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Antigen-Specific Tolerance in Immunotherapy of Th2-Associated Allergic Diseases

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Abstract

Allergic diseases are an increasing health concern, particularly in the developed world. The standard clinical approach to treatment of allergic disease focuses on allergen avoidance and symptom control but does little to address the underlying Th2 bias of disease. Specific immunotherapy (SIT) consisting of controlled administration of allergen, however, has been demonstrated to successfully induce desensitization and tolerance in an antigen-specific manner for a variety of Th2-mediated diseases. This review focuses on the mechanisms by which current SIT approaches induce tolerance as well as discussing attempts to modify the safety and efficacy of SIT. These refinements focus on three major aspects of SIT: the route of antigen administration, modification of the antigen to remove allergenic epitopes and reduce adverse events and choice of adjuvant used to induce tolerance and/or immune deviation from Th2 to Th1 and regulatory T cell (Treg) phenotypes. Synthesis of these recent developments in SIT provides considerable promise for more robust therapies with improved safety profiles to improve resolution of allergic disease and its associated costs.

Keywords

Adjuvants; routes of administration; allergen modification; antigen carriers; treatment

I. INTRODUCTION

Allergic disease is an increasing health concern, particularly in developed nations. Allergic diseases are thought to be induced by natural exposure to allergens leading to a T-helper-2-type (Th2)-dominated immune response characterized by IgE-mediated hypersensitivity to allergen. Th2 cells produce cytokines such as IL-4, IL-5, IL-9 and IL-13 that modulate the responses of innate effector cells such as eosinophils, basophils and mast cells and further propagate the adaptive arm of the Th2 response by inducing allergen-specific B cell activation and class switching to IgE.¹ Allergen-specific IgE binds to Fc RI on the surface of mast cells and basophils, which, upon re-exposure to allergen, are activated and release mediators that cause the development of type I hypersensitivity.² The standard clinical response to allergic disease is one of allergen avoidance and symptom control rather than a curative approach targeted to the underlying Th2 bias of allergic disease. However, specific immunotherapy (SIT) consisting of gradually increasing doses of soluble allergen has been used for over 100 years for the treatment of allergic disease.^{3,4} Despite the long history of

SIT and its promise of ultimately providing an antigen-specific cure for allergic disease rather than lifelong symptom control, advances in SIT have been slow to enter clinical practice. This has been in part due to concerns over the efficacy and safety of SIT in allergic patients, as well as a lack of understanding of the mechanisms underlying the action of SIT. This review will provide an overview of the tolerogenic mechanisms common to SITs and describe three primary areas in which attempts have been made to improve SIT. First, we will discuss the importance of route of administration to the success of SIT, focusing on classical subcutaneous immunotherapy (SCIT), mucosal routes of administration and intralymphatic immunotherapy (ILIT). Second, we will discuss attempts to modify the structure of the antigen in SIT with the goal of decreasing allergenicity associated with adverse events and increasing immunogenicity associated with successful immunotherapy. Finally, we will discuss various formulations/delivery vehicles including use of adjuvants that have been employed in SIT, from classical alum adjuvant to TLR agonists, and the use of delivery platforms including apoptotic cells and particulate carriers of antigen.

II. MECHANISMS OF TOLERANCE

The ultimate goal of SIT is the induction of long-lasting, antigen-specific immunoregulation of Th2-mediated hypersensitivity. Studies of SIT have identified three primary mechanisms by which this is achieved – through immune deviation to a competitive Th1 response, through the induction of a suppressive regulatory T cell (Treg) response, and through the induction of anergy or deletion of Th2 cells (Fig. 1).

Beginning with the earliest categorization of T helper cell subsets, Th1 and Th2 cells have been defined as separate, mutually inhibitory lineages. The relationships between T helper cell subsets have since proven to be considerably more complex, but factors associated with Th1-type immunity have still been shown to antagonize Th2-type responses. Antigen presenting cells (APCs) presenting antigen in the context of IL-12 induce IFN- γ -producing Th1 cells and inhibit the differentiation of Th2 cells.⁵ Similarly, IFN- γ itself has been shown to inhibit Th2 development and Th2-mediated allergic inflammation.^{6,7} Importantly, Th1 cells and associated cytokines promote B cell class switching to IgG isotypes, including IgG4, and inhibit IgE production.⁸ IgG antibodies compete with pathogenic IgE for allergen binding and, as outlined later in this review, have an intrinsic immunosuppressive effect.

Tregs comprise another T helper cell subset that has been demonstrated to play a crucial role in the regulation of allergy. Tregs can be broadly categorized as natural CD4⁺CD25⁺Foxp3⁺ Tregs and inducible, IL-10 producing T regulatory 1 (Tr1) cells. A comparative study of T cell subsets in healthy versus allergic individuals found that Tr1 cells dominated the allergen-specific T cell population in healthy individuals, while allergic patients showed an increased frequency of Th2 cells. Antigen-specific suppression by Tr1 cells in this study was mediated by IL-10, TGF- β , PD-1 and CTLA-4.⁹ The induction of Tregs has been demonstrated in many studies of SIT, where they have been demonstrated to have suppressive effects on almost all aspects of Th2-mediated hypersensitivity.¹⁰ Indeed, a study of beekeepers before and during beekeeping season found that the high dose bee venom exposure beekeepers receive robustly induces IL-10 secreting Tr1 cells, although not CD4⁺CD25⁺ Tregs, while reducing numbers and responsiveness of Th1 and Th2 cells. *In vitro* cultures of isolated PBMCs showed that IL-10, PD-1 and CTLA-4 activity was crucial for the antigen-specific suppression of T cell proliferation and cytokine production in response to bee venom restimulation.¹¹ Additionally, transfer of Tregs inhibited and reversed airway hyper-responsiveness (AHR), lung inflammation and Th2 responses in murine models of allergic airway inflammation.^{12,13} Tregs can directly suppress innate mediators of allergic responses, as they have been demonstrated to inhibit mast cell degranulation and subsequent anaphylaxis through OX40-OX40L interactions.^{14,15} Through

production of IL-10 and TGF- β , Tregs also have the potential to induce production of protective IgA and IgG4 antibodies.^{16–20} Antigen-specific IgA and IgGs, particularly IgG4, are induced by SIT and block interactions between IgE and allergens, inhibiting early phase responses to allergen challenge.²¹ Furthermore, IL-10 has been shown to induce a tolerogenic population of CD45RB^{high} DCs that secrete IL-10 and, in turn, induce the differentiation of Tr1 cells, creating a positive tolerogenic feedback loop.²² IL-10 has also been demonstrated to have direct suppressive effects on T cells, inhibiting the CD28 costimulatory pathway and raising the threshold for T cell activation, resulting in anergy.²³

III. ROUTES OF IMMUNIZATION

It has been well established that the immune response can be highly variable between tissues, with multiple tissue-specific innate and adaptive immune cells regulating these responses. In particular, the presence of different APC populations can be critical to the success of SIT, while the distribution of effector cells such as mast cells can impact adverse reactions to treatment.^{24–26} Therefore, the route of administration of SIT can have significant impacts on the tolerability and efficacy of treatment, as will be discussed below. Following the initial studies by Noon and Freeman, most SIT has been delivered subcutaneously (SCIT) and this is the only route of administration currently approved by the FDA.^{3,4,27} Mucosal routes of administration have also been studied extensively, although of these only sublingual immunotherapy (SLIT) is currently approved for clinical use in Europe. Ultrasonography has allowed for the direct intralymphatic injection of antigen (ILIT), which has the advantage of directly supplying antigen to immune cells.

A. Subcutaneous Immunotherapy

First described in 1911 by Noon and Freeman, subcutaneous immunotherapy (SCIT) is the most commonly used form of SIT and the only type of SIT currently approved by the FDA.^{3,4,27} As such, studies of SCIT have been used to identify many of the mechanisms of SIT outlined above. Indeed, SCIT has been demonstrated to reduce Th2 and increase Th1 cytokine production.^{28,29} Additionally, SCIT skews antibody responses from IgE to protective IgA, IgG1 and IgG4 isotypes.^{21,30} SCIT induces antigen-specific T cell anergy in an IL-10 dependent fashion³¹ and, in mice, it was found that IL-10 was crucial for many of the beneficial effects of SCIT including reductions in airway responsiveness and inflammation, antigen-specific IgE and Th2 cytokines.³² SCIT typically requires a build-up phase of one to three injections per week for the first three to six months of treatment, with maintenance injections every two to eight weeks afterwards for three years or longer.²⁷ SCIT takes advantage of the large proportion of largely tolerogenic Langerhans cells present in the skin, although they are capable of recruiting inflammatory dendritic epidermal cells (IDECs) which, together with tissue resident mast cells, may account for some of the adverse events observed with SCIT.^{24–26} However, on encounter with antigen, IDECs also produce Th1-driving cytokines IL-12 and IL-18, which may contribute to the switch from Th2 to Th1 dominated responses seen in successful SCIT.²⁵ In a recent study demonstrating multiple mechanisms of SIT, SCIT with birch pollen reduced patient symptom scores, medication use and skin prick test (SPT) reactivity.³³ Additionally, there was an early increase in IL-10 secreting Tr1 cells correlating with decline in clinical symptoms. Birch pollen-specific Th2 cells producing IL-5 were increased at this time, suggesting active suppression as a primary mechanism of tolerance at this stage of treatment.³³ Later, in the maintenance phase of treatment, the ratio of Th2/Th1 cells decreased, indicating a shift from Th2 to Th1 immunity. Additionally, allergen-specific IgG, particularly IgG4, increased significantly after the first 3 months of treatment, as did the ratio of pollen-specific IgG4/IgE.³³ A follow-up study found that the Tr1 increase was only transient and declined to baseline over the three years of SCIT. Birch-specific IgG4 levels continued to increase

during SCIT, although total levels and the IgE-blocking capacity of serum IgG declined after cessation of SCIT.³⁴

Two principle disadvantages of SCIT are the frequency of clinical visits needed for injections and the risk of systemic adverse events. Therefore, epicutaneous delivery of antigen by an easily applied patch provides a potentially more convenient alternative to traditional SCIT, while improving the safety profile due to the lack of vascularization of the epidermis. An initial clinical trial of epicutaneous immunotherapy delivering a antigen-filled patch after tape-stripping of the skin in grass pollen allergy showed only moderate efficacy, but was well tolerated, although patch-associated eczema was observed in some patients.³⁵ Nasal challenge was not significantly improved, but improvements were observed in patient symptom scores, verifying reports of an allergist in 1959 performing needle scarification to induce antigen-specific immunotherapy in hay fever patients.^{35,36}

SCIT has been shown to be effective for the treatment of allergic rhinitis, allergic conjunctivitis, allergic asthma and stinging insect hypersensitivity.²⁷ However, SCIT is not recommended for treatment of food allergy due to the high rate of systemic adverse reactions, even during maintenance-phase injections.³⁷ Overall, SCIT has a long and successful track record of inducing tolerance/immune deviation for the treatment of allergic disease. However, the need for frequent injections creates a significant inconvenience that inhibits patient compliance. Furthermore, SCIT is frequently associated with local and occasionally systemic allergen-related adverse events including fatal airway obstruction and anaphylactic shock³⁸ as well as the formation of adjuvant-related granulomas due to inaccurate injections.³⁹ With these disadvantages in mind, other routes of more convenient, safe and effective antigen administration are currently being examined for SIT.

B. Mucosal Immunotherapy

Mucosal surfaces are typically considered to be tolerogenic sites of antigen encounter. Exposure to food and aeroantigens is well tolerated in healthy individuals. Compared to the skin, APCs in mucosal surfaces display a more tolerogenic phenotype. However, despite the generally improved tolerance of antigens in the mucosa, it should be noted that mast cells are present at levels equivalent to the skin, resulting in similar risks for adverse events upon allergen administration to sensitized individuals.²⁴ A number of studies of mucosal SIT have been performed to take advantage of these naturally tolerogenic routes of antigen administration.

1. Sublingual Immunotherapy—SLIT is performed by administration of increasing doses of antigen drops or tablets that are held under the tongue for uptake by oral APCs, primarily Langerhans cells, prior to swallowing.²⁴ Unlike SCIT, SLIT can be administered at home after clinically monitored initial doses, thus providing benefits in convenience and comfort. However, compliance is complicated by the need for SLIT to be more frequent than SCIT treatments, although recent studies indicate that a less frequent, intermittent treatment schedule can increase compliance while maintaining comparable efficacy.⁴⁰ SLIT has entered common clinical use in Europe as an alternative to SCIT, but has not yet been FDA approved. A Cochrane meta-analysis confirms the efficacy and safety of SLIT for the treatment of allergic rhinitis, characterized by reduced symptom scores and medication use, increased antigen-specific IgG and IgG4 and typically only minor local adverse reactions.⁴¹ Similarly, peanut allergic patients treated with SLIT demonstrated significant desensitization to peanut, reduced SPT reactions and increased antigen-specific IgG4, although increased IgG4 was also observed in patients that were not successfully desensitized.⁴² Additional studies of SLIT for dust mite and pollen allergens demonstrated reduced proliferation and Th2 responses from restimulation of allergic patient PBMCs and increased levels of IFN- γ ,

IL-10, IgG1, IgG4, IgA and Foxp3⁺ Tregs.^{43–45} The observed upregulation of Tregs with SLIT matches reports that oral Langerhans cells cultured *in vitro* with grass pollen allergen have impaired maturation and both produce IL-10 and TGF- β and stimulate T cells to produce IL-10 and TGF- β .⁴⁶

Trials comparing SLIT and SCIT have demonstrated that SIT by either route of administration induces similar reductions in symptom scores, antigen-specific IgE and allergic reactions to antigen challenge.^{47,48} However, the relative tolerability of each technique, as well as the involvement of IL-10 and IgG4 in tolerance, remains unclear due to conflicting data.^{47–49} A study of children with house dust mite allergy combined SCIT in the four month build up phase of tolerance with SLIT in the maintenance phase over the next 14 months. Patients treated with this novel schedule did not experience adverse events and quickly demonstrated reduced symptom scores and medication usage and induced protective IL-10, TGF- β and antigen-specific IgG4.⁵⁰ Thus, this combined approach yielded the benefits of rapid, robust tolerance induced by SCIT with the ease and safety of tolerance maintenance with SLIT.

2. Oral Immunotherapy—Unlike SLIT, oral immunotherapy (OIT) is performed by the immediate swallowing of antigen. Like other mucosal routes of immunotherapy, OIT takes advantage of a classically tolerogenic route of antigen delivery. In addition to the tolerogenic oral Langerhans cells believed to play a role in SLIT, populations of Treg-inducing intestinal DCs have been identified and it has been observed that intestinal macrophages can produce IL-10 and TGF- β .^{51–53} Studies of children with milk and egg allergies found that dietary consumption of baked food allergens resulted in a significant increase in tolerance to unheated allergen and a concurrent decrease in allergen-specific IgE and SPT reactivity and increased allergen-specific IgG4.^{54–56} A study of children with peanut allergy demonstrated that after one year of OIT, treated patients tolerated considerably higher doses of peanut by oral challenge. This desensitization was associated with increased peanut-specific IgG4, an increased Treg/effector T cell ratio and decreased IL-5 and IL-13 production from peanut-restimulated PBMCs.⁵⁷ OIT of egg allergic children with egg white powder was correlated with improved tolerance of antigen challenge, decreased basophil activation, decreased antigen-specific IgE and increased IgG4. However, the majority of patients experienced adverse oral and pharyngeal events related to OIT and only 18% of the original cohort passed antigen challenge only two months after cessation of OIT.⁵⁸ A clinical trial comparing the safety and efficacy of SLIT and OIT in peanut-allergic patients found that OIT was more effective at both desensitization and lasting tolerance after the cessation of treatment whereas SLIT was safer, with fewer adverse events and less need for medication after dosing than OIT, which induced not only mild local events but also some systemic adverse events. SLIT and OIT both reduced SPT reactions to peanut and induced peanut-specific IgG4, although this did not correlate with improved clinical outcome.⁵⁹

Oral immunotherapy has been shown to significantly improve patient quality of life.⁶⁰ However, meta-analyses of OIT for food allergy conclude that current evidence of improvements due to OIT is insufficient to justify the increased cost and risk of adverse events compared to allergen avoidance.^{61,62} Overall, further studies are needed to demonstrate the efficacy of OIT, particularly in regards to the longevity of tolerance after treatment.

C. Intralymphatic Immunotherapy

Recent studies have shown the promise of direct administration of allergens to lymph nodes. ILIT directly addresses two principal concerns with conventional SIT – safety and efficacy.

By delivering antigen directly to lymphatic tissue, ILIT is posited to induce a stronger immune response while avoiding injection site and systemic adverse events associated with traditional SIT. Early studies in rodent models demonstrated that vaccines delivered directly to lymph nodes elicited considerably more robust immune responses compared to other routes of immunization.⁶³ The first therapeutic applications of intralymphatic immunogenicity were reported with whole cell vaccines in cancer patients,^{64,65} but were also studied in animal models with vaccines including DNA,⁶⁶ peptides,⁶⁷ and proteins⁶⁸. These studies confirmed that intralymphatic injections induced significantly more robust immune responses, requiring considerably less antigen than other routes of immunization. Additionally, ILIT has been shown to decrease the need for adjuvant compared to SCIT, thereby reducing the risk of adjuvant-related adverse events.⁶⁹ ILIT has only recently been investigated for use in allergic patients. An initial trial of ILIT in patients with hay fever due to grass pollen allergy found that patients receiving ILIT experienced less pain than is induced by venous puncture and had fewer adverse reactions than patients undergoing SCIT.⁷⁰ ILIT reduced symptoms in response to nasal challenge and SPT and decreased grass-specific IgE levels comparably to SCIT, and required only three injections over eight weeks as opposed to 54 injections over three years, resulting in significantly improved patient compliance.⁷⁰ The success of ILIT was corroborated in a small-scale trial of patients with cat dander allergy using a recombinant Fel d 1 modified to enhance presentation through the MHC II pathway.⁷¹ Patients receiving three treatments of ILIT showed improved nasal and skin tolerance to cat dander, increased dander-specific IgG4, but not IgE, and increased IL-10 production by dander-restimulated PBMCs.⁷¹ The early successes of these clinical trials of ILIT highlight the importance of carefully targeted administration of allergens for SIT. The improved safety and efficacy provided by ILIT can be further capitalized on by antigen and adjuvant modifications as will be discussed later in this review.

IV. ALLERGEN MODIFICATION

In sensitized individuals, there is considerable risk in the administration of native allergen for immunotherapy. Whole antigen can bind and crosslink Fc RI on the surface of innate effector cells, initiating adverse events including anaphylaxis. With this risk in mind, diverse approaches have sought to improve the safety of SIT by eliminating or masking conformational epitopes recognized by antibodies, thereby removing the risk of anaphylaxis. In other words, the goal of allergen modification is to minimize allergenicity while retaining immunogenicity, thus allowing for higher doses of antigen to be administered in a shorter time course. These approaches range from simple chemical cross-linking or denaturation of native antigen to the engineering of recombinant peptides and PCR-mutated proteins. Despite the variety of approaches taken, these modified allergens, sometimes referred to as allergoids, all aim to improve the safety profile of SIT while retaining or enhancing protective immunogenicity.

A. Denaturation

The simplest method of removing conformational epitopes from allergens is by heat denaturation. It is well known that cooked foods are generally better tolerated by allergic individuals, likely due to the inability of IgE to bind heat denatured and aggregated antigen.^{54,72–74} Further degradation of protein structure by the addition of a reducing agent such as β -mercaptoethanol to heat denatured antigen further reduces its allergenicity by the reduction of disulfide bonds.^{31,75} Studies have found that heat denatured antigens have reduced ability to sensitize Th2 responses in mice and, in conjunction with an adjuvant such as alum, increase Th1 responses as shown by enhanced IgG2a and IFN- γ production.^{72,75} Studies of milk and egg allergies have shown that allergic children tolerated consumption of baked food allergens and that treatment with dietary baked milk or egg was considerably

more likely to induce tolerance to unheated allergens, a state associated with increased levels of allergen-specific IgG4, likely induced through the oral route of mucosal tolerance.^{54–56}

B. Chemical cross-linking

Treatment with aldehydes such as formaldehyde or glutaraldehyde cross-links proteins, causing the formation of high molecular weight oligomers that obscure native conformational epitopes. Glutaraldehyde-treated antigen has been demonstrated to have significantly reduced IgE binding and concomitant allergenicity, while retaining the immunogenic properties of native antigen.⁷⁶ Aldehyde cross-linked antigens, similar to soluble antigens, are presented by DCs and macrophages, which produce IL-12 and IL-10, driving antigen-specific T cells to Th1 and Treg phenotypes.⁷⁷ Furthermore, clinical trials demonstrated that SCIT with several different aldehyde-treated extracts was well tolerated in allergic patients and successfully induced tolerance as measured by SPT and nasal challenge and also induced favorable antigen-specific IgG4/IgE ratios compared to placebo.^{78–80} A trial of SCIT with formaldehyde-treated olive pollen showed, compared to baseline, decreased basophil activation upon allergen stimulation and an increased IgG4/IgE ratio at eight months after only seven pre-seasonal injections.⁸¹ The reduced allergenicity of aldehyde-cross-linked allergoids therefore allows for the possibility of shortened treatment schedules as greater doses can be safely administered to rapidly induce tolerance.

C. Carbamylation

Carbamylation by isocyanic acid or reaction with acid anhydride reacts with free amino groups (lysine and arginine) on proteins,^{82,83} thereby inhibiting the formation of salt bridges and altering conformational epitopes. These allergoids are monomeric with a consequent low molecular weight that allows for easier bypass of mucosal barriers than aldehyde-reacted allergoids. As with aldehyde cross-linking, disruption of conformational epitopes in monomeric allergoids renders them less readily bound by IgE but retains their immunogenicity, including induction of antigen-specific IgG antibodies.⁸³ A clinical trial with standardized carbamylated allergoid tablets for SLIT of patients with pollen or HDM allergies showed that allergoid SLIT significantly reduces symptom-medication scores and nasal reactivity compared to untreated controls and allows for a shortened or entirely bypassed build-up phase of tolerance compared to standard clinical practice for SIT, thereby allowing faster entry in to maintenance phase dosing.⁸⁴

D. Synthetic Peptides

Synthetic peptides are increasingly used in studies of immunotherapy. The primary advantages of peptide, rather than whole protein or protein extract, is the use of a well characterized antigen and, more importantly, reduced ability to cross-link IgE and induce adverse events, as IgE specificities typically develop against conformational epitopes on tertiary or quaternary structure of proteins which short peptide fragments do not display. However, peptides are capable of being recognized by the T cell receptor (TCR), therefore retaining immunogenicity while minimizing allergenicity. However, it should be noted that IgE responses can develop against linear peptides and, in murine models, can induce fatal anaphylaxis.⁸⁵ Early trials of peptide immunotherapy for cat allergy showed that, in mice and humans, peptides derived from the cat allergen Fel d 1 were able to safely induce T cell tolerance and reduce symptoms from antigen challenge.^{86,87}

Similarly, immunotherapy to bee venom allergen phospholipase A2 (PLA2) was well tolerated in mice and humans and reduced symptoms to antigen challenge. Tolerance induced by PLA2 peptides also displayed hallmarks of protective immune deviation, with decreased production of IL-4 and increased antigen-specific IgG4/IgE ratios.^{88,89} A

deficiency of peptide immunotherapy is that only a subset of T cell epitopes can be covered by a single peptide, thus requiring cocktails of peptides encompassing multiple epitopes for standardized SIT. A clinical trial of SIT with a cocktail of seven peptides generated from the cat allergen Fel d 1 demonstrated reduced allergenicity, but similar immunogenicity compared to whole Fel d 1. SIT with this peptide cocktail induced a trend, albeit non-significant towards reduced skin reactivity compared to placebo control.⁹⁰ A slightly different approach to peptide immunotherapy identified surface peptides of allergens that were not bound by IgE and had only low T cell reactivity, but instead induced protective IgG antibodies that were able to inhibit binding of whole allergen to allergic patient IgE.^{91,92} This intriguing approach warrants further study in allergic disease models to determine what role, if any, T cells may play in the induction or maintenance of tolerance induced with IgG-, but not T cell-reactive peptides.

E. Recombinant Allergens

An obstacle for peptide approaches to immunotherapy is the diversity of epitopes recognized both in individuals and across a population. Therefore, generating sufficient peptides to cover the diversity of individual responses becomes difficult and cost prohibitive. Several attempts have been made to combine the advantages of a defined, safe antigen offered by peptide immunotherapy with the inclusion of all available T cell epitopes offered by approaches such as allergoids and traditional whole protein SIT. One such approach was the creation of a recombinant fusion protein of the two major bee venom allergens, Api m 1 and Api m 2. This fusion protein was found to have reduced binding to IgE and IgG antibodies from bee venom allergic patients and further displayed reduced allergenicity in SPT and basophil mediator release assays.⁹³ However, the fusion protein retained the ability to stimulate T cells derived from allergic patient PBMCs and, given to mice as SCIT prior to sensitization with wildtype Api m 1, prevented the production of Api m 1-specific IgG1, IgG2a and IgE antibodies.⁹³ Another group generated modified forms of the major peanut allergens Ara h 1, 2 and 3 with point mutations of amino acids at critical IgE binding sites. These mutant proteins demonstrated reduced allergenicity as measured by IgE-binding but retained the capacity to stimulate T cell responses, thereby enabling their safe use in SIT to prevent allergic inflammation and anaphylaxis in murine models of allergic asthma and food allergy.⁹⁴⁻⁹⁶ This site directed mutagenesis approach to reducing the allergenicity of whole proteins has also proven successful for pollen and dust mite allergens, inhibiting IgE binding and SPT and basophil reactivity while retaining the ability to stimulate T cells.⁹⁷⁻¹⁰¹

V. ADJUVANTS/DELIVERY VEHICLES

Delivery of antigen alone to naïve T cells is known to induce tolerance. Under the two-signal hypothesis antigen in the context of MHC (signal one), without costimulation (signal two) drives T cells towards an unreactive, anergic phenotype. This has been borne out in studies of non-mitogenic anti-CD3 to induce TCR cross-linking in the absence of costimulation.^{102,103} However, SIT often includes an adjuvant to boost immune responses. Due to the established Th2 bias of patients undergoing SIT, antigen alone may exacerbate allergic responses or simply be insufficient for therapy. Adjuvants are immune modulators that, when administered with antigen, drive the immune response in a desired direction. This adjuvant activity typically takes advantage of the innate immune system to potentiate adaptive immune responses. For the purposes of this review, adjuvants include not only potentiators of effector immune responses but also of regulatory, tolerogenic responses. Co-administration of antigen with the proper adjuvant can drive Th1 responses to compete with Th2-mediated hypersensitivity as well as Treg responses for the active suppression of inflammation as discussed in section II above. The efficacy and known mechanisms of adjuvants ranging from classical alum to TLR agonists and antigen delivery platforms ranging from apoptotic cells to engineered particulate antigen carriers are discussed below.

A. Alum

The adsorption of antigen onto aluminum salts is one of the oldest and most common means of boosting immunogenicity for vaccinations. Because of this adsorption, it was long hypothesized that the adjuvanticity of alum was owed to a depot effect wherein antigen was slowly released over a long period of time, allowing for persisting priming of immune responses and improved safety over soluble antigen. However, this depot effect was found to be tissue-dependent and, more notably, dispensable for the adjuvant effects of alum.^{104,105} Alum injection results in the rapid influx of eosinophils, neutrophils, monocytes, DCs, NK cells and NKT cells.¹⁰⁶ Newly migrated cells, as well as tissue-resident cells such as macrophages and mast cells, produce a variety of proinflammatory cytokines and chemokines, including MCP-1, KC, IL-1b, IL-4, IL-5, IL-6 and histamine. This inflammatory milieu is likely responsible for the strong Th2 bias of alum-induced immune responses seen in mice.¹⁰⁶ Alum binds to the cell membrane of macrophages and DCs, activating the Syk-PI3k pathway, ultimately leading to the release of prostaglandin E2, which, in conjunction with IL-4, induces the production of IgE.¹⁰⁷ Additionally, alum induces the release of uric acid, which has been demonstrated to have an adjuvant effect on its own.^{108,109} Phagocytosis of alum and uric acid particles activates the NLRP3 inflammasome, which induces activation of caspase-1 and production of proinflammatory IL-1 β ,^{109,110} and induces the maturation of APCs which then activate antigen-specific T cells.¹⁰⁹ However, despite this response to NLRP3 inflammasome activation, there is considerable controversy regarding its role in adjuvanticity as both alum and uric acid can exert adjuvant effects independent of NLRP3 through as yet unknown sensing mechanisms.^{106,108}

Despite the Th2 bias of immune responses induced by alum in mice, alum has been a commonly used adjuvant for SCIT in Europe, where clinical trials employing alum adjuvant have been successful in treating allergy.^{21,30,111,112} However, in addition to the aforementioned Th2 predisposition of alum, subcutaneous alum injections are associated with adverse granulomatous events. The granulomas associated with alum injections appear to be due to local toxic effects of alum and consist of activated eosinophils, T cells and monocytes and, in the case of inaccuracy resulting in intradermal rather than subcutaneous injections, can be long-lasting.^{39,113} Because of these limitations of alum, it is important to investigate new adjuvants for SIT of Th2-driven allergic diseases. Such adjuvants would have the goal of skewing towards Th1 responses to compete with the Th2 bias of allergic disease, inducing suppressor cells such as Tregs or inactivating allergen-specific cells through clonal deletion or anergy.

B. TLR Agonists

Toll-like receptors (TLRs) are molecules that recognize pathogen-associated molecular patterns and induce robust immune responses. TLRs recognize lipids, proteins, lipoproteins and nucleic acids from microbes including bacteria, viruses, fungi and parasites.¹¹⁴ Adjuvants targeting TLRs with Th1-inducing activity are desirable for SIT for allergic diseases. Several studies in mice have determined that the use of heat-killed bacteria such as *Listeria monocytogenes* and *Escherichia coli* as adjuvants for SIT inhibits Th2 responses and skews the immune response towards Th1 and Treg type responses, thereby reversing established allergic inflammation.^{94,95,115,116} In particular, several species of probiotic *Lactobacillus*, when used as adjuvants for treatment of murine models of allergic asthma and food allergy, have been shown to preferentially induce IL-12 and IL-10-mediated mixed Th1/Treg responses that inhibit allergic inflammation and Th2 recall responses.^{117,118} In the following section we will discuss SIT approaches using adjuvants targeting specific TLRs.

1. TLR9 Agonists—CpG oligodeoxynucleotides (ODNs) are recognized by TLR9 within the endosomes of APCs. Stimulation through TLR9 induces macrophages and DCs to become activated and upregulate costimulatory molecules such as CD40, CD80, CD86 and MHC class II and produce high levels of IL-12 thereby inducing a potent Th1 response.¹¹⁹ In mice, CpG administered as an adjuvant for SIT induced local and systemic Th1 responses characterized by antigen-specific IgG2a, IgA and IFN- γ production and inhibition of antigen-specific IgE.^{120,121} In a model of allergic airway inflammation, this Th1 skewing effect resulted in the ablation of AHR and lung eosinophilia.¹²¹ CpG ODNs have been used as adjuvants in clinical trials for ragweed allergy SIT with differing effects. Some of the variability in CpG trials is due to the relative efficacy of different types of CpG. B-type CpG has a biologically stable phosphorothioate backbone and is most commonly used clinically. However, A-type CpG is a more potent IFN- γ -inducing adjuvant, but is less stable due to its phosphodiester backbone that is readily cleaved by DNase type I in vivo. A clinical trial stabilizing A-type CpG by association with bacteriophage coat protein-derived virus-like particles demonstrates that it can be a potent adjuvant for SCIT that, less than a year after a 10 week treatment course, ameliorates symptoms of rhinitis and allergic asthma and boosts antigen-specific IgG allergic patients.¹²²

2. TLR4 Agonists—Bacterial-derived lipopolysaccharide (LPS) has been demonstrated to signal through TLR4, resulting in the activation of APCs and subsequent induction of a potent Th1 response.^{123,124} However, the profound toxicity associated with LPS makes it a poor choice for a therapeutic adjuvant. A derivative of LPS, monophosphoryl lipid A (MPL), has been demonstrated to have reduced toxicity while retaining immunostimulatory effects.^{125,126} MPL activates APCs through TLR4, inducing their maturation and upregulation of MHC II and costimulatory molecules such as CD80 and CD86 and the production of IL-12, TNF- α and IL-10.¹²⁷ Stimulation with MPL caused oral Langerhans cells to upregulate IL-10 and the negative costimulatory molecule PD-L1, driving naïve T cells to Th1 and Treg phenotypes as determined by expression of IFN- γ , IL-10, TGF- β and Foxp3.¹²⁸ MPL also acts directly on T cells, inducing their upregulation of CD40L.¹²⁹ Clinical trials using MPL as an adjuvant for SCIT and SLIT found that SIT was well-tolerated and resulted in significant reductions of symptom scores, nasal and SPT reactivity tests and antigen-specific IgE as well as increased antigen-specific IgG, including IgG4, after only a small number of treatments.^{130,131} MPL represents a promising adjuvant for SIT, able to induce potent immune deviation to Th1 and Treg phenotypes with relatively little associated toxicity.

3. TLR2 Agonists—TLR2 agonism with lipopeptides has been shown to induce Th1 and Treg skewing to inhibit Th2 responses. The synthetic lipopeptide Pam3CSK4 was found to signal through TLR2 and be a potent inducer of IL-12 and IL-10 in BMDCs and oral myeloid DCs, which in turn stimulate IFN- γ and IL-10 production by co-cultured naïve CD4⁺ T cells.¹³² T cells co-cultured with Pam3CSK4-treated APCs also display abortive proliferation, indicative of tolerogenic anergy. Treatment of sensitized mice with Pam3CSK4 decreased AHR, airway inflammation and Th2 recall responses in murine models of allergic airway inflammation.^{132,133} Another synthetic lipopeptide, LP40, was also found to signal through TLR2 and induce IL-10, IFN- γ and IL-12 production. Treatment of human PBMCs with LP40 showed an inhibition of antigen-specific IgE and an induction of protective antigen-specific IgG4. Additionally, LP40 inhibited allergen- and *Schistosoma*-induced eosinophilia.¹³⁴ From these studies, lipopeptides have been identified as potential adjuvants to induce T cell inactivation and/or immune deviation to Th1 and Treg phenotypes for the treatment of allergic disease.

C. Inhibitory Immunoglobulins

Intravenous immunoglobulin (IVIG) has been demonstrated to have a profound anti-inflammatory effect in a variety of disease settings. Originally used for antibody replacement therapies, IVIG has seen broad application to a variety of inflammatory disorders.¹³⁵ In murine models, Ig given intravenously or sublingually has been shown to inhibit allergic airway inflammation.^{136,137} Clinical trials of IVIG for the treatment of asthma demonstrated some reduction in symptoms, SPT reactivity and medication use, although this was not consistent across all trials and was associated with a considerable degree of IVIG-associated adverse events, potentially due to the poorly defined, polyclonal nature of pooled human Ig.^{135,138} Despite some of these clinical contraindications, there remains significant therapeutic potential for the use of IVIG and related, better defined modalities in allergic diseases. IVIG was found to inhibit IgE production by B cells *in vitro*.^{139,140} Additionally, IVIG has been demonstrated to induce Tregs and enhance their suppressive function.^{137,141} Many of these suppressive effects of IVIG appear to be due to interactions of IgG with the inhibitory Fc receptor FcγRIIb.¹⁴² Furthermore, the anti-inflammatory properties of IgG are strongly associated with sialylation of the Fc portion of the antibody, causing interactions with SIGN-R1 on APCs leading to upregulation of FcγRIIb.^{143–145} This appears to be a natural mechanism of immunomodulation as stimulation with antigen without costimulation and successful SIT of allergic patients are associated with increased levels of sialylated IgG.¹⁴⁶ Furthermore, studies of the Fc portion of IgG identified tolerogenic, Treg-inducing epitopes (Tregitopes) that, administered with antigen to sensitized mice or human PBMCs, inhibit Th2 cytokine production and induce IL-10 and IFN- γ production.¹⁴⁷

Taking advantage of the tolerogenic nature of the Fc portion of IgG antibodies, some groups have generated chimeric IgGs with peptides inserted into the variable region of the molecule. These Ig-peptide formulations have been used to induce antigen-specific tolerance in murine models of type 1 diabetes and experimental autoimmune encephalomyelitis (EAE). Using this technique, Ig-peptide is bound by Fc γ R on the surface of macrophages and dendritic cells, leading to their presentation of the encoded antigen and production of IL-10.^{148,149} Ig-peptide treatment induces apoptosis of antigen-specific T cells and induces protective IFN- γ in models of diabetes.^{150,151} In keeping with mechanisms described in this review, the deletion of antigen-specific T cells and induction of IFN- γ and IL-10 make Ig-peptide an intriguing candidate for the induction of tolerance in Th2-mediated allergy, although this has not yet been studied.

D. Antigen-Coupled Apoptotic Cells

A number of groups, including our own, have studied the use of antigens coupled to apoptotic cells as tolerogenic carriers – taking advantage of natural mechanisms of apoptotic debris disposal to induce tolerance to associated antigen. Covalently coupling antigen to splenic leukocytes (Ag-SP) using ethylene-carbodiimide (ECDI) prior to i.v. infusion induces antigen-specific tolerance.¹⁵² Ag-SP are flexible carriers of antigen, able to induce tolerance to complex diseases using single peptides, peptide cocktails, whole proteins, food extracts and tissue homogenates.^{153–155} The coupling process induces apoptosis of the leukocyte carriers, thereby targeting antigen for indirect presentation to antigen-specific T cells by host APCs.¹⁵⁶ In graft rejection and autoimmune disease models, as well as *in vitro*, it was demonstrated that this uptake, primarily by splenic marginal zone macrophages and DCs, induces production of IL-10 and subsequent upregulation of programmed death ligand 1 (PD-L1) and induction of Tregs.^{157–159} Therefore, it is hypothesized that Ag-SP targets antigen to natural tolerogenic pathways of apoptotic debris disposal by host splenic APCs.^{160–162} Ag-SP treatment induces defective CD40L expression on antigen-specific T cells, thereby inhibiting the maturation of B cells and other APCs.¹⁶³ The

immunomodulatory molecules indolamine oxygenase (IDO) and CTLA-4 have also been demonstrated to play a role in the induction and maintenance, respectively, of Ag-SP-induced tolerance in models of allograft rejection, EAE and type 1 diabetes.^{164–166} A recent phase I trial treating multiple sclerosis patients with PMBCs coupled to a myelin peptide cocktail found the treatment to be well tolerated in MS patients and a decrease in antigen-specific T cell responses was observed in patients receiving the highest cell doses.¹⁶⁷

In addition to the predominantly Th1 and Th17-dominated autoimmune and graft rejection models discussed above, Ag-SP have been used to prevent hapten-specific IgE responses in mice immunized with alum adjuvant.¹⁶⁸ More recently, Ag-SP were found to induce antigen-specific tolerance in mouse models of allergic airway inflammation and food allergy.¹⁵⁵ In both models, prophylactic treatment with Ag-SP inhibited development of Ag-specific IgE and inhibited eosinophilia and Th2 cytokine responses following acute antigen challenge. Unlike soluble antigen, Ag-SP do not induce anaphylaxis in pre-sensitized mice, likely due to cross-linking of antigen to cells and other antigen molecules causing the obfuscation of conformation-dependent IgE epitopes. Post-sensitization treatment with Ag-SP inhibited Th2 responses in both models, although antigen challenge and serum analysis shortly after treatment revealed increases in antigen-specific IgE that mirrors the transient IgE increases seen in other forms of SIT. Tregs were found to be dispensable for the induction of tolerance in the allergic airway model, but not in the mast cell-dependent food allergy model, perhaps due to the requirement for Treg-mediated suppression of mast cell degranulation via OX40-OX40L interactions in this model.^{14,15} Tolerance induced by intravenous Ag-SP did not appear to skew T cell responses towards Th1 or Th17, instead implying the action of anergy and clonal deletion mechanisms as seen in Ag-SP tolerance in other models.¹⁵⁹ Further research is required to address the long-term therapeutic potential of Ag-SP in allergic disease and to verify the mechanisms defined in Th1/Th17-mediated models.

E. Polysaccharide Particles

A number of studies have used formulations of polysaccharide-derived particles as antigen carriers and adjuvants for the induction of tolerance to treat allergic disease. These biodegradable particles offer the advantages of the antigen depot effect associated with other particulate adjuvants such as alum without the associated non-specific, potentially Th2-inducing, inflammation.

Particulate formulations of chitin, a polymer of N-acetyl-D-glucosamine, and its deacetylated derivative, chitosan, have been extensively studied as adjuvants for use in mucosal SIT. Chitin particles are phagocytosed via the mannose receptor,¹⁶⁹ and subsequently produce IL-12, driving production of IFN- γ .¹⁷⁰ Given orally to sensitized mice, chitin particles alone increase antigen-specific IgG2a and decrease specific IgE and eosinophilia.¹⁷¹ Positively charged, antigen-loaded chitosan microparticles enhance uptake and processing of antigen by mucosal APCs, resulting in increased IFN- γ and IL-10 secretion by T cells *in vitro* and T cell priming in cervical lymph nodes after SLIT in mice.¹⁷² In sensitized mice, therapeutic SLIT with chitosan particles inhibits AHR, lung eosinophilia and Th2 cytokine production.¹⁷² Additionally, chitosan particles can be used for the immunogenic delivery of genes. In a murine model of peanut food allergy, oral administration of chitosan particles bearing DNA encoding the *Ara h 2* gene induced Ara h 2-specific IgA and IgG2a and inhibited Ara h 2-specific IgE and anaphylaxis upon antigen challenge.¹⁷³

Similarly to chitin-derived polysaccharide formulations, mucoadhesive maltodextrin nanoparticles enhance tolerance induction by SLIT. In a murine model of allergic asthma,

SLIT with maltodextrin adjuvant reduced established AHR, lung inflammation and Th2 responses in restimulation cultures while enhancing antigen-specific IgA in the lungs.¹⁷⁴

Particle formulations of another polysaccharide, sepharose, have been studied as adjuvants for SCIT in models of allergic airway inflammation. Mice treated with sepharose particles show reduced lung inflammation, AHR, Th2 recall responses and IgE but increased IFN- and IgG2a production.^{175,176} In direct comparison to alum, subcutaneous injections of sepharose particles induced a greater Th1 response and did not induce the granulomatous tissue reactions associated with alum.¹⁷⁵ Sepharose particles have been shown to induce partial maturation of DCs, a phenomenon associated with improved tolerogenicity.¹⁷⁷ Overall, naturally derived polysaccharide particles offer a promising alternative to alum adjuvants by minimizing adverse events and improving the potential for skewing towards Th1 responses.

F. Poly(lactide-co-glycolide) (PLG) Particles

PLG particles have been extensively studied as antigen carriers and adjuvants for immunization. These particles combine the advantages of being easily manufactured and modified with biodegradability achieved by hydrolysis. Additionally, they are able to hold encapsulated solutions such as drugs and antigens, taking advantage of the biodegradation of PLG to provide a slow, controlled release of encapsulated contents.¹⁷⁸ Several studies have demonstrated the protective Th1-inducing properties of PLG carrying antigen and the potent Th1 adjuvant MPL.^{179–182} However, PLG-associated antigen has been shown to have a significant potential for immune deviation on its own. Intraperitoneal immunization with PLG microparticles induces a Th1-dominant response to encapsulated antigen characterized by antigen-specific production of IgG2a and IFN- γ .¹⁸³ Given subcutaneously, PLG with encapsulated allergen inhibit SPT reactivity and increase allergen-specific IgG2a and IFN- and IL-10 produced in recall cultures.¹⁸⁴ Similarly, oral administration of PLG particles containing encapsulated milk allergen β -lactoglobulin (BLG) reduced BLG-specific IgE levels with 10,000 times less antigen than needed to achieve similar suppression with oral administration of soluble BLG alone, due in part to the protection from digestion provided by PLG. This treatment also inhibited T cell responses as determined by reduction in delayed type hypersensitivity reactions (DTH) and splenocyte proliferation upon BLG restimulation.¹⁸⁵

While other groups have previously examined the potential of PLG particles as Th1-inducing adjuvants for vaccine development, our group has recently begun investigating the tolerogenic potential of PLG particles in Th1/Th17- as well as Th2-mediated inflammatory diseases. Our studies have employed the i.v. route of administration, which directs particles to the splenic marginal zone where they are taken up via the same tolerogenic pathways that mediate tolerance induced by apoptotic Ag-SP^{157,186} (and Harp, *et al.* unpublished data). In a model of relapsing-remitting EAE, i.v. infusion of PLG particles conjugated to encephalitogenic myelin epitopes inhibited disease induction when given prophylactically and inhibited relapse when given during remission.¹⁸⁶

More recently, we have begun to examine the ability of antigen-associated PLG particles to induce tolerance in an OVA/alum induced model of allergic airway inflammation. A previous group found that footpad, i.p. and oral immunization with encapsulated OVA-PLG particles induced OVA-specific IgA and IgG and enhanced DTH and ex vivo splenocyte restimulation responses.¹⁸⁷ However, our studies with i.v. administration of OVA-PLG have found that both surface-bound and encapsulated formulations, given prior to OVA/alum sensitization, induce prophylactic tolerance. Airway inflammation, particularly eosinophilia, is decreased, as are Th2 cytokines in the lungs, OVA-specific IgE and OVA recall responses. No increase in Th1 or Th17 cytokines has been observed, although OVA-specific

IgG2a is increased (Smarr, *et al.* unpublished results). Ag-PLG particles are well tolerated in mice previously sensitized with the antigen in either alum or CFA adjuvant (Smarr, *et al.* and Hunter, *et al.* unpublished results). We hypothesize that the differences observed between studies may stem from the route of administration, as i.v. administration of Ag-PLG targets antigen to tolerogenic pathways in the splenic marginal zone perhaps via uptake by the MARCO scavenger receptor.¹⁸⁶

VI. CONCLUSIONS

SIT represents the only curative treatment for allergic diseases. Traditional SIT has been effective in many cases, but has been constrained by issues with safety, inconvenience and expense. SIT currently requires many treatments administered over months or years to induce protective tolerance and is frequently associated with adverse events requiring either prophylactic or rescue medications such as antihistamines, anti-IgE antibodies or epinephrine. Innovations in the route, antigens and adjuvants employed for SIT provide exciting potential solutions to these problems with considerably improved efficacy and safety profiles. However, many of the advances outlined in this review need considerably more study to fully ascertain and refine the mechanisms by which they promote tolerance. Furthermore, relatively few studies of SIT have employed well-controlled, blinded trials or examined the longevity of tolerance as opposed to short-term desensitization. Information gleaned from these studies will have applications not only for the treatment of Th2-mediated disease, but also for autoimmunity and tissue transplantation. Moving forward, rigorous studies must be performed to provide clinicians with refined tools to safely and efficiently induce antigen-specific tolerance.

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ABBREVIATIONS

Ag-SP	antigen-coupled splenic leukocytes
AHR	airway hyper-responsiveness
BLG	b-lactoglobulin
DC	dendritic cells
DTH	delayed type hypersensitivity
EAE	experimental autoimmune encephalomyelitis
ECDI	ethylenecarbodiimide
IDO	indolamine oxygenase
ILIT	intralymphatic immunotherapy
IVIG	intravenous immunoglobulin
LP40	lipoprotein 40
LPS	bacterial lipopolysaccharide
MPL	monophosphoryl lipid A
ODNs	oligodeoxynucleotides
OIT	oral immunotherapy

PBMC	peripheral blood mononuclear cell
PLA2	phospholipase A2
PLG	poly (lactide- <i>co</i> -glycolide)
SCIT	subcutaneous immunotherapy
SIT	specific immunotherapy
SLIT	sublingual immunotherapy
SPT	skin prick test
TCR	T cell receptor
TLR	Toll-like receptors
Tr1	T regulatory 1 cell
Treg	regulatory T cell

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FIGURE 1.

Mechanisms of SIT for allergic diseases. Antigen in the presence or absence of adjuvant or carrier is processed by APCs and presented to T cells. **A.** In the absence of positive costimulation and/or the presence of negative costimulation via PD-L1/2, Th2 effector cells are driven towards clonal anergy or apoptosis. Additionally, SIT leads to the induction and expansion of Treg, Tr1 and Th1 cells by APCs producing IL-10, TGF- β and IL-12, respectively. **B.** Tregs suppress effector cells such as mast cells, basophils and eosinophils both directly and indirectly through suppression of Th2 cells and induction of IgG4-producing B cells. **C.** Th1 cells produce IFN- γ which inhibits Th2 cell differentiation and induces B cells to produce IgG that competes with pathogenic IgE for antigen.