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Adjuvants for allergy immunotherapeutics

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ABSTRACT

Allergic diseases are reaching epidemic proportions in developed countries. In particular, food allergy is increasing in prevalence and severity, thus becoming an important socioeconomic burden. Numerous cell types and cell populations, which form an intricate and balanced network, are involved in an immune response. This balance is occasionally disturbed, leading to the onset of different diseases, such as allergic diseases. Antihistamines and corticosteroids provide some degree of relief from the symptoms of allergic conditions. However, the only treatment that can revert the disease is immunotherapy. Nevertheless, specific immunotherapy has at least two major drawbacks: it is time-consuming, and it can produce local and even systemic allergic side effects. Immunotherapy's potential goes beyond our current knowledge of the immune response; nevertheless, we can still design strategies to reach a safer immune modulation for treating allergies. This review deals with the use of adjuvants to reduce the undesirable side effects associated with specific allergen immunotherapy. For example, nanoparticles used as immunoadjuvants are offering promising results in preclinical assays.

Keywords:

Anaphylaxis; allergy; immunotherapy; nanoparticle; adjuvant

1. INTRODUCTION, ALLERGIC DISEASES

Allergy represents the most prevalent disease in developed countries with a current rate of up to 30% in industrialized nations. There are robust epidemiological data demonstrating a steady increase in the prevalence of asthma, rhinitis and atopic dermatitis (1), as well as an alarming increase in food allergy around the world (2). These changes are reflected not only by a surge in allergic conditions, but also by an increase in the severity of allergic reactions (3-5) with hen's eggs, cow's milk, and nuts being the foods most frequently associated with anaphylactic reactions. Allergic diseases have a serious impact on quality of life and represent a very significant socioeconomic burden (6, 7) due to healthcare costs (8, 9) and decreased productivity, poor school performance, or absenteeism (1). This is because allergic diseases have a high prevalence in children and young adults. Factors influencing this pattern of increasing risk of developing an allergy include early microbial and dietary exposure, even while within the uterus, that modifies gene expression (10). Other factors affecting microbiota and which could orchestrate a Th2 pro-allergic immune type response are environmental changes, including climate, lifestyle, indoor life, habits and diet. However, the mechanisms underlying the current increase in the prevalence of allergic diseases has not been fully elucidated.

2. ALLERGY, FROM CONCEPTS TO IMMUNE MECHANISMS

The overall biological function of our immune system is to defend the body by recognizing potential chemical or biological attacks. However, the overzealousness of the immune system occasionally causes an inappropriately excessive response, which we describe in terms such as

allergy, atopy, hypersensitivity or anaphylaxis. The term allergy was introduced by von Pirquet in 1906 following his observations of early reactions noted on the skin 24 hours after cowpox vaccinations. In fact, he referred to it as “inadequate immunity” or “allergy” (from the Greek “*allos*”, other and “*ergon*”, work). However, he was dealing in *sensu stricto* with a “delayed hypersensitivity”, an immune process concept that was not understood at that time. Actually, it was in the 1960’s when Gell and Combs described the type I to IV hypersensitivity reactions, and only type I is now considered “allergic”. We currently classify any allergic symptoms (e.g., rhinitis, conjunctivitis, dermatitis, etc.) mediated by immunoglobulin E (IgE) as atopic (which means “out of place” in Greek). These symptoms come from a cascade of reactions involving mucus production, increase robustness of epithelial barriers, and other effects on the musculoskeletal and nervous systems that lead to mechanical, physical and chemical changes whose objectives are to prevent and remove these toxic environmental challenges (11, 12). This immune response consequently manifests as lacrimation, vomiting, diarrhea, coughing, sneezing or itching. These reactions were historically called “anaphylactic” responses, literally, anti-protection (from the Greek “*ana*”, backward and “*phylaxis*”, protection), even though, as mentioned earlier, they are in fact natural responses designed to protect the host. Nowadays, an anaphylactic reaction is a clinical reference to the most severe type I hypersensitivity reaction.

Figure 1 illustrates the IgE-mediated allergic immune response using the two classical common phases: sensitization and elicitation. Allergic sensitization occurs during initial exposure to a specific allergen. The allergen is taken up and processed by antigen-presenting cells (or dendritic cells, DCs, in the present case) which then present allergen peptides to T cells. T cells differentiate into Th2 cells which mainly release the cytokines IL-4, IL-5 and IL-13 resulting in

allergen-specific IgE production by B cells, and the activation and recruitment of basophils and eosinophils. Secreted IgE binds to FcεR receptors on the basophil and mast cells. The elicitation phase occurs after re-exposure to the same allergens when they cross-link IgE bound to mast cells and basophils, resulting in degranulation and release of inflammatory mediators, such as histamine, serotonin, tryptase and cytokines, which trigger different physical mechanisms (increased vascular permeability, muscle contraction, bronchoconstriction, with the concomitant mucous production) aimed at removing the “aggressor”. Alternatively, in the presence of a high dose of allergens, immunocomplexes with macrophage- or neutrophil-bound IgG will stimulate the release of PAF (platelet-activating factor). This is recognized as the “alternative” sensitization pathway and plays a major role when allergens enter the blood stream and form immune complexes with specific IgG. However, most anaphylactic reactions in humans are IgE-mediated because this pathway involves small quantities of antigen (13).

Allergic diseases are mediated by Th2 cytokine responses involving IL-3, IL-4, IL-5, IL-13 and GM-CSF (granulocyte-macrophage colony-stimulating factor), and a reduction in the number of Th1 IFN-γ-producing cells. It is well known that Th2 is counterbalanced by Th1 and regulatory T cells. Consequently, both IFN-γ (Th1) and IL-10 (Treg) cytokines have been shown to increase with allergen-specific immunotherapy (Figure 2). Similar counterbalances have been described between Th17 and Th1, Th17 and Th2, or between Th17 and regulatory T subsets (14). Thus, the immune response is likely to shift from the pro-allergic Th2 considering that cytokines secreted by Th1, Th17 and regulatory T subsets may suppress Th2 cell function. However, other cell types, like NK and Tc-cells, shall also be considered.

3. ALLERGY, FROM STRATEGIES TO THERAPY

The appropriate drugs can, to some extent, help alleviate the symptoms of allergic conditions and may improve patients' quality of life. However, currently, the only treatment that can reverse the disease course is immunotherapy. Nevertheless, in spite of its efficacy, the underlying mechanisms of immunotherapy are not completely understood. Immunotherapy means to "act specifically" (allergen-specific immunotherapy, IT) in order to desensitize the host to a specific allergen; in other words, the host develops a "tolerance" to that allergen. Thus, successful desensitization will mean that a greater amount of allergen is required to induce an allergic reaction or that the allergen no longer induces a reaction (15). IT was traditionally based on a system with an empirical basis. Thus, a century ago, Leonard Noon started to use subcutaneous injections of a grass pollen extract to treat hay fever (16). However, IT is time-consuming, and subcutaneous injections are associated with local and systemic allergic side effects. New forms of immunotherapy with allergens were later introduced in order to improve the immunotherapy safety profile. The administration routes and number of injections were modified in some cases. In other cases, modifications were introduced in the allergenic extracts. Finally, adjuvants have been added in attempts to modulate the immune response to a tolerogenic one, to increase the time interval between injections and/or to enable a particular administration route (Tables 1 and 2).

3. 1. Administration routes

With regard to administration routes, subcutaneous immunotherapy (SCIT) is the best established method. What we currently consider to be classical IT consists of repeated subcutaneous inoculation of doses of allergen over a long period of time. However, in spite

of its efficacy, there are downfalls to the use of SCIT. These include local allergic reactions, anaphylaxis (occasionally) and, near-fatal reactions (rarely) (17, 18). Considering these risks, subcutaneous immunotherapy should only be administered by trained specialists. On the other hand, guidelines recommend at least 3 to 5 years of treatment in order to obtain a long-term immunological response, which requires good patient compliance. SCIT is performed in two phases: (i) starting phase (build-up), a weekly dose is administered with increasing amounts of allergen until the maximum dose is reached; and (ii) maintenance phase, the maximum dose of allergen is administered every four weeks. Other disadvantages related to the use of classical subcutaneous immunotherapy derive from some patients' fear of needles and the risks associated with their use (19). A plausible alternative is the practice of cluster regimens, in an attempt to reduce the number of hospital visits during the build-up phase. Cluster regimens consist of administering multiple injections each day on non-consecutive days, and represent an alternative to conventional up-dosing schedules as they reach the maintenance dose quickly, shortening the build-up phase. These regimens have not been associated with an increased risk of systemic reactions compared to conventional schedules for subcutaneous allergen administration. Moreover, they shorten the time required to reach maintenance therapy, as seen with dust mite and grass-pollen immunotherapy (20).

The use of sublingual immunotherapy (SLIT) has become more extensive over the last 20 years (22). In general, the risk of anaphylactic reaction is smaller in SLIT compared to SCIT and so patients can self-administer at home; but it appears to be less effective than SCIT (23, 24). The need to administer SLIT daily or several times a week also results in poor treatment adherence (25). SLIT with tablets should solve this problem (26, 27). However, in the particular case of IT

for food allergies, where subcutaneous immunotherapy has been ruled out due to the prevalence of severe adverse reactions, SLIT has shown only limited efficacy.

The decision to use either SCIT or SLIT currently depends on the allergist's preference, and not on scientific criteria. The main arguments in favor of the subcutaneous route are because it achieves higher patient compliance and there is indirect evidence that it is more effective than the sublingual route. Nevertheless, the sublingual route could be the selection of choice because it is administered at home and presents less risk of provoking a severe reaction. Studies with appropriate methodologies are required to make a comparison of both administration routes (28).

3.1.a Oral immunotherapy (OIT) has been introduced with good results in terms of efficacy, but with frequent adverse reactions. OIT is being used to treat food-allergic patients, especially for milk, egg or peanut allergies. It comprises a dose-escalation phase, in which an increasing oral dose is administered weekly at the hospital, followed by a maintenance phase, in which the maximum dose (if it was well-tolerated) is administered on a daily basis at home. OIT is now used under research protocols, and can induce desensitization (the food is tolerated while receiving treatment) or, in some patients, a sustained unresponsiveness after discontinuation (29). Epicutaneous and intralymphatic routes are currently being evaluated. Epicutaneous immunotherapy (EPIT) has been shown to be effective in the treatment of grass pollen allergic rhinoconjunctivitis and is being studied for the treatment of food allergies, such as milk or peanut, where its efficacy has also already been proven (30). The intralymphatic route consists of administering three injections of allergenic extract into the inguinal lymph nodes over the course of a year; the technique has proven to be effective and safe in the treatment of cat dander or grass pollen-induced allergic rhinoconjunctivitis (31).

3.2. Modified allergens

Attempts to develop safer, more convenient and more effective strategies include the use of hypoallergenic glycol-conjugates or recombinant allergens, also known as modified allergens, which do not induce IgE-mediated activation (33-34). Another possibility being explored is fusing allergens with proteins containing allergen specific T epitopes (35 , which have the advantage that they do not stimulate the allergen-specific T cells). However, one major disadvantage is that they can cause delayed reactions(36). These B peptides must be linked to a carrier protein (37). When modified allergens are used, the build-up phase can be significantly shortened due to the reduced risk of an adverse reaction. However, there are no studies available comparing the efficacy of using modified allergens compared with native ones. A further benefit of these novel forms is that they can improve the standardization, reproducibility and quality of the extracts (36, 38).

4. Adjuvants in allergen-specific immunotherapy: safety and efficacy.

Adjuvants have been used extensively in conventional prophylactic vaccines for preventing infectious diseases. An adjuvant is any “substance” with the capacity to increase the immunogenicity of the accompanying antigen and/or modulate the quality of the elicited immune response. In this regard, adjuvants can be selected to favor the development of any of the specific T cell branches. Consequently, over the years, adjuvants have been widely used in allergy vaccines in order to enhance and simplify immunization regimens, besides improving the safety profile of allergen-specific immunotherapy treatments (39). However, they can also cause adverse events (40), raising clinical concerns about their use. Recent advances in developing new

forms of adjuvants for immunotherapy have focused on reducing their potential risks while modulating the immunogenicity of the allergen-specific immunotherapy (41). Among others, novel and promising allergen delivery systems with immunomodulatory properties appear to fulfil the ideal requirements for allergy immunotherapy.

Two different ‘generations’ of adjuvants for allergy vaccines have been described: the first generation refers to those molecules that constitute vehicles for binding the allergen. These molecules have the ability to co-stimulate innate cells, including antigen-presenting cells. Second-generation adjuvants are considered specific immunomodulatory molecules capable of inducing specific immunopotentiality of either immune cells or pathways (42).

4.1. First-generation adjuvants

The efficacy of aluminum hydroxide (alum) and calcium phosphate for subcutaneous allergy vaccines has been demonstrated in humans as they can elicit proinflammatory responses after inflammasome activation (43, 44). Alum has been used as an adjuvant in vaccines against infectious diseases since 1926 (45), and for treating allergic conditions since 1937 (46-49). Today, most subcutaneous allergen-specific immunotherapy is performed with $\text{Al}(\text{OH})_3$ as the adjuvant (39), and can generally be regarded as safe in terms of acute local or systemic side effects. However, a number of reports of chronic aluminum toxicity are accumulating and are being inspected by national authorities (46).

Microcrystalline tyrosine (MCT) constitutes a relatively new depot adjuvant formulation that can co-precipitate the allergens, thus decelerating allergen bioavailability, reducing the rate of dissemination from the injection site and the prevalence of systemic reactions (50). It is frequently used in combination with monophosphoryl lipid A (see below), leading to a reduction

in the up-dosing phase. Its safety has been demonstrated in different studies in both animal models and in humans (51-54).

4.2. Second-generation adjuvants

Second-generation adjuvants contain immune modulators that stimulate a Th1 and/or T regulatory response. They include microbial products (Toll-like receptor agonists) and vector-delivery systems (microparticles, nanoparticles, liposomes, virosomes, or immunostimulating complexes, among others) (55).

4.2.a Microbial products (TLR agonists)

Microbial products contain inflammatory “danger” signals, such as those known as “pathogen-associated molecular patterns” (PAMP). The host senses PAMPs as danger molecules, eliciting the release of cytokines and chemokines that initiate an inflammatory defensive response. Immune system cells have special receptors for PAMPs known as PRRs (pattern recognition receptors), including Toll-like receptors (TLRs), Nod-like receptors (NLRs), RIG-I-like receptors (RLRs), and C-type lectin receptors (CLRs) (56-59). One of the main PAMPs is lipopolysaccharide (LPS) found in Gram negative bacteria, a TLR4 agonist. Both natural and synthetic derivatives of LPS with reduced toxicity are currently being developed. Monophosphoryl lipid A (MPL) extracted from *Salmonella enterica* serovar Minnesota is included as an adjuvant, alone or in combination with alum, in some current IT applications (60). MPL has shown efficacy in murine asthma models (39) and in patients with asthma after receiving the first cycle of allergen-specific SCIT for grass pollen combined with MPL (61). The efficacy of this adjuvant relies on the strong induction of allergen-specific IgG antibodies which

may have protective effects by inhibiting immediate-type reactions, as confirmed through the decrease in nasal reactivity after nasal allergen challenge in the case of an MPL-adjuvanted grass pollen allergy vaccine administered using SLIT (62). The LPS extracted from *Brucella ovis* has also been studied due to its low toxicity but maintained capacity to induce Th1 responses to the carried antigens (63). Flagellin, the monomeric protein that forms the bacterial flagellum, is a potent TLR5 agonist and induces the maturation of intestinal DC, activates CD4⁺ T cells *in vivo* and promotes the development of effector Th1 cell responses. Other PRR agonists that induce strong Th1 responses include some unmethylated CpG oligodeoxynucleotides (CpG ODN) which interact with TLR9, or the bacterial second messenger 3',5'-cyclic diguanylic acid, recognized by two distinct PRRs, STING and DDX41 (59, 64). Moreover, the CpG dinucleotides have been proposed via different allergen immunotherapy administration routes (sublingual, intradermal, nasal, subcutaneous) (41). However, the subcutaneous use of a type B CpG ODN with ragweed pollen did not yield reproducible clinical data in a large phase III study (65). Some authors have recently suggested that immunotherapy could be effective even in the absence of allergens, as reported with the use of virus-like particles and CpG-motifs, either as standalone products, or as adjuvants for allergen-specific immunotherapy (42, 66). However, this strategy is a double-edged sword due to the potential side effects associated with inflammation, such as pain, swelling, and cell death.

4.3. Probiotics

Symbiotic bacteria, such as *Lactococcus lactis*, *Lactobacillus plantarum* and *Bifidobacterium bifidum*, have been studied for use in mucosally administered immunotherapy (e.g., intranasal or

sublingual), showing efficacy in animal models through the induction of Th1 responses, regulatory T cells and even reductions in asthma symptoms (39). In a double-blind randomized clinical trial, Tang and coworkers (67) evaluated the effects of a combined probiotic (*Lactobacillus rhamnosus* strain CGMCC, NCC4007) and peanut oral immunotherapy administered to children with peanut allergy. They reported that nearly 90% of the subjects receiving the combined oral immunotherapy were desensitized to peanut allergy, including induction of Treg and Th1 cytokine responses. The efficacy relies on the probiotics' capacity to induce beneficial gut immune modulation, which could promote the patient's sustained unresponsiveness after discontinuing the OIT. Further studies are needed before considering probiotics efficient adjuvants for allergen immunotherapy, especially in children, where the mucosal route, in our experience, appears to be more acceptable for the patient and their parents (68, 69).

4.4. Small Molecule Compounds

The active metabolite of vitamin D₃, 1 α ,25-dihydroxyvitamin D₃ (VD₃), has been described as a potent inducer of T regulatory cells, and thus, a good adjuvant in allergen immunotherapy. A recent study showed that its addition to a conventional immunotherapy vaccine could reduce the allergoid dose needed and, therefore, the cost. Furthermore, this would also help patients adhere to the treatment, hopefully increasing the effectiveness of the immunotherapy (70). Baris and co. (71) evaluated the efficacy, safety and T regulatory cell response of vitamin D as adjuvant for allergen-specific IT; 50 children with bronchial asthma and sensitization to dust mite were randomized into three groups: one receiving SCIT with vitamin D supplementation, and the other two with SCIT alone and pharmacotherapy alone. They reported similar results for the two SCIT

groups, although some more favorable outcomes were observed in those treated with vitamin D supplementation, and both were better than those for pharmacotherapy alone (71).

In a double-blind placebo-controlled study in 50 children, Jerzynsak et al. reported a better response and reduced symptoms of allergic rhinoconjunctivitis after five months of treatment with SLIT grass tablets plus 1000 units of vitamin D compared to those who received SLIT alone (72). It has previously been suggested that vitamin D regulates immune function by inhibiting the differentiation and maturation of human dendritic cells, enhancing IL-10 production by CD4⁺ T cells and up-regulation of Foxp3⁺ CD4⁺ T cells (73-75).

Other synthetic molecules involved in adjuvant immunotherapy are the calcineurin inhibitor cyclosporine A, rapamycin, aspirin and mycophenolate mofetil which seem to enhance IL-10 production (39).

4.5. Particle delivery systems

Particle delivery systems are adjuvants whose aim is to facilitate the work of antigen-presenting cells by increasing the length of contact between the allergen and the patient's mucosa. They extend contact duration via different mechanisms, such as the use of mucoadhesive polymers in the oral mucosa or by increasing the influx of professional APCs to the injection site in the case of SCIT. Particle delivery systems have also shown efficacy in animal models by successfully targeting immunotherapy via parenteral routes (76, 77). Among the systems described in the literature, the use of virus-like particles (VLP) as adjuvants in allergen-specific immunotherapy seems to increase the safety in patients with a high risk of systemic reactions as they appear to limit the bioavailability of active substances that may cause massive mast cell degranulation and,

therefore, systemic reactions during treatment (42, 66). The VLP technology platform has been explored for different administration routes, such as subcutaneous, intralymphatic and intramuscular (42).

4.5.a. Nanoparticles

The use of micro- and nanoparticles based on polymers and macromolecules has received considerable attention in recent years because of their potential to enhance the antigen delivery to the immune system. In fact, these compounds constitute a group of delivery systems with interesting abilities as adjuvants; since they are easily captured and internalized by different immune cells, such as DCs and macrophages, then they can enhance the delivery of the loaded antigen/allergen to the immune system (Figure 2) (70, 76, 77). The terms microparticle and nanoparticle refer here to particles with dimensions best described in micrometers and nanometers, respectively. Size brings real differences at many levels, including formulation techniques, in vivo administration or cellular uptake and intracellular fate (55). Furthermore, nanoparticles (NPs) can be used to load biologically active material (i.e., allergens) and thereby offer the following advantages: (i) protection from hydrolysis and/or enzymatic degradation, (ii) controlled and sustained load release properties, and (iii) co-encapsulation of the allergen and immunopotentiators (i.e., CpG motifs or LPS). Most of these nanoparticles can easily be tailored in order to improve their targeting abilities. A present disadvantage is that only a few materials are currently available for preparing nanoparticles with an acceptable safety profile. For parenteral delivery purposes, some polyester-based nanomedicines [e.g., poly(lactic) acid (PLA) and poly(D,L-lactic-co-glycolic) (PLGA)] or human serum albumin nanoparticles are now

available. For oral delivery purposes, nanoparticles based on either chitosan (78) or Gantrez ANm (79) have been demonstrated to be safe, including in subchronic toxicity studies.

4.5.b. Parenteral administration

Intramuscularly, subcutaneously or intradermally administered micro- and nanoparticles are expected to essentially remain at the injection site and then control the release of the loaded antigen. Therefore, slowly degradable polymers such as polyesters [PLA, PLGA, or poly(epsilon-caprolactone) (PEC)] are suitable for this application as they provide an adequately prolonged release of the loaded allergen. Schöll and coworkers (80) recently demonstrated the capacity of PLGA nanoparticles in immunotherapy to treat birch pollen (Bet-v1) allergy in BALB/c mice. PLGA nanoparticles loaded with Bet-v1, the major birch pollen allergen, enhanced the immunogenicity of the allergen and did not lead to novel sensitization in naïve animals. The authors assumed that vaccination with PLGA nanoparticles can counterbalance an ongoing Th2 response to Bet-v1, readdressing it to a Th1 protective response. In the same way, carbohydrate-modified ultrafine hydroxyapatite-based nanoparticles (aquasomes) also induced a shift in mouse immune response against ovalbumin (used as a model allergen) when administered intradermally. In sensitized mice, these nanoparticles elicited lower serum IgE and histamine levels and a higher survival rate in comparison with alum-adsorbed ovalbumin (81). Another interesting nanopatform for allergen-specific immunotherapy is based on the use of protamines, which are arginine-rich proteins that can spontaneously assemble into nanoparticles with DNA or RNA via electrostatic interactions (82, 83). These protamine-based nanoparticles, combined with CpG sequences, were evaluated as an allergen delivery system using Ara h 2 as a

model allergen. When the protamine nanoparticles were subcutaneously injected into naïve BALB/c mice, a favorable increase in Ara h 2-specific IgG2a antibodies was observed, whereas no Ara h 2-specific IgE was detected (84). Nanoparticles based on tetrafunctional block copolymer 704 have also been proposed for gene delivery (85). When these nanoparticles, which contain a pDNA encoding for the allergen *Dermatophagoides farinae* 1 (Der f 1), were intramuscularly administered to healthy mice they induced strong humoral and cellular responses with a Th-1 profile. However, in asthmatic mice, the same nanoparticles led to a reduction of symptoms and a significant decrease in the level of inflammatory cytokines in bronchoalveolar lavage fluids (85).

4.5.c. Mucosal administration

Important advances in nanotechnology have enabled the development of new nanoparticles for use as allergen delivery systems. These have been investigated in recent years as they offer an alternative administration route, as well as reducing the dose and diminishing allergen exposure to IgE bound to mast cells or basophils (86). The advantages of polymer nanoparticles include their use for either subcutaneous or mucosal routes and the fact they are biodegradable, in contrast to traditional alum mineral salts (45). For allergen delivery, nanoparticles based on poly(DL-lactide-co-glycolide) (PLGA) or its derivatives, chitosan or poly(anhydrides), among other materials, have given encouraging results. Most of these studies involved the use of allergen-loaded nanoparticles administered via a mucosal route.

PLGA microparticles were the first devices proposed to elicit oral tolerance to an allergen (87). In this work, Pecquet et al. demonstrated that the oral administration of β -lactoglobulin

encapsulated in PLGA particles produced a greater tolerance than the free protein (10,000 times higher) (88). In another interesting study, the possibility of increasing the capacity of PLGA nanoparticles to promote Th1 responses was evaluated through their combination with CpG sequences (89). CpG motifs provide steric hindrance that reduces IgE binding and enhances Th1 responses by stimulating TLR9, APCs and NK cells to secrete gamma-interferon and IL-12 (89). The oral administration of PLGA nanoparticles loaded with a peanut extract and CpG over a 4 week period provided long-lasting protection from anaphylaxis to repeated challenges in animals. During this time, animals treated with the nanoparticles displayed lower levels of specific IgE, IgG1 and pro-Th2 cytokines (IL-4, IL-5 and IL-13) than the controls (89).

Nanoparticles based on the methyl vinyl ether-maleic anhydride copolymer (Gantrez AN®) represent another interesting class of potential nanocarriers for oral allergen delivery. These poly(anhydride) nanoparticles have demonstrated their efficacy to induce Th1 immune responses (90, 91), which is mediated by their capacity to activate TLR-2 and TLR-4 and induce DC maturation with a significant up-regulation of the CD40, CD80, and CD86 receptors (92, 93). Using ovalbumin (OVA) as an allergen model, a single oral dose of these poly(anhydride) nanoparticles given to mice induced high serum levels of OVA-specific IgG2a, IgA and IL-10 (94). These nanoparticles were also able to decrease the anaphylactic symptoms of sensitized animals and protect them from death after an intraperitoneal challenge with the native protein, in contrast to the control group (94). Interestingly, the co-encapsulation of LPS in these OVA-loaded poly(anhydride) nanoparticles, but not its binding to their surface, also protected sensitized mice during the challenge (94). In another study using a peanut protein extract, similar poly(anhydride) nanoparticles were prepared and orally administered to mice. After a single

dose, the nanoparticles induced a balanced Th1 and Th2 antibody response and low levels of specific IgE. Moreover, animals treated with peanut protein-loaded nanoparticles displayed lower levels of pro-Th2 cytokines (IL-4, IL-5, IL-6) and higher levels of IFN- γ and IL-10 than control animals (95).

Other interesting materials for the preparation of nanoparticle adjuvants are chitosan and its derivatives. Chitosans, particularly those with a deacetylation of 75–90%, can drive potent cell-mediated immunity, promoting DC maturation by inducing type I interferons and enhancing antigen-specific Th1 responses (96). In addition, chitosan-based nanoparticles possess mucoadhesive properties that increase their contact time at mucosal surfaces (97). Last but not least, the cationic character of chitosan makes it ideal for designing devices used to deliver polyanions, such as DNA or RNA (98). In this context chitosan nanoparticles were proposed for the oral delivery of a pDNA encoding for Ara h 2, a dominant peanut allergen. Compared with the controls (non-immunized mice or mice treated with 'naked' DNA), animals immunized with nanoparticles showed a substantial reduction in allergen-induced anaphylaxis associated with reduced levels of IgE and plasma histamine (98). More recently, chitosan nanoparticles containing a plasmid DNA encoding for Der p1 (a house dust mite allergen), primed a Th1-skewed immune response against this allergenic protein (99). In another interesting publication, in which chitosan nanoparticles were used to deliver a pDNA encoding for *Dermatophagoides pteronyssinus* 2 allergen (Der p 2), Li and co-workers demonstrated the expression of Der p 2 in the epithelial cells of both the stomach and small intestine and the induction of a Th1-type immune response (100).

4.6. Adjuvant limitations

Empirically developed vaccines have been in use for decades. The field is now applying increased use of rational design based on our knowledge of the mechanisms of the protective immune response against allergies and the adjuvants' mechanisms of action. Concerning adjuvants, the use of alum compounds illustrates the need for new safer adjuvants with immunological functions oriented towards an effective therapy. As stated above, alum is still the most commonly used adjuvant in allergy immunotherapy. Its strength is acknowledged despite several important disadvantages, for example it stimulates high and persistent levels of IgE antibodies, limiting its use in long-term therapies (101). Alum's intrinsic physicochemical properties can also limit its use. The adsorption capacity of aluminum is based on a combination of reversible interactions, including electrostatic and van der Waals forces. Therefore, if dealing with simple antigens (e.g., tetanus toxoid) we can predict the adsorption; however, the avidity and kinetics of such interactions are unknown in the case of the antigenic complexes that are currently used in immunotherapy. Furthermore, there is no data available regarding the dose of alum required to achieve adequate adsorption to the antigens, limiting our predictions on toxicity, stability, and the effect of the final product (102) (103).

On the other hand, the mechanism of action of alum remains elusive, raising safety and efficacy concerns over its application (104). Our newly acquired knowledge of the role of antigen-presenting cell receptors for danger signals, which dictates the fate of the immune response, has opened the door to novel, better-characterized adjuvants. Thus, MPL was the first well-characterized TLR agonist used as an immunoadjuvant in allergy immunotherapy (see above). Its immunological function is to orientate the antibody response from IgE to IgG1 and IgG4 (105).

However, MPL does not have a unique, well-defined structure; on the contrary, there are several MPL products available on the market, from synthetic MPL derivatives from *Escherichia coli*, to both natural and synthetic lipid A derivatives from *Salmonella enterica* serovar Minnesota R595. These macromolecules consist of a mixture of lipid A species with different fatty acid chains. Variables such as the number of fatty acids, chain length, origin and the ratio of molecules can affect the physicochemical and adjuvant properties (106). Consequently, further studies into the safety and efficacy of MPL derivatives are required before drawing conclusions regarding the benefits and risks during their application in vaccines and immunotherapies. (107)

Few studies have explored the influence of administration route on the immune response elicited during immunotherapy (105), e.g., MPL studies only focused on parenteral applications. However, rational designs for alternative therapies must consider the neglected natural routes, the mucosal routes (108). Several delivery systems such as polymeric nanoparticles have been developed for mucosal routes with very promising results (109). Yet these systems are still a long way from being authorized. Particularly, in the case of polymeric delivery systems for allergy IT, more investigation into safety during prolonged therapy is required.

The use of probiotics as tolerance-inducing immunomodulators is specially promising. Nevertheless, variations among individuals may reduce the potential applications of probiotics, and further new clinical trials are required to demonstrate the long-term sustained reduction of specific IgE response.

Finally, despite recent advances in adjuvants, the costs of developing new products mean alum is still the primary adjuvant for human vaccines worldwide. This is really discouraging because if alum hadn't been in use for all these years then it would be refused registration by today's

standards on the basis of safety concerns (110). Many adjuvants have been proposed but costs are, so far, a major limitation. Consequently, there is an urgent need for innovations that streamline manufacturing processes and reduce costs in accordance with the needs and priorities of immunotherapy and vaccines. On the other hand, there is an increasing cost-benefit value from the use of adjuvants as if the use of adjuvants would improve efficacy or result in the use of fewer doses and physician visits, cost would not be an issue limiting their use.

A greater knowledge of the mechanisms of action of allergen-specific immunotherapy techniques has fostered the inclusion of novel adjuvants that will improve treatment safety and efficacy (22). Moreover, the contemplation of wider indications besides what we know already, such as the preventive effect on the development of bronchial asthma (111, 112) or identification of the optimal maintenance dose and dose-ranging for common allergens from grass pollen, dust mite allergens or ragweed pollen in large trials studies have constituted important advances in the field of specific allergen immunotherapy (27, 113, 114). However, in our opinion, there is only limited data available in terms of efficacy of the different adjuvants and there are still unmet requirements, such as the performance of *ad hoc* clinical trials to evaluate the different types of adjuvants (traditional and novel ones) in order improve the safety of the immunotherapy and to individualize each patient's indication according to their allergies.

5. Conclusions

As in the case of vaccines against infectious disease, adjuvants in immunotherapy for allergic conditions are expected to enhance and modulate immune responses to allergens, enabling fewer

doses and/or lower dosage levels of allergen. The adjuvant should provide better immunomodulation that would ideally balance the overactivation of Th2 cells and, consequently, the overproduction of IgE, thus achieving a quicker and longer-lasting efficacy (see Table 3 for on-going clinical trials; www.clinicaltrials.gov).

On the one hand, allergen immunotherapy has proven its long-term efficacy, with a protection rate of over 97% in the case of hymenoptera. Allergen immunotherapy has the potential to balance the immune dysregulation causing the allergic inflammation and it has demonstrated its ability to prevent future sensitizations. It is well accepted by patients because it is not a synthetic drug. However, allergen immunotherapy also has several disadvantages: it requires time to build up the immune response, and in the case of subcutaneous immunotherapy it must be administered by trained personal at a healthcare facility, with monthly doses for an average of at least three years. Although allergen immunotherapy may be effective, it also presents a risk since patients can have an allergic reaction at any given dose. Recent developments in allergy research have focused on allergen components, molecular diagnosis and targets for biological drugs, rather than developing new strategies to design safer, more precise, standardized, and faster immunotherapy.

Some important innovations in immunotherapy are currently at different developmental stages as researchers try to achieve: greater extract efficacy, more reproducible production at lower manufacturing costs, safer immunotherapy requiring fewer doses, potential application to food allergies, a lack of sensitizing potential, and the use of IT in a prophylactic manner (36). The influence of probiotics on the immune system is an exciting area of research and, specifically, the role of probiotics as adjuvants in vaccinations or immunotherapy is very promising (115).

Nanoparticles are being studied as the next generation of mucosal adjuvants since they can enhance the delivery of the loaded antigen to gut lymphoid cells thanks to their special bioadhesive properties. NPs may stimulate “protective” Th1 and/or T regulatory responses while avoiding the systemic side effects associated with IgE cross-linking on mast cells and basophils, which is the risk associated with conventional whole allergen extracts. These properties open new possibilities for treating not only infectious diseases but also allergic diseases.

Important advances in developing new forms of adjuvants have been made in the past few years. However, further research is needed to develop an ‘ideal’ adjuvant that shows promising efficacy in modulating immune response, facilitating shorter up-dosing phases (which would increase adherence), reducing adverse reactions during treatment and finally inducing sustained unresponsiveness in allergic patients.

Disclosure of potential conflicts of interest

The authors report no potential conflicts of interest.

Authors’ contributions

CG, JMI, CD, GG, and MF all contributed to the conceptual framework, writing, and revision of the manuscript.

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Figure legends**Figure 1. Sensitization to allergens and development of allergic responses.**

The sensitization pathway starts when an Antigen Presenting cell (APC) recognizes and processes an environmental protein as potentially dangerous, such as inhalants allergens (pollen, animal dander, house dust mites), or food proteins. This phase is known as sensitization. During allergen sensitization, naïve T cells are primed into CD4⁺ T helper type 2 T cells, producing Th2 cytokines as IL4 and IL-13, which are responsible of class switching to the IgE production by B cells. IgE will bind to the surface mast cells and basophils IgE receptor. Upon the next encounter with the allergen, the protein will crosslink two IgE molecules starting a signaling cascade that causing mast cells and basophils degranulation, releasing preformed mediators (histamine, proteases, prostaglandins, thromboxanes) constituting the early-phase response. In addition, mast cells and basophils also produce cytokines and chemokines that recruit inflammatory cells to the site (late-phase). This immune response type 2 (Th2) immunity is counterbalanced by Type 1 (Th1) immunity and T regulatory cells.

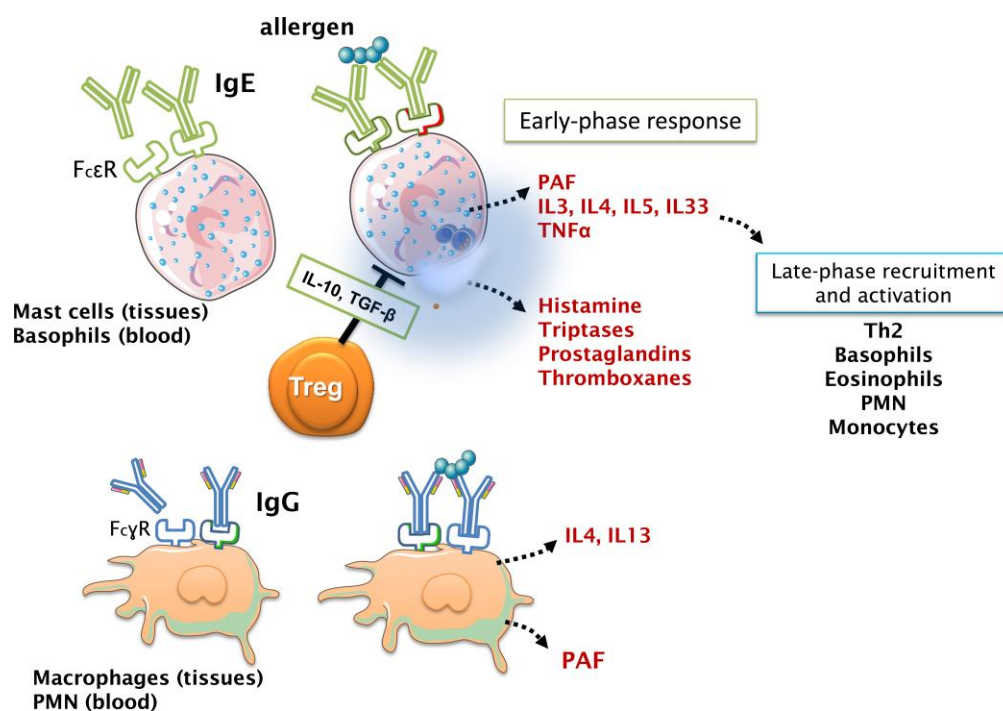


Figure 2. Immunological mechanism of immunotherapy based on nanoparticles carrying allergens.

Dendritic cells (DC) are specialized cells able to shape the direction of the specific immune response from the allergic (Th2) to the inflammatory or to the tolerogenic ones. Currently, the allergen-specific IT is based on the gradual administration of increasing amounts of allergens. It is well documented that high-dose injections with short intervals induce T regulatory cells and suppress Th2 cells. However, it is also possible to reduce Th2 responses by increasing Th1 by the use of appropriate adjuvants. Thus, the uptake of allergens containing suitable nanoparticles increase of affinity of the interaction between DC and Th cells, rendering a Th1 response.

