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REVIEW

Adjuvant Allergen Fusion Proteins as Novel Tools for the Treatment of Type I Allergies

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Abstract

While acute allergic symptoms can be managed by emergency medication, to date, allergen-specific immunotherapy (SIT) with allergen extracts is the only available curative treatment option. However, the risk of anaphylactic reactions, long treatment duration, varying extract quality, and underrepresentation of certain allergens currently prevent many patients from successfully undergoing SIT. Novel strategies are needed to enhance efficacy, safety, and convenience of allergy treatment. Fusion proteins combining allergen and adjuvant into a single molecule can efficiently induce immune responses by targeting the allergen to the relevant immune cells in vivo. Simultaneous co-delivery of both antigen and adjuvant to the same cell in a fixed molecular ratio triggers the uptake and presentation of the conjugated allergen in the context of the adjuvant-induced immune cell activation. This review summarizes the published strategies to improve the treatment of type I allergies using fusion proteins consisting of allergen (peptides) and either (1) immune-activating bacterial (flagellin, MPLA, S-layer, cholera-, and tetanus toxin), (2) viral (PreS, VP-1, TAT), or (3) fungal (FIP-fve) components, (4) immune-activating DNA motifs, (5) forced delivery of allergens to the MHC-II loading pathway, and (6) killing of immune cells expressing allergen-specific IgE by fusion of the allergen to diphtheria toxin.

Keywords Fusion protein · Allergen · SIT · PAMP · PRR

Abbreviations

3Crp	Peptides derived from the major Japanese cedar pollen allergens Cry j 1 and Cry j 2
AH3a42	Ara h 2 peptide
APC	Antigen-presenting cell
AS	Adjuvant system
ASAS	Active systemic anaphylactic shock
BALF	Bronchoalveolar lavage fluid
CLR	C-type lectin receptors
CT	Cholera toxin
CTA	Catalytic cholera toxin A-subunit
CTB	Non-toxic cholera toxin B subunit
CTB-3Crp	Fusion protein consisting of cholera toxin B subunit and peptides derived from the major Japanese cedar pollen allergens Cry j 1 and Cry j 2 (3Crp)
DC	Dendritic cell
DT	Diphtheria toxin
FIP-*fve*	Fungal immunomodulatory protein *fve*
Foxp3	Forkhead-box-protein 3
GLU-3Crp	Fusion protein consisting of rice gluten subacidic unit and 3Crp
HAS	Human serum albumin
HDM	House dust mite
HIV-1	Human immunodeficiency virus type 1
HR1/2/4	Histamine receptor 1/2/4
IFN	Interferon
IL	Interleukin
ILIT	Intralymphatic immunotherapy
ISS–ODN	Immunostimulatory oligodeoxynucleotides
MAT	Modular antigen translocating
MPLA	Monophosphoryl lipid A
NLRs	NOD-like receptors

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ODN	Oligodeoxynucleotides
Ova	Ovalbumin
OsDp2Fve	Fusion protein consisting of the fungal immunomodulatory protein and the major house dust mite allergen Der p 2
PAMP	Pathogen-associated molecular pattern
PRR	Pattern recognition receptor
rCTB-Bet v 1a	Fusion protein consisting of cholera toxin B subunit and Bet v 1a
rFlaA:Artv1	Fusion protein consisting of flagellin A from *Listeria monocytogenes* and the major mugwort pollen allergen Art v 1
rFlaA:Betv1	Fusion protein consisting of flagellin A from *Listeria monocytogenes* and the major birch pollen allergen Bet v 1
rFlaA:Ova	Fusion protein consisting of flagellin A from *Listeria monocytogenes* and the egg white allergen Ova
rSbpA-Bet v 1	Fusion protein consisting of the S-layer protein SbpA of *Bacillus sphaericus* CCM 2177 and Bet v 1
rSbsC-Bet v 1	Fusion protein consisting of the S-layer protein of *Geobacillus stearothermophilus* and Bet v 1
rTAT-Che a 3	Fusion protein consisting of TAT and the main *Chenopodium album* pollen allergen Che a 3
S-layer protein	Bacterial surface layer proteins
SIT	Specific immunotherapy
SlpB-AH3a42	Fusion protein consisting of the S-layer protein SlpB of *Lactobacillus buchneri* CD034 and a peptide derived from the major peanut allergen Ara h 2
TAT	Trans-activating regulatory protein
TNF	Tumor necrosis factor
Th1/2/17	T helper 1/2/17 response
TLRs	Toll-like receptors
Treg	Regulatory T cell
TT	Tetanus toxoid
VP1	Viral protein 1
VP1-2xP5	Fusion protein consisting of a hypoallergenic peptide (P5) of the timothy grass pollen allergen Phl p 1 and VP1

Introduction

Pathomechanism and Current State-of-the-Art Treatment Options for Type I Allergic Diseases

In allergic patients, the combination of both genetic predisposition and environmental factors results in the induction of pathologic Th2 responses typically directed against otherwise harmless environmental antigens. These Th2 responses are characterized by the secretion of typical Th2 cytokines (IL-4, IL-5, and IL-13) driving the differentiation of allergen-specific B cells into IgE-producing plasma cells (Pelaia et al. 2015). Once produced, these allergen-specific IgE antibodies bind to the high affinity IgE receptor Fc epsilon RI located on the surface of mast cells and basophils. Upon reencountering the allergen (e.g., in the gastrointestinal tract or the lungs), the bound IgE antibodies trigger the IgE-mediated degranulation of mast cells, and basophils finally resulting in allergic inflammation.

While the acute symptoms of allergic reactions can be managed by emergency medication (e.g., epinephrine or inhaled corticosteroids), to date, allergen-specific immunotherapy (SIT) is the only available curative treatment option (Passalacqua et al. 2018). Technically, after determining the individual patient's safe starting dose, SIT is accomplished by either subcutaneous or sublingual administration of increasing amounts of crude allergen extracts. The used allergen extracts are derived from allergen sources such as pollen, animal dander, or house dust mites (HDM) that contain a mixture of different allergenic proteins (Passalacqua et al. 2018).

While allergen extracts can be successfully used to treat certain allergies, SIT has disadvantages that prevent many patients from undergoing this type of treatment. The major problems are: (1) risk of anaphylactic reactions, (2) low patient adherence due to long treatment duration, (3) varying extract quality, and (4) underrepresentation of certain allergens in the used extracts due to either low stability or extractability, as well as (5) poor treatment efficacy in polysensitized patients (Klimek et al. 2013).

To address these problems, novel strategies are investigated to enhance efficacy, safety, and convenience of allergy treatment. Among others, the tested strategies include the use of: (1) recombinantly produced allergens, hypoallergens, or peptide-based vaccines, (2) new allergen delivery routes (intranodal and transcutaneous), (3) novel types of adjuvants derived from immune-stimulating bacteria and viruses, as well as (4) new formulations and delivery systems (e.g., allergen-adjuvant fusion proteins, virus-like-particles, DNA vaccination, modified vaccinia virus Ankara-based vaccines, aggregates, nanoparticles, or liposomes) (partly reviewed in: Anzaghe et al. 2018; Curin et al. 2018; Kündig et al. 2015).

This review will describe the published strategies to improve the treatment of type I allergic diseases using fusion proteins consisting of allergen (peptides) either conjugated or fused to immune-activating adjuvants.

Adjuvants: Mode of Action and State of the Art

Adjuvants are substances that are used in combination with vaccines to enhance and prolong immune responses

to certain pathogens (Chiarella et al. 2007). The usage of adjuvants enables dose reduction (reduced number of immunizations), antigen sparing (reduction in quantity of administered antigen), and improves immune responses in populations which do not possess a fully functional immune system such as infants, the elderly, or immunocompromised patients (Di Pasquale et al. 2015). The enhanced immune response mediated by the addition of adjuvants results from the delivery of antigen, the recruitment of antigen-presenting cells (APCs) to the site of inoculation, the activation and maturation of these APCs, as well as the enhanced antigen uptake and presentation (Garlapati et al. 2009). Therefore, the inclusion of adjuvants in vaccines enhances antigen-specific immune responses and increases the formation of immunological memory, respectively. To achieve these effects, adjuvants need to be stable under a broad range of storage conditions (time, temperature, and pH). They should be easy to use and convenient to inject, non-toxic, as well as non-mutagenic, and have low levels of adverse effects (Heegaard et al. 2011; Marciani 2003).

Initially, adjuvants were defined by Alexander Glenny and Gaston Ramon in the 1920s. In 1926, Glenny used aluminum sulfate (Alum) to formulate the first adjuvanted diphtheria vaccine (Bonanni et al. 2011). Besides Alum, six more adjuvants have been included in licensed vaccines in the last 20 years (Di Pasquale et al. 2015). In general, adjuvants can be classified in two main groups: (1) vehicles/delivery systems such as mineral salts, emulsions, liposomes, or virosomes, and (2) immunostimulators that directly or indirectly induce the secretion of cytokines and, therefore, activate the immune system (Garlapati et al. 2009; Heegaard et al. 2011).

Alum is the most commonly used adjuvant and the only one approved for global use (Kwissa et al. 2007). It is included in vaccines such as those for diphtheria, tetanus and typhus, inactivated polio virus, hepatitis B virus (HBV), or human papilloma virus (HPV), and elicits strong antibody responses. Alum mediates a so-called depot effect enabling continuous antigen release, enhanced antigen uptake and presentation, and induces the secretion of pro-inflammatory cytokines via an activation of the NLRP3 inflammasome (Eisenbarth et al. 2008). In addition, Alum was shown to boost humoral immunity by promotion of Th2 responses (Reed et al. 2009). However, the goal in SIT is to promote Th1-based immune responses against the respective allergen. Therefore, the Th2-promoting effects of Alum and its ability to boost B-cell responses in a Th2-driven manner may be potentially dangerous in vaccines for the treatment of allergic diseases.

Two types of emulsions (dispersions of water and oil) can be distinguished: (1) water in oil and (2) oil in water emulsions (Bonanni et al. 2011). Freund's adjuvant is a water in paraffin oil emulsion of killed mycobacteria with Arlacel A first used in the 1930s by Jules Freund (Heegaard et al. 2011; Leroux-Roels 2010). Although inducing strong Th1 responses, it was not approved for use in humans as it is too toxic and results in excessive local adverse effects (Heegaard et al. 2011; Leroux-Roels 2010). Novel water in oil emulsions like Montanadine was tested in clinical trials for malaria, human immunodeficiency virus (HIV), and cancer vaccines (Reed et al. 2009).

In contrast, oil in water emulsions has been successfully used in several vaccines as they are usually well tolerated and very efficient. For example, MF59 which induces recruitment and activation of APCs, chemokine expression, and high antibody titers is used for seasonal and pandemic influenza vaccines (Bonanni et al. 2011; Pellegrino et al. 2015; Reed et al. 2009).

Besides emulsions, another type of vehicle/delivery system is liposomes which are artificial vesicles consisting of lipid layers (Bonanni et al. 2011; Leroux-Roels 2010). Interestingly, licensed products do not contain liposomes but virosomes. Virosomes are spherical lipid vesicles (without viral DNA/RNA) that carry the influenza glycoproteins hemagglutinin and neuraminidase on their surface (Pellegrino et al. 2015). Both viral glycoproteins target APCs and facilitate antigen uptake by APCs via receptor-mediated endocytosis (Leroux-Roels 2010). Vaccines that are currently adjuvanted with virosomes are the hepatitis A vaccine *Epaxal* and the Influenza vaccines *Inflexal* and *Invivac* (Leroux-Roels 2010).

Novel effective adjuvants link innate and adaptive immunity via activation of pattern recognition receptors (PRR). Four different classes of PRRs have been identified so far: (1) Toll-like receptors (TLRs), (2) C-type lectin receptors (CLRs), (3) retinoic acid inducible gene I like receptors, and (4) NOD-like receptors (NLRs) (Takeuchi and Akira 2010). All these PRRs recognize pathogen-associated molecular patterns (PAMPs) to initiate signaling pathways that lead to the synthesis of cytokines including type I interferon (IFN), interleukin (IL)-6, tumor necrosis factor (TNF)-α, or IL-12 (Kawai and Akira 2008). Immunostimulators that are used as adjuvants include PAMP structures and immunologically active substances. Among the immunostimulators, the TLR agonists are the most commonly used adjuvants. The glycolipid monophosphoryl lipid A (MPLA) is a non-toxic derivate of lipopolysaccharide and was the first TLR ligand approved for human use (Reed et al. 2009). It directly stimulates the immune system by activation of NF-κB, mitogen-activated protein kinases, APC maturation, and stimulation of Th1 responses (Blanco-Pérez et al. 2019; Pellegrino et al. 2015). MPLA is part of the HBV and HPV vaccines and also licensed in Europe for allergy treatment because of its ability to dampen Th2 responses (Kwissa et al. 2007; Reed et al. 2009).

Novel adjuvants for immunotherapies also include TLR9 ligands such as CpG as it was shown that CpG oligodeoxynucleotides (ODN) can shift Th2 to Th1 responses. Accordingly, CpG induces the secretion of the Th1 cytokines IL-1, IFN-α, TNF-α, and IL-12. Thus, a phase II study with a ragweed allergen conjugated to CpG (TLR9 ligand) demonstrated reduced symptoms of allergic rhinitis in patients during ragweed season (Marshall et al. 2001). Besides TLR ligands, other immunologically active substances are used as adjuvants. Those include cytokines, bacterial toxins, and glycolipids (Reed et al. 2009).

Moreover, allergoid preparations conjugated to non-oxidized mannan from *Saccharomyces cerevisiae* were shown to have promising immune-modulating properties (reviewed in Benito-Villalvilla et al. 2018). Allergoids, which are already used in the clinical practice, are allergen extracts that are chemically cross-linked with glutaraldehyde reacting with the primary amine groups of the allergen proteins to generate high molecular aggregates (Benito-Villalvilla et al. 2018). For the conjugation of non-oxidized mannan to the allergoids, glutaraldehyde can also act as a linker by covalently connecting both molecules via a glutaryl-diimine group (Benito-Villalvilla et al. 2018).

The mannan residues target the conjugated allergoids to CLRs expressed on mouse and human dendritic cells (DCs) where they mediate an enhanced uptake of the allergoids, increase DC expression of programmed death ligand 1, and induce an increased secretion of both IL-6 and IL-10 (Sirvent et al. 2016). The mannan-allergoid stimulated DCs were in turn shown to promote the induction of both allergen-specific IFN-γ-producing Th1- and IL-10-coproducing $CD4^+CD25^{high}CD127^-Foxp3^+$ regulatory T-cell (Treg) subsets capable of suppressing allergen-specific Th2 responses and increasing production of blocking allergen-specific IgG2a antibodies in mice (Sirvent et al. 2016). In addition, the mannan-allergoid conjugates were shown to be hypoallergenic due to reduced IgE binding, resulting in reduced mast cell and basophil activation from allergic donors (Sirvent et al. 2016). Dose-finding phase II clinical trials using either grass pollen or mite allergoids conjugated to mannan are currently ongoing (Benito-Villalvilla et al. 2018).

Well-known adjuvants such as Alum or MF59 are often combined with other components to further modulate the desired immune response. Several of these adjuvant systems (AS) have been evaluated for human use (Bonanni et al. 2011), whereas AS01 (malaria and tuberculosis) and AS15 (cancer immunotherapy) are tested in clinical trials, AS03 (Influenza) and AS04 (HBV, HPV) are already used in licensed vaccines (Bonanni et al. 2011). Over 30 currently licensed vaccines have an adjuvant component in their formulation (Bonanni et al. 2011).

Overall, the currently approved adjuvants available for use in humans include aluminum salts, oil in water emulsions (such as MF59, AS03, AF03, and MPLA), virus-like-particles, and virosomes (Pellegrino et al. 2015; Reed et al. 2009).

Adjuvant Allergen Fusion Proteins Have Distinct Advantages Compared to the Non-fused Mixture of Both Components

Pure protein antigens by themselves have a rather low immunogenicity (Petrovsky and Aguilar 2004). The immunogenicity of allergen molecules is further decreased if hypoallergenic molecules or allergen-derived peptide sequences are employed to prevent side effects (Rajakulendran et al. 2018). Since IgE-mediated activation of mast cells and basophils is caused by recognition of the three-dimensional structure of the allergen by allergen-specific IgE antibodies, these modifications of the allergens' three-dimensional structure can abrogate recognition of conformational IgE epitopes. To increase the immunogenicity of such vaccine antigens, the proteins/peptides are currently applied together with adjuvants. However, the co-administration of antigen and adjuvants can have undesired side effects such as local inflammation at the injection site, systemic toxicity of the adjuvant [e.g., observed for TLR7-ligands (Vidal and Alomar 2008)], and the induction to detrimental (antigen-specific) immune responses by bystander activation (Salazar-Gonzalez et al. 2007).

The next step to more efficiently leverage the immune-modulating properties of the applied adjuvants is to combine adjuvant and antigen into a single molecule. Compared to a non-conjugated mixture of allergen and adjuvant, such allergen:adjuvant fusion proteins have some key advantages (also summarized in Fig. 1): (1) they efficiently target the antigen to the relevant immune cells in vivo (usually APCs) by binding to receptors specific for the used adjuvant. (2) Once they have reached their target cells, such constructs simultaneously co-deliver both antigen and adjuvant to the same cell in a fixed molecular ratio, resulting in reproducible levels of immune system activation and a reduced risk of potentially detrimental bystander activation. (3) Binding of the adjuvant part of the allergen:adjuvant fusion protein was repeatedly shown to not only trigger immune cell activation but also uptake of the respective fusion proteins. Under these conditions, uptake of the fused antigen always takes place in the context of the adjuvant-mediated immune cell activation, resulting in both qualitative and quantitative differences in processing and presentation of the fused antigen (Khan et al. 2007; Schülke et al. 2014; Shirota et al. 2001).

Because of the resulting ability to administer lower doses of the respective fusion proteins, allergen:adjuvant

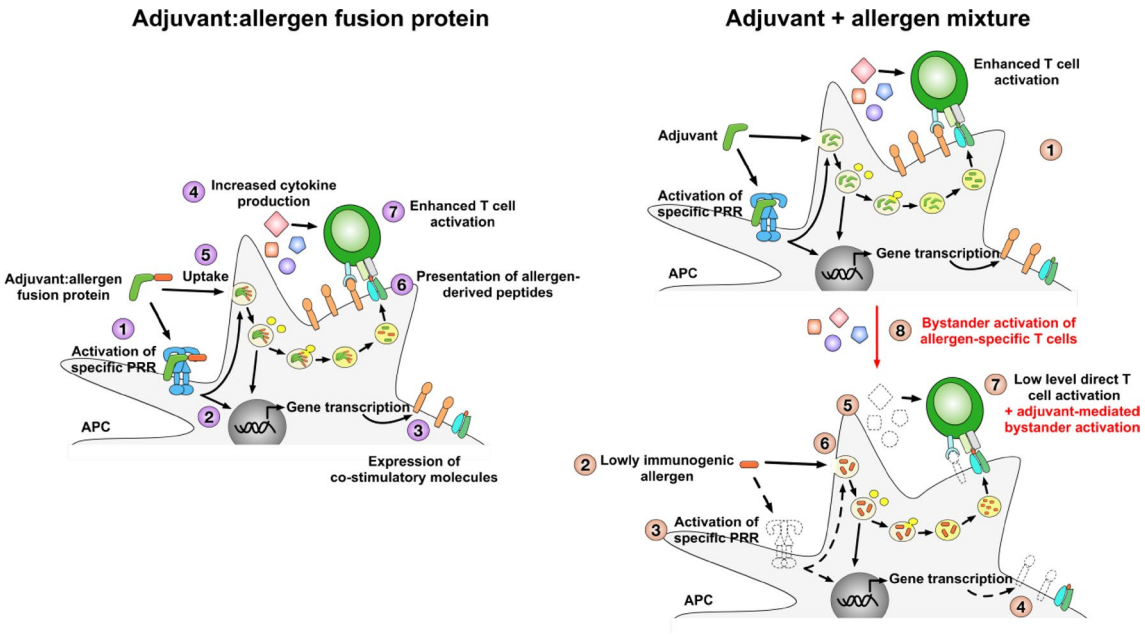

Fig. 1 Advantages of adjuvant:allergen fusion proteins over the non-fused mixture of both components. The application of adjuvant:allergen fusion proteins enables the stimulation of one target APC with both components in a fixed molecular ratio. Thereby, fusion proteins activate antigen-presenting cells by binding to pattern recognition receptors specifically recognizing the adjuvant part (violet 1), resulting in activating signal transduction via the PRR to the APCs nucleus (2), APC activation, increased pro-inflammatory gene transcription and expression of co-stimulatory molecules (3), as well as production of cytokines (4). Furthermore, adjuvant-mediated APC activation may also increase constitutive uptake of the fusion protein (5), resulting in subsequent processing and presentation of allergen-derived peptides via MHC-II molecules (6) in the context of the adjuvant-mediated APC activation. Taken together, these effects of the fusion protein may result in enhanced activation of allergen-specific T cells (7). In contrast to this, stimulation with the mixture of both single components does not ensure that both components are taken up by the same APC. Here, one APC may be stimulated by and take up the adjuvant, resulting in induction of adjuvant-specific immune responses (orange 1), while the low immunogenic allergen may be processed by a different APC (2). Since the allergen does not activate PRRs on the surface of the APC (3), it likely does not result in the expression of co-stimulatory molecules (4) and cytokines (5). The allergen is still taken up, processed, and presented via MHC-II (6), but, due to the lack of co-stimulatory molecules and activating cytokines, only low-level allergen-specific T-cell activation is observed (7). In addition, allergen-specific immune responses may also be induced by bystander activation where the necessary immune-activating cytokines are provided to the APC by a second APC that was activated by the adjuvant (8)

fusion proteins have the potential to increase both safety and efficacy compared to the mixture of both components. Accordingly, allergen:adjuvant fusion proteins were repeatedly shown to strongly increase the immunogenicity of the fused antigen, resulting in the efficient induction of protective immune responses against the fused antigen (Kastenmüller et al. 2011; Schülke et al. 2011; Song et al. 2015). Technically, such allergen:adjuvant fusion proteins are generated either by recombinant DNA technologies (if the fused adjuvant is a protein) or chemical conjugation using different chemical linker groups (e.g., for immune-stimulating CpG motifs and TLR4 ligands).

In the following part of the review, the published strategies employing allergen:adjuvant fusion proteins will be described grouped according to the used adjuvant classes (for an overview of all strategies, see Fig. 2 and Table 1).

Description of the Published Strategies

Immune-Activating Bacterial Components

TLR4-Ligand MPLA

In own studies, we described the generation and characterization of a fusion protein consisting of the detoxified TLR4-ligand MPLA and the model allergen ovalbumin (Ova) (Schülke et al. 2016). MPLA was chemically coupled to Ova using 1,1´-carbonyldiimidazole (CDI) as a linker resulting in the formation of a stable carbamate linkage between both components (Schülke et al. 2016). Biochemical analysis suggested a coupling rate of MPLA to Ova of 1:1 accompanied by a 22-fold increased hydrodynamic radius compared to non-conjugated Ova. This might lead to an aggregation of the fusion protein, possibly

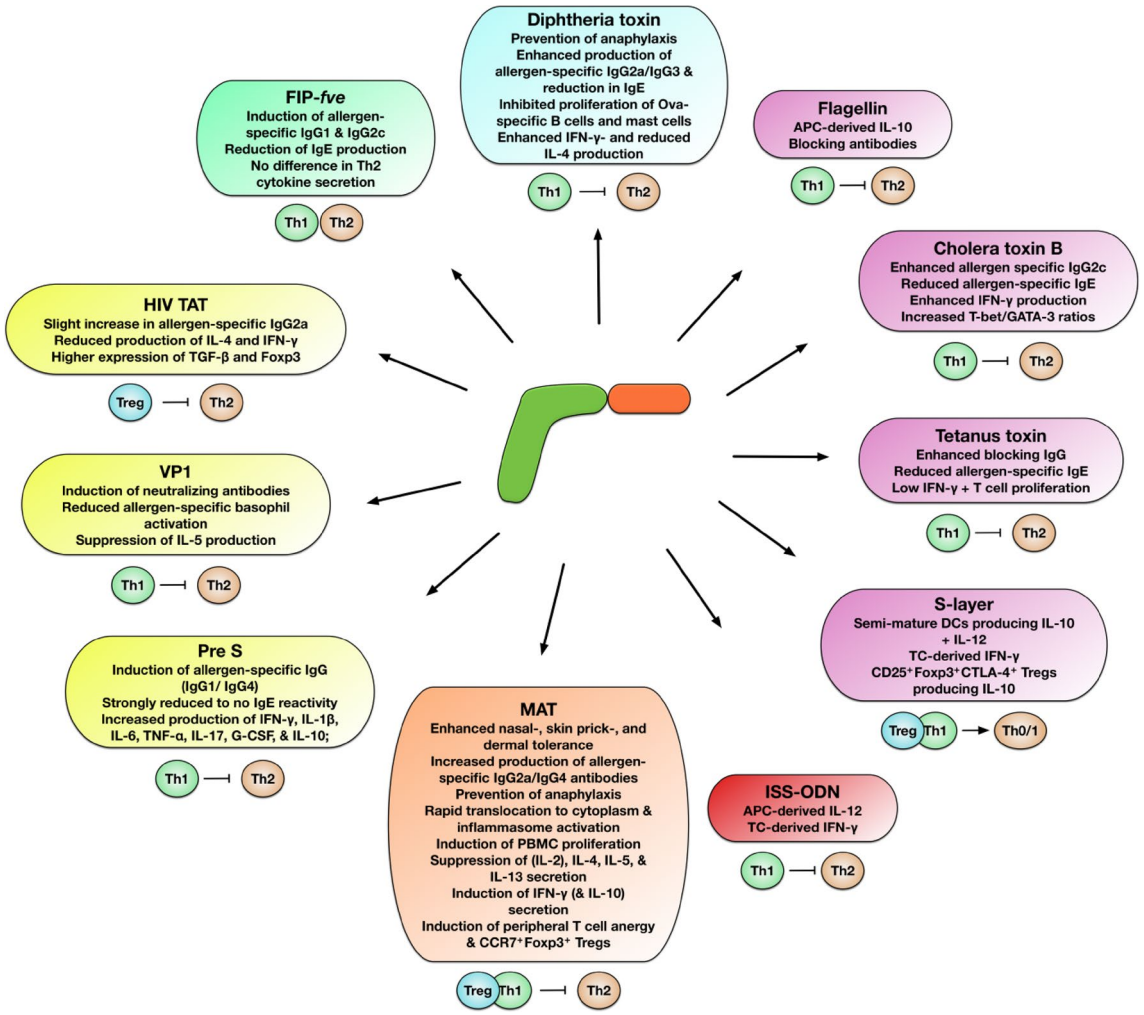

Fig. 2 Immunological effects of the described adjuvant:allergen fusion proteins. The immunological effects of the discussed adjuvant:allergen fusion proteins are summarized for the different adjuvant strategies (violet: bacterial proteins, red: immune-stimulatory ODNs, orange: forced delivery of allergens to the MHC-II loading pathway, yellow: viral proteins, green: fungal proteins, and blue: killing of IgE-bearing cells by fusion of the allergen to diphtheria toxin), whereas the overall types of immune responses induced are indicated below the respective boxes

mediated by the formation of micelle-like structures by the fatty acid chains of MPLA (Schülke et al. 2016).

When the immune-modulating properties of MPLA:Ova on innate and adaptive immune responses were investigated using an in vitro co-culture system of bone marrow-derived myeloid DCs (mDCs) and allergen-specific DO11.10 $CD4^+$ T cells, cytokine measurement showed the following results: MPLA:Ova boosted antigen-specific Th1 (IFN-γ), Th2 (IL-5 and IL-13), and Th17 (IL-17A) cytokine production as well as mDC-derived production of pro- (IL-1β, IL-6, and TNF-α) and anti-inflammatory (IL-10) cytokines compared to a significantly lower production of these cytokines when equimolar amounts of the non-fused mixture of MPLA + Ova were used (Schülke et al. 2016).

Since such strong DC and T-cell (TC) activation without a distinct bias towards a defined T-cell subset (e.g., towards Th1-biased immune responses) is likely detrimental causing inflammation-related side effects, the generated MPLA:Ova fusion protein was suggested not to be a suitable vaccine candidate for allergy treatment (but might hold potential for the treatment of other diseases, e.g., cancer requiring a strong stimulation of the host's immune system to overcome tumor tolerance).

TLR5-Ligand flagellin

Flagellin, a bacterial motility protein forming the body of the bacterial flagellum, activates both membrane-bound TLR5 (Hayashi et al. 2001) and cytoplasmic ICE protease-activating factor, also termed NLR family CARD-containing four protein (Franchi et al. 2006). Flagellin has repeatedly demonstrated its potential as mucosal adjuvant to mediate

Table 1 Summary of the published strategies to improve the treatment of type I allergies using allergen:adjuvant fusion proteins

Used adjuvant	References	Used allergen (source)	Investigated species	Immunological effects
Immune-activating bacterial components				
MPLA	Schülke et al. (2016)	Ova (hen's egg)	Mouse	Boosting of antigen-specific Th1, Th2, and Th17 cytokine production, as well as mDC-derived production of pro- and anti-inflammatory cytokines
Flagellin	Kitzmüller et al. (2018)	Bet v 1 (birch pollen)	Human/mouse	TLR5-dependent maturation of human monocyte-derived dendritic cells, reduced IgE-binding capacity, enhanced T-cell stimulatory capacity, increased production of allergen-specific IgG1, IgG2a, and IgE antibodies in mice
	Schülke et al. (2011)	Ova (hen's egg)	Mouse	Strong mDC activation, enhanced uptake into mDCs, significantly higher mDC-derived pro- and anti-inflammatory cytokine secretion, suppression of Th2 responses both in vitro and in vivo, increased metabolic activation of mDC, IL-10-dependent suppression of Th2 responses, mTOR-dependent IL-10 production and activation of mDC metabolism
	Schülke et al. (2014)	Ova (hen's egg)	Mouse	
	Schülke et al. (2018a)	Bet v 1 (birch pollen)	Mouse/human	
	Schülke et al. (2018b)	Wt & hypoallergen Art v 1 (mugwort pollen)	Mouse/human	
ISS–ODN	Tighe et al. (2000)	Amb a 1 (ambrosia pollen)	Mouse/rabbit/monkey	Induction of Amb a 1-specific IgG2a and suppression of IgE antibodies, IFN-γ-secretion from spleen cells
	Shirota et al. (2000)	Ova (hen's egg)	Mouse	Suppression of airway eosinophilia, Th2 cytokine secretion in BALF, and airway hyperresponsiveness
	Shirota et al. (2001)	Ova (hen's egg)	Mouse	Enhanced uptake of the CpG-conjugated antigen into the cytoplasm of DCs
	Marshall et al. (2001)	Amb a 1 (ambrosia pollen)	Human	IL-12-dependent suppression of Amb a 1-induced Th2 responses, induction of IFN-γ production and enhanced T-cell proliferation
	Santeliz et al. (2002)	Amb a 1 (ambrosia pollen)	Mouse	Reversion of allergen-induced airway hyperresponsiveness and suppressed eosinophilia in BALF, strong increase in IFN-γ production, significantly increased production of Amb a 1-specific IgG1 and IgG2a antibodies
S-layer	Jahn-Schmid et al. (1996)	Bet v 1 (birch pollen)	Human	Shift from Th2- to Th0/Th1-cytokine production in human allergen-specific Th2 lymphocytes upon immobilization of Bet v 1 on S-layer self-assembly products or cell wall preparations
	Bohle et al. (2004)	Bet v 1 (birch pollen)	Human	Retained ability of the fusion protein to self-assemble with correctly folded Bet v 1 conformation, but reduced capacity to induce histamine release from blood basophiles, increased IFN-γ- and IL-10-, but no detectable IL-5-secretion from PBMCs isolated from birch pollen-allergic individuals
	Gerstmayr et al. (2007)	Bet v 1 (birch pollen)	Human	Induction of IFN-γ and IL-10 production in Bet v 1-specific Th2-biased T-cell clones, retained capacity to activate mediator release from basophils, induction of semi-mature DCs from human monocyte-derived DC preparations (enhanced surface expression of CD40, CD80, CD83, and CD86, increased secretion of IL-10 and IL-12) inducing the IL-12- and IFN-γ-dependent differentiation of naive $CD4^+$ T cells into either IFN-γ-producing T cells coproducing IL-4 or IL-10-producing $CD25^+Foxp3^+CLTA\text{-}4^+$ regulatory T cells capable of active suppression
	Gerstmayr et al. (2009)	Bet v 1 (birch pollen)	Human	Retained ability to induce proliferation of Bet v 1-specific T-cell clones, significantly reduced IgE-binding and mediator-release capacity compared to Bet v 1 alone
	Anzengruber et al. (2017)	Ara h 2 (peanut)	Human/rabbit	Retained reactivity with IgE from peanut allergic patients, no mediator release from IgE-sensitized RBL cells at concentrations up to 100 ng/ml, induction of strong Ara h 2-specific IgG responses in rabbits (inhibition rates to natural Ara h 2 between 10 and 30%)

Table 1 (continued)

Used adjuvant	References	Used allergen (source)	Investigated species	Immunological effects
Cholera toxin B subunit	Bublin et al. (2007)	Bet v 1a (birch pollen)	Mouse	Reduction of Bet v 1-specific IgE production and basophil degranulation while enhancing IFN-γ and Bet v 1-specific IgG2 production upon challenge with birch pollen extract, higher T-bet/GATA-3 ratios in lungs
	Takagi et al. (2008)	Cry j 1 and Cry j 2 (cedar pollen)	Mouse	Reduced Cry J-specific IgE production, lower histamine release, and reduced sneezing in mice sensitized and challenged with cedar pollen extract
	Hoang et al. (2015)	Four major T-cell epitopes of Cry j 1 and six major T-cell epitopes from Cry j 2 (cedar pollen)	Human	Maintained reactivity of Cry j 1 and Cry j 2 T-cell epitopes with anti-Cry j 1 and anti-Cry j 2 antibodies from cedar pollen-allergic patients
Tetanus toxoid	Fanuel et al. (2018)	Der p 1 (house dust mite)	Mouse/human	Reduced rDer p 1-specific IgE, but enhanced rDer p 1 specific IgG production in mice, low IFN-γ production and reduced T-cell proliferation of PBMCs isolated from house dust mite-allergic patients
Immune-activating viral components				
Hepatitis PreS	Niespodziana et al. (2011)	Fel d 1 (cat)	Mouse/rabbit/human	No IgE reactivity and 1000-fold reduced capacity to activate basophils, induction of Fel d 1-specific IgG in immunized mice and rabbits inhibiting the binding of allergic patients' IgE to unmodified Fel d 1
	Marth et al. (2013)	Bet v 1 (birch pollen)	Human/rabbit	No IgE reactivity and basophil activation, reduced T-cell activation, reduced IL-5 secretion, increased IL-10 and IFN-γ production of PBMC-derived T cells from allergic patients, high level of IgG production in fusion protein-immunized rabbits inhibiting patient IgE binding to the unmodified allergen
	Focke-Tejkl et al. (2015)	Peptides of Phl p 1, Phl p 2, Phl p 5, and Phl p 6 (grass pollen, BM32)	Human/mouse	Strongly reduced allergenic activity, significantly reduced pro-inflammatory cytokine secretion and T-cell proliferation of PBMCs isolated from grass pollen-allergic patients, induction of allergen-specific IgG antibodies in mice inhibiting the binding of patients' IgE to all four major grass pollen allergens
	Cornelius et al. (2016)	Peptides of Phl p 1, Phl p 2, Phl p 5, and Phl p 6 (grass pollen, BM32)	Human	Induction of PreS-specific IgG antibodies, inhibiting HBV infection in vitro
	Zieglmayer et al. (2016)	Peptides of Phl p 1, Phl p 2, Phl p 5, and Phl p 6 (grass pollen, BM32)	Human	Significant decrease in total nasal symptom score, dose-dependent decrease in total ocular symptom score and skin prick test without systemic immediate type side effects, significantly increased production of allergen-specific IgG responses without allergen-specific IgE production, reduced T-cell activation and allergen-induced basophil activation
	Niederberger et al. (2018)	Peptides of Phl p 1, Phl p 2, Phl p 5, and Phl p 6 (grass pollen, BM32)	Human	Clinical improvements regarding symptom medication, visual analog scale, rhinoconjunctivitis quality-of-life questionnaire, and asthma symptom scores without reaching the reaching the primary end point, induction of allergen-specific IgG, no induction of allergen-specific IgE
Rhinovirus viral protein 1	Edlmayr et al. (2009)	Phl p 1 (grass pollen)	Human	Increased immunogenicity of the Phl p 1 peptides, reduction in allergenicity of the fusion protein in basophils isolated from human grass pollen-allergic patients, induction of allergen-specific Th1-responses and suppression of IL-5 production from splenocyte cultures;

Table 1 (continued)

Used adjuvant	References	Used allergen (source)	Investigated species	Immunological effects
HIV-TAT	Salari et al. (2017)	Che a 3 (goosefoot pollen)	Mouse	Retained immunogenicity, no differences in Che a 3-specific IgG and IgE- and only slightly higher IgG2a levels in rTAT-Che a 3-immunized mice, lower IL-4- and IFN-γ-levels in re-stimulated splenocytes from rTAT-Che a 3-immunized mice, significantly higher TGF-β-mRNA and protein expression, higher Foxp3-mRNAs expression
Immune-activating fungal components				
Fungal immunomodulatory protein *five*	Su et al. (2012)	Der p 2 (house dust mite)	Mouse	Significantly increased Der p 2-specific IgG2c- and earlier IgG1-production upon subcutaneous immunization, reduction of Der p 2-specific IgE production
Targeting of allergens to the MHC-II loading pathways using the MAT				
MAT	Crameri et al. 2007	Asp f 1 (aspergillus) Bet v 1 (birch pollen) Phospholipase A2 (bee venom) Der p 1 (house dust mite) Fel d 1 (cat)	Human	Rapid translocation of allergen:MAT constructs into the cytoplasm of PBMCs inducing PBMC proliferation, decreased secretion of IL-4-, IL-5-, and IL-2 secretion as well as increased secretion of IFN-γ and IL-10 in PBMC cultures from allergic donors
	Martinez Gomez et al. (2009)	Fel d 1 (cat)	Mouse	Increased production of Fel d 1-specific IgG2a antibodies upon intralymphatic immunization, significantly increased production of IL-2 and IFN-γ and lower IL-4 and IL-10 production from splenocyte cultures, no anaphylaxis upon in vivo application of the fusion protein and efficient prevention of cat fur extract-induced anaphylaxis in sensitized mice, reduced capacity to induce degranulation of basophils isolated from cat-allergic patients
	Senti et al. (2012)	Bet v 1 (birch pollen)	Human	No adverse events, significantly increased nasal tolerance, significant increases in skin prick- and dermal tolerance compared to placebo controls, increased cat dander-specific IgG4 production, no significant differences in cat dander-specific IgE levels, increased IL-10 production from PBMCs from MAT-Fel d 1-treated patients
	Zaleska et al. (2014)	Bet v 1 (birch pollen)	Human	Efficient internalization of MAT-Fel d 1 and aggregate formation in monocytes, T cells, B cells, and NK cells in vitro, increased cleavage of pro-caspase-1 into caspase-1 and rapid IL-1β secretion, higher IFN-γ and IL-10 and lower IL-5 and IL-13 secretion from PBMCs of cat-allergic patients, increased IL-10 levels and full peripheral T-cell unresponsiveness upon intralymphatic immunization in a phase I/IIA clinical trial, reduced numbers of Fel d 1-tetramer-specific T cells in the circulation of MAT-Fel d 1-treated patients, increased $CCR7^{+}Foxp3^{+}$ Treg cells, significantly increased HR2/HR1 ratios
Killing of immune cells expressing allergen-specific IgE molecules by fusion of the allergen to diphtheria toxin				
Diphtheria toxin	Lee et al. (2001)	Ova (hen's egg)	Mouse	Prevention of anaphylaxis inhibited proliferation of Ova-specific B cells and mast cells, enhanced Ova-specific IgG2a production, production of IFN-γ, and suppression of IL-4 secretion from splenocyte cultures
	Lee et al. (2004)	Ova (hen's egg)	Mouse	Demonstration of the immune-modulatory effects to be antigen-specific, increased production of Ova-specific IgG2a/IgG3 (likely preventing anaphylaxis) & decreased production of Ova-specific IgE antibodies

RBL rat basophil leukemia, *GATA-3* GATA-binding protein 3, *T-bet* T-box-containing protein expressed in T cells, *TGF-β* transforming growth factor beta

protective immunity (Honko et al. 2006; Huleatt et al. 2007; Lee et al. 2006), inducing strong Th1-biased pro-inflammatory immune responses (Vijay-Kumar et al. 2008).

One major advantage of flagellin is its protein nature allowing for the generation of fusion proteins of flagellin and antigen using recombinant DNA technologies. Such fusion proteins enable the efficient targeting of antigens to and simultaneous activation of $TLR5^+$ APCs (Huleatt et al. 2007).

Kitzmüller et al. (2018) recently described the enhanced immunogenicity, reduced allergenicity, and intrinsic adjuvanticity of flagellin C:Bet v 1 fusion proteins in human monocyte-derived DCs and T cells from allergic patients. They fused a truncated version of the *Salmonella* flagellin C to either the N- or C-terminus of the major birch pollen allergen Bet v 1 (Kitzmüller et al. 2018). In immunological tests, both fusion proteins efficiently matured human monocyte-derived DCs (upregulation of CD80, CD83, and CD86) in a TLR5-dependent manner while showing reduced IgE-binding capacity (Kitzmüller et al. 2018). Moreover, both fusion proteins displayed an enhanced T-cell stimulatory capacity, resulting in retained T-cell proliferation and increased secretion of IL-13 and IFN-γ compared to Bet v 1 alone (Kitzmüller et al. 2018).

In vivo application of the fusion proteins to mice resulted in the production of allergen-specific IgG1, IgG2a, and IgE antibodies (Kitzmüller et al. 2018). Only antibodies induced by vaccination with the fusion protein in which flagellin was fused to the C-terminus of Bet v 1 were able to inhibit the binding of patients IgE to Bet v 1, suggesting that the sequential order of allergen and adjuvant contributes to the fusion proteins immunological characteristics (Kitzmüller et al. 2018).

In own studies, we evaluated the immune-modulating properties of fusion proteins consisting of the TLR5 ligand rFlaA from *Listeria monocytogenes* and either Ova from hen's egg as a model allergen (FlaA:Ova) (Schülke et al. 2010, 2011, 2014), the major mugwort pollen allergen Art v 1 (rFlaA:Artv1) both as a hypoallergenic variant and as the unmodified parent molecule (Schülke et al. 2018b), or the major birch pollen allergen Bet v 1 (rFlaA:Betv1) (Schülke et al. 2018a).

Compared to the non-fused mixture of both components, mouse bone marrow-derived mDCs were strongly activated (upregulation of CD40, CD69, CD80, CD86, B7-DC, and B7-H1), displayed highly increased surface levels of the target receptor TLR5, and secreted both pro- (IL-1β, IL-6, TNF-α) and anti-inflammatory (IL-10) cytokines when stimulated with these fusion proteins (Schülke et al. 2011, 2018a, 2018b). Upon co-culture with either Ova-, Art v 1-, or Bet v 1-specific $CD4^+$ T cells, the fusion protein-activated DCs efficiently suppressed both allergen-induced Th1 and Th2 cytokine secretion in vitro (Schülke et al. 2011, 2018a, 2018b) in an IL-10-dependent manner, since the suppression of T-cell-derived cytokine secretion could be reversed using either IL-10-neutralizing antibodies or IL-10-deficient mDC as APCs for the co-cultures (Schülke et al. 2011). In addition, rFlaA:Artv1 and rFlaA:Betv1 were shown to induce similar patterns of activation in human monocyte-derived DCs (Schülke et al. 2018b) and peripheral blood mononuclear cells (PBMCs) from birch pollen-allergic patients (Schülke et al. 2018a), respectively.

Mechanistically, mDCs stimulated with either rFlaA:Ova or rFlaA:Betv1 were shown to more efficiently take up the respective fusion proteins compared to the mixture of both components. This stronger uptake was accompanied by an increased resistance to microsomal digestion (Schülke et al. 2018a). Analysis of the contributing signaling pathways in stimulated mDCs revealed that stimulation with either rFlaA:Betv1 (Schülke et al. 2018a) or rFlaA:Artv1 (Schülke et al. 2018b) resulted in an increased metabolic activity of the stimulated mDCs characterized by a high rate of glycolysis followed by lactic acid fermentation, known as the Warburg effect (Finlay and Cantrell 2011).

This activation of mDC metabolism was shown to depend on activation of the mammalian target of rapamycin (mTOR) pathway, since pretreatment of the mDCs with the specific inhibitor of mTOR dose-dependently suppressed both the activation of the Warburg effect, mDC metabolism, and the induction of anti-inflammatory IL-10 secretion by rFlaA:Betv1 (Schülke et al. 2018a). In contrast to this, pro-inflammatory cytokine secretion (IL-1β and IL-6) was unaffected by mTOR inhibition (Schülke et al. 2018a). Interestingly, these findings show that immune-modulatory IL-10 secretion and, therefore, the DC-tolerizing capacity of the tested flagellin A-containing allergen fusion proteins were linked to the activation of mDC metabolism.

In vivo vaccination with the different flagellin fusion proteins (but not the corresponding mixture of both single proteins) efficiently protected mice from Ova-induced gastrointestinal allergy Schülke et al. (2011, 2014), prevented allergic sensitization to Bet v 1 (Schülke et al. 2018a), and suppressed the induction of Art v 1-specific Th2 responses (Schülke et al. 2018b). Immunologically, the protective effect of rFlaA:Ova was associated with a reduced production of Th2 cytokines in intestinal homogenates, the suppression of both systemic T-cell immune responses and Ova-specific IgE production, as well as an induction of Ova-specific IgG2a antibodies (Schülke et al. 2011).

In summary, the available studies using the TLR5-ligand flagellin as a fusion partner suggest that flagellin:allergen fusion proteins have a potent immune-modulatory capacity, resulting in the induction of immunosuppressive IL-10 secretion and the production of blocking IgG antibody subclasses.

Immunostimulatory Oligodeoxynucleotide TLR9 ligands

Immunostimulatory oligodeoxynucleotides (ISS-ODN) containing TLR9-activating unmethylated CpG motifs are a common component in bacterial but not in mammalian DNA (Shirota et al. 2000). These ISS-ODNs have the potential to induce strongly Th1-biased immune responses by causing macrophages and DCs to produce Th1-promoting cytokines such as IFNs, IL-6, IL-12, and IL-18 while also driving the surface expression of co-stimulatory molecules (Chu et al. 1997; Klinman et al. 1999; Marshall et al. 2001; Roman et al. 1997; Sun et al. 1998; Tighe et al. 2000). Moreover, CpG ODNs also have the capacity to directly activate B-cell proliferation and immunoglobulin production (Hartmann and Krieg 2000; Krieg et al. 1995; Liang et al. 1996; Liang and Lipsky 2000; Santeliz et al. 2002). Due to their potent immune-activating potential, CpG ODN application can also result in adverse effects in vivo such as increases in serum TNF-α levels and mortality (Cowdery et al. 1996; Sarmiento et al. 1994; Sparwasser et al. 1997a, 1997b). Therefore, their usage as adjuvants so far has been limited.

Several studies have been testing the potential of such CpG ODNs conjugated to allergen to either prevent or reverse allergic responses. Tighe et al. (2000) described the coupling of a 22-mer ISS-ODN to the major ragweed allergen Amb a 1. This ISS conjugate was characterized as a mixture of ten bands with apparent molecular weights between 50 and 120 kDa (Tighe et al. 2000). The average coupling rate was four molecules of ISS-ODN per molecule of Amb a 1 (Tighe et al. 2000). Intradermal immunization of BALB/c mice with the Amb a 1-ISS conjugate resulted in high levels of IgG2a antibodies and strongly IFN-γ-secreting spleen cells, suggesting the induction of Th1-biased immune responses, while non-conjugated Amb a 1 induced both IgG1 and IgE production and IL-5 secretion from T cells indicative of a Th2 response (Tighe et al. 2000). Co-injection of Amb a 1 mixed with ISS-ODN also induced detectable but much weaker Th1 responses in mice, while controls using an Amb a 1-non-ISS conjugate did not have comparable immune-modulating effects (Tighe et al. 2000).

Moreover, in a therapeutic approach, injection with the Amb a 1-ISS conjugate both induced de novo Th1 responses in Amb a 1-sensitized mice and suppressed IgE antibody formation after challenge with Amb a 1 (Tighe et al. 2000). In addition, the Amb a 1-ISS conjugate was shown to be less allergenic, since approx. 30-fold higher concentrations of conjugate were necessary to induce levels of histamine release from human basophils of patients with ragweed allergy that were comparable to the ones induced by Amb a 1 (Tighe et al. 2000). The increased immunogenicity of the Amb a 1-ISS conjugate also extended to both rabbits and cynomolgus monkeys where the conjugate, but not Amb a 1 alone or admixed with ISS-ODN, induced significantly enhanced IgG antibody responses (Tighe et al. 2000).

In a very similar approach, Marshal and coauthors reported ISS–ODN 1018 conjugated to Amb a 1 (coupling rate 3–5 molecules of ISS–ODN per molecule of Amb a 1) to suppress Amb a 1-induced Th2 responses while inducing IFN-γ production and enhancing T-cell proliferation in PBMC preparations from ragweed allergic patients (Marshall et al. 2001). Here again, the simple mixture of ISS-ODN with Amb a 1 did not have the capacity to suppress Amb a 1-specific Th2 responses (Marshall et al. 2001). In line with the preferential induction of Th1-responses by the ISS–ODN:Amb a 1 conjugate, analysis of cytokine mRNA expression from PBMCs isolated from ragweed allergic patients stimulated with the ISS–ODN:Amb a 1 conjugate showed a substantially lower expression of IL-1α-, IL-4-, IL-5-, and IL-8 mRNA, but enhanced levels of IL-2-, IL-15-, and IFN-γ mRNAs compared to Amb a 1 stimulation (Marshall et al. 2001).

Using an IL-12 neutralizing monoclonal antibody, the IFN-γ production induced by the ISS–ODN:Amb a 1 conjugate was shown to be dependent on IL-12 (Marshall et al. 2001). Interestingly, the reduction of Amb a 1-specific IL-4 and IL-5 production by the ISS–ODN:Amb a 1 conjugate was unchanged in the presence of anti-IL-12 (Marshall et al. 2001).

Santeliz et al. (2002) tested whether intradermal delivery of an Amb a 1-ISS conjugate had the potential to reverse established asthmatic airway responses in mice. In Amb a 1-sensitized and ragweed extract challenged mice, intradermal treatment with the Amb a 1-ISS conjugate significantly reversed allergen-induced airway hyperresponsiveness and suppressed eosinophilia in bronchoalveolar lavage fluid (BALF) upon intratracheal challenge with ragweed extract, paralleled by a strong increase in IFN-γ production from Amb a 1-stimulated splenocytes and significantly increased production of both Amb a 1-specific IgG1 and IgG2a antibodies (Santeliz et al. 2002). This shift towards Th1-biased immune responses was neither observed in animals treated with Amb a 1 alone nor an Amb a 1-non-ISS control (Santeliz et al. 2002). Interestingly, the inhibition of allergen-driven airway hyperresponsiveness by the Amb a 1-ISS conjugate did occur in the absence of any effect of the Amb a 1-ISS conjugate on Amb a 1-specific IL-5 production (Santeliz et al. 2002).

Shirota et al. (2000) described immune-stimulating CpG motifs conjugated to the model allergen Ova (coupling rate: 8.3 molecules CpG to 1 molecule of Ova) to inhibit airway eosinophilia 50- to 100-fold more efficiently than co-administration of a non-conjugated mixture of CpG and Ova. The suppression of airway eosinophilia, Th2 cytokine secretion in BALF, and airway hyperresponsiveness was paralleled by reduced Th2 responses from Ova-specific T cells in the

draining lymph nodes (suppression of IL-4 and IL-5 secretion but no detectable IFN-γ secretion) and a significantly increased induction of IL-12-dependent Th1 responses in vitro (Shirota et al. 2000). The authors suggested the CpG-Ova conjugate to be taken up, processed, and presented by APCs in the context of the CpG-mediated APC activation (Shirota et al. 2000). Here, the resulting IL-12 production drives both Ova-specific Th1 differentiation and suppression of Ova-specific Th2 responses.

In further studies, Shirota et al. (2001) were able to show that stimulation of spleen cells from Ova T-cell receptor transgenic mice with the CpG-Ova conjugate, but not Ova or CpG alone or as a non-conjugated mixture, resulted in strongly increased IFN-γ secretion, while IL-4 secretion was low. In their system, the enhanced Th1-promoting capacity of the CpG-Ova conjugate was not caused by an observed polymerization of Ova, but due to an enhanced uptake of the CpG-conjugated antigen into the cytoplasm of DCs (Shirota et al. 2001). Using fluorescence labeling, Shirota et al. (2001) reported the conjugation of CpG to Ova to increase uptake of the conjugated antigen by approx. 100-fold compared to the non-conjugated mixture. Moreover, murine DCs stimulated with the CpG-Ova conjugate were also observed to express increased levels of co-stimulatory molecules (CD40, CD86, and MHC-II) and secrete the Th1-promoting cytokine IL-12 (Shirota et al. 2001). These results suggest the enhanced Th1-promoting capacity of the investigated CpG-Ova conjugate to be caused by a CpG-guided increase in antigen uptake into DCs and the subsequent presentation of antigen-derived peptides in the context of CpG-mediated APC activation (Shirota et al. 2001).

In summary, the different studies investigating immune-stimulating CpG motifs conjugated to either Amb a 1 or Ova consistently report the induction of Th1-biased immune responses characterized by APC-derived IL-12 secretion as well as high levels of TC-derived IFN-γ, resulting in the suppression of allergen-specific Th2 responses.

S-Layer

Bacterial surface (S-)layer proteins are promising antigen carriers in vaccine development. S-layers are regularly structured, two-dimensional crystalline arrays of (glyco) protein subunits that make up the outermost layer of many bacteria and archaea (Anzengruber et al. 2017; Bohle et al. 2004; Sára and Sleytr 2000; Sleytr et al. 1999, 2007b, 2014). Accordingly, in vitro, isolated S-layer proteins self-assemble into either flat mono- or double-layer sheets, open-ended cylinders, or closed vesicles with particle-like dimensions of 0.5–2 µm (Breitwieser et al. 2002; Sleytr et al. 2007a). Their immunogenicity can be attributed to their highly organized repetitive structure (Bohle et al. 2004), their potential to act as B-cell epitopes (Anzengruber et al. 2017), as well as their dual role as both carrier and adjuvant (Ilk et al. 2011).

Early work by Jahn-Schmid et al. (1996) suggested that bacterial S-layers/S-layer proteins may be suitable carriers/adjuvants to induce Th1-biased allergen-specific immune responses. They demonstrated that immobilization of the major birch pollen allergen Bet v 1 on either native or cross-linked S-layer self-assembly products or cell wall preparations obtained from *Bacillus sphaericus* CCM 2177 and *Thermoanaerobacter thermohydrosulfuricus* L111-69 and L110-69 resulted in the shift from Th2- to Th0/Th1-cytokine patterns in human allergen-specific Th2 lymphocytes (Jahn-Schmid et al. 1996).

Based on this work, Bohle et al. (2004) reported the generation and immunological characterization of a fusion protein consisting of the C-terminus of the truncated S-layer protein of *Geobacillus stearothermophilus* and the major birch pollen allergen Bet v 1 (rSbsC-Bet v 1). rSbsC-Bet v 1 was shown to retain its ability to self-assemble and contain Bet v 1 in a correctly folded conformation including all relevant Bet v 1-specific B- and T-cell epitopes, but displayed a significantly reduced capacity to induce histamine release from blood basophiles (Bohle et al. 2004).

When PBMCs from birch pollen-allergic individuals were stimulated with rSbsC-Bet v 1, the fusion protein induced both IFN-γ and IL-10 secretion, but no detectable IL-5 production (Bohle et al. 2004). Further analysis demonstrated rSbsC-Bet v 1 to induce the production of Th1-promoting IFN-γ and immunosuppressive IL-10 in both Bet v 1-specific T-cell lines and Th2-cell clones (Bohle et al. 2004). Similar but less pronounced effects were obtained for cultures stimulated with the non-fused rSbsC (Bohle et al. 2004). Therefore, rSbsC-Bet v 1 was shown to favor the differentiation of allergen-specific T cells into Th1/Th0-like effector cells (Bohle et al. 2004).

Further work on rSbsC-Bet v 1 was published by Gerstmayr et al. (2007). The rSbsC-Bet v 1 fusion protein was shown to preserve Bet v 1-T-cell epitopes, and the potency to induce IFN-γ and IL-10 production in Bet v 1-specific Th2-biased T-cell clones while displaying the capacity to activate basophils in mediator-release assays (Gerstmayr et al. 2007). Human monocyte-derived DC preparations stimulated with SbsC-Bet v 1 were shown to have a semi-mature phenotype characterized by enhanced surface expression of CD40, CD80, CD83, and CD86 (compared to stimulation with Bet v 1 alone) and a strongly increased secretion of the DC-derived cytokines IL-10 and IL-12 (Gerstmayr et al. 2007). Functionally, DCs matured with SbsC-Bet v 1 induced the IL-12- and IFN-γ-dependent differentiation of naive $CD4^+$ T cells into IFN-γ-producing T cells coproducing IL-4, suggesting a Th0 phenotype (Gerstmayr et al. 2007). Naive T cells stimulated by these DCs differentiated into IL-10-producing $CD25^+Foxp3^+CTLA\text{-}4^+$ regulatory

T cells capable of active suppression, thus promoting the simultaneous differentiation of Th0/Th1 cells and regulatory T cells (Gerstmayr et al. 2007). Preliminary studies of the authors suggested that rSbsC-Bet v 1 may mediate its effects via an activation of the innate immune receptor TLR2 (Gerstmayr et al. 2007). Indeed, TLR2 ligands have been reported to promote Treg differentiation by induction of IL-10-producing tolerogenic DCs (Gerstmayr et al. 2007; Hoarau et al. 2006; Netea et al. 2004).

In a follow-up study, Gerstmayr et al. (2009) compared the IgE-binding and mediator-release capacities of non-assembled and self-assembled variants of a fusion protein consisting of a C-terminally truncated form of the S-layer protein SbpA from *Bacillus sphaericus* CCM 2177 and Bet v 1 (rSbpA-Bet v 1) using sera from birch pollen-allergic patients. Both rSbpA-Bet v 1 variants retained the ability to induce proliferation of Bet v 1-specific T-cell clones and were characterized by a significantly reduced IgE-binding and mediator-release capacity compared to Bet v 1 alone (Gerstmayr et al. 2009). However, if the ability of SbpA to self-assemble was retained, allergenicity was further reduced and the T-cell stimulatory capacity of monocytes and DCs was further increased (Gerstmayr et al. 2009). Interestingly, Ilk and coauthors were able to show that such S-layer:allergen fusion proteins can also be expressed and purified using the Gram-positive *B. subtilis* expression system, thereby eliminating the need for laborious and cost-intensive endotoxin removal (Ilk et al. 2011).

Finally, Anzengruber et al. (2017) described the immune-modulating properties of a fusion protein consisting of the S-layer protein SlpB of *Lactobacillus buchneri* CD034 and a peptide derived from the major peanut allergen Ara h 2 (SlpB-AH3a42). The used Ara h 2 peptide AH3a42 consisted of formerly identified immunodominant B-cell epitopes as well as one T-cell epitope (Anzengruber et al. 2017). While the SlpB-AH3a42 fusion protein was still recognized by IgE in sera from peanut allergic patients, it did not induce mediator release from IgE-sensitized rat basophil leukemia cells at concentrations up to 100 ng/ml (Anzengruber et al. 2017). Moreover, immunization of rabbits with either SlpB-AH3a42 or natural Ara h 2 resulted in the induction of strong Ara h 2-specific IgG responses. Ara h 2-specific IgG levels induced with SlpB-AH3a42 were shown to be approximately 60% lower than the responses induced with natural Ara h 2 (Anzengruber et al. 2017). When sera from SlpB-AH3a42-immunized rabbits were used to inhibit IgE-binding to the natural allergen, inhibition rates were between 10 and 30%, suggesting that the SlpB-AH3a42 fusion protein was able to induce blocking antibodies interfering with binding of IgE to the antigen (Anzengruber et al. 2017).

In summary, the available studies using different S-layer proteins as immune-modulating components in fusion proteins show that these bacterial proteins have strong adjuvant capacities resulting in the suppression of allergen-specific Th2 responses while inducing both Treg and Th1 responses both in vitro and in vivo.

Cholera Toxin B Subunit

Cholera toxin (CT) is the main virulence factor of *Vibrio cholerae,* a Gram-negative bacterium that can cause cholera, an acute life-threatening dehydrating diarrhea (Baldauf et al. 2015). CT is an 84 kDa protein that consists of a catalytic A-subunit (CTA) and a non-toxic homopentameric B subunit (CTB) (Lönnroth and Holmgren 1973; Sánchez and Holmgren 2008; Zhang et al. 1995). CTB is both able to bind to GM1 ganglioside receptors that mediate the entry of the toxin into the cell and induce strong immune responses without the toxicity associated with the CTA subunit (Baldauf et al. 2015; Holmgren et al. 1994; Sanchez and Holmgren 2011). In line with this, several studies have shown the strong adjuvant effect of CTB on co-applied antigens mainly when it is administered intranasally (Blanchard et al. 1998; Sánchez and Holmgren 2008). CTB has been used as experimental adjuvant in allergy and diabetes treatment, as well as for the prevention of bacterial and viral infections (Czerkinsky et al. 1996; Guo et al. 2014; Tinker et al. 2014).

Different studies have conjugated CTB to allergens to facilitate both the delivery and presentation of the conjugated allergen in the mucosa. Bublin et al. (2007) generated a fusion protein of the major birch pollen allergen Bet v 1a and CTB (rCTB-Bet v 1a). The rCTB-Bet v 1a protein was produced in *E. coli* and purified using a $[His]_6$-tag under denaturing conditions (Bublin et al. 2007). Intranasal treatment with the fusion protein in Bet v 1-immunized mice resulted in a reduction of Bet v 1-specific IgE production and basophil degranulation while enhancing IFN-γ and Bet v 1-specific IgG2 production upon challenge with birch pollen extract (Bublin et al. 2007). Pretreatment with the fusion protein induced a higher T-box-containing protein expressed in T cells/GATA-binding protein 3 ratio in lungs compared with either rBet v 1 or CTB alone, suggesting the fusion protein to shift immune responses towards more Th1-biased responses (Bublin et al. 2007).

Two other studies generated fusion proteins of CTB with Cry j 1 and Cry j 2, the major Japanese cedar pollen allergens, one of the most common IgE-mediated allergies in Japan (Sugimura et al. 1996). Takagi et al. (2008) generated two fusion proteins using two dominant T-cell epitopes and one subdominant T-cell epitope, linked together and named 3Crp. 3Crp was then expressed in rice seed as a fusion protein with either CTB (CTB-3Crp) or a rice gluten subacidic unit (GLU-3Crp). In a mouse model fed with either CTB-3Crp or GLU-3Crp, a 50-fold reduction in the dose necessary to generate tolerance by CTB-3Crp was observed in comparison with GLU-3Crp, paralleled by a reduced

C3rp-specific IgE production, lower histamine release, and reduced sneezing in mice sensitized and challenged with cedar pollen extract (Takagi et al. 2008).

In another study, Hoang et al. (2015) fused four major T-cell epitopes of Cry j 1 and six major T-cell epitopes from Cry j 2 with CTB and expressed the resulting fusion protein in *E. coli*. Despite the fusion to CTB, the Cry j 1 and Cry j 2 T-cell epitopes maintained their antigenicity as determined by reactivity of the fusion protein with anti-Cry j 1 and anti-Cry j 2 antibodies from cedar pollen-allergic patients (Hoang et al. 2015). Hoang et al. (2015) focused on the expression and purification of the fusion protein without describing immunological effects for this fusion protein.

Collectively, these studies show that CTB-allergen-fusion proteins have the potential to induce potent allergen-specific immune responses and to modify the induced immunological response towards a Th1 type in comparison to the non-fused mixture of allergen and CTB.

Tetanus Toxoid

A recent study investigated the adjuvant effect of tetanus toxoid (TT) upon fusion to the major house dust mite (HDM) allergen *Dermatophagoides pteronyssinus* group 1 (Der p 1) (Fanuel et al. 2018). Using a bioinformatic approach, a hypoallergenic region of Der p 1, lacking T-cell epitopes was fused with a partial (155 amino acid) fragment of TT, expressed in *E. coli*, purified using $[His]_6$-tag, and subsequently investigated for its application as a B-cell epitope vaccine for immunotherapy (Fanuel et al. 2018). In accordance with the other adjuvant:allergen fusion proteins, mice immunized with the fusion protein showed a reduced level of rDer p 1 specific IgE, but enhanced levels of blocking rDer p 1 specific IgG antibodies, suggesting the ability of the antibodies generated by vaccination with the fusion protein to inhibit the binding of allergens to specific IgE antibodies (Fanuel et al. 2018). The lack of T-cell epitopes in the Der p 1-TT fusion protein was confirmed by a low IFN-γ production from and low T-cell proliferation of PBMCs from HDM-allergic patients (Fanuel et al. 2018). This lack of T-cell reactivity was hypothesized to reduce possible side effects during the immunotherapy (Fanuel et al. 2018).

Immune-Activating Viral Components

PreS

PreS is a T-cell epitope derived from the HBV, which efficiently triggers T-cell responses against itself (Niespodziana et al. 2011). By fusing PreS to allergen-derived peptide sequences, PreS was hypothesized to modulate immune responses towards neutralizing allergen-specific immune responses without the risk of triggering allergic reactions.

So far, PreS-based fusion proteins have been described incorporating different peptides from the cat allergen Fel d 1 (PreS-2xP1, PreS2xP5, PreSP1-P5) (Niespodziana et al. 2011), the major birch allergen Bet v 1 (2PA-PreS, 2PB-PreS, 4PA-PreS, 2PAPB-PreS) (Marth et al. 2013), and different grass pollen allergens (BM32 including Phl p 6-B, Phl p 5-D, Phl p 2-B, Phl p 1-B) (Cornelius et al. 2016; Focke-Tejkl et al. 2015; Niederberger et al. 2018; Zieglmayer et al. 2016).

Immunologically, these PreS–peptide fusion proteins were shown to induce robust IgG responses, especially of the IgG1 and IgG4 subtype, while either completely lacking allergen-specific IgE reactivity or having only very low residual IgE reactivity (Focke-Tejkl et al. 2015; Marth et al. 2013; Niespodziana et al. 2011). This lack of IgE reactivity was explained by only using peptide sequences from IgE-reactive parts of the respective allergen molecules resulting in the disruption of allergen conformation (Cornelius et al. 2016). Another interesting observation was that in mice vaccinated with PreS-P1-P5 absorbed to aluminum hydroxide, Fel d 1-specific IgG1 levels could be increased after boosting with the P1 peptide only (without PreS) (Niespodziana et al. 2011).

In addition, both BM32 and the different Bet v 1-PreS-fusion proteins displayed a lack of basophil activation from allergic patients compared to the respective allergen extracts (Focke-Tejkl et al. 2015; Niespodziana et al. 2011). In PBMCs from Bet v 1-allergic patients, an increase in IFN-γ, IL-1β, IL-6, TNF-α, IL-17, granulocyte colony-stimulating factor, as well as IL-10 levels was observed when stimulated with the corresponding Bet v 1–PreS fusion proteins compared to Bet v 1 alone (Marth et al. 2013). Stimulation of PBMCs derived from birch pollen-allergic donors with allergen-derived peptides alone induced significantly less T-cell proliferation than either the full-length allergen or the fusion proteins (Marth et al. 2013), strongly implying that, in the allergen–PreS fusion proteins, PreS is the main stimulator of T-cell responses. Interestingly, in a similar experimental approach with grass pollen-allergic patients all these cytokines showed decreased levels when BM32 instead of grass pollen extract was used (Focke-Tejkl et al. 2015).

These differences in immune responses induced by the different allergen–PreS fusion proteins may be explained by the distinct three-dimensional structures of the fusion proteins resulting from the different options of how to combine the respective peptides with the PreS protein (Focke-Tejkl et al. 2015).

PreS fusion proteins induced a robust production of allergen-specific IgG antibodies upon immunization of mice (Fel d 1) (Niespodziana et al. 2011), rabbits (Fel d 1, Bet v 1, BM32) (Focke-Tejkl et al. 2015; Marth et al. 2013; Niespodziana et al. 2011), and humans (BM32) (Niederberger et al. 2018; Zieglmayer et al. 2016). For BM32, in humans,

this answer predominantly consisted of allergen-specific IgG1 and IgG4 antibodies (Niederberger et al. 2018; Zieglmayer et al. 2016). Notably, upon immunization with BM32 in a clinical study, allergen-specific IgG4 levels constantly increased over the course of the study, while IgG1 levels fluctuated between repeated immunizations (Niederberger et al. 2018).

Inhibition of patient IgE binding to the corresponding allergen by rabbit sera (obtained after immunization with the allergen–PreS fusion proteins) was observed for all PreS-fusion proteins. The inhibition of IgE binding was comparable, if not better, to inhibition by rabbit IgG obtained after immunization with the corresponding allergens (Focke-Tejkl et al. 2015; Marth et al. 2013; Niespodziana et al. 2011). The authors suggested the higher inhibitory capacity of the rabbit sera to be caused by the higher frequency of IgE-binding site peptide-specific IgG antibodies compared to the mixed antibody response towards the whole allergen molecule observed in corresponding immunizations with allergen extracts (Marth et al. 2013). Another benefit of BM32 is the parallel induction of PreS-specific IgG antibodies, which are important in preventing HBV infection. In an HepAd38 cell model, IgG from either BM32- or Engerix-B immunized rabbits were shown to equally prevent the expression of HB antigens in vitro (Cornelius et al. 2016). Whether this is a common feature of all PreS fusion proteins remains to be investigated.

Finally, BM32 has been tested in clinical studies. While not reaching the primary end point in one of the studies (improvement in daily symptom medication score during peak grass pollen season) (Niederberger et al. 2018), it was well tolerated and improved clinical symptoms in grass pollen-allergic patients (Niederberger et al. 2018; Zieglmayer et al. 2016). Subcutaneous injections with BM32 resulted in reduced T-cell proliferation of PBMCs from allergic patients as well as a reduced basophil activation after a challenge with timothy grass pollen allergen (Zieglmayer et al. 2016). In addition, allergen-specific IgE levels did not increase during the 2-year treatment period with BM32, while allergen-specific IgG levels increased significantly and seasonal increases of allergen-specific IgE levels decreased significantly (Niederberger et al. 2018). Intriguingly, a treatment with lower doses of BM32 (i.e., 20 versus 40 µg) resulted in a stronger decrease in symptom medication scores, which was attributed to a better affinity maturation of allergen-specific antibodies in lower vaccination doses (Niederberger et al. 2018).

In summary, the different allergen–PreS fusion proteins were shown to conserve T-cell reactivity towards the fused allergen peptides while exhibiting T-cell reactivity towards PreS and to efficiently induce allergen-specific IgG responses. Moreover, all tested allergen–PreS fusion proteins showed a strongly reduced allergenic potency. Therefore, this type of fusion protein may hold potential to improve allergy treatment.

HIV Trans-activating Regulatory Protein

Salari et al. (2017) investigated the sublingual immunotherapeutic potential of a recombinant HIV type 1 (HIV-1) trans-activating regulatory protein (TAT)-fusion protein with the main allergen of *Chenopodium album* pollen (Che a 3) in a murine rhinitis model (rTAT-Che a 3). The TAT protein transduction domain is a highly cationic 11-amino acid peptide derived from HIV-1 rich in arginine and lysine. The electrostatic interaction between the cationic TAT protein and the negatively charged cell membrane of eukaryotic cells can result in immune-modulating effects due to enhanced adherence and internalization (Salari et al. 2017). The fusion protein rTAT-Che a 3 retained its immunogenic potential as shown by IgE reactivity with sera of allergic patients (Salari et al. 2017). This finding correlated with Salari et al. (2017) observing only a slightly altered protein structure of the fusion protein rTAT-Che a 3 compared to native Che a 3 protein. Moreover, no differences in Che a 3-specific IgG1- and, IgE levels and only slightly higher IgG2a levels were demonstrated in rTAT-Che a 3-immunized mice compared to animals only immunized with Che a 3 (Salari et al. 2017).

In terms of immune modulation, both IL-4- and IFN-γ-levels in re-stimulated splenocytes from the rTAT-Che a 3-immunized mice were found to be significantly lower than in those from the rChe a 3 mice (Salari et al. 2017). In line with these effects, splenocytes from rTAT-Che a 3 sublingually immunized mice were shown to have significantly higher expression of transforming growth factor β-mRNA and protein as well as Foxp3-mRNAs, suggesting rTAT-Che a 3 to enhance Treg-mediated immune responses (Salari et al. 2017).

Rhinovirus Viral Protein 1

Since both allergies and rhinovirus infections are major triggers of asthma bronchiale, Edlmayr et al. (2009) investigated the immunomodulatory properties of a combination vaccine based on rhinovirus viral protein 1 (VP1), a rhinovirus surface protein that plays a key role in the infection of respiratory cells, and a hypoallergenic peptide (P5) of the timothy grass pollen allergen Phl p 1 fused either to the VP1N-terminus (VP1-P5), or both N- and C-terminal sites of VP1 (VP1-2xP5).

Fusion to VP1 resulted in both increased immunogenicity of the P5 peptides as well as a reduction in allergenicity of the fusion protein in basophils isolated from human grass pollen-allergic patients in comparison to native Phl p 1 allergen (Edlmayr et al. 2009). In addition, both VP1-P5

and VP1-2xP5 induced allergen-specific Th1-based immune responses while suppressing IL-5 production from splenocyte cultures (Edlmayr et al. 2009). Moreover, it was confirmed that the antibodies induced by immunization of mice with VP1-2xP5 were both cross-reactive to natural group 1 pollen allergens from several grass species and sufficient to inhibit human rhinovirus infection in HeLa cells (Edlmayr et al. 2009).

While these results clearly demonstrate the potential of virus-derived proteins as immune-modulatory components as part of adjuvant:allergen fusion proteins, up to now, there is no report describing the effectiveness of these VP-1grass pollen peptide fusion proteins in humans.

Immune-Activating Fungal Components

Fungal Immunomodulatory Protein fve

Su et al. (2012) characterized the immune-modulatory properties of a fusion protein consisting of the major HDM allergen Der p 2 (*Dermatophagoides pteronyssinus*) and the fungal immunomodulatory protein *fve* (FIP-*fve)* from the golden needle mushroom (*Flammulina velutipes*), known for its Th1-directed adjuvant properties. Technically, the fusion protein (OsDp2Fve) was expressed in a rice suspension cell culture and induced under sucrose starvation to evade poor solubility of Der p 2 (Su et al. 2012). The structural integrity of Der p 2 in OsDp2Fve was confirmed by IgE reactivity with sera from human Der p 2 allergic patients (Su et al. 2012). In splenocyte cultures, the lymphoproliferative effect of OsDp2Fve was strongly increased, while the hemagglutination/lymphoagglutination properties were reduced compared to FIP-*fve* alone (Su et al. 2012).

In vivo subcutaneous immunization of C57BL/6 mice with OsDp2Fve significantly increased Der p 2-specific IgG2c- and lead to earlier Der p 2-specific IgG1-production while reducing Der p 2-specific IgE production compared to the mixture of Der p 2 + FIP-*fve* (Su et al. 2012). Upon re-stimulation of splenocyte cultures from mice immunized with OsDp2Fve with 10 µg/ml Der p 2, T cells produced similar levels of Th2 effector cytokines as compared to T cells from Der p 2-immunized (Su et al. 2012). Despite these promising effects, the obtained fusion protein was shown to be only approx. 75% pure (Su et al. 2012). Therefore, the contribution of contaminating substances to the obtained results cannot be excluded, complicating the interpretation and evaluation of the presented results.

Targeting of Allergens to the MHC-II Loading Pathway Using the Modular Antigen Translocator

The modular antigen translocating (MAT) molecule was designed for intracellular targeting of allergens to the major histocompatibility class II (MHC-II) pathway (Crameri et al. 2007). It is composed of a $[His]_6$-tag for protein purification, the tat-translocation sequence from HIV-TAT, and the allergen of interest fused C-terminally to the first 110 amino acids of the human invariant chain (Crameri et al. 2007). The TAT-derived translocation peptide allows for the efficient translocation of extracellular proteins into the cytoplasm, where the proteins are targeted to endosomal/lysosomal compartments by the invariant chain fragment (Crameri et al. 2007). This targeting of the fused allergens to endosomal/lysosomal compartments is thought to increase loading of allergen-derived peptides onto MHC-II molecules and, therefore, antigen presentation (Crameri et al. 2007).

Crameri et al. (2007) generated different MAT constructs incorporating the allergens Asp f 1 from *Aspergillus fumigatus*, Bet v 1 form birch pollen, phospholipase A2 from honey bee venom, Der p 1 from HDM, and Fel d 1 from cat saliva. The MAT constructs were shown to rapidly translocate into the cytoplasm of PBMCs inducing PBMC proliferation, while pure allergen molecules were taken up to a much lower extend and induced 10- to 100-fold lower rates of proliferation (Crameri et al. 2007). When PBMC cultures from allergic donors were stimulated with the MAT constructs, a decrease in IL-4-, IL-5-, and IL-2 secretion as well as an increase in IFN-γ- and IL-10 secretion was observed in comparison to stimulation with the respective His-tagged or TAT-tagged allergens alone (Crameri et al. 2007). These results suggest that the different allergen:MAT constructs were able to induce a shift from allergen-specific Th2 to Th1 responses, a phenotype also observed in successfully desensitized allergic patients (Crameri et al. 2007).

Based on these results, Martinez Gomez et al. (2009) tested the immunogenicity and safety of MAT-Fel d 1in a mouse model of anaphylaxis. Intralymphatic immunization into the inguinal lymph nodes of CBA mice with MAT-Fel d (30 pmol) resulted in an increased production of Fel d 1-specific IgG2a antibodies and, therefore, strongly increased IgG2a/IgG1 ratios as well as significantly increased production of IL-2 and IFN-γ and lower (but not significantly reduced) IL-4 and IL-10 production from splenocyte cultures re-stimulated with 10 µg/ml Fel d 1 (Martinez Gomez et al. 2009). In comparison to this, subcutaneous immunization with MAT-Fel d 1 (300 pmol) did not induce comparable IgG2a production, suggesting that intralymphatic injection resulted in a stronger Th1 polarization than the subcutaneous route (Martinez Gomez et al. 2009).

In a mouse model of intralympatic immunotherapy, three vaccinations were shown to induce the production of Fel d 1-specific IgG2a antibodies and to efficiently prevent cat fur extract-induced anaphylaxis in sensitized mice (Martinez Gomez et al. 2009). Moreover, in contrast to cat fur extract or rFel d 1, application of MAT-Fel d 1 into sensitized mice did not result in anaphylaxis (Martinez Gomez et al. 2009).

In line with this reduced allergenic potential of MAT-Fel d 1, MAT-Fel d 1 displayed a 100-fold reduced capacity to induce the degranulation of basophils isolated from cat-allergic patients (Martinez Gomez et al. 2009).

MAT-Fel d 1 was further investigated in a double-blind clinical trial by Senti et al. (2012). Compared to saline-in-alum placebo, intralymphatic immunotherapy (ILIT) with MAT-Fel d 1 in 20 cat dander allergic patients significantly increased nasal tolerance by 74-fold upon challenge with cat dander extract while eliciting no adverse events (Senti et al. 2012). Moreover, treatment with MAT-Fel d 1 also resulted in significant increases in skin prick and dermal tolerance compared to placebo controls (Senti et al. 2012). Only three intralymphatic injections with MAT-Fel d 1 within 2 months were needed to achieve these effects (Senti et al. 2012).

Immunologically, ILIT with MAT-Fel d 1 resulted in a 5.66-fold increase in median cat dander-specific IgG4 levels, (no allergen-specific IgG1 production was detected) which was not observed in the placebo group (Senti et al. 2012). There were no significant differences in cat dander-specific IgE levels between the MAT-Fel d 1 and placebo groups (Senti et al. 2012). MAT-Fel d 1 treatment furthermore stimulated regulatory responses as PBMCs from MAT-Fel d 1-treated patients produced more IL-10 at the end of the study, while neither treatment with MAT-Fel d 1 nor placebo resulted in significant changes in IL-2-, IL-4-, IL-5-, IL-17-, or IFN-γ levels (Senti et al. 2012). Therefore, this first-in-human study suggests MAT-Fel d 1 to be both a safe and efficacious vaccine, inducing immune tolerance after only three intralymphatic injections.

Finally, Zaleska et al. (2014) further investigated the mechanisms underlying the immune-modulating effects of MAT-Fel d 1. In vitro MAT-Fel d 1 was shown to be efficiently internalized into T cells, B cells, NK cells, and most prominently monocytes, forming aggregates inside the cells upon uptake, while non-fused Fel d 1 alone was poorly internalized (Zaleska et al. 2014). This uptake was associated with an increased cleavage of pro-caspase-1 into caspase-1 and rapid IL-1β secretion, suggesting inflammasome activation (Zaleska et al. 2014). In PBMCs from cat-allergic patients, MAT-Fel d1 induced higher IFN-γ and IL-10 secretion, while production of the Th2 cytokines IL-5 and IL-13 was reduced compared to stimulation with Fel d 1 alone (Zaleska et al. 2014). In line with the previous results obtained with MAT-Fel d 1, this suggests a shift from Th2- to Th1-biased immune responses.

In a single-center, double-blind, placebo-controlled, randomized, two-arm phase I/IIA clinical trial 12 cat-allergic patients received three injections every 4 weeks of alum-adsorbed MAT-Fel d 1 (1, 3, 10 µg) directly into a superficial subcutaneous lymph node in the groin area (Zaleska et al. 2014). Eight control patients received three intralymphatic injections of an alum suspension as placebo (Zaleska et al. 2014). Patients treated with MAT-Fel d 1 had increased IL-10 levels 1 week after the last injection and displayed a full peripheral T-cell unresponsiveness upon re-stimulation of PBMCs with Fel d 1 1 year after completing the study, while PBMCs from placebo-treated patients proliferated in response to Fel d 1 stimulation (Zaleska et al. 2014). These results suggested the induction of an allergen-specific T-cell tolerance in MAT-Fel d 1-treated patients. Moreover, Zaleska et al. (2014) observed reduced numbers of Fel d 1-tetramer-specific T cells in the circulation of MAT-Fel d 1-treated patients, but not placebo-treated patients, 1 week after the last treatment that were correlated with an increase in $CCR7^{+}Foxp3^{+}$ Treg cells (Zaleska et al. 2014). One year after completion of the study, histamine receptor two (HR2)/HR1 ratios were significantly increased in MAT-Fel d 1-treated patients compared to either patients before treatment or the placebo group, while there was no difference in HR4 expression between the two groups (Zaleska et al. 2014).

Therefore MAT-Fel d 1 was shown to efficiently suppress allergic responses in human cat allergics by multiple effector mechanisms: (1) early T-cell activation followed by full peripheral T-cell unresponsiveness 1 year later, (2) the induction of allergen-specific T regulatory cells, (3) decreased circulating Fel d 1 tetramer-positive cells, and (4) increased HR2/HR1 ratios (Zaleska et al. 2014).

Killing of Immune Cells Expressing Allergen-Specific IgE Molecules by Fusion of the Allergen to Diphtheria Toxin

Lee et al. (2001) generated a fusion protein consisting of a truncated form of the *corynebacterium*-derived diphtheria toxin (DT; containing only the ADP-ribosylation catalytic domain and the transmembrane domain) and an Ova fragment containing allergenic determinants that were previously reported to induce hypersensitivity reactions in mice. In theory, the toxicity of DT in this fusion protein could be leveraged to kill cells expressing surface-bound allergen-specific IgE molecules (e.g., sensitized mast cells) in vivo and thereby preventing allergic reactions.

To test this approach, a mouse model of active systemic anaphylactic shock (ASAS) was used (Lee et al. 2001). In this model, mice were i.p. inoculated with a mixture of Ova, aluminum hydroxide, and *B. pertussis*. ASAS was then induced by intravenous injection of Ova or Ova-DT (Lee et al. 2001). Ova-DT completely prevented the development of ASAS (Lee et al. 2001). In line with a suggested switch from a Th2- to Th1-biased immune response, Ova-DT also inhibited the proliferation of Ova-specific B cells and mast cells, enhanced Ova-specific IgG2a levels in serum, as well as the production of IFN-γ from splenocyte cultures, while the splenocyte-derived production of IL-4 was reduced

(Lee et al. 2001). In a follow-up study by the same authors, the same model was used to determine the specificity of the effects observed by Ova-DT (Lee et al. 2004). For this, BALB/c mice were either sensitized to Ova or human serum albumin (HSA), and subsequently treated with Ova-DT. As expected in this experimental setting, Ova-DT suppressed only Ova-, but not HSA-induced anaphylaxis, showing the effects of Ova-DT to be antigen-specific (Lee et al. 2004). Moreover, when Ova-sensitized mice were boosted with Ova 14 days after the last treatment with Ova-DT, an increased production of Ova-specific IgG2a/IgG3 as well as a decreased production of Ova-specific IgE antibodies was observed (Lee et al. 2004). The induced Ova-specific IgG2a/IgG3 antibodies are likely to block anaphylaxis by neutralizing the allergen in vivo.

These effects of Ova-DT treatment were paralleled by a reduced survival of isolated IgE-expressing B cells ex vivo when stimulated with Ova-DT, suggesting that Ova-DT targeted B cells bearing Ova-specific IgE, and killed them by DT-mediated cytotoxicity (Lee et al. 2004). Therefore, the presented studies suggest that the inclusion of DT in allergen-containing fusion proteins can efficiently suppress allergic reactions by depleting cells expressing allergen-specific IgE molecules on their surface.

Conclusion and Future Aspects

In theory, fusion of allergens with immune-activating components has the potential to more robustly induce immune responses directed against the fused allergen while also (by choosing the right adjuvant) modulating immune responses away from the allergy-causing Th2 phenotype. The strategies described in this review combining allergen molecules with immune-activating molecules are quite diverse ranging from the inclusion of immune-activating bacterial (flagellin, MPLA, S-Layer, cholera- and tetanus toxin), viral (PreS, VP-1, TAT), or fungal (FIP-*fve*) components, immune-activating DNA motifs (ISS-ODN), the forced delivery of allergens to the MHC-II loading pathway (MAT), to killing of immune cells expressing allergen-specific IgE molecules by fusion of the allergen to DT. However, so far, all those studies have shown the resulting allergen:adjuvant fusion proteins to display enhanced immune-activating potential compared to either the respective allergen molecules alone or the non-fused mixture of both single components. Therefore, such fusion proteins have the potential to improve the currently laborious and time-consuming treatment of allergic diseases.

While the immunological effects of such allergen:adjuvant fusion proteins are well described (induction Th1/Treg responses characterized by production of either IL-10, IFN-γ, or both, suppression of allergen-specific Th2 responses), the activated cell types, underlying mechanisms, and the signaling pathways contributing to the observed immune activation are still not completely clear and need further studies for the future safe application of such constructs in human patients. Moreover, while some fusion proteins have already undergone first clinical trials, other constructs have not been tested in humans so far.

Indeed, the only antigen:adjuvant fusion protein that is already licensed is Sipuleucel-T (PROVENGE®) (Sheikh et al. 2013). It consists of a fusion protein of the non-classical adjuvant granulocyte/macrophage colony-stimulating factor and the prostate cancer antigen prostatic acid phosphatase. PROVENGE® is used to treat hormone-resistant prostate cancer by ex vivo stimulating differentiated monocytes from patient blood and re-administrating the activated cells into the patient where they are described to effectively initiate immune responses against tumor tissue (Sheikh et al. 2013). Clinically, PROVENGE® was shown to increase the mean survival of patients with hormone-resistant prostate cancer by 4.1 months (Sheikh et al. 2013). Although these results refer to the treatment of cancer and not allergic diseases, they, nevertheless, demonstrate the clinical potential of such fusion proteins. Therefore, further studies are needed to better understand the mode of action and evaluate the therapeutic potential of these fascinating vaccine candidates.

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest relevant to this manuscript.

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