

Is Genetic Vaccination against Allergy Possible?

Richard Weiss^a Sandra Scheiblhofer^a Maximilian Gabler^a Fatima Ferreira^a
Wolfgang W. Leitner^b Josef Thalhamer^a

^aDepartment of Molecular Biology, Division of Allergy and Immunology, University of Salzburg, Salzburg, Austria;

^bDermatology Branch, NCI/NIH, Bethesda, Md., USA

Key Words

Type I allergy · DNA vaccination · Genetic immunization

Abstract

Genetic immunization has proven a powerful method to induce antiallergic immune responses. The underlying functional principle has been described to be based on the recruitment of allergen-specific Th1 cells, CD8+ cells and the establishment of a Th1 cytokine milieu, which prevent the development of a Th2-biased response in a protective setup and can balance an ongoing Th2-type response in a therapeutic situation. Genetic immunization with plasmid DNA offers innovative solutions to the major problems associated with protein immunization, such as crosslinking of pre-existing immunoglobulin E on mast cells/basophils or induction of de novo synthesis of immunoglobulin E by the protein immunization itself. It easily enables the routine production of hypoallergenic vaccines, which do not translate native allergens, thus avoiding potential anaphylactic side effects. DNA vaccines can also be applied as mixtures of single vaccines, making them interesting candidates for treatment based on component-resolved diagnosis, followed by an individualized therapy with the relevant allergens. In addition to the description of up-to-date allergen gene

vaccine approaches, this review gives an overview of animal studies dealing with the following topics: danger signals as the inherent adjuvant properties, methods to optimize the vaccine immunogenicity, modulation of the immune response, nonparenteral applications and low-dose vaccination strategies.

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Introduction

Since the early 1990s, DNA vaccination has evolved rapidly as a new technique for induction of immunity as well as for plasmid-based immunotherapy. Many experiments in animal models and a rising number of clinical studies have demonstrated the broad applicability and efficacy of this method in vaccination against infectious diseases and tumors [for a review, see ref. 1–4]. Because of their capacity to induce Th1-biased immune responses, so far, the majority of studies have been focused on DNA immunization against viral diseases or cancer. However, over the last decade, an increasing number of publications reported on successful preventive and – in some cases – also therapeutic approaches in rodent models of type I allergy. An overview of DNA-based studies with respect to prevention and/or treatment of allergy is given in table 1.

Table 1. Overview of plasmid immunization studies against type I allergies

Encoded allergen(s)	Method	Modifications	Prevention	Therapy	Ref.
Ara h 1, Ara h 4	i.d.		n.d.	n.d.	100
Ara h 2, ovomucoid	i.m., i.d.		+/-	n.d.	101
Ara h 2	oral	chitosan nanoparticles	+	n.d.	66
Art v 1a	i.d.	optimized codon usage	n.d.	n.d.	59
Bet v 1a	i.d.	appending and/or coimmunization of CpG motifs	n.d.	n.d.	16
Bet v 1a	i.d.		+	+	17
Bet v 1a	i.d.	hypoallergenic fragments, hypoallergenic mutant	+	+	9
Bet v 1a, Bet v 1d	i.d.	appending and/or coimmunization of CpG motifs	n.d.	n.d.	15
Bet v 1a, Phl p 2, β -Gal	gene gun	i.d. coinjection or prepriming with CpG motifs	n.d.	n.d.	18
β -Gal	i.d.		+	+	10
β -Gal	i.d.	transfer of CD4+/CD8+ T lymphocytes	+	n.d.	11
β -Gal	gene gun		+	n.d.	64
β -Lactoglobulin	i.m.		+	n.d.	24
Cry j 1	i.m., gene gun		+	n.d.	63
Cry j 2	i.m.	CD4+ T-cell epitope	+	n.d.	76
Der f 11	i.m.	depletion/transfer of CD4+/CD8+ T lymphocytes	+	+	12
Der f 11	i.m.	CpG, bestatin, bupivacaine, lipofectine, alum	+	n.d.	41
ProDer p 1	i.m.	optimized codon usage	+	n.d.	19
Der p 1	i.m.	codon optimization, Igk light chain leader	+	+	21
Der p 1		enzymatically inactive	n.d.	n.d.	22
Der p 1	i.m.	fusion of CD4+ and CD8+ immunodominant T-cell epitopes	n.d.	+	23
Der p 1	i.m.	epitopes	n.d.	+	75
Der p 1, Der p 2	i.m.	mixture of T-cell epitopes	+	n.d.	20
Der p 2	i.m.		+	n.d.	27
Der p 5	i.m.	transfer of CD8+ T lymphocytes	+	n.d.	13, 14
KBG ¹	i.m.	IFN- γ and/or IL-12 plasmids	+	n.d.	25
Ova	i.m.	fusions with GM-CSF, IFN- γ , IL-2, IL-4, IL-12 or IL-1 β	n.d.	n.d.	60
Ova	i.m.	fusion with IL-18	+	+	28
Ova	i.m.	fusion with IL-18	n.d.	n.d.	61
PLA2	i.d.		+	+	43
PLA2	s.c.	PLGA microspheres	+	n.d.	67
-	i.m.	Flt3-ligand plasmid	n.d.	+	94
-	i.m.	α CD3sFv-IL-18 fusion	n.d.	+	95
-	i.m.	IL-4 double mutant	n.d.	+	97
-	i.m.	calcium-activated chloride channel	+	n.d.	98
-	i.d.	IL-5 with foreign Th epitope	+	n.d.	96

i.d. = Intradermal; n.d. = not done; i.m. = intramuscular; KBG = Kentucky blue grass; IFN = interferon; GM-CSF = granulocyte macrophage colony-stimulating factor; s.c. = subcutaneous; PLGA = poly(lactide-co-glycolide).

¹ Applied as recombinant protein.

Th1-Biased Immunity – The Key to DNA-Based Allergy Vaccination

Type I allergy is characterized by the induction of Th2-biased immune reactions against otherwise harmless environmental antigens derived from grass, tree and weed pollens, house dust mites, cockroaches, domestic pets, various moulds, stinging insects, certain drugs, foods and latex. Due to complex genetic and environmental factors, atopic persons are prone to mount a Th2-type immune

reaction, which is defined by the formation of immunoglobulin (Ig)E (and IgG1 antibodies in the mouse) and the production of Th2-type cytokines such as interleukin (IL)-4, IL-5 and IL-13 [5–8].

The unique features of DNA vaccines, including the possibility to induce Th1-biased immune responses, led to the development of new approaches for protective and therapeutic allergy treatment.

A Th1-type immune reaction is triggered by the innate immune system via antigen-presenting cells (APCs),

secreting certain cytokines such as interferon (IFN)- α , IFN- β and IL-12, and thus stimulates the production of IFN- γ by natural killer (NK) cells and T cells. This milieu counteracts a class switch to IgE. In the mouse, it leads to class switching to IgG2a, which therefore represents an indirect indicator for the induction and action of Th1 cells.

The antiallergic principles of DNA vaccines could be clearly attributed to the recruitment of IFN- γ -producing CD4+ and CD8+ cells and the establishment of a balanced Th1/Th2-type cytokine milieu. Neither blocking antibodies nor T regulatory cells or other tolerance-related responses play a major role in effective protective and therapeutic antiallergic DNA-based reactions [9].

A survey of the principle mechanisms involved in DNA immunization and its antiallergic effects is presented in figure 1.

In 1996, Raz et al. [10] published the first study demonstrating that immunization with a plasmid encoding the model allergen β -galactosidase (β -Gal) induced a Th1-type response that dominated over an ongoing protein-induced Th2 response in an antigen-specific manner. Both CD4+ and CD8+ T cells from DNA-immunized animals have been shown to contribute to the suppression of IgE antibody production by affecting the primary response and/or by propagating the Th1 memory response in a passive cell transfer system [11, 12].

The first evidence for the antiallergic effect of DNA vaccines with a clinically relevant allergen was achieved by intramuscular injection of rats with plasmid DNA encoding the dust mite allergen Der p 5 [13, 14]. DNA-based immunization with this construct not only resulted in its long-term expression and the induction of specific immune responses, but also prevented the induction of IgE synthesis. This study also demonstrated the efficacy of DNA vaccines to counteract allergen-induced pathological changes of the lung. Prevacination with plasmid DNA encoding the allergen led to a significant decrease in histamine release in bronchoalveolar lavage (BAL) fluids and airway hyperresponsiveness (AHR) in rats challenged with aerosolized allergen. This suppression was persistent and could also be transferred into naive rats by CD8+ T cells from gene-immunized animals.

Since then, a variety of model allergens as well as clinically relevant molecules have been used for DNA-based immunization against type I allergy. Among them, Bet v 1, the major allergen of birch pollen, belongs to the most extensively studied molecules [9, 15–18]. These studies have demonstrated that DNA-based immunization with a construct encoding Bet v 1 mounted Th1-biased spe-

cific immune responses and protected against allergic sensitization. Moreover, in therapeutic approaches, treatment of sensitized animals with DNA vaccines decreased IgE production, IgE-dependent mediator release and reduced anaphylactic activity as measured by passive cutaneous anaphylaxis assays, thus clearly indicating that DNA immunization induces a sustaining shift from a Th2-type response towards a balanced Th1/Th2-type response.

Similar results have been demonstrated with DNA vaccines encoding allergens of house dust mite [12, 19–23] and a DNA vaccine coding for the major milk allergen bovine β -lactoglobulin [24].

Most of the cited studies have demonstrated the effect of DNA vaccination by measuring immunological parameters. However, there is an increasing number of papers dealing with the influence of DNA vaccines on lung parameters, because allergic asthma is one of the most serious problems encountered by atopic patients. It is characterized by airway obstruction, mucus hypersecretion and inflammation of the airways, including infiltration and activation of eosinophils in the bronchial mucosa. Several publications showed that DNA-based vaccines offer an intriguing long-term solution to counteract the early events in allergic inflammation of the lung [13, 19–21, 23, 25–28].

While it is well known that DNA vaccination is a powerful method to induce CD8+ T-cell responses, concerns have been raised, that induction of CD8+ immune responses in the lung might lead to exacerbation of lung pathology in asthma. These reservations mainly derive from studies investigating the role of viral infections in asthma. It has been shown that viral infection can sensitize mice to inhaled antigen [29]. Respiratory viruses can recruit T cells nonspecifically to the lung [30] where they may enhance inflammation by releasing cytokines that recruit mast cells, basophils and eosinophils. Indeed, there is evidence that the local Th2 environment in the lung of asthmatic individuals can switch virus-specific CD8+ T-cell responses to IL-5 production, thereby inducing airway eosinophilia [31].

Although these data advise caution on the generation of CD8+ T-cell responses in the asthmatic lung, there is also evidence for inhibition of IgE and normalization of airway responsiveness by CD8+ T-cell-derived IFN- γ or dendritic cell IL-12 induction [32–36].

Additionally, several investigators showed that increased numbers of CD8+ T cells in the lungs of DNA-vaccinated mice correlated with a reduced influx of inflammatory cells [20]. A direct involvement of CD8+ T

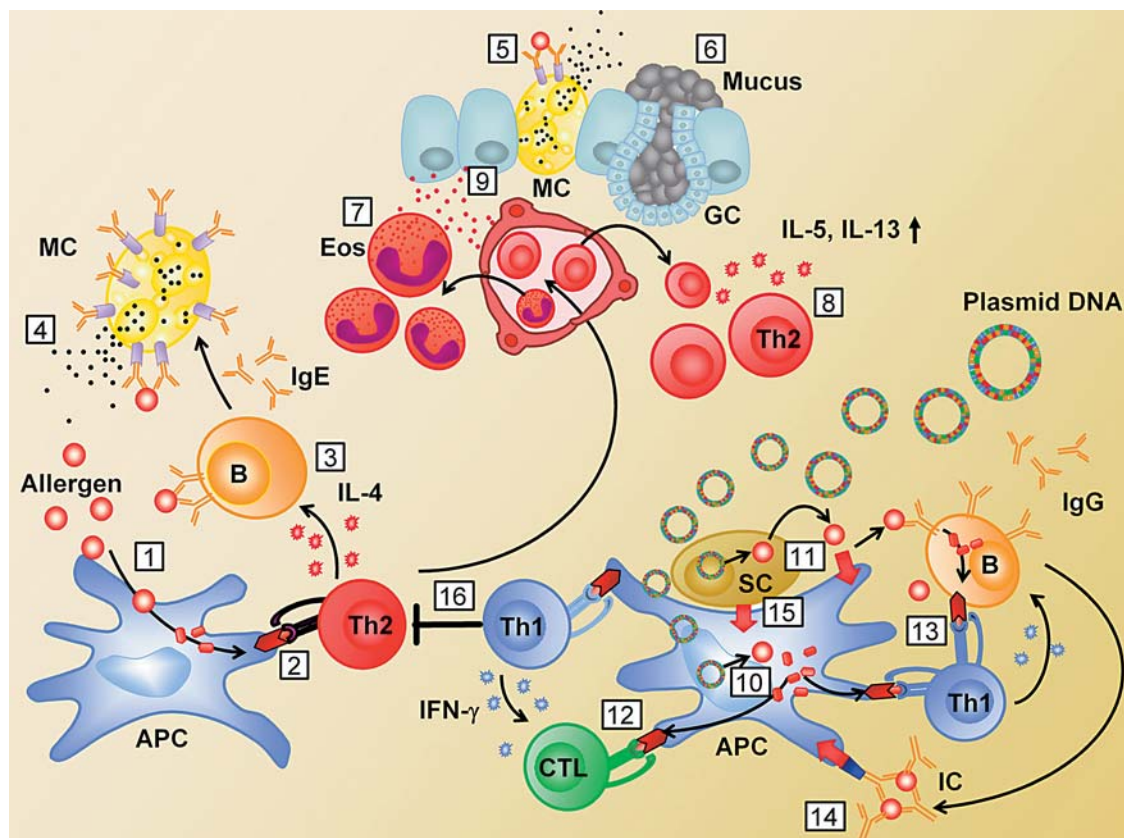


Fig. 1. Overview of the mechanisms of DNA-based vaccination in allergy. In atopic individuals, allergen is taken up by APCs (1), processed into peptides and presented to T helper cells in the context of MHC class II (2). Induction of antigen-specific Th2 cells results in the secretion of Th2-type cytokines such as IL-4 and IgE class switching of B cells (3). Secreted IgE molecules bind to mast cells (MC) via FcεRI receptors and crosslinking with allergen leads to mediator release (4) causing the typical symptoms of type I allergy. In the bronchus, the early-phase allergic response is characterized by binding of allergen to specific IgE on mast cells, followed by degranulation (5). Mast cell mediators cause mucus production by goblet cells (GC) (6) and induce infiltration of eosinophils (Eos) (7) as well as Th2 lymphocytes, which upon activation secrete high levels of IL-5 and IL-13 contributing to eosinophil maturation and infiltration and exacerbation of asthma (8). Degranulation of eosinophils leads to damage of the vascular and bronchial epithelium (9). Plasmid DNA encoding an allergen applied by intradermal or intramuscular injection is taken up by APCs, which transcribe and translate the information into the respective allergen (10). Additionally, somatic cells (SC), e.g. skin keratinocytes, can take up

plasmid DNA and secrete allergen, which in turn can be taken up by APCs (11). Both intracellularly synthesized antigen as well as antigen acquired from extracellular spaces can be presented on MHC I as well as MHC II via crosspriming or autophagy. Presentation of antigenic peptides on MHC II leads to activation of antigen-specific Th1 cells, which secrete IFN-γ. Presentation of peptide in the context of MHC I in conjunction with Th1 cells induces cytotoxic T lymphocytes (CTL) (12). Th1 cells also drive expansion of B cells and class-switch to IgG (13). Allergen-specific IgG is assumed to compete with IgE (blocking antibody hypothesis). Immune complexes (IC) of IgG and allergen also serve as strong activators of APCs, thereby amplifying the immune response (14). Additionally, uptake of plasmid DNA and expression of the foreign antigen may drive transfected cells into apoptosis. The allergen-rich, highly immunogenic apoptotic vesicles are then taken up by APCs (15). Together with the intrinsic CpG motifs, these danger signals contribute to the induction of a Th1-biased immunity, which can prevent the formation of allergen-specific Th2 cells or even counterbalance existing Th2 reactions (16).

cells in the prevention of lung pathology was demonstrated by transfer [13] or depletion studies [28].

Taken together, the available data clearly demonstrate that DNA-based vaccines can, at least in the mouse model, efficiently protect from IgE expression, experimental

airway inflammation and AHR by induction of Th1-biased immune reactions. Secretion of IFN-γ and subsequent suppression of Th2 cytokines seems to be the central mechanism involved in the antiallergic response. So far, the available data demonstrated that expression of

significant amounts of Th1-related cytokines such as IFN- γ in the BAL elicited no detrimental effects on lung pathology, suggesting that DNA vaccination is a safe method not only to prevent from induction of serum IgE but also from airway sensitization by exposure to aerosolized antigen.

The Inherent Adjuvant Effect of DNA Vaccines

The induction of Th1-biased immune responses after intramuscular or intradermal DNA immunization, which was in striking contrast to the immune response triggered by protein injection, could be explained by immunostimulatory properties of certain DNA sequences. Unmethylated bacterial DNA obviously represents an old evolutionary danger signal for the mammalian immune system, which is displayed by defined CpG motifs. These motifs trigger Th1-biased immune responses by acting on professional APCs, which secrete a panel of cytokines including IFN- α/β , tumor necrosis factor- α , IL-6 and IL-12, thereby stimulating NK cells to produce IFN- γ [37].

The cytokine milieu present at the time of initial T-cell activation appears to be the most important among the factors contributing to T-cell differentiation [38]. IL-12 facilitates differentiation towards a Th1-type via activation of signal transducer and activator of transcription 4, and IFN- γ promotes Th1 cell development via enhancing IL-12 production by macrophages and expression of IL-12 receptors on CD4 $^{+}$ T cells, while simultaneously suppressing Th2 cell differentiation.

CpG motifs belong to pathogen-derived molecular structures which are recognized by APCs via a set of pattern recognition receptors, including the members of the Toll-like receptor (TLR) family [for a review, see ref. 39]. Immune activation of B cells, dendritic cells and NK cells by CpG DNA has been shown to be dependent on TLR9 [40].

Stimulation via CpG motifs attracted attention because it was believed to allow for the modulation of the Th1/Th2 balance according to the respective requirements. With the intention to utilize the Th1-inducing capacity of these immunostimulatory sequences for genetic desensitization, some studies included additional CpG motifs, either by appending a CpG-rich sequence to the allergen gene and/or by coimmunization of the allergen-encoding vector together with CpG oligodeoxynucleotides (ODN) [15, 16]. Both approaches increased the Th1 bias of the immune response with respect to humoral as well as cellular responses.

Evaluation of various adjuvants for protein and DNA-based immunization with the mite allergen Der f 11 re-

vealed CpG motifs as a necessary adjuvant for recombinant Der f 11 to inhibit IgE responses, whereas the DNA vaccine alone induced profound suppression of IgE [41].

The immunomodulatory effect of coinjected or coapplied CpG motifs turned out to be complex and partly double-edged, as has been described by studies investigating the effect of CpG coimmunization or prepriming on Th2 responses induced by gene gun or protein immunization. The influence of added CpG motifs depended on the nature of the antigen and on immunization schedules. In one study, a combination of CpG + DNA immunization was more effective in antagonizing Th2 responses than the combination of CpG + protein immunization [18]. In another study, the results indicated that coimmunization of CpG ODN with protein antigen might be equally effective as DNA-based immunization in attenuating the development of anaphylactic hypersensitivity in subsequently sensitized mice [42].

In the context of DNA immunization, in most cases, the vaccine-inherent, CpG-mediated Th1 bias seems to be strictly antigen dependent. Immunization with plasmid DNA encoding a certain antigen leads to a Th1-type response against this molecule, but does not influence a Th2-type response triggered by protein immunization against a different antigen [9, 17]. This antigen-specific Th1 bias of gene vaccines has been demonstrated by numerous studies using mock vectors (which contain the same amount of immunostimulatory plasmid DNA as the vaccine itself) in the control groups. However, in a study which evaluated a DNA vaccine directed against bee venom phospholipase A (PLA), immunization with mock DNA has been shown to protect from the development of allergic reactions [43]. DNA immunization into the skin, before or after sensitization with PLA plus alum, led to reduced PLA-specific IgE and IgG1 titers, pointing to an antigen-independent immune modulation. Similarly, Jarman and Lamb [23] showed that in a therapeutic airway model using the house dust mite allergen Der p 1, vaccination with mock DNA led to an antigen-independent suppression of CD4 $^{+}$ Th2-mediated inflammation and eosinophilia comparable with a Der p 1 encoding plasmid construct.

Beyond their role within plasmid DNA vaccines, CpG motifs have been demonstrated as useful immunomodulators for protein immunization. Several publications have shown that immune responses induced by coadministration of CpG ODN together with protein are similar to those induced by genetic immunization, at least with respect to the triggered Th type of the immune response.

Coapplication of CpG motifs was able to prevent Th2-biased reactions or to shift already established Th2-type responses towards Th1 [44]. Moreover, protection from asthma could be measured in murine models [45–47]. Immunotherapeutic approaches using CpG motifs have been further developed by chemical conjugation of CpG motifs to allergens [48, 49].

However, it must be emphasized that CpG motifs can also trigger severe side effects when applied frequently and in high doses [50–53]. Therefore, the use of additional Th1 stimuli and enhancers of immunity should be more carefully studied, especially in the light of future clinical application.

CpG Motifs and the Unknown Danger Signal

Based on the described properties, unmethylated CpG motifs of plasmid DNA were unanimously considered as the major reason for the general immunogenicity of DNA vaccines and for the induction of a Th1-type immune response by intramuscular or intradermal genetic immunization with high amounts of DNA [54]. Accordingly, the induction of a Th2-biased immune response by epidermal application of low doses of plasmid DNA with a biolistic device has been attributed to the lack of sufficient immunostimulatory DNA [54].

However, recent publications question these hypotheses. The assumed role for CpG-DNA/TLR9 interactions as the cause of plasmid immunogenicity has been upset by two independent studies demonstrating that DNA vaccines are equally effective in wild-type and TLR9-deficient mice with respect to the induction of Th1-biased immune response [55, 56]. These results confirm data from our lab using methylated plasmid DNA for immunization. Since the immunostimulatory capacity of bacterial DNA is restricted to unmethylated CpG motifs [39, 57], intradermal injection of these methylated vaccines should result in impaired immunogenicity and a reduced Th1 bias compared with unmethylated vaccines. However, we observed similar humoral and cellular antigen-specific Th1-biased immune responses with methylated vaccines, thus confirming the assumption from the TLR9 knockout studies that CpG motifs are not the only danger signal delivered with DNA vaccines (unpublished data). Additionally, we could demonstrate that the Th2-inducing capacity of the gene gun could not be attributed to a lack of immunostimulatory DNA sequences, but is rather associated with the bombardment of gold particles itself [58].

Taken together, these results suggest that DNA vaccines elicit immune responses by multiple mechanisms

and they clearly indicate that plasmid DNA contains an additional, still unknown TLR9-independent pathogen-associated molecular pattern.

Approaches to Optimize the Immunogenicity of Allergy Gene Vaccines

Similar to immunization with protein antigens, two major parameters determine the immunogenicity of gene vaccines, i.e. the nature of the translated antigen and the danger signals triggered upon injection. Additional aspects specific for the immunogenicity of DNA vaccines are the level of gene expression within transfected cells, the way of antigen presentation, the mode of antigen application and the inherent adjuvant capacity of the gene vaccine itself.

In contrast to immunization with protein, gene vaccines open a vast range of possibilities to modulate the immune reaction and to induce tailor-made responses. The following paragraphs describe recent attempts to develop improved DNA vaccines for allergy treatment in animal model systems.

Recoding of Allergen Genes

The expression level of antigens encoded by DNA vaccines is predominantly influenced by the composition of the plasmid vector and the degree of correspondence between the codon usage of the host and the codons of the respective gene. Because usually the codons of plant allergen genes are suboptimal concerning mammalian codon usage, protein expression in the mammalian host can be severely diminished. Several studies have proven that this problem can be solved by adapting the sequence of the allergen gene to mammalian codon usage [19, 21, 59].

Immunization with plasmids encoding the mugwort allergen Art v 1 revealed no or only marginal immune responses. Analysis of the Art v 1 sequence showed that the codon usage strikingly differed from those prevalent in mammals. Therefore, each codon of Art v 1 was replaced with the codon displaying the highest frequency for the respective amino acid in mammals, thus creating a synthetic recoded gene. Compared with the wild-type gene, protein expression with the recoded gene was increased about 180 fold, and intradermal DNA immunization resulted in strong immunogenicity [59]. Similar approaches were made using plasmid DNA encoding the mite allergen precursor ProDer p 1 with optimized codon usage [19], and an optimized construct containing mouse

Ig κ light chain leader sequence followed by codon optimized mature Der p 1 [21].

Allergen-Cytokine Fusion Molecules and Coapplication of Cytokine Genes

Due to the clear attribution of Th2-type responses to the pathology of allergy, most attempts to use regulatory molecules for balancing the allergic response are naturally focused on cytokines known for their Th1 bias, such as IL-12, IL-18 and IFN- γ .

Maecker et al. [60] investigated DNA vaccines expressing different ovalbumin (Ova)-cytokine fusion molecules for their influence on the Th1/Th2 bias of the induced immune reaction. While an Ova-IL-12 fusion construct or a fusion of Ova with 9 amino acids of IL-1 β elicited increased IFN- γ secretion and reduced IL-4 secretion from memory T cells, increased cytotoxicity and little or no antibody production, Ova-IL-4 fusion displayed high anti-Ova antibodies and increased Th2 cytokine production. In a similar study focused on IL-18, it was demonstrated that a DNA vaccine encoding an Ova-IL-18 fusion construct could protect from IgE induction and AHR. Protection appeared to be mediated by IFN- γ and CD8 $^{+}$ cells because treatment of mice with neutralizing anti-IFN- γ monoclonal antibody or with depleting anti-CD8 monoclonal antibody abolished the protective effect. In contrast to a DNA vaccine encoding Ova alone, the Ova-IL-18 construct could also successfully reverse established AHR in a therapeutic setting [28]. This was confirmed by Kim et al. [61] who also used an Ova-IL-18 construct and showed that the fusion construct was more efficient in inducing Th1-type immune responses than a mixture of plasmids encoding Ova and IL-18, respectively.

Coapplication of plasmids expressing IFN- γ and/or IL-12 was also found to bias the immune response against protein allergens from Kentucky blue grass towards a Th1-type reaction, thereby protecting from IgE induction and lung pathology [25].

These data indicate that allergen-cytokine fusion molecules or coapplication of cytokine expression vectors can be used to alter the type of immune reaction in an antigen-specific manner. Furthermore, the use of cytokines can enhance the Th1 polarization and thus increase the protective and/or therapeutic efficacy of DNA vaccines. However, as mentioned above, the risks associated with Th1-increasing stimuli must be considered, and overwhelming Th1 responses should be strictly avoided.

Immunization via Different Routes and Modes

The vast majority of studies using DNA vectors for preventive and/or therapeutic approaches against type I allergy have been performed by injecting the plasmid DNA in saline solution into skin or muscle. However, the high amount of plasmid DNA necessary for induction of an immune response via intradermal or intramuscular injection remains one of the major drawbacks for transfer of DNA-based desensitization methodology into the clinic. By using a biolistic device such as the gene gun, immune responses can be elicited with at least 100-fold lower quantities of plasmid DNA compared with needle injection. Unfortunately, immunization with gene gun is linked to Th2-biased immune responses (at least at the serological level), rendering this method questionable for any application concerning protection and/or therapy of allergic diseases [18, 58, 62, 63]. Conversely, Ludwig-Portugall et al. [64] showed prevention of long-lasting IgE antibody production by gene gun-mediated DNA immunization using the model allergen β -Gal. The authors speculate that different experimental settings might account for the discrepancy between their results and the work of Toda et al. [63]. Whereas Toda et al. [63] looked for antibody production at early time points after the sensitizing injection of protein in alum, Ludwig-Portugall et al. [64] describe long-term suppressive activity of gene gun-mediated vaccination after repeated challenge with β -Gal protein. Additionally, the nature of the antigen/allergen used in these studies, as well as different plasmid vectors, could possibly contribute to the observed outcome. However, recent observations from our laboratory point to a clearly negative influence of gene gun immunization on allergic conditions. Immunization with vectors encoding the birch pollen allergen Bet v 1a or the grass pollen allergen Phl p 5 resulted in massive and sustained IgE production with crosslinking activity. Moreover, subsequent intranasal provocation with allergen induced lung eosinophilia, which was even enhanced by gene gun prevaccination.

Carrier-Mediated DNA-Based Immunization

Biodegradable polymer and particulate carriers have been shown to have considerable potential for the delivery of peptides, proteins and DNA in animal models and are of special interest for the delivery via mucosal routes [65].

A promising immunoprophylactic strategy using oral DNA immunization to modulate peanut allergen-induced murine anaphylactic responses has been described by Roy et al. [66]. Oral administration of DNA nanoparticles produced by complexing plasmid DNA with chitosan, a natural biocompatible polysaccharide, resulted in gene expression in the intestinal epithelium. Mice receiving nanoparticles containing the gene encoding the peanut allergen Ara h 2 produced secretory IgA and serum IgG2a. Compared with nonimmunized mice or mice treated with mock DNA, mice immunized with the DNA vaccine on nanoparticles showed a substantial reduction in allergen-induced anaphylaxis associated with reduced levels of IgE, plasma histamine and vascular leakage.

Biodegradable poly(lactide-co-glycolide) microspheres, known as vaccine carriers capable of maturing APCs to stimulate T-cell-mediated immune responses, have been tested for their potential to downregulate an allergic response in vivo [67]. Plasmid DNA encoding bee venom allergen (PLA2) was either encapsulated into poly(lactide-co-glycolide) (anionic) microspheres or adsorbed to polyethylenimine-containing (cationic) microspheres. However, no allergen-specific protection from PLA2/alum sensitization could be observed, as all groups, independent of the type of plasmid or microspheres, displayed protection from anaphylactic shock. The interpretation of the presented data is limited by the fact that the author presented no data from sensitized-only animals.

Nevertheless, the use of micro- or nanoparticles represents an interesting approach for the development of non-parenteral DNA vaccines for allergy treatment.

Preparing the Step from Mouse to Man

Due to their inherent features, DNA vaccines lack some of the disadvantageous properties which are responsible for the unwanted side effects of protein vaccines during specific immunotherapy against allergic disease. Anaphylactic reactions after DNA immunization are limited by the fact that only nanogram amounts of the allergen are translated at the site of injection. Moreover, in contrast to immunization with protein, DNA immunization does not trigger new 'therapy-induced' IgE production.

Nevertheless, any approach with DNA vaccines for the treatment of allergic diseases must aim at the highest possible safety standards. Therefore, besides aspects concerning the safety of DNA vaccines in general, the development of hypoallergenic strategies represents a major task for allergen gene vaccine research.

According to the definition of a 'hypoallergen', gene vaccines encoding allergens must avoid translation of native allergenic determinants while T-cell epitopes of the encoded allergen must not be destroyed to guarantee the recruitment of allergen-specific Th1 cells. These requirements can be met by different approaches, which will be addressed in the following chapters.

Hypoallergenic Allergen Derivatives – Fragments, Mutants and Minigenes

DNA vaccines coding for two hypoallergenic fragments or for a hypoallergenic mutant of the birch pollen allergen Bet v 1a were evaluated for their antiallergic efficacy. Mice pretreated intradermal with any of the two DNA vaccines were protected against allergic sensitization with Bet v 1a protein. In contrast to the DNA vaccine encoding the wild-type or mutated Bet v 1, plasmids encoding the fragmented hypoallergenic Bet v 1 derivatives induced no Bet v 1-specific antibody responses. However, antibodies were directed against the recombinant fragments, indicating that the three-dimensional structure of the isolated fragments differs from the wild-type molecule. The fragment vaccine not only displayed a protective capacity but was also able to suppress an established Th2-type response [9].

Another approach for avoiding anaphylactic side effects during immunotherapy is the use of epitope vaccines instead of full length allergens. Similar to peptide immunotherapy [68–71], minigene vaccines can elicit specific CD4+ and/or CD8+ T-cell induction and memory [72–74]. Prevaccination with a mix of minigene vectors encoding T-cell epitopes from the mite allergens Der p 1 and Der p 2 led to increased levels of IgG2a, induction of IFN- γ and suppression of IgE after sensitization with Der p extract, suggesting that minigene vaccination indeed induced Der p-specific Th1 cells that were reactivated upon challenge with the extract and could bias the immune response towards a Th1-type reaction [20]. Draghi et al. [75] used minigene constructs encoding either a single CD8+ T-cell epitope or a region (p111–139) containing CD4+ as well as CD8+ epitopes and could demonstrate induction of IFN- γ -secreting T cells as well as cytotoxic T lymphocytes in naïve animals. Treatment of sensitized animals with the construct containing multiple epitopes elicited partly independent Th1 populations; however, simultaneously, the pre-existing memory Th2 population was boosted. No suppression of IgE or IL-5 in BALs could be observed. Interestingly, although CD8+ cells induced in these sensitized animals secreted high levels of IFN- γ , they displayed no cytotoxic activity,

in contrast to CD8+ cells induced in naïve animals. In another publication, the same group used the identical minigene plasmid (p111–139) to demonstrate reversal of established allergic airway inflammation and eosinophilia. Again, no suppression of IgE was observed; however, in contrast to their previous experiment, they found a reduction in Th2 cytokines but no induction of IFN- γ [23].

A DNA vaccine encoding a CD4+ T-cell epitope from the Japanese cedar pollen allergen Cry j 2 together with an invariant chain (Ii) for delivery of the epitope peptide into the major histocompatibility complex (MHC) class II pathway has been developed either by replacement of the core class II-associated invariant chain peptide with a peptide corresponding to the major Cry j 2 CD4+ T-cell epitope or by fusion of the Ii with the peptide sequence at the C terminus [76]. Repeated intramuscular immunization with either plasmid induced no antibody production against Cry j 2 but epitope-specific T-cell responses predominantly of the Th1 type and inhibited the IgE response to Cry j 2 sensitization.

However, with respect to the diversity of MHC I and II within a non-inbred population, it must be emphasized that the use of single peptides for allergen-specific immunotherapy would be questionable. With only a few exceptions (e.g., the mugwort allergen Art v 1), allergic patients generally recognize different T-cell epitopes of a given allergen in different combinations [77, 78]. Peptide-based approaches must consider these facts. As demonstrated with peptide immunotherapy for the treatment of cat allergy, the thorough investigation of the proper complex mixture of T-cell epitopes is a major requirement for successful treatment [79].

Generation of Hypoallergenic Vaccines by Forced Ubiquitination

Fragmentation, mutation and minigene approaches have certain disadvantages. Detailed knowledge of the three-dimensional structure and the immunogenic T-cell epitopes is necessary for the design of a successful vaccine, requiring a case-by-case evaluation of individual allergens, thereby making the generation of such vaccine types time consuming and costly.

We have recently established a new method to fragment any allergen into peptides by using forced ubiquitination. Linkage to ubiquitin effectively channels the gene product into the pathway for proteasomal degradation, thereby promoting Th1-biased immune responses. This method has been demonstrated to efficiently induce cytotoxic responses [80, 81]. We adapted this concept to

DNA-based immunization with DNA vaccines encoding ubiquitinated allergens. Immunization with these constructs induced no detectable antibodies against the native protein, but still elicited Th1 memory responses that protected from subsequent induction of IgE, reduced airway eosinophilia, goblet-cell metaplasia and endothelial damage after airway provocation to a similar extent as the respective conventional DNA vaccine encoding the conformationally intact allergen. Additionally, we observed significant suppression of an ongoing IgE response in a therapeutic setting. Both the protective as well as the therapeutic approach prove that ubiquitination enables the routine conversion of any given allergen into an effective hypoallergenic derivative.

From Component-Resolved Diagnosis to 'Blended Gene Vaccines'

Whereas classical specific immunotherapy still largely relies on poorly defined allergen extracts for diagnosis and therapy, the growing availability of recombinant allergens has led to the concepts favoring component-resolved diagnosis as well as component-resolved immunotherapy. These approaches eliminate the risk of cross-sensitization with related proteins in extracts and allow for treatment according to the patient's sensitization profile [82].

It has been demonstrated that mixtures of plasmids encoding different antigens can induce immune responses against the individual encoded antigens [83–87]. However, in some cases, also antigenic competition could be observed [88, 89].

We found that intradermal application of a mixture of plasmids encoding allergens of the 17-kDa tree pollen-related family, such as Bet v 1 (birch), Mal d 1 (apple), Cor a 1 (hazel) and Aln g 1 (alder), elicited Th1-biased immune responses against the individual vaccine compounds which were comparable with those induced by immunization with each single construct alone. Various degrees of crossreactivity between the different allergens could be detected on the B- and T-cell level. Prevacination with the DNA multi-vaccine protected from the induction of IgE after sensitization with both a mixture of the four recombinant allergens as well as the individual recombinant allergens. Moreover, immunization with either Bet v 1 or Cor a 1 DNA vaccine induced a highly crossprotective immune response. Prevacination with only one of the two single DNA vaccines led to complete protection from IgE production after sensitization with a mixture of all four recombinant allergens.

A DNA multi-vaccine encoding Bet v 1 and its related food allergens Mal d 1 (apple), Dau c 1 (carrot) and Api g 1 (celery), and also a DNA vaccine mixture coding for the profilins Art v 4 (mugwort), Lyc e 1 (bell pepper) and Cap a 2 (tomato) elicited similar results (unpublished data).

However, complex mixtures of DNA vaccines raise the problem that the minimal necessary plasmid dose per individual vaccine is multiplied by the number of components in the vaccine formulation. Therefore, increasing the number of single compounds increases the amount of plasmid DNA and thus the concentration of CpG motifs with the risk of entering a dangerous dose range. On the other hand, keeping the total amount of plasmid DNA constant would mean to dilute the single vaccine compounds to a suboptimal dose range. This problem limits the number of different allergen genes within a DNA multi-vaccine to approximately 10–15.

We have addressed this problem by using DNA vaccines based on viral replicases, which have been shown to prevail over conventional DNA vaccines at extremely low doses [90, 91]. The principle behind DNA replicon vaccines comes from alphaviruses which encode a replicase enzyme to efficiently replicate the viral genome inside the host cell cytoplasm. In theory, cells only need to pick up a single molecule of this self-replicating vaccine to produce sufficient amounts of antigen for the induction of an immune response. The immunogenicity of this type of self-replicating vaccine seems to be achieved by mimicking a viral infection of the transfected cells. This might trigger powerful antiviral host pathways that would enhance the immune response to the antigen encoded by the self-replicating vaccine.

Additionally, this type of vaccine induces removal of transfected cells by apoptosis and gains its immunogenicity via danger signals independent from CpG motifs [92, 93]. Immunization with as little as 1 µg of a replicase-based plasmid encoding the timothy grass pollen allergen Phl p 5 resulted in Th1-biased immune responses leading to 90% reduction in functional IgE, 50% reduction in eosinophilia in BALs, and alleviation of lung pathology following allergen provocation. These results are comparable with the antiallergic effects achieved by immunization with 100 µg doses of a conventional Phl p 5 DNA vaccine (unpublished data).

Antigen-Independent Approaches

Similar to the approach of coapplication of a DNA vaccine together with regulatory molecules containing adjuvant properties, the injection of plasmid DNA can be used to express molecules which trigger antigen-independent modulatory effects on allergic immune responses. These approaches are sometimes misleadingly described as DNA vaccination but they represent a form of somatic gene therapy with the intention of influencing an allergic response by the transient expression of modulator molecules. Most studies are focused on attempts intended to favor Th1-type reactions.

Edwan et al. [94] used a plasmid DNA-encoding Flt3 ligand, a growth factor for dendritic cells that induces Th1 cell response. Injection of this construct resulted in complete reversal of established AHR, increased levels of IFN-γ, IL-10 and IgG2a, as well as reduced IL-4 and IgE in Ova-sensitized mice. In another Th1-promoting approach, Kim et al. [95] used a fusion gene in which anti-CD3 single-chain Fv cDNA, the smallest unit of antibody recognizing the CD3 epsilon moiety of the T-cell receptor, was covalently linked to mature IL-18 cDNA. When administering this plasmid to Ova-sensitized mice, they could show increased levels of Ova-specific IgG2a, as well as enhanced IFN-γ secretion and suppression of IL-4 in *in vitro* restimulated splenocytes.

In contrast to these Th1-enhancing approaches, other groups attempted to block Th2 reactions via induction of antibodies against IL-5 [96], or by using plasmids expressing Th2 cytokine antagonists to suppress ongoing Th2 responses [97], or by directing antibodies against a regulator of soluble gel-forming mucins, a critical component of mucus in the airways [98].

These studies demonstrate that an ongoing Th2-biased immune response can be partly blocked or skewed into a Th1 direction by application of unspecific stimulators of Th1 immunity. However, care has to be taken since in predisposed individuals, systemic induction of Th1 stimulators could trigger Th1-mediated autoimmune diseases through a bystander effect as demonstrated in an experimental mouse model of experimental allergic encephalomyelitis [99].

Conclusion and Perspectives

Taken together, the studies discussed in this review provide strong evidence for genetic immunization as a powerful method to induce antiallergic immune responses.

es in both protective experimental setups as well as in therapeutic approaches. The underlying functional principle can be described as the recruitment of allergen-specific Th1 cells and the establishment of a Th1 cytokine milieu. Both prevent from the development of a Th2-biased response in a protective setup and balance an ongoing Th2-type response in a therapeutic situation. The inherent features of DNA vaccines also promote the induction of CD8+ cells which play a supportive role as bystander cells producing IFN- γ , thus enhancing the Th1 cytokine milieu.

Genetic immunization with plasmid DNA also offers innovative solutions to the major problems associated with protein immunization, such as crosslinking of pre-existing IgE on mast cells/basophils or induction of newly induced IgE by the protein immunization itself. Beyond the concepts of creating hypoallergenic allergen derivatives by fragmentation, fusion or mutation, which proved to be feasible but must be evaluated case by case in a costly and time-consuming way, DNA immunization allows for the simple production of hypoallergenic vaccines, e.g. by forced ubiquitination or the application of replicon DNA vaccines. These DNA vaccines do not translate or secrete native allergens, thus avoiding the above mentioned side effects of protein vaccines. Using these principles we have developed vectors, which allow the routine production of hypoallergenic DNA vaccines with any given gene of interest, independent of the nature of the protein.

DNA vaccines can also be applied as mixtures of single vaccines, making them interesting candidates for treatment based on component-resolved diagnosis followed by an individualized therapy with the relevant allergens. The production of recombinant proteins is costly and complicated due to frequent problems concerning the expression systems and the quality of the proteins. In contrast, the production of plasmid DNA vaccines is a simple procedure which can be easily standardized and results in a highly pure product. Additionally, there is no difference in the production protocol for different gene vaccines, i.e. a plasmid DNA vaccine encoding a birch pollen allergen can be produced following the same protocol as one encoding a grass pollen allergen. These facts enable the easy production and approval of hundreds of different allergen gene vaccines, which could serve as the basis for individualized mixtures for allergy treatment. Whenever a new allergen and its respective gene are found, a DNA vaccine can be produced and added to the existing vaccine library.

Due to the fact that the antiallergic properties of DNA vaccines are primarily carried out via the action of Th1 cells, they induce a marked crossprotective capacity among sequence-related allergens which share common T-cell epitopes. This feature opens the possibility to minimize the number of single compounds of multi-vaccine mixtures because selected single vaccines can protect against whole groups of allergens, as has been demonstrated with members of the 17-kDa tree pollen allergen family.

Gene vaccines are the youngest generation in the field of vaccine research. Most of the recently closed or running clinical trials served to evaluate the safety and tolerability of the vaccines. The results indicated no induction of severe adverse effects during all trials pointing to a high safety profile of DNA vaccines. In the next decade, newly developed and optimized DNA vaccines will replace the first DNA vaccine generation in clinical trials with the focus on efficacy.

Corresponding to the urgent need and the global importance, cancer and infectious diseases such as malaria, AIDS and tuberculosis are on top of the hierarchy with respect to the clinical use of gene vaccines in the near future. These new attempts also intend to overcome some of the shortcomings of the first trials, which were mainly caused by suboptimal parameters, e.g. concerning the injection mode and/or the restricted focus on certain genes. In many cases, proprietary constraints were the reasons for a priori suboptimal trial design.

The impressive antiallergic efficacy of DNA vaccines seems to justify the continuation into clinical trials with allergy gene vaccines in the near future. However, it is advisable to carefully prepare this step by a thorough comparative evaluation of the many different vaccination approaches in an animal model in order to acquire objective selection criteria for allergen gene vaccine candidates.

Acknowledgements

This work was supported by the Austrian Science Fund, grant numbers S8811, S8813, and S8802.

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