic T cell epitopes of Cry j 2 and/or Cry j 1	Oral	Synthetic peptides (mice: 4 oral doses in 2 weeks)	Preclinical	Suppression of Th2 and Th1 responses	128-130
	T cell epitopes of Cry j 2 and/or Cry j 1 by Transgenic chicken	Oral	Transgenic chicken egg white (mice: 5 days/week for 4 consecutive weeks)	Preclinical	Suppression of Th2 responses	131
	Birch or grass pollen extract	Intralymphatic	Extract + Al(OH)3 3 injections in 3–4 week intervals	Phase II	Induction of high affinity of IgG4 antibody and Th1 deviation	133,134
	rBet v 1, rBet v 1B2	Epicutaneous	Allergen + Th1 adjuvant (mice: 8 weekly EP treatments; guinea pigs: 3 biweekly patches)	Preclinical	Th1 deviation and reduction of IgE	144-146

ID: intradermal, IM: intramuscular, SC: subcutaneous, SL: sublingual, EP: epicutaneous

Safety and clinical efficacy of the ultra-short vaccination regimen have been investigated in a number of clinical trials.[43-46] The ultra-short birch allergoids vaccinated birch allergic patients showed significantly improved combined symptom scores and medication scores.[46] The immunological mechanisms associated with this approach include the induction of Tregs/Tr1 cells and the production of IgG antibody.[46] In a clinical study for grass allergy, allergoids/MPL treatment resulted in significantly reduced skin reactions, reduced basophil reaction and induction of allergen-specific IgG1 and IgG4 antibodies.[47] Currently, a Phase III clinical trial designed to evaluate the efficacy and safety of the allergoids/MPL birch immunotherapy in birch pollen-induced rhinoconjunctivitis is ongoing in Europe (EudraCT number: 2016-002781-31).

4.2. Purified natural allergens and Th1 adjuvants

It was found that oligomannose-coated liposomes (OMLs) have adjuvant effects.[48] After taking up OMLs, antigen presenting cells upregulate the production of IL-12 and expression of CD80/86 costimulatory molecules. Thus, OMLs preferentially induce Th1 immune response against the capsulated antigens.[49] Ishii et al. entrapped purified Cry j 1 protein with OMLs and showed that two injections of such Cry j 1/OMLs induced robust Th1 cytokine response and IgG2a (a Th1 type antibody) production in mice. Total IgE levels were suppressed in Cry j 1/OMLs treated mice after Cry j 1/Alum challenge in both prophylactic and therapeutic models.[50]

CpG motif containing oligodeoxynucleotide (ODN), which is a toll like receptor 9 agonist, has adjuvant effects which could boost Th1 immune response.[51] Kaburaki et al. showed that CpG ODN conjugated purified Cry j 1 protein elicited strong Th1 cytokine response and Cry j 1 specific IgG2a response in mice.[52] *In vitro* CpG-Cry j 1 culture resulted in production of IL-12 by splenocytes. CpG conjugation reduced the binding to serum IgE from JRC allergic patients, indicating the IgE epitopes on the Cry j 1 protein were masked by CpG. Thus, CpG-Cry j 1 treatment suppressed the Cry j 1 specific IgE levels after allergen challenge.[52] Similarly, Suzuki et al. conjugated a T cell epitope from Cry j 2 protein with the CpG ODN. This T cell epitope-CpG treatment suppressed Cry j 2 specific IgE reaction and attenuated the clinical symptoms upon allergen challenge in mice.[53] IL-4 and IL-5 production was found significantly reduced in the T cell epitope-CpG treated mice.

These approaches using new adjuvant and purified allergen or T cell epitope have not yet been evaluated in humans.

5. Recombinant hypoallergenic derivatives and synthetic peptides

The side effects of the traditional AIT are highly associated with the natural allergenicity of pollen extract products. In addition, the extract products from different manufacturers vary in the composition and potency of the individual allergens.[54,55] Extracts that lack therapeutically relevant allergens would compromise AIT efficacy. Moreover, therapeutically irrelevant allergens in the crude natural pollen extract may induce new IgE antibodies in patients receiving AIT, a process called *de novo* sensitization that may exacerbate allergic responses and are one potential safety concern of using such crude extracts.[56] With the accumulated knowledge of pollen molecular characteristics and allergenicity, it now becomes feasible and reasonable to generate immunotherapeutic products by either recombinant proteins or synthetic peptides, which have better lot to lot consistency and improved reproducibility during the manufacturing process over the natural extract-based AIT products.[57]

5.1. Recombinant proteins

Recombinant grass and birch allergens had been generated and evaluated in clinical trials. In a randomized, double-blind, placebo-controlled (RDBPC) Phase II trial, it was found that a single allergen SCIT using either recombinant Bet v 1 (rBet v 1, expressed in *E coli.*) or purified Bet v 1 (nBet v 1) has the same efficacy and similar safety profile as those of birch extract treated subjects.[58] In this trial, a maximal dose of 15 μg rBet v 1 or nBet v1 was reached during the buildup phase. Unlike the birch pollen extract, rBet v 1 and nBet v 1 protein treated subjected did not develop novel sensitization (Bet v 2 specific IgG1 and IgE), indicating the defined component allergen did not induce unnecessary IgE responses.[58] Nevertheless the allergenicity of the rBet v 1 allergen remained unchanged. As a result, the treatment requires an inconvenience dose escalation and long-term maintaining injections regimen to reduce side effects, same as the extract based SCIT. Alternatively, rBet v 1 in tablet formulation has been investigated in a RDBPC SLIT clinical trial for birch allergy.[59] After 5 months rBet v 1 tablets, treatment group showed reduced clinical symptoms.

Hypoallergenic rBet v 1, which has eliminated IgE binding but remained IgG and T cell epitopes, has been generated.[60-62] SCIT treatment with alum absorbed hypoallergenic rBet v 1 induced protective IgG production, improved clinical symptoms, reduced skin reaction, and more importantly, suppressed IgE production during the seasonal birch pollen exposure.[63,64] Because of the reduced allergenicity of the hypoallergenic rBet v 1, subjects tolerated to a maximum dose of 80 μg allergen per injection during the buildup phase, which was higher than dose of wild type rBet v 1.

One concern is that due to the intact T cell epitopes, the recombinant allergens or hypoallergenic derivatives could be associated to the T cell mediated late phase allergic reactions.[65-67] Thus, further efforts are needed to improve this safety issue of the recombinant wild type or hypoallergenic allergens as immunotherapeutics.

5.2. Contiguous overlapping peptides

Hypoallergenic allergen derivatives could be generated as recombinant proteins with genetic modification or as synthetic peptides. Contiguous overlapping peptides (COP) are allergen-derived long peptides, whose peptide sequences overlap, thus covering the full protein sequence of an allergen. T cell epitopes (usually linear) are unchanged and IgE binding epitopes are eliminated in COPs because the epitopes cannot recapitulate their natural conformational structure. Initial studies on bee venom immunotherapy showed that the COPs (from a major bee venom allergen) stimulated T cell proliferation but reduced

the IgE binding to serum from the bee venom hypersensitive patients.[68-70] In a phase I study, COP treatment induced Th1-deviation, IL-10 production, T cell hyporesponsiveness, and allergen-specific IgG4 production in bee venom hypersensitive patients.[68]

AllerT, which consists of three Bet v 1 derived 49-71 amino acid COPs, was designed to treat birch allergy.[71] It was found that AllerT did not bind to human IgE nor induce basophil activation in both mice and human, and AllerT treatment protected mice from birch allergy anaphylaxis after pollen exposure.[71] Because of the reduced allergenicity, AllerT has the potential to be safer than extract-based immunotherapies. Safety and immunogenicity of AllerT has been evaluated in a RDBPC phase I/IIa study testing preseasonal administration of AllerT with alum adjuvant in a short regimen spanning two months.[72] Treatment (15 subjects received AllerT SCIT) entailed a four dose escalation injections in the first day, three weekly injections, and a final injection on day 51.[72] AllerT treatment resulted in increases in IL-5 and IL-10 and Bet v 1 specific IgG4 production in 4 weeks, and IgG4 levels were found to be sustained for at least three years.[72] Potential efficacy was also evaluated in a single season dose finding Phase II clinical trial (50 μg COP group n = 79 and 100 μg COP group n = 82).[73] AllerT SCIT at a dose of 50μg/injection showed significantly improved rhinoconjunctivitis symptom and medication scores, quality of life, nighttime nasal scores, as well as increased Bet v 1-specific IgG4 production.[73] The AllerT SCIT for birch allergy is currently undergoing testing in a large clinical trial (EudraCT number: 2016-000076-23) in Europe.

The safety endpoints were met in these birch allergy COP-based clinical studies. The mechanism of this approach is likely through the induction of blocking IgG4 antibody and activation of Th1 cells and IL-10 producing cells. T cell epitopes are essential for the Th1 deviation and the induction or activation of IL-10 producing Tr1 T cells. However, as with the recombinant allergen products described in the section 5.1, one concern is that T cell epitopes also can possibly trigger the T cell mediated late phase allergic responses, especially in moderate to severely allergic patients.[68,72]

While COPs include both IgG and T cell epitopes, an alternative peptide-based approach called Synthetic Peptide Immuno-Regulatory Epitopes (SPIREs) entails administering a mixture of short 8 to 17 amino acid length T-cell epitope peptides derived from various major allergens. SPIRE peptide immunotherapy candidates have been designed and evaluated in cat, grass, and house dust mite (HDM) allergies, but not in tree-pollen allergies.[74-76] SPIREs have shown a reasonable safety profile in humans when administrated intradermally. Indications of efficacy have been demonstrated in several RDBPC clinical studies, but only in evaluating symptomology through repeated allergen exposure in dedicated allergy chambers. The clinical relevance of this approach has come into question because of two recent studies that failed to confirm the efficacy reported in the allergy chambers, including a Phase III trial for cat allergy and a Phase II trial for HDM allergy (EudraCT number: 2012-001733-13 and 2014-001662-94).

5.3. B cell epitope vaccine

Valenta's laboratory has developed a B cell epitope-based allergy vaccination approach.[77] Initial work by Vrtala et al. demonstrated that breaking the Bet v 1 protein into two fragments by using a recombinant technique abolished IgE recognition sites and remained the T cell epitopes.[78] Such recombinant Bet v 1 hypoallergenic derivatives, when intranasal administrated, induced significantly fewer clinical symptoms and lower mast cell and eosinophil activation than wild type rBet v 1 protein.[60] Later from different clinical trials, it was demonstrated that recombinant hypoallergenic derivatives were associated with T cell mediated late phase side effects.[65-68,72] To eliminate the side effects caused by T cells, this group further truncated the allergen sequences and only kept the IgG binding B-cell epitopes in their immunotherapy products.[79-82] Thus, the rationale of this approach is to generate IgG epitopes from the IgE binding region of an allergen, as a result, the hypoallergenic derivatives could induce blocking IgG antibody without priming an IgE reaction.

B cell epitopes, between 25–40 amino acids, were derived from IgE binding sites of allergens. To provide T cell help for making IgG antibodies, Bet v 1 B-cell epitopes were chemically coupled with the hapten protein Keyhole limpet hemocyanin (KLH).[81] Alternatively, the chemical coupling was replaced by recombinant fusion protein consisting of a viral protein as a carrier such as the VP1 from human rhinovirus or the protein PreS domain from hepatitis B virus and the B-cell epitopes.[80,83-85] Without a secondary structure, these B cell epitopes neither recognize IgE antibodies from birch allergenic patients nor induce basophil activation and skin test reactions. In animal models, these B-cell epitopes induced functional allergen-specific IgG antibodies, which exhibited IgE blocking activity as indicated by inhibition of IgE binding to serum from allergic patients and by blocking basophil activation by allergens.[81] Prophylactic and therapeutic immunization of this Bet v 1 B-cell epitopes in KLH carrier induced a strong Bet v 1 specific IgG antibody response in recipient mice and protected mice from allergic lung inflammation upon allergen challenge.[86] Administration of PreS fused Bet v 1 B cell epitope vaccine induced strong allergen-specific blocking IgG antibodies in rabbits. Incubation of this fusion protein with birch allergic patient PBMCs reduced IL-5 and increased IL-10 and IFN-γ production.

The Bet v 1 B-cell epitope immunotherapy has not yet been tested in a clinical study. In a phase II trial for grass allergy, the B-cell epitope immunotherapy was found safe and effective in an allergen exposure chamber challenge.[80] Nevertheless, a concern remains about whether solely inducing blocking IgG antibodies without tolerance induction through T cell immunity is sufficient for long-term efficacy.

5.4. PEG conjugated recombinant hypoallergenic protein

Commercially available pollen extracts have variable levels of patient-relevant allergens, and thus any given preparation may not have sufficient relevant allergen required for achieving therapeutic effects in AIT. When used in treating JRC induced SAR by SCIT or SLIT, the JRC pollen extract (CPE) has been standardized for the amount Cry j 1 protein. 2000 JAU/ml (Japanese allergy unit), which is the highest concentration of the CPE, contains 1.5-4.2 μg Cry j 1 protein, much lower than the recommendation of SCIT dose (5-20μg/injection) [87]. Thus,

there is the need to improve upon the extract-based approach for treating JRC allergies. Fujimura et al. generated a recombinant fusion protein consisting of the two major JRC allergens, Cry j 1 and Cry j 2. To break the Cry j 1 and Cry j 2 conformational structures, the majority of cysteine residues were replaced with serine residues.[88] The modified fusion protein was then conjugated with polyethylene glycol (PEG) and used to treat CPE sensitized monkeys. After four weekly subcutaneous injections of the PEG-conjugated fusion protein administered at a 1mg/0.5ml dose, the production of Cry j 1 specific IgG was significantly increased when compared to the baseline prior to treatment and significantly reduced Cry j 1 specific IgE antibody production. In Cry j 1 sensitized mice, four injections of 100 or 20 μg PEG-allergen fusion protein elicited a Th1 skewed immune response while inhibiting Cry j 1 specific IgE and Th2 cytokine production. Safety and efficacy of the PEG-Cry j 1/2 hypoallergenic fusion protein has not yet been evaluated in human patients.

6. DNA vaccine

DNA vaccines are bacterial plasmid vectors expressing an antigen gene for *in vivo* administration and represents a novel strategy to treat tree pollen allergies. The concept of naked DNA immunization was established in the early 1990s.[89-91] Since then, this technique has been extensively studied in a variety of disease models in preclinical and clinical studies.[92-94] Although promising efficacy profiles have been demonstrated in preclinical and Phase I/II clinical trials, DNA vaccines are not yet a validated technology for preventing or treating human diseases. DNA vaccines preferentially activate Th1 cells and suppress the production of Th2 cytokines and IgE antibody,[95-98] thus indicating their potential as a therapeutic for treating tree pollen induced allergies.

Over the past two decades, DNA vaccine approaches have been tested in several preclinical studies as potential birch or JRC pollen allergy treatment. Hartl et al. demonstrated that a DNA vaccine expressing the full length Bet v 1a allergen induced a strong Th1-biased immune response in mice.[99,100] In both prophylactic and therapeutic models, Bet v 1a DNA vaccinated mice exhibited increased production of Th1 type antibody IgG2a and IFN-γ, decreased production of Th2 type antibody IgG1, and suppressed basophil activation, indicating a shift from Th2 dominant towards a Th1/Th2 balanced immune response. Similarly, Toda et al. demonstrated that a Cry j 1 encoding DNA vaccine elicited a predominant Th1 type immune response and suppressed IgE response through intramuscular injection in a mouse JRC allergy model. Interestingly, gene gun inoculation failed to achieve such Th1 polarizing effects, indicating the routes of gene immunization are critical for the protective effects by DNA vaccines.[101]

One of the unique features of DNA vaccination is the ability to control trafficking of the endogenously produced allergens allowing allergens to be targeted to specific sub-cellular compartments (lysosomes/endosomes for MHC class II presentation, proteasomes for MHC class I presentation, or extracellular secretion). Toda et al. improved their DNA technique by including an invariant chain (Ii) with a $CD4^+$ T cell epitope, p247-258 of the Cry j 2 allergen. This MHC class II targeting DNA vaccine induced the epitope-specific Th1 T cell response without eliciting IgG antibody production in the recipient animals. Possibly due to the induced cellular response, Cry j 2 specific IgE response was suppressed in the animals after the Cry j 2 allergen injections.[102] Weinberger et al. studied the immunological consequence of including different sub-cellular compartment targeting sequences with Bet v 1 allergen in mice.[103] Unmodified, MHC class I or class II targeting forms, or extracellular secretion form of Bet v 1 encoding DNA plasmids were generated. Vaccination with these four variants of plasmids protected mice from Bet v 1 allergic sensitization, as indicated by reduced Th2 cytokines and IgE production and suppressed airway hyperresponsiveness and lung inflammation. Lysosomal or proteasomal degradation enhanced the allergen presentation by APCs to MHC class II or class I molecules and reduced availability of free allergens in circulation, leading to a vaccine candidate with increased immunogenicity and improved safety relative to untargeted allergens.[103]

6.1. LAMP-based DNA vaccine for JRC allergy

Lysosomal-associated membrane protein-1 (LAMP-1) is a resident protein of the lysosomes that functions to maintain lysosomal membrane integrity.[104,105] Testing in several infectious disease models has indicated that fusion of LAMP-1 with an antigen of interest in DNA plasmids results in lysosomal-class II pathway trafficking of the target antigens and subsequent enhancement of target antigen immunogenicity.[106-108] Based off these observations, our group generated two LAMP-based DNA vaccines, CryJ1-LAMP and CryJ2-LAMP, which encode Cry j 1 and Cry j 2, respectively and investigated their application in treating JRC-induced allergy. The CryJ1-LAMP and CryJ2-LAMP plasmids immunized mice exhibited a robust Th1-biased immune response, as indicated by high titers of IgG2a and low IgG1 and IgE.[109] Thus, the MoA of this LAMP-based DNA vaccination seems to be skewing of Th2 allergenic reaction to a Th1 response. It is interesting to note that the LAMP-based DNA vaccination is likely independent on Tregs and IL-10 producing cells, as neither depletion of Tregs nor blockage of IL-10 receptor compromise the immunogenicity of the DNA products in mice.[109]

In a Phase IA and IB study, the safety and immunological effects of the CryJ2-LAMP DNA vaccine was evaluated in six non-atopic and eighteen JRC- and/or Mountain Cedar (MC)-atopic Japanese expatriates living in Hawaii.[110] This is to our knowledge the first clinical trial for treating allergies with a DNA vaccine. The CryJ2-LAMP vaccine was well tolerated by both non-atopic and JRC- and/or MC-atopic subjects in a biweekly four-dose intramuscular immunization regimen (4mg or 2mg plasmid DNA per injection) and no severe adverse events were found. The majority of JRC and/or MC atopic subjects experienced negative conversion of skin reaction against the JRC or MC extract and the negative skin conversion was maintained through the end of the trial in most subjects.[110] There were several limits in this trial, such as small study size, lack of placebo control, and lack of JRC and/or MC pollen exposure. However, data from this study of applying DNA vaccines in allergies indicate that DNA vaccine might be immunologically effective as a therapeutic for tree pollen allergies.

Currently, the CryJ2-LAMP and CryJ1-LAMP DNA vaccines are undergoing investigation in a RDBPC Phase II trial in Japan (clinicaltrials.gov, NCT03101267).

7. Alternative delivery routes for AIT

Besides SCIT and SLIT, several alternative allergen delivery routes have been studied for treating tree pollen allergies. These include oral administration, intralymphatic injection, and epicutaneous patch.

7.1. Oral immunotherapy (OIT)

Oral immunotherapy (OIT), which was initially developed for treating food allergies, has more recently been studied for treating tree-pollen allergies in several forms in both animal models and clinical trials. Compared to SCIT or SLIT, a relative large amount allergens could be orally administrated during OIT. Due to the presence of a large number of immune cells in the gut associated lymphoid tissues, immune tolerance could be induced by OIT.[111] In 1980s, OIT clinical trials had been conducted in north Europe to treat birch pollen allergy.[112,113] However, efficacy and safety of these birch pollen extract based OIT were not satisfied. In recent years, several OIT approaches for JRC allergy have been investigated in Japan.

7.1.1. Galactomannan coupled Cry j 1 OIT

It has been demonstrated that conjugation of Cry j 1 with galactomannan by the Maillard reaction reduces the allergenicity of Cry j 1 protein.[114,115] The conjugated galactomannan may mask the IgE binding epitopes of Cry j 1, thus inhibit IgE binding of JRC allergic patients' sera. Galactomannan conjugation has also been shown to protect Cry j 1 protein from enzymatic digestion in the stomach as orally administered Cry j 1-galactomannon co-localize with dendritic cells in the gut lumen.[115] This approach has been evaluated in several clinical trials for treating JRC allergy.[116-119] Safety and efficacy of the Cry j 1-galactomannan OIT had been demonstrated in two open-label Phase I/II trials which have different administration regimens.[116,117,119] No severe adverse events were reported in the subjects receiving Cry j 1-galactomannan OIT. Subjects in OIT group showed improved quality of life score and reduced total symptom scores, medication score, and total symptom-medication scores throughout the pollen season, as well as increased levels of allergen-specific serum IgG4 and IL-10 production in PBMCs when compared to the control group.

The efficacy and safety Cry j 1 allergen extract galactomannan conjugate OIT has been further evaluated in a recent RDBPC Phase II trial. 55 subjects with moderate or severe rhinoconjunctivitis caused by JRC pollen were involved in this study (27 active, and 28 placebo).[118] During the buildup phase, which started about two weeks prior to the start of the JRC pollen season, subjects received one Cry j 1-galactomannan conjugate capsule orally (187.5ug Cry j 1) for 6 days (morning), two capsules (375ug) for the next 6 days (morning and evening), and three capsules (562.5ug) for the last 6 days (morning two and evening one). During the maintenance phase, four capsules (750μg) were given twice daily for 51 days (morning two and evening two). Treatment was started in middle of January 2014 and ended at the end of March 2014. Cry j 1-galactomannan conjugate treatment group exhibited partially improved clinical symptoms (mean total symptom and medication score) during the entire pollen season (both JRC and Japanese cypress) and significantly lower mean medication score over the placebo group.[118] The overall efficacy of this OIT regimen demonstrated in this RDBPC trial was lower than that of from the previous open-label trials. The investigators suggested several possible explanations 1) placebo effects, 2) early started pollen season, and 3) greater amount of pollen in 2014 than the previous years.[118]

7.1.2. Transgenic rice OIT

Takaiwa group has developed a transgenic rice seed-based OIT approach for treating allergies caused by HDM, JRC pollen, or birch pollen.[120-126] Initially, a transgenic rice line was generated to produce a fusion protein consisting of two mouse immune-dominant T cell epitopes from Cry j 1 and Cry j 2 allergens.[121] The transgenic gene was under a seed-specific promoter and the fusion protein was expressed and accumulated in the endoplasmic reticulum derived protein storage vacuoles (Protein Bodies) of the endosperm. Mice, which were fed with powder of the transgenic rice seeds (200mg powder, consists of about 70μg fusion protein, once a day for 4 weeks), were protected from JRC pollen extract challenge. OIT Mice exhibited reduced production of Th2 cytokine and IgE production, as well as decreased histamine release and nasal sneezes. To optimize the transgenic rice seed OIT, this group generated a serial of transgenic rice plants encoding multiple human T cell epitopes, full length protein, or derivatives of Cry j 1 and Cry j 2, with and without adjuvants.[122-124]

The most recent strain of the transgenic plant expresses a fusion hypoallergenic protein consisting of three COPs of Cry j 1 and a reassembled Cry j 2 protein.[124] This destructed fusion protein lacks IgE binding and has reduced allergenicity. In a prophylactic model, feeding mice with the seed of this transgenic rice prevents allergy development after JRC pollen extract challenge, both JRC specific Th1 and Th2 cytokines and IgG and IgE antibodies were suppressed. In a recent study, the fusion protein in the protein bodies from the transgenic rice seeds was concentrated by thermostable alpha-amylase treatment at 90°C to remove the starch from the milled rice seeds.[127] The 12.5-fold concentrated protein body products contains >70% fusion protein, exhibits higher resistant to enzymatic digestion than those in the milled seed powder, and remains stable at room temperature storage for at least 10 months. In addition, the concentrated fusion Cry j 1 and Cry j 2 hypoallergenic protein shows same immunological effects in suppressing JRC pollen extract induced IgE production and T cell proliferation as those in the milled seed powder, providing an alternative formulation of OIT.[127]

Using the same transgenic technique, a hypoallergenic derivative of Bet v 1 transgenic rice was generated, but its immunological activity has not yet been evaluated.[125] This transgenic rice based OIT has not been evaluated in the clinic, and its immunological effects have only been demonstrated in the prophylactic, but not in the therapeutic setting in mouse models. Considering that rice is one of the major food sources in Japan, this approach might be easily accepted by JRC allergic

patients in Japan, if the clinical efficacy is approved in future. Alternatively, a potential of this transgenic rice OIT is that it can be used as a prevention vaccine for JRC induced SAR.

7.1.3. T cell epitope OIT

Using a mouse model for JRC allergy, Hirahara et al. demonstrated that oral administration of a peptide p246-259, an immunedominant T cell epitope from Cry j 2 allergen, induced tolerance in Cry j2 sensitized mice, productions of Th1 cytokine IFN-γ and Th2 cytokine IL-4 and Cry j 2-specific IgG2a/IgG2b, IgG1, and IgE antibodies were suppressed.[128] In a later study, Murasugi et al. found that 4 doses of 200μg p246-259 oral administration protected mice from Cry j 2 induced allergic reaction, including sneezing and airway obstruction.[129] Later this group showed that OIT with a synthesized peptide containing three T cell epitopes from Cry j 1 and Cry j 2 allergens induced tolerance to Cry j 1 and Cry j 2 sensitization in mice.[130] This T-cell epitope OIT has not been further tested in human yet.

Kawabe et al. generated genetically modified chickens which produce a fusion protein containing seven T cell epitopes from Cry j 1 and Cry j 2 allergens in the egg white.[131] Oral administration of the T cell epitope containing egg white suppressed Cry j 1 specific IgE production in JRC extract sensitized mice. Upon intranasal challenge with JRC pollen extract, transgenic egg white fed mice exhibited lower number of nasal sneezing than normal egg white fed mice. The transgenic chicken egg white treatment also improved the lung inflammation after the intranasal JRC pollen extract challenge.[131]

7.2. Intralymphatic immunotherapy (ILIT)

Intralymphatic immunotherapy (ILIT) seeks direct injection of allergens into the lymph nodes. Theoretically this approach has two advantages over the SCIT. First, direct delivery of allergens into the secondary lymph organs increases the efficacy of antigen presentation, thus enhances the immunogenicity of allergen, resulting in rapidly achieved benefits of treatment with reduced dose of allergen and numbers of injections. In addition, intralymphatic injection reduces the exposure of allergens to mast cells and basophils, thus reducing the side effects. In mouse models of bee venom or cat allergy, ILIT was found to be more efficient than SCIT in inducing allergen-specific IgG and T-cell responses and only ILIT was able to induce Th1-dependent IgG2a antibody production.[132]

In human, three injections of ILIT were found to be effective in inducing tolerance grass pollen and cat dander extract.[133,134] In two recent RDBPC controlled clinical trials, birch or grass SAR patients were treated with three inguinal lymph node injection (0.1ml per injection in 3- or 4-week intervals) of active allergen (ALK Alutard, 1000SQ-U aluminum hydroxide adsorbed birch or grass pollen) or placebo.[135,136] Twenty eight subjects received active allergen ILIT in these two trials and it seems that these subjects tolerated the ILIT well and no severe adverse events were elicited. Active ILIT patients experienced improved SAR symptoms. After nasal allergen challenge, the clinical nasal symptoms were reduced and the nose inflammatory responses were decreased in active ILIT patients. In symptom improved subjects, the affinity of allergen-specific IgG4 antibody was significantly higher than that from subjects without symptom improvement. These studies demonstrated that ILIT is a safe and effective treatment for tree-pollen allergies. However, the efficacy of this approach needs to be further evaluated in large scale and long term clinical trials. In addition, it is uncertain to what degree patients will accept this injection route.

7.3. Epicutaneous AIT (EPIT)

Epicutaneous AIT (EPIT) has been clinically tested as an alternative route of allergen delivery for more than 70 years.[137] The rationale in support of using this route is that the skin provides an antigen presenting cell, such as local Langerhans cells, enriched environment for rapid and efficient antigen presentation. Safety and clinical efficacy of EPIT for grass allergy or peanut-induced food allergies were evaluated in several RDBPC clinical trials.[138-141] EPIT requires administration on physically disrupted skin (*stratum corneum* disruption), for example, by type-stripping or abrasion treatment. Abrasion has a stronger effect in epidermal barrier disruption, but also results in a higher frequency of systemic reactions than the type-stripping skin preparation.[142] Alternatively, EPIT could be administrated through intact skin, which requires a long-term daily treatment. One potential advantage of EPIT is that the skin patches can be self-administered, resulting high adherence to treatment (more than 96%), although the convenience of daily dosing in the real-world setting remains to be determined.[139,141] The MoA of EPIT is not fully understood yet. In one clinical study, increased peanut-specific IgG4 levels and IgG4/IgE ratios were observed in peanut EPIT-treated subjects and a trend of reduced Th2 cytokine production and reduced basophil activation were found.[139] In an animal model for peanut allergy, EPIT induced allergen-specific regulatory T cells, which mediate the long-term immunological effects of treatment.[143]

EPIT for treating tree-pollen allergies has not yet been investigated in a human study. Using a mouse model for Bet v 1 induced allergic asthma, Siebeneicher et al. evaluated the immunological effects of epicutaneous delivery of Bet v 1 allergen combined with different adjuvants.[144,145] Recombinant Bet v 1, when epicutaneously administrated on *stratum corneum* disrupted skin with a Toll-like receptor 7 agonist, induced Th1 cytokine IFN-γ and IgG2a antibody. This therapy regimen exhibited efficacy in both prophylactic and therapeutic experiments, as indicated by suppression of lung inflammation and airway type-reactivity upon multiple intranasal challenges.[144] In a following EPIT study, a hypoallergenic recombinant protein Bet v 1B2, which is a folding variant of Bet v 1, was found to be superior to the wild type recombinant Bet v 1 in suppressing IgE production and lung inflammation.[145] Because of its reduced IgE binding capacity, the hypoallergenic Bet v 1B2 allergen is believed to be a safer candidate for EPIT. Recently, in a guinea pig model, Cabauatan et al. found that EPIT using recombinant Bet v 1, when combined with adjuvant heat-labile toxin, derived from *E.coli*., induced blocking IgG antibody, which inhibits the allergen binding of IgE from birch allergic patients.[146] These results from animal studies suggest EPIT might be an effective route of AIT administration for tree-pollen allergies.

8. Summary

Pollen allergies have become a significant public health burden in industrialized nations and their prevalence rates are believed to continue increasing in the foreseeable future. There is an unmet need to develop a safe, effective, and convenient allergen-specific therapies for treating pollen allergies. To this end, a variety of novel approaches have been developed. In the case of tree pollen allergies, the novel approaches include recombinant hypoallergenic derivatives, synthetic peptides, DNA vaccines, transgenic plants, different adjuvant formulations, and alternative delivery routes. These novel strategies have shown early promise and the mechanisms seem to be supported by a significant amount of animal data. Some of these approaches have shown to be safe with indications of potential effectiveness in Phase I/II trials and are thus very encouraging. It now remains for these approaches to be tested in RDBPC Phase III clinical trials to show safety and efficacy in natural field settings. In addition, continuous efforts on optimizing the formulation of allergens, delivery method, and administration doses/frequency will further improve the safety and efficacy profiles, as well as the patient's compliance to the therapy.

9. Expert opinion

Key points:

- Absence of true biomarker makes progress of therapy difficult to assess.
- Clinical studies for AIT are challenged by "placebo effect".
- Phase III studies are dependent upon "park studies" rather than controlled environmental chamber studies where the pollen exposure is defined and controlled.
- There is a relatively low bar for improvement in symptoms for regulatory approval (approximately 20 – 30% improvement) so a successful PIII study should lead to licensure.
- Conventional AIT is based on immune system attenuation whereas DNA vaccines mechanism is believed to be active immune system "re-education."

The allergy challenge is complicated by several factors that are independent of the therapeutic method. Allergy lacks a method for a clinically relevant biomarker assay. For example, specific IgE, while often a compelling means to monitor allergy status, does not necessarily correlate with symptomology. The most relevant assay for allergy remains skin prick testing but this assay format requires a skilled allergist to deliver and evaluate the response. In the clinical setting, evaluating symptom responses of test subjects is not straightforward. Study subjects use arbitrary scoring of symptoms as a means of evaluating test treatments. This has resulted in a very pronounced placebo effect in many clinical studies which has made determination of symptom relief difficult to impossible. In the case of tree pollen, subjects for a Phase III study are expected to be exposed to natural allergen in a so-called "park test". This adds additional variability to the interpretation of the data as pollen levels vary annually and exposure to open air pollen will include non-related pollens which certainly can influence the observed symptoms in the study subjects. Thus, any approach whether the standard AIT method or one of the newer peptide or DNA vaccine approach faces a number of challenges in the clinical setting to prove clinical relevance.

Desensitization is the approach that allergists are familiar with and have employed for many years. It suffers from poor patient compliance and a general overall success rate of about 30%. The newer therapeutic approaches center on one of two primary mechanism of action: tolerance induction or Th1 activation. In the case of the former, the goal is to essentially turn the immune system off with respect to a specific allergenic target. With Th1 activation, the objective to re-educate the immune system such that the allergen no longer induces a Th2 response but rather switches to the antigenic Th1 pathway. The next generation of AIT methods have focused on using better defined allergens or peptide derivative in combination with adjuvants or additives intended to favor immune system attenuation. Despite the innovation in the newer AIT designs, new product approvals have been limited to oral or sublingual delivery of allergen formulations. The concept of an allergy vaccine therapy using DNA vaccines and in particular, LAMP-based formulations provides an intriguing new alternative. This approach has generated potent Th1 responses in animal models and showed promising initial responses in early human clinical studies. Vaccine immunotherapy also has the added safety advantage of treating subjects without direct exposure to allergen. Given the overall goal of creating a permanent solution to treating allergy, this latest approach may provide the long-lasting symptom relief that has eluded allergy researchers for decades.

Disclosure of potential conflicts of interest

Y.S, E.R., and T.H. are employees of ITI.

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