

## Clinical Commentary Review

# Allergens and Adjuvants in Allergen Immunotherapy for Immune Activation, Tolerance, and Resilience

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## Learning objectives:

1. To appreciate the differences an adjuvant makes in allergen immunotherapy (AIT).
2. To understand why the selection of adjuvants is pivotal for patient safety and treatment success.
3. To be informed about novel adjuvant- and vector strategies applicable in allergy.

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Allergen immunotherapy (AIT) is the only setting in which a vaccine is applied in patients allergic exactly to the active principle in the vaccine. Therefore, AIT products need to be not only effective

but also safe. In Europe, for subcutaneous AIT, this has been achieved by the allergoid strategy in which IgE epitopes are destroyed or masked. In addition, adjuvants physically precipitate

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**Abbreviations used**

AIT- Allergen immunotherapy  
 alum- Aluminum hydroxide  
 BLG- Beta-lactoglobulin  
 DC- Dendritic cell  
 EU- European Union  
 IC- Immune complex  
 MPLA- Monophosphoryl lipid A  
 RA- Retinoic acid  
 SCIT- Subcutaneous allergen immunotherapy  
 SLIT- Sublingual allergen immunotherapy  
 Treg- Regulatory T cell  
 TLR- Toll-like receptor

the allergen at the injection site to prevent too rapid systemic distribution. The choice of adjuvant critically shapes the efficacy and type of immune response to the injected allergen. In contrast to  $T_H2$ -promoting adjuvants, others clearly counteract allergy. Marketed products in Europe are formulated with aluminum hydroxide (alum) (66.7%), microcrystalline tyrosine (16.7%), calcium phosphate (11.1%), or the  $T_H1$  adjuvant monophosphoryl lipid A (5.6%). In contrast to the European practice, in the United States mostly nonadjuvanted extracts and no allergoids are used for subcutaneous AIT, highlighting not only a regulatory but maybe a “historic preference.” Sublingual AIT in the form of drops or tablets is currently applied worldwide without adjuvants, usually with higher safety but lower patient adherence than subcutaneous AIT. This article will discuss how AIT and adjuvants modulate the immune response in the treated patient toward immune activation, modulation, or—with new developments in the pipeline—immune resilience. © 2020 The Authors. Published by Elsevier Inc. on behalf of the American Academy of Allergy, Asthma & Immunology. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>). (J Allergy Clin Immunol Pract 2020;■:■-■)

**Key words:** Adjuvant; Allergen immunotherapy; Allergoid; Aluminum hydroxide; Microcrystalline tyrosine; Monophosphoryl lipid A; Vaccine

## INTRODUCTION

The development of vaccines against infectious agents and their toxins paralleled the development of immunotherapy against allergens (allergen immunotherapy [AIT]), which historically were regarded as toxins, too.<sup>1</sup> Extracts of biological allergen sources are still used today for AIT, even if we know that an extract besides the specific molecular allergens contains nonallergenic entities. There are still some fundamental discrepancies among these distinct approaches (Table 1).<sup>2</sup>

First, AIT needs to be safer. Molecular allergy diagnosis has greatly improved patient selection, making AIT a best practice example of precision medicine.<sup>3</sup> Still, at least 1 systemic reaction occurred in 2.1% of 4316 treated patients,<sup>4</sup> mostly in subcutaneous AIT (SCIT).

Therefore, in AIT safety is a greater concern than in infectious vaccines because patients are allergic exactly to the active principle in

the vaccine and may react systemically because of preexisting allergen-specific IgE antibodies on effector cells. Consequently, usually accelerating doses are applied in the build-up phase, whereas in the maintenance phase the dose stays the same over years. The build-up phase can be shortened by rush or cluster applications in both, allergens without or formulated with adjuvants. In addition, so-called allergoid technologies have been introduced into European AIT manufacturing, in which allergens are chemically denatured, for instance, by formaldehyde, calcium cyanate, or glutaraldehyde.<sup>5</sup> Glutaraldehyde, for instance, polymerizes monomeric allergens to giant aggregates, thereby hiding away IgE epitopes, enlarging their distance, and thus preventing IgE cross-linking. Therefore, with allergoids the build-up phase can be shortened.<sup>6</sup> In a European survey of SCIT and sublingual AIT (SLIT), allergoids were significantly less associated with a risk for anaphylactic reactions than were unmodified natural extracts in adults ( $P = .001$ )<sup>4</sup> and in children.<sup>7</sup> Safety, therefore, addresses mechanisms preventing IgE cross-linking by allergens at the effector-cell site.

In addition, precipitation of the soluble therapy allergen in a depot at the injection site lowers the risk of systemic distribution and lowers the risk of systemic IgE-mediated reactions (Figure 1, A). Most adjuvants provide depot effect, although their name (derived from the Latin word *adjuvare*—“help”) refers to their immunostimulatory capacity (see below). All adjuvants presently applied in marketed European AIT products confer depot effect (Table II).<sup>5</sup> Notably, when the alum depot in mice was surgically removed from the injection site 120 minutes later, the T- and B-cell immune responses were comparable to the animals to the injected control group.<sup>8</sup> Therefore, deposition of the allergen is rather a measure for safety than for effective immunostimulation.

Second, AIT needs to be effective in a robust  $T_H2$  setting. In contrast to the usually prophylactic vaccines against infectious diseases, AIT is a therapeutic intervention. Because prophylactic vaccines initiate a first-time immune response to an antigen in naive immune cells, a *de novo* immune response can usually be built up easily. Numerous mouse models, although representing only an approximation to the human setting, propose that preventive effects against allergy can be easily achieved, whereas a successful proof-of-concept in therapeutic mouse models is usually more difficult.<sup>9</sup> The reason is that in atopic allergy the preexisting  $T_H2$  immune response is very robust, requiring application schemes over the range of 3 to 5 years in human patients to finally establish a protective immune response, be it immune activation against the allergen, or immune tolerance. In consequence, AIT with extracts is rather regarded as an immunomodulatory process than a vaccination. In the future, the usage of recombinant allergens fused to viral proteins and displayed on virus-like particles may allow the introduction of immunization schemes with allergens that are closer to vaccines<sup>10</sup> and, depending on the carrier, possibly at the same time confer protection to the virus itself.<sup>11</sup> The long duration of AIT and high frequency of injections is directly connected to the limited compliance of patients,<sup>12</sup> which is more of a problem in SLIT than in SCIT in real-life studies.<sup>13</sup> Therefore, optimal adjuvants should improve the efficacy of AIT by leading to faster relief of clinical symptoms and, with lower number of treatments, result in a better patient adherence.

## ADJUVANTS WITH A ROLE IN TOLERANCE INDUCTION

The basic principle of AIT is the application of logarithmically increasing doses of allergen by rush, for instance, with an

**TABLE I.** The major differences between vaccines against infectious agents and vaccines against allergens (AIT)

| Characteristic   | Vaccine against infectious diseases                                                                                                                                    | Allergen immunotherapy                                                                                                 |
|------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------|
| Type             | Prophylactic                                                                                                                                                           | Therapeutic                                                                                                            |
| Adjuvants        | Yes, in most cases                                                                                                                                                     | Yes in EU, no in the United States                                                                                     |
| Application site | SC, ID, IM, IL, EC, oral                                                                                                                                               | SCIT: SC in Europe, SC and ID in the United States, SLIT: sublingual, EC for food only; other routes experimental only |
| Doses            | Same doses in all applications of a vaccine                                                                                                                            | Stepwise increase to maintenance dose                                                                                  |
| Injection scheme | Prime and boost, usually 3-4 shots, 6 mo later 1 shot                                                                                                                  | Treatments 3-5 y, 4-41 shots depending on product                                                                      |
| Memory boosts    | Single booster applications years later, depending on type of vaccine.<br>Some vaccines induce lifelong memory, like the live attenuated vaccinia vaccine <sup>2</sup> | No clear recommendation, usually repetition of whole AIT course to boost memory function                               |

EC, Epicutaneous; ID, intradermal; IL, intralymphatic; IM, intramuscular; SC, subcutaneous.

infusion pump, cluster build-up, or depot injections, followed by booster injections until clinical tolerance is achieved. The trick is to saturate the specific IgE immunoglobulins and exploit them for antigen uptake and introduction of an immune response, rather than eliciting immediate-type allergic side reactions (Figure 1, A). The varying immunization protocols in the maintenance dose range from 1 to 2 monthly applications to more vaccine-like schemes with only 4 injections a year, but all are recommended for a duration of 3 to 5 years. It seems plausible that aqueous extracts and adjuvanted extracts initiate divergent immune responses, but protective immune responses can be achieved by various mechanisms.

## ESTABLISHMENT OF T<sub>H</sub>2 PATHWAYS IN ALLERGY

Allergens are exceptional antigens because they naturally stimulate T<sub>H</sub>2 immune responses, characterized by IgE formation, upregulation of IgE receptors, and T<sub>H</sub>2 inflammation composed of mast cells, eosinophils, and—in the periphery—basophils. Typically, the IgE response results from very low doses of antigen exposure. These responses are facilitated on an atopic background with elevated IL-4, IL-13, and total IgE levels, combined with permeable cutaneous and mucosal barriers. The decisive steps of T<sub>H</sub>2 skewing are made at the entrance sites where some allergens disturb the epithelial layer via enzymatic function. Activated epithelia release thymic stromal lymphopoietin, the initiator of the T<sub>H</sub>2 cascade, and IL-33, involved in pruritus and amplification of the inflammatory response. Other allergens such as Der p 2 activate inflammatory pathways via their ability to interact with LPS, or like Der p 13, are sensed by serum amyloid A1, a pattern recognition receptor that activates T<sub>H</sub>2 pathways in the lung.<sup>14</sup>

However, allergens extracted at the mucosa are complex mixtures and often promote a T<sub>H</sub>2 environment via synergistic mechanisms. Many laboratories have observed that recombinant allergens actually have no cognate sensitizing capacity. It is thus pivotal that allergens bring in bioactive danger signals that are important cofactors in the sensitization process, with the present environmental pollutants and microbiota of pollen likely helping as adjuvants.<sup>15</sup> Allergens and cofactors thus synergize to robustly promote T<sub>H</sub>2 pathways.

## MECHANISMS OF ADJUVANTICITY: DANGER SIGNALS

The purpose of adjuvants in AIT is to skew the robust T<sub>H</sub>2 immune bias toward the cytosolic inflammatory pathway for

enhanced antigen cross-presentation and IgG production (Figure 1, B), or toward the vacuolar pathway with a clear T<sub>H</sub>1 shift and active tolerance (Figure 1, C). Adjuvants in AIT products in the European market, in human and veterinary applications,<sup>16</sup> mechanistically combine these strategies.<sup>5</sup>

Immunoactivatory adjuvants such as alum, calcium phosphate, or microcrystalline tyrosin (Figure 1, B) all form crystals that physically equip the allergen with strong danger signals (exception: specific chemical modifications; for instance, exchange of hydroxyl ions in alum with phosphate groups inhibits the crystallization process, resulting in an amorphous state of aggregation).<sup>17</sup> Formulation with these adjuvants supports interaction with the lipid layers of dendritic cell (DC) membranes, as has been shown for alum.<sup>18</sup> Via scavenger receptor-mediated endocytosis, these adjuvants lead antigens into phagolysosomes, which get mechanically disrupted.<sup>19</sup> The escaped antigens subsequently enter the so-called cytosolic pathway, and get degraded into peptides in the proteasome, which get loaded onto recycled HLA-I molecules in the endoplasmic reticulum and presented to T cells on the surface (HLA-I presentation pathway). During this process, alum activates the NALP-inflammasome and leads to caspase-1 activation and the release of proinflammatory IL-1 $\beta$  and IL-18.<sup>20</sup> Alum adjuvants can induce IL-4 production and T<sub>H</sub>2 responses independently of IL-4 or IL-13,<sup>21</sup> but can be reprogrammed by nanoparticle technology.<sup>22</sup> IFN- $\beta$  signaling is possibly induced by danger-associated molecular patterns-sensing pathways such as toll-like receptor (TLR) or nucleotide-binding oligomerization domain-containing protein 1 activation.<sup>23</sup>

The danger signal gets amplified via cross-presentation to specific immune cells, enhancing the vaccine efficacy and improving T- and B-cell immunogenicity.<sup>24</sup> Of note, alum belongs to the so-called T<sub>H</sub>2 adjuvants, which in fact enforce T<sub>H</sub>2 immunity but lead to allergen tolerance at some stage, especially associated with formation of antibodies that block IgG (IgG<sub>1</sub> and IgG<sub>4</sub>)<sup>25</sup> (see below).

Immune activation toward T<sub>H</sub>1 by monophosphoryl lipid A (MPLA), a compound derived from the LPS of gram-negative bacteria, exploits the TLR4 pathway, which introduces allergens into the vacuolar pathway and counteracts the T<sub>H</sub>2 immune bias<sup>26</sup> (Figure 1, C). As compared with LPS, MPLA has attenuated inflammatory potency.<sup>27</sup> It slows down the phagolysosomal fusion and thus antigen degradation. This supports enhanced antigen presentation. The MPLA pathway induces IL-27, IL-10, IL-4, TNF, IFN- $\gamma$ , TGF- $\beta$ , and TLR4 according to a recent transcriptome analysis during a clinical trial.<sup>28</sup> In

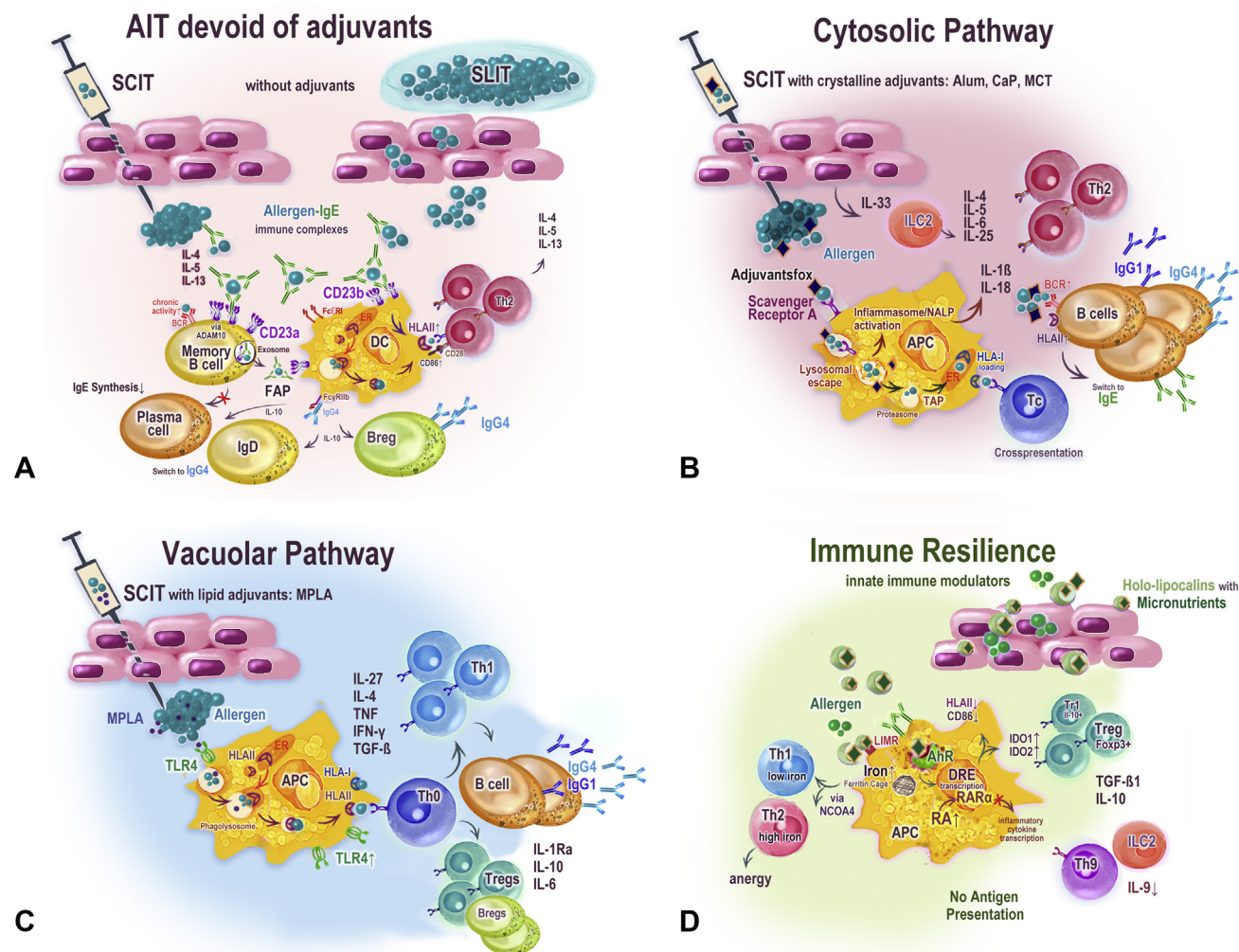


**FIGURE 1.** Immunologic mechanisms of immune activation, tolerance, and resilience. **(A)** Mechanisms of AIT devoid of adjuvants. When allergen is applied via SLIT or SCIT at a high concentration, it forms allergen-IgE ICs, which bind to the low-affinity IgE receptor CD23. In a  $T_H2$  cytokine-high environment, CD23/IgE-ICs get internalized, recycled, and shipped to neighboring DCs. The APCs then start IgE-facilitated antigen presentation (FAP), and IgE synthesis is downregulated, in favor of a switch to IgG4. IgG4 via FcγRIIb binding interferes with this activator pathway and drives macrophages into a regulatory phenotype secreting IL-10. **(B)** Mechanisms of SCIT with nanocrystalline adjuvants. Allergens or allergoids noncovalently adjuvanted with alum, CaP, and MCT rather support antibody (IgE, IgG1, IgG4) than cellular immune responses. DCs recognize and internalize the nanocrystalline vaccine by pattern recognition receptors. Lysosomal escape of the allergen prompts its proteasomal processing. Cross-presentation renders HLA-I display of peptides also to cytotoxic T cells. These adjuvants trigger also the inflammasome, rendering proinflammatory cytokines IL1-β and IL-18. **(C)** Mechanisms of SCIT with adjuvants driving allergens into the vacuolar pathway. MPLA is an agonist of TLR4. Phagocytosed allergen/MPLA is degraded in the phagolysosomes from where peptides are presented on HLA-II and via cross-presentation on HLA-I. This induces relatively strong cellular and humoral responses. Overall, MPLA skews the immune response toward Treg-cell and  $T_H1$  pathways, rendering more IgG1 and IgG4 without boosting IgE. **(D)** Mechanisms of immune resilience. The lipocalin BLG binds via lipocalin-interacting-membrane receptor (LIMR). After internalization BLG releases its ligands: *Iron* enters the labile iron pool and can be stored in the “ferritin cage,” preventing activation of APCs; siderophores from the iron complex can bind to the AhR, which stabilizes Foxp3 expression in Treg cells; RA is transformed and translocated to the nucleus, where it represses transcription of inflammatory cytokines. AhR, Aryl hydrocarbon receptor; APC, antigen-presenting cell; CaP, calcium phosphate; MCT, microcrystalline tyrosin.

this dose-finding trial, triggered by the European Therapy Allergen Ordinance, MPLA allowed higher cumulative allergen doses than previously used.

The induction of allergen-specific IgG antibodies is since long a hallmark in AIT, independent of the usage of adjuvants, and these antibodies may be more than biomarkers of compliance.<sup>29</sup>

IgG1 may, via its variable allergen-specific domain, be equally effective in allergen neutralization as IgG4. However, the constant domains of IgG1 and IgG4 allow interactions via different FcγRs and thus decide the cooperation, activation, or inhibition of inflammatory cells. A study on grass pollen SCIT proposed nasal IgG4 as a valid and reliable biomarker of AIT: IgG4

**TABLE II.** Adjuvants in European marketed AIT products for SCIT

| Compound                  | Abbreviation | Introduced for AIT in | Market share, % | Mean no. of injections in 3-5 y |
|---------------------------|--------------|-----------------------|-----------------|---------------------------------|
| Aluminum hydroxide        | Alum         | 1937                  | 66.7            | 41                              |
| Microcrystalline tyrosine | MCT          | 1970                  | 16.7            | 40.5                            |
| Calcium phosphate         | CaP          | 1980                  | 11.1            | 26                              |
| Monophosphoryl lipid A    | MPLA         | 1999                  | 5.6             | 12                              |

inhibited IgE-facilitated binding of allergen-IgE complexes to B cells, correlating with an increase in IL-10<sup>+</sup> regulatory T (Treg) cells and symptom improvement.<sup>30</sup> IgG<sub>4</sub>, but not IgG<sub>1</sub>, turned macrophages into an immunoregulatory phenotype, characterized by reduced expression of the activatory IgG receptor FcγRIIa but upregulated inhibitory FcγRIIb, and in addition resulted in IL-10 and CCL-1 secretion.<sup>31</sup>

IgG<sub>4</sub> therefore has unique properties in skewing the immune response into a beneficial circle, at least in allergy, characterized by IL-10 secretion, stimulating B cells to higher IgG<sub>4</sub> production.<sup>32</sup> Of note, by the same mechanisms, IgG<sub>4</sub> has an unfavorable role in cancer.<sup>33</sup> Regulatory B cells thus play a central role in the efficacy of AIT by formation of IgG<sub>4</sub> and (mucosal) IgA, and again IL-10, as demonstrated in house dust mite-specific AIT.<sup>34</sup> It has been shown that not only follicular helper T cells support B cells but that *vice versa* regulatory B cells also control follicular helper T-cell maturation.<sup>35</sup>

## HOW THERAPEUTIC ALLERGENS WORK WITHOUT OR WITH ADJUVANTS

### Mechanisms of therapeutic allergens devoid of adjuvants

Without antigen, the IgE membranous B-cell receptor in memory B cells is chronically active.<sup>36</sup> When allergen is applied in the form of SLIT or SCIT, it enters the tissue at a high concentration, prompting binding of allergen to resident IgE antibodies to form allergen-IgE immune complexes (ICs). The low-affinity receptor CD23 expressed mainly on B cells and DCs acts as a decoy receptor for IgE and counteracts the loading of IgE to the high-affinity IgE receptor FcεRI. B cells and DCs react differently to IgE-ICs.<sup>37</sup>

IgE-switched memory B cells, which in the allergic patient are exposed to elevated levels of IL-4, IL-13, and IgE, express higher levels of low-affinity IgE receptor CD23 (CD23a) on their surface. This prompts oligomerization of CD23 into trimers, thereby also enhancing the binding avidity to the allergen-IgE ICs. Overall, after internalization and recycling of the CD23/IgE-ICs in the memory B cell,<sup>38</sup> the IgE synthesis gets down-regulated.<sup>39</sup> In contrast, naive IgD<sup>+</sup> B cells, which express higher levels of CD23, have a higher capacity to switch toward IgE or IgG<sub>4</sub> synthesis than the memory B cells.

When the sheddase disintegrin and metalloproteinase domain-containing protein 10 cleaves CD23 from the B-cell surface, the IgE-ICs can via exosomes be shipped to neighboring antigen-presenting cells such as DCs.<sup>40</sup>

Taken together, allergen-IgE ICs fixed via overexpressed CD23 on primary human B cells act noninflammatory. AIT reduces again the CD23 expression on switched B cells.<sup>41</sup>

DCs, monocytes, and other antigen-presenting cells express CD23b (and CD23a) on their surface. They may encounter allergen-IgE ICs directly, or may be instructed by B cells via exosomes to start IgE-facilitated antigen presentation. After

internalization, IgE-ICs are led into degradative pathways, resulting in CD86 and HLA-II upregulation, display of antigenic peptides on HLA-II, and activation of T<sub>H</sub>2 helper cells as a source of IL-4, IL-13 for isotype switch, as well as IL-5. The induction of IgE in the onset of AIT is a well-known phenomenon, and is followed by IgG<sub>1</sub> and IgG<sub>4</sub> formation during the course of AIT. IgG<sub>4</sub> via FcγRIIb binding is able to interfere with this activator pathway and drives macrophages into a regulatory phenotype secreting IL-10,<sup>32</sup> switch factor for IgG<sub>4</sub> and inducer of Treg and B cells.

### Mechanisms of allergens adjuvanted with nanocrystalline adjuvants

Allergens or allergoids noncovalently adjuvanted with alum, calcium phosphate, and microcrystalline tyrosine and injected subcutaneously form retard depots (Figure 1, B).

In a sensitized individual, IgE displayed by CD23 on DCs senses injected allergens, prompts DC activation, and brings this exogenous antigen into the HLA-II pathway, subsequently rendering B- and T-cell activation (as illustrated in Figure 1, A). This adaptive pathway rather supports antibody (IgE, IgG<sub>1</sub>, IgG<sub>4</sub>) than cellular immune responses.<sup>42</sup> However, when antigens are tightly ligated to alum nanoparticles, the direct antigen delivery to B cells, B-cell receptor signaling, antigen processing, and expression on HLA-II on B cells are enforced, too.<sup>43</sup>

Nanocrystalline adjuvants have an additional function: DCs or other antigen-presenting cells recognize the injected vaccine in an innate manner by pattern recognition receptors,<sup>44</sup> for instance scavenger receptor A, and internalize it into phagosomes, which fuse to phagolysosomes. By mechanical forces, nanocrystals prompt lysosomal escape of the allergen into the cytoplasm and its proteasomal processing. Trimmed peptides are then with the transporter associated with antigen processing, transported to the endoplasmic reticulum and loaded to HLA-I molecules. This so-called cross-presentation renders HLA-I display of peptides also to cytotoxic T cells.<sup>20</sup> Equally important is the triggering of the inflammasome by these adjuvants, rendering synthesis and secretion of caspase-dependent proinflammatory cytokines, such as the IL-1 family members IL-1-β and IL-18.<sup>45</sup> IL-18 induces IgE isotype switch and synthesis and T<sub>H</sub>2 responses.<sup>46</sup> In addition, innate cells are a source of IL-4, IL-6, and IL-25, cytokines responsible for a resulting T<sub>H</sub>2-cell bias *in vivo*.<sup>20,42</sup>

### Mechanisms of adjuvants driving allergens into the vacuolar pathway

As already illustrated in Figure 1, A, in the allergic individual, IgE displayed by CD23 senses injected allergens and therefore in an adaptive manner prompts HLA-II presentation, and then B-cell and T-cell activation. The fate of allergen adjuvanted with MPLA is illustrated in Figure 1, C.

The adjuvant MPLA is the lipid A moiety derived from bacterial LPS and is an agonist of TLR4, a pattern recognition receptor. Therefore, phagocytosed allergen after fusion with

lysosomes stays in the phagolysosomes where this exogenous antigen is degraded and peptides directly loaded onto HLA-II antigens contributed from the endoplasmic reticulum.<sup>20,47</sup> MPLA therefore strongly supports antigen processing and presentation in HLA-II context, but also supports cross-presentation on HLA-I and relatively strong cellular responses besides humoral responses. Overall, MPLA skews the immune response toward T<sub>H</sub>1 pathways, rendering more IgG<sub>1</sub> and IgG<sub>4</sub> without boosting IgE,<sup>47</sup> and to Treg-cell pathways by inducing IL-1–receptor antagonist (IL-1Ra) and IL-10 expression, and via IL-6 supports antibody production.<sup>48</sup>

### Mechanisms of spiked allergens toward immune resilience

To train resistance to type I allergies in an innate manner is a novel concept in the immunotherapy of allergy (Figure 1, D). Micronutrients such as iron (Fe<sup>3+</sup>) complexed with siderophores, or retinoic acid (RA), are pivotal for immune cell function and in the acquisition of tolerance. Relatively large dietary amounts have to be taken in to reach sufficient levels at the target cells. This can be circumvented by targeted micronutrition using lipocalins or lipocalin-like molecules as Trojanic horses, with molecular pockets that can accommodate these ligands.<sup>49</sup> All lipocalin ligands also bind into the pocket of PR10 molecule Bet v 1.<sup>50</sup>

The lipocalin beta-lactoglobulin (BLG) (alias RA-binding protein) binds via lipocalin-interacting-membrane receptor<sup>51</sup> or via mIgM of naive B cells. It does not bind preformed IgE because ligands RA and quercetin-iron complexes mask IgE epitopes.<sup>52,53</sup> After internalization, BLG releases its ligands, which reach diverse intracellular pathways with a role in tolerance:

1. Iron enters the labile iron pool and can be stored in the “ferritin cage” in cytosol and mitochondria from where it can be released by autolysosomal degradation under instruction by nuclear receptor coactivator 4.<sup>54</sup> The intracellular load of complexed iron (ferric iron) is pivotal for preventing any activation of antigen-presenting cells, especially macrophages.<sup>52,53</sup> At the T-cellular level, T<sub>H</sub>1 cells have lower intracellular iron reserves and thus are greatly affected by iron-deficient conditions, whereas T<sub>H</sub>2 cells have a greater iron reservoir, which promotes their survival under iron-deficient conditions. The greater need of iron during immune activation is also mirrored by the expression of transferrin receptor upon T-cell activation, whereas its downregulation is characteristic for T-cell anergy.<sup>55</sup>
2. Siderophores from the iron complex can bind to the cytosolic aryl hydrocarbon receptor,<sup>53</sup> which is translocated into the nucleus where it controls the transcription of dioxin-responsive element. Depending on the ligand, the aryl hydrocarbon receptor in DCs induces expression of the enzymes indoleamine 2,3-dioxygenase 1 and 2, boosts and stabilizes Foxp3 expression in Treg cells including Tr1, suppresses HLA-II and CD86 expression, and enhances TGF- $\beta$ 1 and IL-10 secretion and expansion of Foxp3<sup>+</sup> Treg cells and IL-10<sup>+</sup> Tr1 cells.<sup>56</sup>
3. RA released from BLG in the cytosol is transformed into less bioactive metabolites by enzymes of the CYP26 family, a process coordinated by intracellular retinoid-binding proteins, before RA is translocated to the nucleus, where RA through

its receptor RAR $\alpha$  represses transcription of inflammatory cytokines and represses the program of the CD4<sup>+</sup> T<sub>H</sub>9-cell subset to secrete proallergic IL-9.<sup>57</sup>

### NEW ADJUVANTS AT THE HORIZON

Other adjuvants in the developmental pipeline, which all are experimental or in clinical trials and have not reached the market,<sup>5</sup> include addition of TLR-9 agonists such as CpG-Oligodesoxynucleotides or other immune modulators to the allergens and formulation into nano- or microparticles or as liposomes, hypoallergens, or allergen fusion proteins. Interestingly, in the end, the most sophisticated approaches among allergen engineering are formulated with a classical adjuvant, most often alum. An alternate concept is the presentation of allergens on particles, making them on the one hand attractive for DC phagocytosis and on the other hand less allergenic (also here, often regular adjuvants are used in addition in the final formulation). Probably also the above-listed adjuvants on the European market,<sup>5</sup> which absorb the allergens by noncoherent forces, form convolutes with particle characteristics. In addition, allergoid chemistry transforms allergens into macromolecules by intermolecular chemical links. In allergen aggregates, IgE epitopes are often masked or their spacing enlarged, impairing the cross-linking capacity of the injected allergen. For instance, when ovalbumin<sup>58</sup> or peanut allergens Ara h 1 and Ara h 2<sup>59</sup> were fused on virus-like particles, the distant spacing prohibited IgE cross-linking, but supported their immunogenicity. Of note, epitope spacing below 50 Å may form proallergic molecular patterns, for example, on allergen dimers, trimers, or multimers, and rather promote allergy.<sup>60</sup>

In addition, glycoconjugates have been followed in AIT for a long time and their beneficial properties in AIT are proven. Besides classical PEGylation, allergens were in a 1-step procedure transformed into allergoids coupled to mannan, an alternate carbohydrate with tolerogenic property. Exactly in this example the formulation with the standard adjuvant alum did in fact impair the tolerogenic effect of the conjugate.<sup>61</sup> Overall, it seems that the choice of the right adjuvant is “the art of AIT,” at least in Europe.

We propose an alternative innate strategy to achieve immune resilience by delivery of micronutrients via spiked lipocalins and lipocalin-like allergens to immune cells<sup>52,53</sup> (Figure 1, D).

### DIFFERENT AIT PRACTICES IN THE UNITED STATES AND THE EUROPEAN UNION

The European and US schools of AIT were compared recently in a joint publication by the Paul-Ehrlich Institute, Germany, and US institutions.<sup>62</sup> At the time of this publication, there were 4 US-licensed manufacturers. The most striking differences in European Union (EU) and US practices are (1) in most cases the absence of adjuvants in the AIT licensed in the United States (Figure 1, A) and (2) the fact that allergen extracts are customized for each individual patient in the US allergist’s office, aseptically mixing up to 4 extracts from stocks. Respective protocols can be retrieved or training obtained in the AAAAi compounding corner, USP Chapter 797: Allergen Extract Compounding Training Module, at the website of the AAAAi.<sup>63</sup>

The stock allergen extracts can be aqueous, glycerinated, lyophilized, acetone precipitated, or (rarely) alum precipitated. The stocks must contain phenol concentrated to at least 0.25% or if lower, a glycerin concentration of at least 20%. Then, the stocks are diluted with 1 of the following diluents: 50% glycerin  $\pm$  phenol, phenol saline, or saline solution containing HSA and phenol (for alum-mixed extracts, phenol saline diluent has to be used). Phenol is added because of its bacteriostatic characteristics, and glycerin and HSA are stabilizers for the proteins. HSA also inhibits the adsorption of allergen to glass or plastic vials. Although the fact that phenol is also able to denature proteins has been criticized, denaturation of allergen proteins may diminish IgE epitopes and contribute to enhanced safety. All steps must be “ideally” done in compliance with United States Pharmacopeia requirements.

Allergen standardization is means to keep the potency and composition of allergen extracts constant to avoid severe reactions from the injection site, especially when switching from one batch to the other. The presence of adequate allergen T- and B-cell epitopes in the extracts is an absolute prerequisite for specificity<sup>64</sup> and thus efficacy.<sup>65</sup> When allergen-IgE complexes are formed, specificity of the immunotherapy is guaranteed and the anti-inflammatory cascade turned on (Figure 1, A). The standardization of a few allergen extracts in the United States is based on intradermal titration as a basis for Bioequivalent Allergy Units to equilibrate the potency between extracts of different allergen source materials. Thus, the potency is based on IgE epitopes not T-cell epitopes, however, probably correlating in most instances. Manufacturers are supposed to copy the Food and Drug Administration standards. In Europe, skin prick testing has been used for the same purpose. However, the biological activity correlates well with the molar content of major allergens; therefore, their content had been partly implemented. Allergen molecules are determined to improve standardization in US-licensed AIT extracts, and in part labeled on the vials. However, standardized and nonstandardized products admitted by the Food and Drug Administration may be used together. For Food and Drug Administration approval, products have to show an at least statistically minimal clinical effect. However, in practice, there will never be stability data on the mixtures used by different practicing allergists. In addition, the efficacy of extracts mixed in the office of practitioners cannot be transferred to those from clinical trials.

In the United States, injections can be given intradermally or subcutaneously, whereas in the EU SCIT is strictly applied subcutaneously. After slow or cluster build-up, the maintenance injections are made every 2 to 4 weeks for 3 to 5 years. SLIT has more recently entered the American scene, with allergen tablets and off-label used extracts in liquid drops. The differentiation of SLIT-tablet (with any of the 4 presently US-approved tablets) and SLIT-drops (with aqueous drops) was recently proposed,<sup>66</sup> referring to their potentially distinct immune mechanisms. Whether SLIT-tablet and SLIT-drops have different efficacies has not been compared head-to-head yet in randomized clinical trials.<sup>66</sup> In addition, the introduction of a SLIT-tablet containing a single allergen opposes the general US practice of mixing several therapeutic allergens in one application. It is however accepted that both SLIT application forms are safer than SCIT, in adults and children.<sup>67</sup>

In Europe, 8 manufacturers share the market for SCIT and almost equally SLIT products. Only products for sublingual application (SLIT), and insect venom SCIT,<sup>68</sup> are not

adjuvanted; for all other SCITs, ready-to-use vials formulated with adjuvants (mostly alum) can be purchased from the industry. Of the products admitted in Europe, 6 contain phenol, 2 mannitol, and 1 glycerin, at least as far as revealed by the AIT industry.<sup>5</sup> Since the introduction of the Therapy Allergen Ordinance in 2008 in Europe by Paul-Ehrlich Institute,<sup>69</sup> significant differences in selected primary end points are required in the clinical trials for marketed AIT products.

The main difference is that in Europe allergoids are available besides aqueous extracts, with a convincing safety profile.<sup>7,70</sup> Many countries around the world need to find compromises in clinical practices between the European and US school of AIT, as recently reported, for instance, for Mexico.<sup>71</sup>

## FROM THERAPEUTIC TO PROPHYLACTIC AIT?

Altogether, AIT in the narrower sense is regarded a therapeutic intervention. However, AIT modifies an ongoing allergic immune response and may prevent the relapse of disease. AIT also counteracts the progression of allergic symptoms (so-called “Etagenwechsel”) from allergic rhinitis to asthma.<sup>72,73</sup> Therefore, AIT is probably a best practice example for secondary prevention.

Prophylaxis per definition is an active treatment that optimally precludes sensitization and subsequently the onset of symptomatic disease. In allergy, prophylactic strategies are so far not state-of-the-art as in vaccinology, probably due to the unpredictability about which allergens will later become relevant for a child, and also because the immunization schemes in AIT are already challenging for the parents to this end. In some countries, children aged between 4 and 6 years have already received up to 126 infectious vaccines.<sup>74</sup> In a unique approach, a clinical prophylactic trial was made in atopic children with a sublingual formulation of house dust mite peptides, which induced IgG, but no IgE, responses.<sup>75</sup> The outcomes of this study on the allergic march remain to be investigated. In this context, the choice of an optimal adjuvant seems especially critical for approaching prophylactic AIT in naive children.

## INNATE LESSONS FROM PRIMARY PREVENTION FOR AIT

Primary prevention is an ideal scenario against the allergy epidemic. This term is most often used in the epidemiologic context and refers to natural exposure, for instance, the farm effect against allergy, asthma, and atopic sensitization. Besides farm dust, drinking cow's milk is considered protective not only against allergies but also against infections.<sup>76</sup> Raw milk contains a plethora of compounds for which a role in counteracting allergy has been discussed,<sup>77</sup> such as bovine IL-10 (76.8% amino acid sequence homology and binds the human IL-10 receptor), TGF- $\beta$ , of which 5 isoforms exist among which bovine and human TGF- $\beta$ 2 have a 100% amino acid homology, bovine IgG antibodies to allergens relevant for humans, lactoferrin, lactoperoxidase, and lysozyme, which act antibacterial, and maybe alkaline phosphatase, which may act anti-inflammatory because it detoxifies bacterial LPS by dephosphorylation of the lipid A moiety<sup>77</sup>; furthermore micro-RNA, polyunsaturated fatty acids, and oligosaccharides are the focus of investigations.

A major whey compound in cow's milk is the lipocalin BLG, characterized by an intramolecular pocket able to transport ligands. We have collected evidence that BLG in a receptor-mediated process carries several micronutrients into immune

cells, such as RA<sup>49</sup> and minerals in the form of iron-siderophore complexes (catechin and quercetin),<sup>53</sup> and thereby counteracts allergic inflammation. Based on this principle, a lozenge was created that, in an innate manner, was able to diminish allergic symptoms to pollen allergens not only in a prophylactic and therapeutic mouse model (Afify et al, unpublished data, 2020) but also in a double-blind placebo-controlled human clinical trial (Bartosik et al, unpublished data, 2020).

The generated knowledge could be expanded to the PR10 allergen Bet v 1, which behaved similar to the lipocalin BLG in terms of a strong absorbance capacity for iron-siderophore complexes<sup>50</sup> as well as RA.<sup>52</sup> Like BLG, Bet v 1 spiked with ligands downregulated allergic inflammation. Therefore, spiking of AIT products with ligands such as RA and/or iron-siderophore complexes may improve AIT efficacy, or may be introduced into novel micronutritional concepts.<sup>78</sup>

These results highlight that allergens can be used to carry micronutrients into regulatory cells, leading to a shutdown of cytokine production and in immune resilience against allergic sensitization and symptoms,<sup>53,79</sup> without boosting specific immunity (Figure 1, D). This is in agreement with the concept that “trained immunity and tolerance” during AIT involves the whole spectrum of innate cells previously not in our focus, such as innate lymphoid cells and innate lymphoid cell 2 regulatory cells, monocytes, DCs, and DC regulatory cells.<sup>80,81</sup>

Therefore, it should be considered whether processing and defatting of allergen extracts during AIT manufacturing may jeopardize their natural ligand load and thereby counteract the tolerogenic potency of therapy allergens.

## UNMET NEEDS

The long duration of AIT is likely related to the intrinsically bad immunogenicity of the allergens, which only in part can be improved by today's adjuvants. The frequency of injections and other applications is still a major cause for insufficient patient compliance and adherence in all forms of AIT. To this end, it is uncertain whether alternate routes such as intralymphatic immunotherapy will gain acceptance in the future, even though safer and with probably higher efficacy.

On the basis of evidence of allergoids and adjuvants in Europe, one might be tempted to encourage changes in the regulatory approval process especially in the United States, which would enhance development of new allergy immunotherapy adjuvants. However, approval of every allergen with each adjuvant on the basis of the current US system would take an incredibly long time and require extraordinary financial resources. Following the Therapy Allergen Ordinance, European products are presently undergoing clinical trials to demonstrate dose-efficacy relations for continued market allowance,<sup>82</sup> efforts that significantly impair the capacities to develop new products at present and in the next years.

Overall, continuous development of novel AIT strategies would be urgently needed. This refers not only to sophisticated engineering of allergens, hypoallergens, or fusion proteins but also to a need to develop novel display systems such as virus-like particles, which provide repetitive epitopes and thus danger signals. Also, the spiking of allergens is a promising concept taking into consideration the restoration of nutritional deficiencies of immune cells, leading to non-responsiveness—immune resilience—in patients.

## MANAGEMENT RECOMMENDATIONS

Both AIT schools, in Europe and the United States, use divergent, but successful practices in terms of mixing allergens (the United States and the EU), allergoid technologies, and the usage of adjuvants (EU), and therefore exploit different immunologic mechanisms (Figure 1, A-C). European studies indicate that allergoids are substantially safer than native allergens, proposing the introduction of allergoids also in the United States. Although the preference for a certain application route, subcutaneous or sublingual, will largely depend on the patient's age, personality, and lifestyle. SCIT generally has a higher adherence rate than SLIT. Adjuvants enhance safety by depot function and train immunity and tolerance. The potential adverse effects of various adjuvants must be carefully monitored and affect regulatory agency approval.<sup>5</sup> In light of the general debate on vaccines, biodegradability and biocompatibility of adjuvants may be special concerns of the patients. Among European adjuvants, calcium phosphate and microcrystalline tyrosin are substances naturally occurring in the organism, and MPLA allows the lowest frequency of injections, closest to that of infectious vaccines. The immunomodulatory potency of alum could be reprogrammed to favorable T<sub>H</sub>1 adjuvanticity by nanoparticle technologies.<sup>22</sup> In the future, allergen extracts could be spiked with immunomodulatory ligands to improve their efficacy toward immune resilience (Figure 1, D).

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