

Design of Protective and Therapeutic DNA Vaccines for the Treatment of Allergic Diseases

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Abstract: The DNA vaccine revolution has opened a vast scope of novel approaches for protective and therapeutic treatments of type I allergy. This review gives an overview on the current status of allergy DNA vaccines and presents advances in the design of vaccine constructs.

An immense number of concurring studies have proven the stimulation of Th1 cells and the induction of a balanced Th1/Th2 cytokine milieu as the fundamental mechanisms underlying the anti-allergic effects of DNA vaccines. Basic vaccine formulations thus can be optimized by improving the cellular immunogenicity *via* co-administration of cytokines, co-expression or co-application of immunostimulatory DNA sequences or adapting the codon usage. The latter is a frequent and major reason for impaired vaccine expression (e.g. translation of plant allergen genes in mammal cells).

Because of unwanted side effects during conventional specific immunotherapy with allergen extracts, safety is increasingly demanded for both, protein and DNA vaccines for allergy treatment. We discuss the creation of hypoallergenic DNA vaccines based on deliberate allergen gene fragmentation, the use of mutations and the routine production of hypoallergenic DNA vaccines by forced ubiquitination. Furthermore, allergen-expressing DNA replicon vaccines are introduced, which enable a drastic reduction of the vaccine dose without loss of anti-allergic efficacy. Finally, the development of DNA multi vaccines and fusion vaccines for protective and therapeutic applications against certain groups of allergens is addressed.

Keywords: DNA vaccine, genetic immunization, allergy, CpG motifs, codon optimization, ubiquitination, DNA replicons

GENERAL MECHANISMS OF DNA VACCINES

The immunological potential of DNA inoculation was first demonstrated back in 1962, when Atanasiu reported that subcutaneous administration of polyoma virus DNA transforming sequences into newborn hamsters resulted in the generation of antiviral antibodies and the induction of tumors [1]. However, it took almost 30 years until immunologists realized the impact of this phenomenon for the development of new vaccines.

In 1990 Wolff *et al.* investigated the expression of different reporter genes after injection of RNA and DNA expression vectors into mouse skeletal muscle [2]. The term "genetic immunization" was introduced in 1992 by Tang *et al.* when they demonstrated that *in vivo* transfection of epidermal tissue with plasmid DNA coated onto gold beads with the gene gun resulted in transcription and translation of genes followed by the proper presentation of gene products to the immune system and the induction of antibodies [3]. Considering the similarity of these events with the endogenous pathways involved in anti-viral responses, it was originally postulated that this new method would primarily be suitable for vaccination against virus infections, and indeed Ulmer *et al.* first showed that intramuscular (i.m.) injection of plasmid DNA encoding influenza

nucleoprotein A was able to induce strong cytotoxic T lymphocyte (CTL) responses in mice that protected from a subsequent challenge with a heterologous influenza A strain [4]. However, within several years after these first publications, a large number of reports have demonstrated the enormously broad range of possible applications, not only including vaccines against viruses such as HIV [5, 6], Hepatitis B and C [7, 8], herpes simplex [9, 10], rabies [11], and ebola [12] but also against bacterial [13-15] and parasitic infections [16-21]. Genetic immunization also seemed to offer new perspectives for the treatment of cancer [22-26] and even allergy [27-30].

Basic Immunological Principles

Although the precise mechanisms relating to the induction of an immune response *in vivo* following DNA immunization have not been fully elucidated yet, the principle events involved in antigen processing, presentation and recognition are indisputable – for details see Fig. (1).

Exogenous antigens are taken up by scavenger cells like macrophages and dendritic cells (DCs). These cells function in the induction and expansion of an immune response by presenting peptide fragments of the internalized antigen on their surface in the context of major histocompatibility complex (MHC) class II molecules: Endocytosed proteins are transported into endocytic compartments where they denature at low pH and are degraded by various proteases into peptides of 12-20 amino acids length. Only a small

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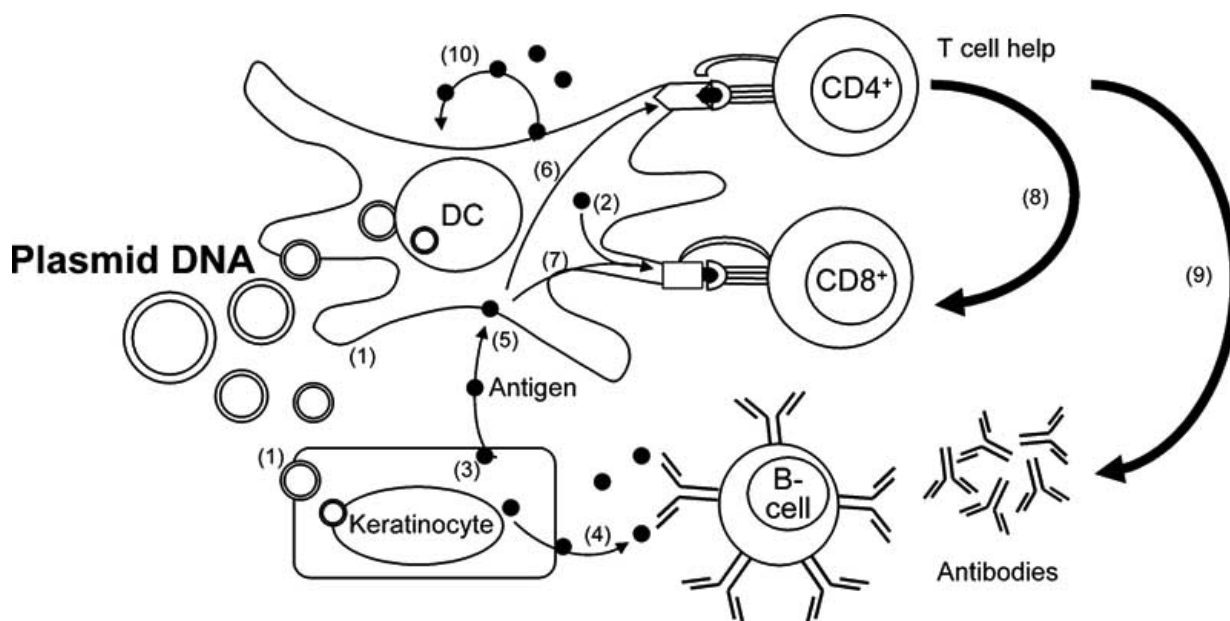


Fig. (1). After saline DNA injection DCs can take up plasmid from extracellular spaces (1), additionally skin keratinocytes (or myocytes after i.m. injection) take up DNA. In the cells, the DNA vaccine is transcribed and translated, and the encoded protein is presented to the immune system in the context of MHC class I molecules (2). Furthermore, depending on the nature of the antigen, the protein can be secreted into extracellular spaces by keratinocytes (3). This soluble antigen can become available for B-cells (4) and DCs (5), followed by processing *via* the endosomal pathway and presentation on MHC II (6). Alternatively, antigen that has been taken up by DCs can also be presented on MHC I *via* a process called cross-priming (7). Upon activation of antigen specific T-helper cells, these cells both stimulate CD8⁺ T cells (8) as well as B cells (9). In addition, DCs may also take advantage of their own antigen production (10).

B cell processing and presentation on MHC class II molecules as well as MHC class I presentation by keratinocytes have been neglected for this figure. Both cell classes play no major role in the initiation of the response because neither naïve B cells nor keratinocytes display co-stimulatory competence.

fraction of suitable peptides binds to MHC class II molecules. These peptide-class II complexes are transported to the cell surface and are recognized by specific CD4⁺ T cells.

Not only exogenous antigens but also proteins produced within cells are presented to the immune system. A finite quantity of newly synthesized proteins is thought to be degraded within the cytoplasm by the proteasome. The resulting peptides are delivered into the lumen of the endoplasmic reticulum (ER) *via* the transporter associated with antigen presentation (TAP), bound to MHC class I molecules and delivered to the cell surface. CD8⁺ lymphocytes recognize this MHC class I/peptide complex consisting of the MHC class I molecule and an embedded 8-12 amino acid long peptide antigen.

Whereas strictly separated pathways for processing exogenous and endogenous antigens would explain the difficulties in inducing CTL responses with recombinant protein vaccines, specialized antigen presenting cells (APCs) seem to be capable of class I-restricted presentation of antigens acquired from extracellular spaces. Immature DCs as well as macrophages have been described to simultaneously process and present MHC I as well as MHC II epitopes of a single protein antigen *via* a process termed cross-priming [31-34]. In the context of DNA vaccination, transfected muscle or skin cells may effectively shuttle antigen to APCs to achieve CTL induction *in vivo*. Cross-

priming processes may improve interaction of antigen-specific CD4⁺ and CD8⁺ T cells, resulting in enhanced immune mechanisms due to activation of both T cell subsets.

In addition to the presentation of an antigen by a professional APC, activation of T cells also requires the co-delivery of certain co-stimulatory molecules like B7.1 (CD80), B7.2 (CD86) and CD40. Lack of these signals results in anergy or activation induced cell death by apoptosis [35]. Usually, APCs can only provide the proper co-stimuli when they have been activated by so called "danger" signals. A number of publications have demonstrated that DCs are the most effective cell type to respond to various "danger" signals [36-38]. In general, such signals are the result of tissue and/or cell damage, but also molecules of bacterial origin such as lipopolysaccharide (LPS) and even bacterial DNA itself can act as danger signals as will be discussed in detail below. The latter seem to reflect the interaction of certain pathogens with the immune system in an evolutionary context. Once activated, DCs can capture antigens in the periphery and subsequently migrate to T cell areas of lymphoid organs such as lymph node or spleen, where activation of T cells takes place.

Since most of the early DNA vaccination studies have been performed by i.m. injection it was difficult to explain the activation of both branches of the immune system. Because Wolff *et al.* observed long-term gene expression and

translation of protein in transfected myocytes [39], it has initially been proposed that these cells act as APCs and present plasmid derived protein to CTLs, whereas released antigen would be responsible for the activation of T helper and B cells. However, since myocytes lack the necessary co-stimulatory molecules, presentation by this cell class would be expected to cause immunological ignorance or tolerance. Indeed, several publications demonstrated that the induction of cell-mediated immune responses after DNA vaccination required antigen presentation by professional APCs [40-44]. These cells can either be directly transfected by the DNA vaccine or take up soluble antigen from transfected bystander cells such as myocytes or keratinocytes (or from the lymph drainage into the lymph node) and present the acquired protein on MHC class II and also MHC I through cross-priming.

Modification of DNA vaccines provides the unique opportunity to favor either the cytosolic or endocytotic pathway of antigen processing, thereby eliciting the desired type of immune response or improving the immunogenicity [15, 45-47].

"ANTI-ALLERGIC" PROPERTIES OF DNA VACCINES

In principle, allergic diseases are disorders of the immune system associated with a dysregulation of the Th1 and Th2 lymphocyte subsets [48-50]. It has been postulated that with a declining incidence of infectious diseases due to vaccination, the use of antibiotics, and other public health practices, a major source of Th1 immune provocation has been lost, with a consequent increase in the Th2 bias of immune responses towards environmental allergens (hygiene hypothesis) [51, 52]. DNA vaccines offer a challenging new approach for specific immunotherapy (SIT) to redirect the allergic immune response.

Plasmid DNA alone has been found to drive a Th1 response. This effect has been attributed to immunostimulatory DNA sequences containing unmethylated CpG motifs. The immunostimulatory properties of bacterial DNA were first discovered in the 1980s by Tokunaga and Yamamoto [53, 54]. They showed that DNA purified from *Mycobacterium bovis* induced limited anti-tumor activity due to natural killer (NK) cell activation and IFN- α/β production. Immunostimulatory DNA sequences were found to contain unmethylated CpG dinucleotides within a palindromic hexamer following the formula: 5'-purine-purine-CG-pyrimidine-pyrimidine-3' [55]. CpG dinucleotides occur less frequently in eukaryotic DNA compared to prokaryotic genomes. Additionally, less than 5% of the cytosines on prokaryotic CpG dinucleotides are methylated, but 70-80% of CpG dinucleotides in eukaryotic genomes contain a methylated cytosine [56, 57]. Studies with synthetic phosphodiester and phosphorothioate oligodeoxynucleotides (ODNs) revealed that base sequence as well as backbone-chemistry influence immune activation, with effects on cytokine production, proliferation, and expression of surface molecules [58]. With regards to developing human vaccines, it has to be noted that CpG motifs are species specific [59-62]. Nevertheless, recent studies by Kandimalla *et al.* demonstrate that species-specific recognition of CpG motifs can be overcome by

substituting the C in CpG with a bicyclic heterobase [1-(2'-deoxy-beta-d-ribofuranosyl)-2-oxo-7-deaza-8-methyl-purine] [63, 64].

Signal Transduction Induced by CpG Motifs

APCs like macrophages or DCs have to be activated by "danger" signals in order to be able to generate adaptive immune responses since they lack the highly specific antigen receptors of T and B cells. They recognize pathogen derived molecular structures *via* a set of pattern recognition receptors (PRRs). Examples of PRRs are the mannose receptor [65], CD14 [66] and several members of the Toll-like receptor (TLR) family.

Immune activation of B cells, DCs, and NK cells by CpG DNA has been shown to be dependent on TLR-9 [67]. CpG mediated intracellular signaling requires endocytosis of CpG ODN and acidification of endosomes [68]. However, the mechanisms of ODN uptake by lymphocytes remain unclear. While it has been previously shown that uptake of ODNs is a sequence-independent active process [69], a recent publication indicated that TLR-9 expression enhances the uptake of CpG DNA but not non-CpG DNA [62]. Signal transduction involves the myeloid differentiation factor MyD88, IRAK and TRAF6, activation of I κ B kinase, mitogen-activated protein kinases, the stress kinases N-terminal c-Jun kinases JNK1/2 and p38 and, finally, results in transcriptional activation of multiple genes involving ATF-2, AP-1 and NF- κ B ([70-72]. Many immunoregulatory genes contain NF- κ B responsive elements in their promoter regions and it has been shown that inhibition of NF- κ B also abolishes CpG mediated immune cell activation [73].

Cellular Responses to CpG DNA

CpG DNA has been shown to activate a variety of immune cell species. It directly induces professional APCs (macrophages and DCs) to secrete a panel of cytokines, including type-1 interferons (IFN- α/β), TNF- α , interleukin (IL)-6 and IL-12 [72, 74-77], and indirectly stimulates NK cells through IFN- α/β and IL-12 to produce IFN- γ [78]. CpG DNA also directly activates B cells to proliferate or secrete antibodies [55] and can stimulate T cells directly or indirectly, depending on their activation state [79]. Finally, CpG-induced cytokines result in enhanced antigen presentation by APCs [80] and, in humans, facilitate Th1 cell development by promoting the expression of IL-12 receptor on these cells [81].

As summarized in Fig. (2), the activation of immune cells by CpG DNA initiates a complex network of cell-cell interactions and cytokine production cascades that result in an overall enhancement of immune functions in an antigen-independent manner. Thus, CpG DNA has been shown in a multitude of independent studies to be a potent adjuvant when applied prior to or together with protein or DNA vaccines.

Effects of CpG DNA on the Th1/Th2 Cytokine Balance

Helper T cells differentiate into several functionally different subsets, among them Th1 and Th2 lymphocytes, which are characterized by their cytokine profile [82]. Th1

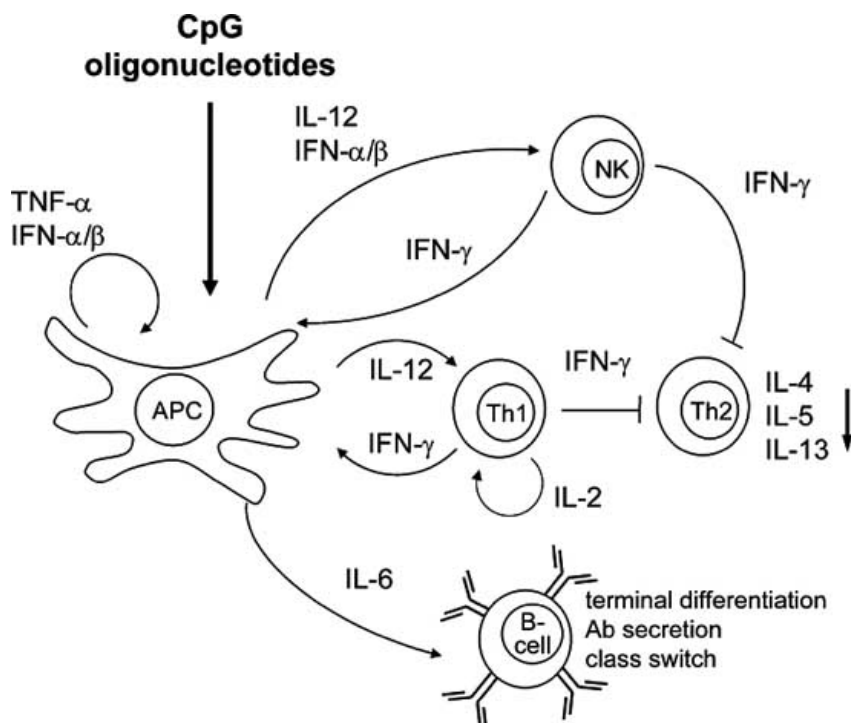


Fig. (2). A simplified diagram of the most relevant events occurring in response to CpG DNA. The term "APC" refers to either macrophages or dendritic cells or both. Arrows indicate the positive feedback loops between macrophages and/or dendritic cells and NK or Th1 cells mediated by IL-12 and IFN- γ .

cells produce IFN- γ whereas IL-4, IL-5 and IL-13 are products typical for Th2 cells. Although several factors have been shown to influence the differentiation of Th1 and Th2 cells, the cytokine milieu present at the time of initial T cell activation appears to be the most important [83, 84]. While IL-12 facilitates differentiation towards a Th1 type *via* activation of STAT4, IL-4 allows for Th2 differentiation *via* activation of STAT6. These cytokines - directly or indirectly - also control and stabilize the Th1/2 balance of an immune response. 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. In contrast IL-4 and IL-13 partially inhibit IL-12 production. This consequently suppresses the development of Th1 cells.

Certain transcription factors seem to have causal roles in the gene expression programs of Th1 and Th2 cells. T-box transcription factor T-bet has been demonstrated to play a central role in Th1-cell development *via* remodeling of the locus encoding IFN- γ as well as inducing selective responsiveness to the growth factor IL-12. In contrast, the zinc-finger transcription factor GATA3 seems to be crucial for inducing transcriptional competence of the Th2 cytokine cluster including genes for IL-13, IL-4 and IL-5 [85].

Both helper T cell subsets are associated with distinct effector functions: A Th2 profile leads to immunoglobulin class switching to IgG1 and IgE in mice and IgG4 and IgE in humans and, therefore, mediates IgE and eosinophil/mast cell-dependent immunity. This type of response is important for ridding parasite infections but is also implicated in hypersensitivity responses which characterize asthma [86], allergic conjunctivitis [87], and anaphylaxis [88].

In contrast, Th1 immune reactions are characterized by Th1-antibody production by B cells (IgG2a in mouse), activation of NK cells and phagocytic cells, and the development of CTL-mediated immunity against viral infections or malignant cells.

The Complex Adjuvant Role of CpG Motifs in Genetic Immunization

Because plasmids used for genetic immunization represent bacterial DNA containing various amounts of immunostimulatory CpG motifs, generation of Th1 type responses is a general property of DNA vaccines. Thus, DNA vaccines concurrently function as immunogens and adjuvants.

We and other groups have shown that increasing the number of certain CpG motifs in plasmids used for genetic immunization enhances immunogenicity and the Th1 bias [89-91]. The double-edged role of CpG motifs is demonstrated by the fact, that certain motifs can suppress the adjuvant effect of immunostimulatory CpG DNA (e.g. CpG motifs within adenoviral DNA). Removal of such neutralizing CpG motifs from immunization vectors enhanced both, IgG2a/IgG1 ratio as well as CTL activity [91].

A unique feature of CpG DNA based adjuvants, even compared to other bacterial-derived adjuvants, is their high potential to induce IL-12, the central mediator of Th1 reactions [92, 93]. This is of special interest with respect to treatment of allergy and tumor immunotherapy. However, the general downstream events of CpG DNA and other bacterial products, such as LPS are remarkably similar, and

CpG DNA has been found in multiple studies to induce TNF- α *in vitro* and *in vivo* [72, 75, 94] and hence, it cannot be ruled out that administration of CpG DNA may carry potential risk factors. In certain experimental systems CpG DNA has been shown to induce septic shock symptoms or to promote the development of autoimmunity. Administration of bacterial DNA or CpG oligos caused a rapid increase in TNF- α serum levels and, in galactosamine sensitized mice, resulted in lethal septic shock after 12 – 14 hours [72, 94]. Furthermore, CpG DNA and LPS synergized in the induction of TNF- α [78].

The situation concerning the risk of autoimmune induction by CpG motifs is even more complex. The studies cited below were performed with different experimental systems and provide data ranging from induction of autoimmune reaction to protection against autologous responses. Immunization of NZB/W mice with E.coli DNA but not with calf thymus DNA resulted in elevated levels of anti DNA-antibodies. However, increased anti-DNA antibodies only seem to be no indication for functional autoimmunity as demonstrated with NZB/W mice, which are prone to a systemic lupus erythematosus-like syndrome and, therefore, are frequently used as an animal model for autoimmune disease. Despite the fact that bacterial DNA immunization yielded increased levels of antibodies to autologous DNA, this treatment even improved survival and reduced the autoimmune symptoms in these animals [95]. On the other hand, CpG DNA induced experimental autoimmune encephalomyelitis by activation of Th1 effector cells specific for myelin basic protein and this effect was dependent on the CpG DNA-mediated induction IL-12 [96]. However, no toxicity has ever been reported in normal animals treated with therapeutic doses of DNA vaccines or CpG ODNs. In addition, hundreds of human volunteers have been exposed to genetic vaccines without serious adverse consequences.

As a conclusion from the numerous experiments with CpG motifs, it can be stated that since the discovery of the immunostimulatory properties of bacterial DNA twenty years ago, much has been learned about structure/function relationships of different CpG motifs, about their action on individual immune cell subpopulations and the overall efficacy of CpG DNA as a Th1 adjuvant.

It has been suggested by a series of independent studies that CpG based adjuvants may be more attractive for clinical applications compared to other microbial derivatives because of their comparably lower toxicity while simultaneously providing large amounts of IL-12. Although research of CpG adjuvants for allergy immunotherapy is still in its infancy [88, 97], a multitude of experiences with CpG adjuvants in different fields of research justifies the high expectations many scientists have on these approaches. Systematic evaluation of their adjuvant potential compared to toxicity will determine their applicability in human medicine.

FIRST PROOFS OF PRINCIPLE FOR THE ANTI-ALLERGIC PROPERTIES OF DNA VACCINES

Allergic diseases result from immune responses against otherwise harmless environmental antigens, characterised by the generation of Th2 T cells, which produce IL-4 and IL-5 and promote the differentiation of B cells into IgE secreting

cells. These IgE antibodies bind to high affinity Fc ϵ receptors on basophils and mast cells. Allergen exposure leads to binding of allergen molecules by surface IgE and crosslinking of the Fc ϵ receptors, causing activation and degranulation of basophils and mast cells. The latter release a variety of preformed proinflammatory and vasoactive compounds such as histamine, prostaglandins, leukotrienes and cytokines, leading to an inflammatory response.

Whereas the majority of current therapeutic efforts against allergic diseases intend to control the symptoms triggered by basophil and mast cell degranulation, a more profound approach would aim at preventing the generation of Th2 immune responses against the allergen or to induce a Th1-like balancing response against the allergen. During classical SIT with allergen extracts formation of "blocking IgG antibodies" can mask allergic epitopes and prevent allergen induced cross-linking of IgE [98-100]. Immunological changes observed in context with successful immunotherapy also include immune deviation towards a Th1 type response characterized by increased levels of IgG2a and IFN- γ [101-104], and the induction of immunological tolerance as shown by increased levels of IL-10 secreting CD4+CD25+ T-cells [105-107].

In contrast, the anti-allergic effects of DNA vaccines could be clearly attributed to their Th1 inducing capacity as there is no evidence for the induction of IL-10 secreting T regulatory cells [108]. In 1996, the first study suggesting that immunization with DNA coding for allergens might provide a novel type of immunotherapy for allergic diseases was published [109]. The authors compared antigen-specific antibody isotypes and cytokine secretion by CD4+ T cells in BALB/c mice immunized i.d. with either recombinant β -galactosidase or plasmid DNA encoding β -galactosidase (pCMV-LacZ). Whereas pCMV-LacZ induced mainly IgG2a, protein in saline or alum induced IgG1 and IgE β -gal-specific antibodies. In addition, splenic CD4+ Th cells isolated from DNA-immunized mice secreted IFN- γ but not IL-4 and IL-5, whereas Th cells from β -gal-injected mice secreted IL-4 and IL-5 but not IFN- γ after *in vitro* stimulation with antigen. These results demonstrated that DNA immunization induced a Th1 response, whereas protein immunization induced a Th2 response to the same antigen. Priming of mice with pCMV-LacZ prevented IgE antibody formation to a subsequent intraperitoneal (i.p.) β -gal in alum injection in an antigen-specific manner. Most importantly, i.d. immunization with pCMV-LacZ of β -gal in alum-primed mice caused a 66-75% reduction of the IgE anti- β -gal titer in 6 weeks. Additionally, pCMV-LacZ induced specific IgG2a antibody titers and IFN- γ secretion by Th cells in the β -gal in alum-primed mice. The data clearly indicated that genetic immunization induced a Th1 response that dominated over an ongoing protein-induced Th2 response in an antigen-specific manner.

The first demonstration of the anti-allergic effect of DNA vaccines with a clinically relevant allergen was performed with the dust mite protein Der p 5 [110]. Intramuscular injection of rats with plasmid DNA encoding Der p 5 not only resulted in its long-term expression and the induction of specific immune responses, but prevented the induction of IgE synthesis, histamine release in bronchoalveolar fluids and airway hyperresponsiveness in rats challenged with

aerosolized allergen. Moreover, this suppression was persistent and could be transferred into naive rats by CD8⁺ T cells from gene-immunized animals.

In recent years, a number of clinically relevant allergens have been tested in DNA vaccine studies. In our laboratory, the immune responses after DNA immunization with the major allergen of birch pollen Bet v 1a have been characterized [30, 111]. Evaluation of the immune responses induced by plasmid DNA encoding Bet v 1a (pCMV-Bet) and recombinant Bet v 1a in a mouse system was performed *via* i.d. and i.p. injection, respectively. The primary humoral response following a single immunization with pCMV-Bet was weak, but could be boosted to high antibody levels by two additional injections. On the other hand, proliferation assays of splenocytes and measurement of cytokine levels indicated a cellular response even after the first injection of plasmid DNA. After two immunizations with pCMV-Bet, the ratio of IgG1 to IgG2a indicated a clear Th1 subclass profile. IgE was not detectable at any time during the immune reaction. Accordingly, IL-4 levels were markedly reduced in sera as well as in supernatants of stimulated splenocytes, whereas levels of IFN- γ were elevated. Immunization with recombinant Bet v 1a protein elicited a strong Th2 -type response, including IgE production, high titers of IgG1, and IL-4 production in both serum and supernatants of proliferation cultures.

A comparison of DNA vaccines encoding different isoforms of Bet v 1 elicited differences in the immunogenic nature of isoforms [111]. Mice were injected i.d. with plasmid DNA encoding the genes of Bet v 1a (pCMV-Bet a) and Bet v 1d (pCMV-Bet d). The two isoforms induced a similar humoral response characterized by the lack of IgE production and a ratio of IgG1 to IgG2a indicating Th-1-type responses. Antisera against both isoforms were highly cross-reactive. Quantification of IFN- γ and IL-4 in sera revealed remarkably different cytokine profiles during the course of the immune response. In contrast to pCMV-Bet a, pCMV-Bet d caused no significant allergen-specific proliferation and induced only marginal levels of the key cytokines.

DNA vaccination was also performed with the gene of the major cow's milk allergen bovine β -lactoglobulin (BLG) employing a construct, which allowed the production of a partially secreted product. The primary response in mice immunized with BLG-encoding plasmid was of the Th1 type. A native form of BLG was only partially recognized by sera of DNA vaccinated mice, whereas sera of BLG-in-alum-immunized mice displayed a broader range of recognition. The study also revealed an inhibitory effect of DNA immunization on the Th2 response induced by subsequent immunization using BLG adsorbed to alum. This inhibition was vastly dependent on the time of pre-administration of the genetic vaccine, with an optimal effect when DNA immunization occurred at least 21 days before protein administration. The preventive effect was characterized by inhibition of BLG-specific IgE, induction of specific IgG2a and a decrease of the specific IgG1/IgG2 ratio, accompanied by an increase in IFN- γ and IL-10 secretion. Prevention was shown to persist even after a booster immunization with alum-adsorbed BLG [112].

The question whether DNA vaccines encoding only T cell epitopes suppress allergic reactions as effectively as

plasmids encoding whole allergens was evaluated with a construct encoding five murine T cell epitopes from the house dust mite allergens Der p 1 and Der p 2 [113]. Mice were immunized i.m. three times at weekly intervals, sensitized twice and then had to inhale Der p extracts intranasally six times at weekly intervals. Vaccinated mice showed a significantly attenuated induction of Der p-specific immunoglobulin E synthesis and more prominent Der p-specific IgG2a antibody response compared to controls. Analysis of the cytokine profile after Der p stimulation of the lymph-node cells revealed that the level of the mRNA expression of the IFN- γ gene was higher in the vaccinated mice than in controls. Histologic studies showed a greatly reduced infiltration of inflammatory cells in lung tissue of the gene-vaccinated mice in comparison to controls.

Another allergen of the house dust mite, the high-molecular-weight Der f 11, was also used to develop a DNA vaccine [114]. Vaccination of mice with plasmid DNA encoding Der f 11 induced Th1 responses characterized by IgG2a responses and spleen cell secretion of IFN- γ . In contrast, sensitization with recombinant Der f 11 and alum induced Th2 responses characterized by IgE responses and spleen cell secretion of IL-4 and IL-5. DNA vaccination with Der f 11 prevented the induction of IgE responses and reduced on-going IgE responses. Sensitization of plasmid-immunized mice after depletion of CD8⁺ T cells still showed suppression of IgE responses. Adoptive transfer of either CD4⁻ or CD8-depleted spleen cells from plasmid-vaccinated mice reduced IgE responses thus indicating that obviously both CD4⁺ and CD8⁺ T cells play an important role in the immunomodulation of IgE responses by DNA vaccines.

Recently, Jacquet *et al.* evaluated the outcome of DNA immunization with a construct coding for the major mite allergen precursor ProDer p 1 [115]. The DNA vaccine was constructed on the basis of a synthetic cDNA encoding ProDer p 1 with optimized codon usage. The immunogenicity of ProDer p 1 DNA in mice was compared with that of purified natural Der p 1 or recombinant ProDer p 1 adjuvanted with alum. Subsequently, vaccinated mice were exposed to aerosolized house dust mite extracts to provoke airway inflammation. Naive mice immunized with ProDer p 1 DNA developed Th1 immune responses characterized by high titers of specific IgG2a antibodies, low titers of specific IgG1 and the absence of anti-ProDer p 1 IgE. Natural Der p 1 or recombinant ProDer p 1 adsorbed to alum induced pronounced Th2 allergic responses with strong specific IgG1 and IgE titers. Spleen cells from DNA ProDer p 1-vaccinated mice secreted high levels of IFN- γ and displayed low production of IL-5. In contrast, both forms of the protein allergen stimulated typical Th2-type cytokine profiles characterized by high levels of IL-5 and low levels of IFN- γ . Whereas eosinophilia in bronchoalveolar lavages was clearly observed in Der p 1-immunized animals, ProDer p 1 DNA immunization as well as ProDer p 1 protein vaccinations prevented airway eosinophil infiltrations.

Der p 1 is a cysteine protease and may cause unpredictable catalytic effects when used as a vaccine. In order to avoid unwanted effects, gene vaccines expressing enzymatically inactive forms of Der p 1 have been designed [116].

An interesting immunoprophylactic strategy using oral allergen-gene immunization to modulate peanut antigen-induced murine anaphylactic responses was adopted by Roy *et al.* [117]. Oral administration of DNA nanoparticles consisting of plasmid DNA complexed with chitosan, a natural biocompatible polysaccharide, resulted in transduced gene expression in the intestinal epithelium. Mice receiving nanoparticles, which contained the gene encoding the dominant peanut allergen Ara h 2 produced secretory IgA and serum IgG2a. In comparison to non-immunized mice or mice vaccinated with "naked" DNA, mice immunized with nanoparticles displayed a substantial reduction in allergen-induced anaphylaxis associated with reduced levels of IgE, plasma histamine and vascular leakage. The results indicated that prophylactic oral allergen-gene immunization with chitosan-DNA nanoparticles may be a useful strategy against food allergy.

THE STATE OF THE ART OF ALLERGEN-DNA VACCINES

One of the greatest advantages of DNA vaccines is the opportunity to easily modify them by inserting special DNA sequences which enable modulation and tailor-made immune reactions. Over the last years, a number of studies have introduced new methods to increase the immunogenicity of DNA vaccine formulations, enhance the Th1 bias and increase the safety of anti-allergic plasmid vaccines. Nevertheless, there are still numerous possibilities to further improve DNA vaccines with regard to the development of clinically administrable genetic therapeutics against type 1 allergies.

Harmonization of the Codon Usage

The pattern of codon usage differs between different genes and different organisms. This bias in codon usage has been attributed to a variety of selective evolutionary pressures, most notable of which is translational efficiency. Codon usage has been correlated with gene expression levels, tissue-specific patterns of expression, the degree of evolutionary conservation of proteins, and the overall or regional nucleotide composition of the genome. Codon usage is least well understood in higher organisms. Because the codons of (plant)-allergen genes are usually suboptimal with regard to the codon usage of mammalian cells, these allergens are often only poorly expressed in the mammalian host.

The expression level of antigens encoded by DNA vaccines is influenced predominantly by two parameters: the vector itself and the codon usage. With respect to the former, a panel of optimized vectors with sophisticated features has been developed in the past decade and most of them are now commercially available. In contrast, however, the aspect of codon usage has largely been ignored. Sub-optimal codon usage after transfection of mammalian cells with heterologous genes (such as genes encoding plant allergens) can severely diminish protein expression. This problem was demonstrated with DNA vaccines encoding the major mite allergen precursor ProDer p 1 [115, 118] and the major mugwort allergen Art v 1 [119]. Constructs encoding the plant allergen Art v 1 revealed no or only marginal immune responses. Immunogenicity of the Art v 1 DNA vaccine

could not be enhanced by well-established immune modulations such as the addition of a eukaryotic leader sequence, co-injection of a GM-CSF encoding plasmid as adjuvant or cationic liposome-mediated i.d. gene delivery [15, 120, 121]. Analysis of the gene sequence for the allergen Art v 1 revealed that the codon usage frequencies strikingly differed from those prevalent in mammals [122]. Therefore, we replaced each codon of Art v 1 with the codon displaying the highest frequency for the respective amino acid in mammals. This resulted in a synthetic (re-coded) gene encoding the amino acid sequence of Art v 1 with the mammalian codon usage. The optimized Art v 1 gene has a high GC content (71%) and a distinct codon bias for guanosine and cytosine at the third codon position (86.9% G or C at the third codon position) compared to the wildtype Art v 1 gene (53% GC and only 35.5 % G or C at the third codon position). Compared to the wild-type gene, protein expression with the re-coded gene was increased about 180-fold and i.d. DNA immunization displayed strong immunogenicity. The type of immune response induced by the re-coded DNA vaccine fulfilled the criteria necessary for allergen DNA vaccines i.e. a Th1-biased antibody profile with high IgG2a titers, no or only marginal IgE levels and antigen-specific IFN- γ expression. Despite its strong immunogenicity the re-coded construct did not induce release-mediating IgE antibodies as measured by a rat basophil leukemia cell release assay [119].

Th1/Th2 Modulation of Allergy DNA Vaccines

The rationale of therapeutic DNA vaccines against allergies is based on the induction of a Th1 type cellular immune response, thereby suppressing or converting the allergic Th2 type response. For this purpose, Th1 promoting immunization strategies, such as the additional delivery of immunostimulatory DNA sequences (CpG motifs), Th1-stimulating cytokines or ubiquitination of the allergen are promising approaches. However, as discussed above, the potential risk of autoimmune reaction when combining several Th1 stimulating agents has to be closely monitored.

We have shown that co-injection of CpG- ODNs along with a plasmid DNA vaccine represents a simple approach to increase the Th1 bias of an allergy DNA vaccine. The number of CpG motifs in a plasmid DNA vaccine can also be increased by appending a CpG-rich sequence to the gene of interest. We have attached a CpG motif encoding the amino acid sequence Tyr-Asn-Asn-Val-Asn-Val-Asp-Val-Asp-Val to the carboxyterminus of the Bet v 1a gene. This sequence contains four CpG motifs and does not disturb the correct folding of the protein. Both approaches increased the Th1 bias of the immune response with respect to humoral as well as to cellular responses [30, 111].

Depending on the immunization schedule, the strength of non-specific Th1-mediating signals, such as the number of immunostimulatory CpG motifs within the backbone and/or gene of interest, can have different influences on the protective and therapeutic efficacy of DNA vaccines [112]. This may also account for the recently reported antigen-independent anti-allergic properties of a DNA vaccine directed against bee venom Phospholipase A (PLA), one of the major honey bee venom allergens for humans. In this study, the authors employed a mouse model using empty or

PLA sequence-carrying DNA plasmids in order to assess long-term prevention of allergic reactions by DNA vaccination [123]. Immunization into the skin with either DNA construct before or after sensitization with PLA plus alum led to reduced PLA-specific IgE and IgG1 titers, with concomitant rise in IgG2a and IgG3. Splenocytes recovered at 5-6 months after the last DNA administration showed a sustained IFN- γ and IL-10 secretion and reduced IL-4 production. Re-challenge with PLA boosted IFN- γ and IL-10 secretion, suggesting the reactivation of quiescent memory Th1 lymphocytes. Mice from the prophylactic groups were fully protected against anaphylaxis, whereas in the therapeutic groups 65% of the animals recovered. Interestingly, Th1-polarized immune responses were also noticeable in mice vaccinated with empty plasmid 32 weeks before sensitization with another antigen (ovalbumin) pointing to an antigen-independent immune modulation by CpG motifs [124, 125].

Cytokines attached to the antigen may influence APC or responding T cells to drive the immune response toward a Th1 or Th2 direction, and/or potentiate it in an antigen-specific manner. To evaluate this concept for DNA immunization against the model allergen ovalbumin, expression vectors containing the ovalbumin gene either alone, or linked to cytokine genes including GM-CSF, IFN- γ , IL-2, IL-4, IL-12, or a sequence encoding nine amino acids of IL-1 β were constructed and i.m. injected into mice [126]. Lymphocytes from mice vaccinated with OVA-IL-12 and OVA-IL-1 β constructs produced more IFN- γ and less IL-4 during *in vitro* re-stimulation assays than did other groups. All constructs elicited OVA-specific cytotoxic responses, which were maintained or even increased over 16 weeks. The OVA-IL-12 and OVA-IL-1 β peptide constructs elicited the strongest cytotoxic responses at 2 weeks post-injection.

A similar approach was performed to enhance the efficacy of therapeutic DNA vaccines and to enable the treatment of established airway hyperresponsiveness with a plasmid containing OVA cDNA fused to IL-18 cDNA [127]. Vaccination of mice either with this fusion construct or with an OVA cDNA-containing plasmid protected mice from subsequent induction of airway hyperreactivity correlating with increased IFN- γ and reduced OVA-specific IgE production. As treatment of mice with neutralizing anti-IFN- γ mAb or with depleting anti-CD8 mAb abolished the protective effect, protection seemed to be mediated by IFN- γ and CD8 $^{+}$ cells. Moreover, vaccination of mice with preexisting hyperresponsiveness with the OVA-IL-18 fusion DNA, but not with the OVA cDNA plasmid, reversed established hyperreactivity, reduced allergen-specific IL-4, and increased allergen-specific IFN- γ production.

However, recent reports indicated a two-edged role for IL-18 which can also stimulate IL-4 production from basophils and CD4 $^{+}$ T cells as well as IL-13 production by mast cells [128, 129]. In addition, IL-18 has also been shown to indirectly induce B cell isotype switching to IgE and together with its effects on Th2 cytokine production, has been demonstrated to play a role in allergic inflammation [130].

Another approach utilized the Th1-inducing capacity of viral vector vaccines. In this study, a replication-deficient

adenovirus vector was used to evaluate the *in vivo* efficacy of preventive and therapeutic genetic immunization in a mouse model of type I allergy [131]. Immunization with recombinant adenovirus expressing the model antigen β -galactosidase induced a Th1 immune response with predominantly IgG2a antibodies, a high frequency of IFN- γ producing T cells and large numbers of cytotoxic T lymphocytes. Preventive immunization with the viral vector abolished the production of specific IgE after subsequent immunization with recombinant β -gal protein, and changed the Th2-biased immune response into a Th1-orientated reaction. However, therapeutic administration of the viral vector expressing β -gal after sensitization with β -gal protein neither significantly inhibited ongoing IgE production nor induced a noticeable Th1 immune response.

DNA Vaccines Encoding Hypoallergenic Allergen Derivatives

A major drawback of SIT is the frequent occurrence of side effects, essentially anaphylactic reactions caused by cross-linking of pre-existing IgE antibodies on mast cells. Moreover, the injection of high doses of antigen can lead to anaphylaxis during the course of treatment *via* induction of „therapy-induced“ IgE antibodies.

DNA vaccines in general avoid these unwanted side effects for the following reasons: The amount of translated antigen after DNA immunization into the skin is at least three to five orders of magnitude lower than the amount of protein injected during a typical SIT protocol. Due to the Th1-biased immune response type of i.d. or i.m. application of DNA vaccines no *de novo* synthesis of IgE antibodies occurs. Furthermore, while SIT with allergen extracts potentially induces allergic reactions against impurities of the extract, this risk is ruled out by the high purity of allergens expressed by DNA vaccines.

Nevertheless, before entering clinical trials, the safety of anti-allergic DNA vaccines has to be increased. Translation of native allergenic determinants must be avoided in order to prevent anaphylactic responses. However, in order to guarantee the recruitment of allergen-specific Th1 cells, T cell epitopes of the encoded allergen must not be destroyed.

Using the major birch pollen allergen Bet v 1, we evaluated two approaches to meet these requirements: (a) cutting the allergen gene into overlapping fragments, which lack any antigenic determinant of the native allergen, but display the original repertoire of T cell epitopes, and (b) employing artificial hypoallergenic derivatives. For this purpose, DNA vaccines coding for two hypoallergenic Bet v 1 fragments [132] as well as for an artificial hypoallergenic Bet v 1 mutant [133] have been constructed.

Immunization with both types of vaccines protected in an antigen-specific manner from IgE production and IgE-mediated cell release after repeated sensitization with recombinant allergen. Compared to the wild-type DNA vaccine which induced a strong humoral response, the DNA vaccine encoding the hypoallergenic mutant of Bet v 1 triggered a reduced and transient antibody response against wild-type Bet v 1, thus indicating the altered allergenic and immunogenic properties of the mutant allergen. The vaccine encoding the two hypoallergenic fragments of Bet v 1

stimulated no antibody response against Bet v 1 confirming that the fragments do not display allergy-associated epitopes present on the entire wild-type Bet v 1 molecule. However, the fragment vaccine triggered the recruitment of Bet v 1-specific Th1 cells as indicated by IgG2a antibodies, IgE suppression and antigen-specific IFN- γ production after sensitization with the native allergen. A therapeutic approach with sensitization followed by DNA immunization with the fragment-DNA vaccine revealed a remarkable and sustained antigen-specific reduction of IgE and IgE-mediated release, thus confirming the excellent therapeutic capacity of this type of DNA vaccine [29].

NEW APPROACHES AND "NEXT GENERATION" ALLERGEN-DNA VACCINES

Knowledge-based approaches to reduce anaphylactic properties of proteins by fragmentation or deliberate mutation, as described above, are time consuming and their suitability must be tested on a case-by-case basis.

Routine Production of Hypoallergenic DNA Vaccines

Therefore, we used ubiquitination of DNA vaccines as an alternative to develop a routine approach for destroying IgE-binding epitopes on allergens in order to avoid recognition by pre-existing IgE antibodies. Simultaneously, any T cell epitope of the allergen would be preserved. This approach takes advantage of the fact that ubiquitination leads to proteasomal degradation of translated gene products. Resulting peptides are then transported by the Tap1/Tap2 heterodimer into the ER, where they associate with MHC-I molecules and β 2-microglobulin, which are in turn delivered to the cell surface for recognition by CD8⁺ T cells. However, presentation of these peptides is obviously not restricted to MHC class I. A growing number of studies have identified significant amounts of peptides derived from cytosolic and nuclear proteins which were presented on MHC class II by autophagy [134].

Under normal conditions only a small percentage of plasmid-encoded proteins undergo ubiquitination following DNA immunization. Stably linking ubiquitin to the antigen of interest has been demonstrated to efficiently channel the hybrid protein into the polyubiquitination pathway. As a result, the immune response is shifted towards a Th1-type and, simultaneously, antibody reactions are abolished while cytotoxic T cell responses are enhanced [135]. To take advantage of this mechanism, we constructed a DNA vaccine encoding a Bet v 1-ubiquitin fusion molecule. Immunization with this vector yielded no antibody production (confirming the expected degradation of the allergen), but resulted in a 100% suppression of IgE antibodies and IgE-mediated cell release in a protective experimental design. In a therapeutic setting, IgE production and IgE-mediated cell release after sensitization were markedly reduced. Treatment with the DNA vaccine thus clearly demonstrated the anti-allergic efficacy of hypoallergenic ubiquitin-fusion constructs [136, 137].

Vaccine Dose Reduction with Self-Replicating Vaccines

The high amount of plasmid DNA necessary for i.m. or i.d. needle injection still remains the major shortcoming of

DNA vaccines for allergy treatment. Using a biolistic device (such as the gene gun) enables to induce strong immune responses with 100fold lower quantities of plasmid DNA compared to needle injection. Unfortunately, immunization with gene gun results in a serological Th2-biased immune response type with the production of high amounts of IgG1 and even IgE antibodies [138-140]. This renders biolistic devices useless or at least questionable for any application concerning protection and/or therapy of allergic diseases.

The principle of self-replicating RNA offered a solution to the dose problem and led to the development of a novel type of genetic vaccines. The genome of alphaviruses encodes a replicase enzyme used by the virus to efficiently replicate the viral genome inside the host cell cytoplasm. In theory, host cells only need to pick up a single molecule of this self-replicating RNA and still produce sufficient amounts of antigen to induce an immune response. It has been previously shown that antigen production in cells transfected with self-replicating RNA is rather independent of the amount of RNA or DNA used for transfection [141]. Similar to infection of cells with alphaviruses, transfection of mammalian cells with self-replicating RNA based on alphaviral replicase, causes host cell apoptosis [142]. This is believed to be a protective response of the host cell attempting to limit viral spread. Therefore, it is assumed that self-replicating RNA vaccines achieve their high effectiveness by mimicking a viral infection of the transfected host cells. This might trigger powerful antiviral host pathways that would enhance the immune response to the antigen encoded by the self-replicating RNA.

RNA stability still remains a problem for these vaccines during *in vitro* production. To facilitate and simplify vaccine production and handling as well as the storage requirements for RNA, the sequence of the self-replicating RNA molecule is reverse-transcribed into DNA and cloned into a conventional expression plasmid vector. The self-replicating RNA is then produced by cells transfected with this plasmid. DNA replicons have successfully been applied in tumor models [142, 143] with humoral and cellular immune reactions displaying Th1-biased profiles. The Th1 type response was not induced by CpG motifs within the plasmid DNA because the amount of plasmid DNA used for immunization with DNA replicons (10 ng – 1 μ g plasmid DNA) is far below the effective concentration of Th1-inducing CpG motifs. The anti-viral defense pathways of mammalian cells and the activation of antigen presenting cells *via* immunostimulatory signals displayed during apoptosis, obviously display a Th1-biased danger signal [144, 145].

I.d. injection of nanogram quantities of a DNA replicon vaccine encoding β -galactosidase was sufficient to trigger a Th1-biased immune response as indicated by IgG2a antibody and IFN- γ expression and a lack of IgE and IL-5 production. FACS analysis revealed both CD4⁺ as well as CD8⁺ cells as the source of IFN- γ production. Moreover, two injections of DNA replicons were sufficient to protect against the induction of an allergic immune response after sensitization with recombinant β -galactosidase. Vaccination induced 100% inhibition of cross-linking IgE antibodies as detected with a sensitive rat basophil leukemia cell release assay. The protective efficacy of DNA replicons is not restricted to

immunodominant antigens such as β -galactosidase, but could also be demonstrated with the clinically highly relevant allergen Bet v 1 (unpublished data).

DNA replicons are the latest generation of DNA vaccines and three aspects render them highly interesting candidates for effective and safety-optimized vaccine approaches against allergy: (a) The very small amounts of plasmid DNA necessary for the induction of anti-allergic immune responses will increase the acceptance of DNA vaccination for allergy treatment, (b) the induction of apoptosis of cells transfected with the DNA replicon vaccine leads to "self-removal" of the vaccine after triggering the immune response and (c) DNA replicon vaccines gain their immunogenicity *via* viral danger signals and induction of anti-viral immune response types. The latter aspect attracted attention since clinical trials with DNA vaccines indicated poor immunogenicity of the constructs in humans in several cases [146]. Differences in the responsiveness of mice and primates to plasmid DNA-related danger signals *via* Toll-like receptors are assumed to be the major reason. In humans, monocytes as well as monocyte-derived DCs do not respond to CpG stimuli [147]. Therefore, toll-like receptor-independent danger signals make DNA replicon vaccines promising candidates for highly immunogenic human vaccines.

DNA Multi-Vaccines

The approaches described above are not restricted to the application of DNA vaccines encoding a single allergen. I.d. injection of a plasmid DNA mixture of the 17kD tree-pollen related family (Bet v 1, Mal d 1, Cor a 1 and Aln g 1) elicited immune responses comparable to that induced by immunization with the single constructs. Both, humoral and cellular responses indicated a Th1-biased reaction with increased IFN- γ and decreased IL-5 expression and various degrees of cross-reactivity between the different allergens. This cross-reaction also proved to have cross-preventive capacity, e.g.: DNA pre-vaccination with Cor a 1 revealed a similar protective efficacy against the challenge with the allergen mixture as pre-immunization with the plasmid mixture. Similar results were found with a DNA multi-vaccine encoding Bet v 1 and its related food allergens Mal d 1, Dau c 1 and Api g 1, and in a separate experiment, with a vaccine mixture coding for the profilins Art v 4, Lyc e 1 and Cap a 2 (unpublished data).

Complex mixtures of DNA vaccines raise the problem that the necessary dose of DNA vaccine is multiplied by the number of components in the vaccine formulation. We addressed this point by creating fusion vaccines containing several allergens arranged in a string-of-bead manner, either by using the respective entire genes (gene A – gene B – gene C) or by splitting genes into fragments (gene A1 – gene B1 – gene C1 – gene A2 – gene B2 – gene C2). With the latter approach we additionally tried to increase the hypoallergenicity of the vaccine by partially destroying the native conformation of the encoded allergens. Preliminary experiments demonstrated the feasibility of this attempt in principle. The whole-gene fusion-vaccine induced antibody responses equal to the multi vaccine or the respective single-gene vaccine. Fusion of gene fragments reduced the antigenicity with respect to IgG responses, indicating the expected loss of native B cell epitopes. Furthermore, both

attempts led to allergen-specific immune responses with the desired Th1 bias pointing to the maintenance of the T cell stimulatory capacity (unpublished data).

CONCLUSION AND PERSPECTIVES

The first decade of DNA vaccines demonstrated the usefulness of this novel vaccination approach and the proof of principle for protective vaccines against numerous infectious diseases in animal models. Amazingly, DNA vaccines not only showed efficacy against highly immunogenic pathogens but they also appeared to be practicable for difficult conditions such as cancer and allergies. One of the advantages of this vaccine type proved to be the "clean" immune response that is induced, i.e., the absence of side effects both in animals and apparently also in humans based on the limited data available so far. However, in the case of weak immunogens it will be necessary to provide the vaccine with additional "information" *via* cytokines, application of co-stimuli and "danger" signals to include strong adjuvant-type signals within the vaccine.

Interestingly, even very simple DNA constructs encoding allergens that are translated in the cytosolic compartment of the cell (because they lack signal sequences for export of the translated gene product) can be highly immunogenic and effective in the treatment of allergies. However, various studies have shown that there are still opportunities for improvement not only in terms of efficacy (e.g. re-coding has hitherto been widely ignored) but also regarding the safety of such vaccines.

The presence of allergen-specific IgE antibodies on mast cells or the induction of an unwanted (IgE) response to allergens by vaccination itself is able to cause anaphylactic responses. We have demonstrated that this serious problem can be avoided by fragmenting the sequence for the allergen and thus destroying its three dimensional structure. However, this approach needs a careful biochemical investigation for each individual allergen fragment in order to determine that no antibodies are induced against native B cell epitopes and that crucial T cell epitopes are still present. Ubiquitination represents an elegant alternative to this time-consuming procedure and seems to offer a general and easy approach for the destruction of potentially anaphylactic properties of DNA vaccines.

An aim of anti-allergic DNA vaccines is the induction of a strong Th1 type cellular immune response, thus suppressing or converting the Th2 type response that is responsible for the high IgE production. Therefore, approaches that drive a Th1 response are potentially useful, such as the additional delivery of immunostimulatory DNA sequences (CpG motifs), Th1-stimulating cytokines or ubiquitination of the allergen. However, it should be emphasized that the intention must not be to trigger devastating Th1 responses. Animal models should be used to carefully investigate combinations of strong Th1 stimuli also under the light of a potential risk to induce autoaggressive reactions. Some studies suggest that allergen specific Th1 responses might lead to acute or chronic Th1 mediated inflammatory diseases at the sites of reoccurring antigen exposure [148-150]. However, those conclusions are based on experiments that transfer relatively large amounts

of monoclonal TCR transgenic T helper 1 cells and challenge with allergen within a short time interval after application of the T cells. There is no evidence that allergen specific T helper 1 cells which are directly generated in the vaccinated animals would also lead to Th1 associated immunopathology after frequent exposure to allergen. Also, numerous phase I/II clinical trials in the fields of cancer, HIV, malaria, and hepatitis B have proven the safety and tolerability of DNA vaccines [151-154].

Better understanding of the signals that trigger innate immune responses will allow us to design more carefully directed strategies. Ideally, such strategies should only induce the desired immune response while avoiding unwanted responses such as the excessive production of inflammatory cytokines. Replicase-based DNA vaccines seem to fulfill this demand by activating innate pathways in the same way as certain viral infections, but without the problems associated with viral vectors. The triggering of pattern recognition receptors [155] and immunostimulatory apoptosis [144], thus stimulating the innate immune system simultaneously with DNA vaccination may give DNA vaccines the necessary "bite" to induce optimized protective and/or therapeutic immune responses. Moreover, a 100-1000fold reduction of the necessary vaccine dose may help to increase the acceptance of DNA vaccines for clinical application in allergology.

Combining the advantages of single optimizing approaches within one construct (e.g. DNA replicons encoding re-coded and ubiquitinated fusion vaccines covering whole groups of allergens) will lead to novel DNA vaccines.

Concluding, the present body of evidence justifies the hope that DNA vaccines will evolve into a valuable alternative to protein-based specific immunotherapy in the future.

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ABBREVIATIONS

i.m.	=	Intramuscular
i.d.	=	Intradermal
i.p.	=	Intraperitoneal
CTL	=	Cytotoxic T lymphocyte
MHC	=	Major histocompatibility complex
ER	=	Endoplasmic reticulum
APC	=	Antigen presenting cell
LPS	=	Lipopolysaccharide
SIT	=	Specific immunotherapy
NK	=	Natural killer
ODN	=	Oligodeoxynucleotide
PRR	=	Pattern recognition receptor

TLR	=	Toll-like receptor
DC	=	Dendritic cell
IFN	=	Interferon
IL	=	Interleukin
BLG	=	β -Lactoglobulin
PLA	=	Phospholipase A

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