Review Article

# DNA Immunization as an Efficient Strategy for Vaccination

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## Abstract

The field of vaccinology provides excellent promises to control different infectious and non-infectious diseases. Genetic immunization as a new tool in this area by using naked DNA has been shown to induce humoral as well as cellular immune responses with high efficiency. This demonstrates the enormous potential of this strategy for vaccination purposes. DNA vaccines have been widely used to develop vaccines against various pathogens as well as cancer, autoimmune diseases and allergy. However, despite their successful application in many pre-clinical disease models, their potency in human clinical trials has been insufficient to provide protective immunity. Several strategies have been applied to increase the potency of DNA vaccine. Among these strategies, the linkage of antigens to Heat Shock Proteins (HSPs) and the utilization of different delivery systems have been demonstrated as efficient approaches for increasing the potency of DNA vaccines. The uptake of DNA plasmids by cells upon injection is inefficient. Two basic delivery approaches including physical delivery to achieve higher levels of antigen production and formulation with microparticles to target Antigen-Presenting Cells (APCs) are effective in animal models. Alternatively, different regimens called prime-boost vaccination are also effective. In this regimen, naked DNA is utilized to prime the immune system and either recombinant viral vector or purified recombinant protein with proper adjuvant is used for boosting. In this review, we discuss recent advances in upgrading the efficiency of DNA vaccination in animal models.

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## Introduction

DNA vaccination is a relatively recent development in vaccine methodology. Although, DNA vaccine is a highly controversial issue, genetic material has been used for therapeutic purpose for the past fifty years. Scientists like Griffith had transferred DNA into cells of living animals in the early 1930. In 1943, Oswald Avery proved that DNA carries genetic information. After 1950, experiments were conducted using purified genetic material. Such experiments provided the evidence that direct injection of DNA results in the expression of the inoculated gene in the host even in the absence of vector. Regarding the DNA vaccine it was accidentally discovered by scientists Tang and Johnson (Express Healthcare). Among the many forms of nucleic acid vaccine that can be

constructed, circular DNA plasmids are the simplest [1, 2]. DNA vaccination involves immunization with a circular DNA plasmid that contains the gene (or genes) that code for an antigen. Indeed, injection of free DNA (naked DNA) stimulates effective and long time immune responses to the protein (antigen) encoded by the gene vaccine, which is being considered "the third generation vaccines". When plasmid DNA is injected into an individual, the plasmid is taken up by cells and its genetic information is translated into the immunizing protein. This enables the host immune system to respond to the antigen [3].

DNA vaccines have become an attractive approach for generating antigen-specific immune responses because of their stability and simplicity of delivery [4, 5]. DNA vaccines can be easily prepared in large scale with high purity, repeatedly administered and are highly stable relative to proteins and other biological polymers [4]. This strategy not only offers a relatively safe modality capable of inducing both cytotoxic T lymphocytes and antibodies, but also allows engineering of artificial immunogens and co-expression of immunomodulatory proteins. The resulting *in vivo* production of the protein after naked DNA injection, can involve biosynthetic processing and post-translational modifications (i.e., native protein form) [3]. The efficiency of DNA vaccination against a pathogen can be affected by the choice of antigen and insertion of multiple antigens. In designing vaccine regimens, it is necessary to consider dose, adjuvants, time of injections and routes of vaccination [6]. However, these vaccines are still experimental and have been applied to a number of bacterial, viral and parasitic models of disease as well as to numerous tumor models.

The active development of this technology only began after Stephen Johnston's group at the University of Texas, Southwestern Medical Center demonstrated that plasmid DNA can induce the formation of antibodies against an encoded protein in 1992. Johnston's group was able to show that when mice are inoculated with plasmid DNA encoding human growth hormone, the mice produce antibodies against the hormone. Then, another research group reported that a protective cell-mediated immune response against influenza virus was generated after immunization with plasmid DNA encoding an influenza virus protein. This study demonstrated that DNA-based immunization stimulates both components of the immune system and helped to establish that DNA immunization is capable of inducing a protective response against infection (DNA vaccine).

In spite of advantages of DNA vaccine strategies, a number of theoretical safety concerns may be considered for DNA vaccines. These include the fate of the plasmid in the vaccinated animals, the risk of the integration of vaccine DNA sequences into the genome of the host and the risk of inducing an anti-DNA immune response. These safety cases should be considered in vaccine design [7].

Two DNA vaccines were recently approved to be used in animals (horse and fish) pointing to the potential of this technology [8]. The reasons for the failure of DNA vaccines to induce potent immune responses in humans have not been completely elucidated. However, some explanation including low levels of antigen production, inefficient cellular delivery of DNA plasmids and insufficient stimulation of the innate immune system can be considered. Efforts to improve these aspects of DNA vaccines have significant effects in their action [8, 9].

Several strategies have been applied to increase the potency of DNA vaccines, such as targeting antigens for rapid intracellular degradation [10,11], directing antigens to APCs by fusion to ligands for APC receptors [12], fusing antigens to chemokines [13] or to a pathogen sequence [14], co-injecting cytokines [15, 16], co-stimulatory molecules [17] and co-administration with CpG oligonucleotides [18]. Recently, the other important considerations are the utilization of HSP as an adjuvant with or without different delivery systems [19, 20].

Two basic strategies that have been used to

increase DNA-vaccine potency are physical delivery to achieve higher levels of antigen production and formulation with microparticles for targeting Antigen-Presenting Cells (APCs). Both approaches are effective in animal models, but have yet to be evaluated in human clinical trials [8]. Also, another effective approach is the prime-boost vaccination, which has generated high levels of T-cell memory in animal models. Two important features of prime-boost immunization that have been demonstrated by investigators are utilization of DNA vaccines as priming vehicles and boosting with the relevant recombinant protein and/or attenuated viruses as booster. Reversing the order of immunization and changing the nature of the boosting virus result in a failure of protection. Prime-boost vaccines can elicit immune responses that differ in magnitude, quality, and balance of cellular and humoral responses from those elicited by single components and thus provide further enhancement for DNA immunizations [21, 22].

## Plasmid DNA Design

The plasmid DNA constructs used for vaccination, have five main features in common [23,24] as follows: 1) a bacterial origin of replication that facilitates amplification of large quantities of plasmid DNA for purification; 2) a prokaryotic selectable marker gene, such as an antibiotic resistance gene; 3) eukaryotic transcription regulatory elements; these are usually strong viral promoter/ enhancer sequences which direct high levels of gene expression in a wide host cell range. Importantly, they also possess DNA sequences which encode the antigenic protein or peptide of interest; 4) a polyadenylation sequence to ensure that the transcribed mRNA is appropriately terminated and 5) the presence of unmethylated CpG motifs, which have T-helper cell type1 (Th1) immunostimulatory activity. These CpG motifs are 6-base unmethylated DNA sequences which have in common a cytosine preceding a guanosine, flanked by two 5' purines and two 3' pyrimidines. In their unmethylated form, these DNA motifs have been demonstrated to be potent stimulators of several types of immune cell [8,23,24]. Monocytes and macrophages are stimulated to produce a range of cytokines including IL-12 and TNF-α and in turn, these cytokines induce the lytic activity of natural killer (NK) cells and stimulate their secretion of IFN-γ. CpG motif may also rapidly activate murine B cells to secrete IL-6 and IgM, as well as to proliferate. These motifs are present at much lower frequencies in vertebrate DNA and are almost always methylated. Therefore, the immunostimulatory activity of bacterial DNA is likely to enhance the host immune response against invading pathogens. In DNA vaccine, plasmids with this immunostimulatory activity may act to mobilize the immune response against the DNA encoded antigen. Therefore, DNA vaccines possess endogenous adjuvant activity that is antigen independent and potently induce Th1 type immune responses [18, 23, 24].

The DNA vaccine constructs are created by the insertion of DNA encoding a desired antigen into a eukaryotic plasmid expression vector. The purified plasmid DNA is inoculated directly into the host cells, following delivery by a number of different routes. The immunizing protein is then expressed in transfected cells *in vivo* under the control of the plasmid expression vector promoter and consequently, an immune response is elicited to the expressed antigen [25].

## Stimulation of Immune Responses by DNA Vaccination

The DNA vaccine applied either intramuscularly or intradermally is mostly taken up by muscle cells or keratinocytes, respectively. However, these cells are unable to initiate primary T cell responses. Recent studies have provided evidence for the involvement of Dendritic Cells (DC) in priming naive T cells after DNA vaccination. Resting DC can be activated by inflammatory cytokines, bacterial products, and certain viruses, resulting in up-

regulation of MHC class II molecules and co-stimulatory molecules [26]. Unmethylated CpG motifs in non-vertebrate DNA have been described to have a potent adjuvant effect [18].

Vaccination with a DNA construct encoding a protein that is not secreted induces strong long-lived CD4+ T cell responses that are initiated in the draining lymph nodes by a small number of DC that express the antigen. Although, DNA vaccination results in direct transfection of only a very small proportion of DC, it leads to general activation of all DC found in the draining lymph nodes, thus, providing optimal conditions for effective T cell activation [26, 27]. The overall immune responses induced by DNA vaccination are depicted in figure 1 and further explanation is described in details in the following sections.

***Antibody responses elicited by DNA vaccination***

The most successful DNA vaccination trials have demonstrated significant antibody response against target antigen (Figure 1). Many factors have been reported to affect the efficacy and nature of the DNA-elicited antibody response such as the route of DNA delivery, the DNA expression vector and the form of the DNA encoded antigen. All these factors apply whether the antigen is secreted, intracellular, or membrane associated [23, 27]. However, it appears that the most important factor influencing DNA-raised antibody responses is the expressed antigen by itself. Some antigens such as the influenza haemagglutinin antigen are able to elicit potent and long-lived antibody responses in mice, whereas others such as the *HIV* or SIV envelop antigen, raise only a transient, low-level antibody response in mice [23, 27]. The difference in antibody responses may reflect basic differences in the physical structure of antigens and how they interact with the immune system [23, 27].

***T-helper cell responses elicited by DNA vaccination***

The T helper cells act by providing help in the form of cytokines to B cells and cytotoxic T cells. At least, two different types of Th cells are thought to exist in humans and mice. These are Th1 and Th2 which support two different types of immune response. Th1 response characterized by IFN-γ synthesis and IL-2 production controls cellular immune responses, while Th2 response characterized by IL-4 production is associated with humoral immune responses. The Th1 cells stimulate the development of cytotoxic T cells, activate phagocytic cells and assist B cells to make IgG2a antibody, which acts to opsonise invading microbes. The Th2 cells activate non-phagocytic defenses such as mast cells and assist B cells to produce IgE and IgG1 antibody [6, 16].

The dominance of either Th1 or Th2 response is dependent on the nature of the antigen. However, there is an argument to suggest that Th1 and Th2 are not distinct cell subtypes, but instead reflect different cytokine expression patterns [6, 16]. Aberrant Th1 responses may result in the development of autoimmune disease, while aberrant Th2 responses may support the development of allergic conditions. It appears that the predominance of either Th1 or Th2 immune response may greatly influence the outcome of a particular disease process. Therefore, the ability to bias the immune response towards one of the T helper cell responses would be very valuable [6, 16].

Furthermore, DNA vaccination is able to raise responses biased towards either Th1 or Th2, depending on the method of vaccine delivery including saline inoculations of DNA or gene gun delivery (Figure 1). Most reports have demonstrated that saline DNA inoculations by the intradermal or intramuscular routes stimulate predominantly Th1 immune responses, whereas gene gun inoculations stimulate Th2 responses. The type of response initiated by saline DNA inoculations may also be modified by changing the form of the antigen or by the co-inoculation of cytokine or other immunostimulant [23, 27]. The mechanisms that support the different types of T helper cell generated by DNA vaccination remain incompletely understood. Following DNA inoculation, it seems that different

migration patterns of APCs, the site of antigen presentation and the nature of APCs may influence whether a Th1 or Th2 response develops [23, 27].

***Cytotoxic T cell responses elicited by DNA vaccination***

In contrast to many other types of vaccine, DNA vaccines are extremely efficient in eliciting cytotoxic T cell (CTL) activity (Figure 1). This is probably due to the fact that vaccine immunogens are presented by MHC class I molecules, a prerequisite for the activation of CD8+ cytotoxic T lymphocytes. There are many reports of potent and persistent cytotoxic T cell activity following DNA inoculation which is possibly due to high expression levels of antigenic protein and thus achieving high levels of MHC class I display [23,27].

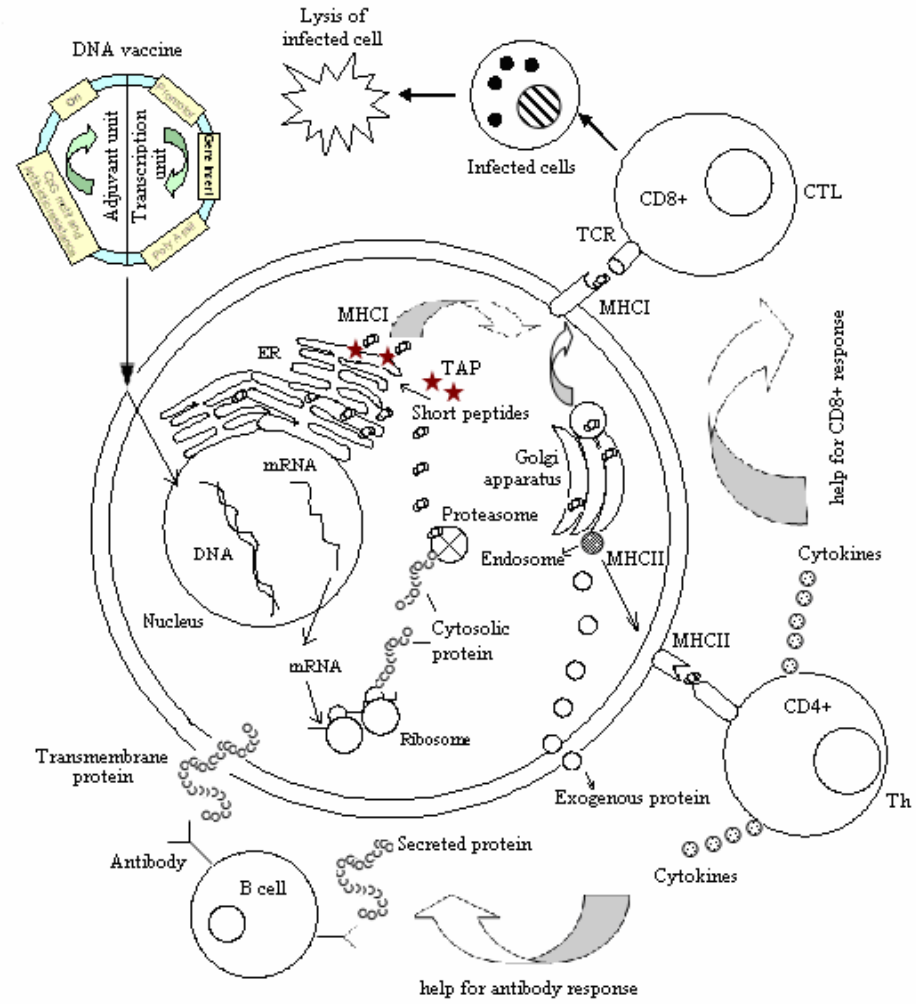


Figure 1. Molecular pathways of DNA vaccine by presenting the antigen to the T cells through the MHC class I and class II molecules. In endogenous pathway, the DNA plasmid enters the cell and nucleus, where the gene is transcribed into messenger RNA (mRNA). Then, mRNA is translated into protein by ribosomes in the rough endoplasmic reticulum (ER, not shown). In the cytosol the protein is cleaved by proteasomes, and the short peptides (contaning 8 to 10 amino acids) are transported into the ER with transport associated proteins (TAP1 and TAP2) and bind to MHC class I molecules. After binding, the complex is transported through the Golgi apparatus to the cell surface, where it can be recognized by cytotoxic T cells (CD8+) and stimulation of cell-mediated immunity occurs. In exogenous pathway, antigen-presenting cells take up extracellular proteins by either endocytosis or phagocytosis. MHC class II molecules in ER pass through the Golgi apparatus and enter acidified endosomes in which the foreign protein has been fragmented into peptides (Endolysosomal degradation pathway). The MHC–peptide complex is then brought to the cell surface, where it can be recognized by helper T cells (CD4+). Specific helper T cells recognize this antigen peptide/MHC class II molecule complex and are activated to produce help in the form of cytokines. These cytokines have many activities, depending on their types, helping B cell to produce antibody and helping cytolytic T lymphocyte (CTL) responses

## Enhancement of DNA Vaccine Potency

There are several possible distinct approaches for increasing the potency of DNA vaccines as follows: 1) modification of the plasmid DNA vector to increase expression levels or to target antigen expression to specific intracellular or extracellular locations has resulted in increased immunogenicity *in vivo*; 2) prime-boost vaccination strategies; 3) inclusion of adjuvants, such as different proteins, compounds or even DNA plasmids encoding immunologically active proteins such as cytokines, chemokines and co stimulatory molecules; 4) DNA delivery facilitation into the target cells [19, 28-30].

### *Expression system design*

A protein expressed by a DNA vaccine often displays the native conformation (with the relevant post-translational modifications such as glycosylation, proteolytic processing and lipid conjugations) that are required to stimulate antibody responses to conformational epitopes, a feature essential for eliciting and neutralizing anti-viral humoral immune responses.

As DNA vaccines deliver antigenic information to the protein synthesis machinery of the cell (comparable to a virus infection), genetic vaccination is also exceptionally potent in stimulating T-cell responses [19]. Expression system design is a central challenge in the construction of DNA vaccines that should deliver immunogens with the aim of eliciting a broad range of specific immune effector functions. To facilitate co-delivery of an extended spectrum of antigenic and immune-stimulating information, complex expression systems using polycistronic cassettes, bidirectional promoters, fusion constructs, or multiple, independent transcription units on a single plasmid have been incorporated into DNA vaccines [31]. There are excessive examples but few are mentioned in the following paragraphs.

In *Human papilloma virus* infections (HPV), a fusion DNA vaccine constructed with *Human papilloma virus* type 16 (HPV16) E7 and E6 genes induced effective cellular immune responses [32]. In this line, the HPV16 L1/E7 fusion constructs not only induced L1-specific antibodies but also L1 and E7 specific CTL responses after DNA immunization [33]. In other study, the enhancement of DNA vaccine potency by co-administration of tumor antigen (HPV16E7 gene) and DNA encoding serine protease inhibitor-6 (SPI-6) was investigated. SPI-6 inhibits granzyme B and thus may provide a method for delaying apoptotic cell death in dendritic cells. It was demonstrated that intradermal co-administration of DNA encoding SPI-6 with DNA constructs encoding HPV16E7, generated E7-specific CD8+ T cell immune responses and E7-specific anti-tumor effects. The results showed that DNA vaccines combining strategies that enhance MHC class I and II antigen processing with SPI-6 have potential clinical implications for control of viral infection and neoplasia [34].

Improvement of a potent vaccine against cutaneous leishmaniasis has been considered for a long time. In an investigation reported by our group, the protection elicited by the intramuscular injection of two plasmid DNAs encoding *Leishmania major* cysteine proteinase type I (CPb) and type II (CPa) was evaluated in a murine model of experimental cutaneous leishmaniasis. The BALB/c mice were immunized either separately or with a cocktail of the two plasmids expressing CPa or CPb [35]. When the cpa and cpb genes were co-injected, the long lasting protection against parasite challenge was achieved. Analysis of the immune response showed that protected animals developed a specific Th1 immune response which was associated with an increase of IFN-gamma production. This was the first report demonstrating that co-injection of two genes expressing different antigens induced a long lasting protective response, whereas the separate injection of cysteine proteases genes was not protective [35].

In a study performed by Ahmed et al [36], four DNA based candidate vaccines encoding to immunodominant *Leishmania* antigens (LACKp24, TSA, LmSTI1 and CPa) were

examined. When these candidates were tested under similar experimental conditions, all of them were able to induce similar partial protective effects in the BALB/c mice model of experimental cutaneous leishmaniasis, but none could induce a full protection. In order to improve the level of protection, DNA based vaccinations with different cocktails of plasmids encoding to the different immuno-dominant *Leishmania* antigens were applied [36]. A substantial increase of protection was achieved when the cocktail is composed of all of the four antigens. The full protection was only achieved after a challenge with a low parasitic dose in the dermis of the ear. The mixture of immunogens induced specific Th1 immune responses against each component. Therefore, such an association of antigens increased the number of targeted epitopes by the immune system with the outlook that the responses are at least additive, if not synergistic [36].

***Prime-boost vaccination***

The DNA vaccines have often been used as priming vaccines in prime-boost regimens that use other vaccine modalities such as recombinant proteins and viral vectors to improve overall immune responses. The rationale for this approach is to use DNA to prime certain antigen-specific immune responses [including cytotoxic T lymphocytes (CTL)] which are then boosted with a large bolus of antigen in the form of a recombinant protein or as a live viral vector [8, 37]. Some studies performed by using this strategy have been described in following parts:

In our laboratory, prime-boost vaccination using cysteine proteinases type I and II of *Leishmania infantum* (*L. infantum*) has been applied to show the protective immunity in different animal models including murine as well as dog against visceral leishmaniasis. In the case of murine model, The BALB/c mice were immunized twice in a 3 weeks interval with cocktail of plasmids DNA encoding type I (cpb) and II (cpa) cysteine proteinases. The DNA immunization was then followed by a boost with rCPA/rCPB in addition to CpG ODN and Montanide 720 as adjuvant. Analysis of the immune response showed that prime-boost vaccination mainly elicited a Th1 immune response [38, 39]. We showed that prime boost vaccination with C-terminal extension of cysteine proteinase type I (CTE) of *L. infantum* displayed both type 1 and type 2 immune signatures in the BALB/c mice [40].

In addition, our group studied further prime boost regimen against experimental canine visceral leishmaniasis using a combination of DNA and protein immunization with cysteine proteinases type I and type II of *L. infantum*. Analysis of cytokine mRNA level suggested that vaccinated dogs had elevated IFN-γ mRNA in Peripheral Blood Mononuclear Cells (PBMC), whereas there was a consistent increase in the level of IL-10 in the control groups and some vaccinated dogs [41]. The level of total IgG and IgG2, but not IgG1, to rCPA and rCPB was significantly higher in the vaccinated group than the control groups. It was also shown that with the exception of one, all the dogs in the vaccinated group in comparison to control dogs had strong DTH responses. Therefore, the combination of DNA and recombinant protein vaccination using CPs could be instrumental to control (VL) in dogs [41].

Comparison of potential protection induced by three vaccination strategies (DNA/DNA, Protein/Protein and DNA/Protein) against *Leishmania major (L. major)* infection using Signal Peptidase type I (SPase) in BALB/c mice has demonstrated that all three strategies induced a parasite specific Th1 response and conferred partial protection against parasite challenge. Furthermore, DNA/DNA strategy developed more effective protective responses than the other two approaches and induced 81% reduction in *L. major* parasite load [42]. This study indicates that the type of antigen and some other factors will determine the out-come of vaccination strategies.

Viral-based vectors are also used extensively for prime-boosting strategies to enhance the antigen-specific immune response. The viral vectors such as *Alpha*

*virus, Adeno virus, or Vaccinia virus* expressing *Mycobacterium tuberculosis* (*M. tuberculosis*) antigens have been tested [43]. The most promising results have been obtained using the modified *Vaccinia virus* Ankara (MVA) expressing Ag85A which gave remarkable immunogenicity and was able to warrant enhanced protection when used in prime-boosting strategies together with BCG. The results obtained in pre-clinical animal models with these new experimental vaccines have been limited, since only a few of them have been shown to be superior to BCG. It has been suggested that the protective activity induced by BCG in animal models such as mice, guinea pigs and rabbits might be exceptionally high and may not necessarily mimic what happens in humans [43].

Heterologous prime-boost vaccination has been determined as another approach for vaccines designed against malaria (*Plasmodium falciparum* infection). It has been shown that DNA vaccines are efficient priming vaccines but do not boost efficiently [44]. In this study, two different vaccine vectors encoding the same antigen were given sequentially. Viral vectors can be given first (priming) or second (boosting). Three carriers that have been clinically tested are DNA, modified *Vaccinia virus* Ankara (MVA) and attenuated *Pox virus* FP9. These were once used to vaccinate chickens against fowlpox. The insert included thrombospondin related adhesive protein (TRAP), a well characterized pre-erythrocytic antigen and a string of T-cell epitopes (called ME for multiple epitope). These ME-TRAP vaccines were given in prime-boost sequence as DNA then MVA, or FP9 then MVA. This approach has induced high T-cell responses and some protection, manifested by a substantial delay to parasitaemia in sporozoite challenge studies [44].

***DNA vaccine adjuvants***

Adjuvants are usually defined as compounds that can increase and /or modulate the intrinsic immunogenicity of an antigen [9]. A general approach to improving DNA vaccines is through the use of adjuvants including DNA plasmids encoding immunologically active proteins such as cytokines, chemokines and co-stimulatory molecules. It is likely that expressed cytokines provide additional T- and B-cell helper responses, whereas, expression of chemokines may result in attraction and/or activation of APCs. With respect to the effect of co-stimulatory molecules, it has been postulated that expression of these proteins in non-APCs may confer transient APC function to these cells [19, 45].

Simple mixtures of DNA vaccines with adjuvants are sometimes effective, but appropriate formulation may be required. For example, certain aluminum salts (such as aluminum phosphate) when mixed with DNA vaccines enhance antibody responses, whereas others (such as aluminum hydroxide) conversely inhibit responses as a consequence of electrostatic interaction between the negatively charged DNA and positively charged adjuvant. This negative effect can be overcome with appropriate formulation to prevent such binding [46].

The inherent adjuvant effect of unmethylated CpG motifs within DNA vaccines is likely to contribute to their effectiveness. The potential simplicity of utilizing CpG effects has led many investigators to test modified vectors and/or mixtures of CpG-containing oligonucleotides with DNA vaccines [19]. In the case of CpG motifs within the vector, the flanking nucleotide sequence is likely to be critical. Also, the presence of neutralizing motifs that can interfere with active motifs complicates their utility [47]. For mixtures of DNA vaccines with CpG oligonucleotides, it appears that the oligonucleotides interfere with transfection of plasmid DNA and consequently reporter gene expression is reduced [47]. Delivery of DNA directly to the cytoplasm of cells, through the use of electroporation, can abrogate the inhibitory effects of CpG oligonucleotides, indicating that the competition between plasmid DNA and CpG oligonucleotides is

marked at the level of DNA uptake by cells [48]. Hence, appropriate formulation and/or delivery of DNA plus CpG oligonucleotides will be required to take advantage of the immunostimulatory effects of CpG. For example, E7+ODN (CpG-oligodeoxynucleotide) co-injection could be an effective approach to induce E7-specific protective immune responses as a possible immunotherapeutic strategy for cervical cancer [49].

At the present, it has been found that HSP promote immunogenic APC function, elicit a strong CTL response, and prevent the induction of tolerance. These findings point to the potential of using HSPs as a T cell adjuvant to induce CTLs targeting viral pathogens or cancer cells [50]. Linkage of antigens to HSPs (HSP70, calreticulin, HSP60, Gp96) represents a potential approach for increasing the potency of DNA vaccines. For example, it has been shown that vaccines containing full length *Human papilloma virus* type 16 E7 (HPV16 E7) fused to *M. tuberculosis* HSP70 dramatically increased the frequency of E7-specific CD8+ T cells by at least 30 fold relative to vaccines containing the wild-type E7 gene. Indeed, this fusion converted a less effective vaccine into one with significant potency against established E7-expressing tumors [4]. Also, linkage of calreticulin (CRT, a family of heat shock proteins located in the endoplasmic reticulum) to HPV16 E6 can generate a significantly enhanced E6 specific CD8+ T cell response in vaccinated mice [51].

Furthermore, a potential preventive and therapeutic HPV DNA vaccine has been generated by using human Calreticulin (CRT) linked to HPV16 early proteins, E6 and E7 and the late protein L2 (hCRTE6E7L2) [51]. Vaccination with hCRTE6E7L2 DNA vaccine induced a potent E6/E7-specific CD8+ T cell immune response, resulting in a significant therapeutic effect against E6/E7 expressing tumor cells. In addition, vaccination with hCRTE6E7L2 DNA generated significant L2-specific neutralizing antibody responses, protecting against pseudovirion infection [52].

An expression system for DNA vaccines has been described in which a fusion protein with an N-terminal, viral J-domain that captures HSPs is translated in-frame with C-terminal antigen encoding sequences [31]. This system supports enhanced expression of chimeric antigens (of >800 residues in length) with an extended half life (>8 *hr*). When used as a DNA vaccine, it delivers antigen together with the intrinsic adjuvant activity provided by bound HSPs. The immunogenicity of the antigens produced by this expression system results in priming CD8+ T-cell responses [31]. Therefore, two major features that characterize the system are as follows: (1) it enhances expression and half life of the protein fused to the HSP-binding domain, and (2) it provides intrinsic adjuvant activity [32].

Protective DNA vaccination using gp96-peptide fusion proteins against the intracellular bacterial pathogen *Listeria monocytogenes* has been demonstrated in a mouse model. Analyses of the cellular immune response revealed profound epitope-specific IFN-γ and cytotoxic T cell responses [53, 54]. We also tested the level of humoral and cellular immune responses by HPV16 E7 plus gp96 co-injection as DNA immunization strategy. Assessment of lymphoproliferative and cytokine responses against recombinant E7 protein (rE7) showed that DNA vaccination including E7 and gp96 induces Th1 response. It was indicated that co-delivery of naked DNA E7+gp96 plasmid was immunologically more effective than E7 DNA and it could be an effective approach to induce E7-specific immune responses as a potential vaccine candidate for cervical cancer [55].

Various adjuvants with different formulations have been used in vaccine design against infectious or non-infectious diseases [56]. In an investigation, the immunological memory was compared after a protein vaccination with DNA vaccination in sheep. The used antigen was the protective antigen (PA83) of *Bacillus*

*anthracis*. Sheep were vaccinated three times with either PA83 plus alhydrogel or with one of four different plasmid DNA (pDNA) formulations which all encoded either the full-length PA83 or its domain 4. Two pDNA formulations included Vaxfectin™ adjuvant and the other two were injected in PBS without adjuvant. Initially, the antibody titres of protein vaccinated sheep were significantly higher than the titres of pDNA vaccinated sheep. After 5 months, the antibody titres of protein vaccinated sheep had dropped remarkably while the titres of all four pDNA vaccinated groups were either stable or increased. Humoral responses of sheep immunized with pDNA formulated with Vaxfectin™ adjuvant were higher than the responses of the corresponding groups that received pDNA in PBS [56].

***DNA vaccine delivery***

Some of the potential barriers to DNA transfection are as follows: 1) lack of widespread distribution of DNA within the inoculated tissue; 2) rapid degradation of unprotected DNA; 3) inefficient uptake of DNA by cells (either directly through the plasma membrane or by endocytosis); 4) degradation of DNA within the endosome/lysosome; and 5) inefficient uptake of DNA by the nucleus, particularly in non-dividing cells where the nuclear membrane remains intact [19, 57]. It has been proved that only a small proportion of the injected material is internalized by cells and results in successful transfection (i.e. production of antigen by cells of the vaccinated animal). In general two basic strategies for increasing DNA vaccine potency include physical delivery such as electroporation and formulation with microparticles such as formulations based on a polymer [e.g. polylactide co-glycolide (PLG)] or a cationic lipid [8, 19, 58].

Electroporation (EP) is a technique for intracellular delivery based on the brief application of electrical signals to target cells. Exposure of target cells to electrical fields of sufficient magnitude and duration can transiently destabilize their cell membranes. During this state, substances present in the extracellular environment that cannot efficiently cross the cell membrane (e.g. DNA) can be taken up inside the cells at high levels [59]. As a result of EP application, cell membrane integrity is rapidly re-established, and the cells resume normal function. The EP has proved to be a particularly potent method for DNA delivery in tissues relevant to DNA immunization (skeletal muscle and skin). An EP-induced increases in DNA expression compared to conventional injection, have been observed in a wide range of animal models with adequate increases in immune response.

Recently, an EP-mediated delivery of plasmid DNA has been shown to be effective as a boosting vaccine in mice primed with DNA alone, possibly owing to the high level of antigen production obtained by the EP-booster vaccine. Interestingly, this regimen was more effective than the one consisting of two doses of DNA with EP [59]. Further work will be required to determine the mode of action of this prime-boost approach. If it is confirmed to work in large animals, this approach might be very attractive because it would eliminate the need for two different types of vaccine in prime-boost strategy (i.e. plasmid DNA would be used in both the prime stage and the boost stage) [59].

The DNA vaccine is the most promising AIDS vaccine since it could provide a safe and protective immunity against *HIV* [60]. Hence, DNA vaccine that has similar qualities and induces a strong immune response similar to that of the live attenuated vaccine will be probably a successful AIDS vaccine [60]. In this regard, the fact that the DNA vaccine with EP can induce antibodies and a T cell response by continuous injections-which are as powerful as the live attenuated vaccine makes it an excellent candidate. Therefore, there are high expectations for DNA vaccine with EP to develop successful AIDS vaccines commercially available in the near future.

In order to develop an effective DNA vaccine with EP, the vaccine candidate should

be evaluated thoroughly in terms of protective immunity in a small number of volunteers before entering large-scale phase IIb-III efficacy trials. More importantly, even before considering any clinical trials in humans, the efficacy test should be evaluated in the appropriate SIVmac-rhesus macaque challenge model that closely resembles the human case [60].

Viral vectors have been evolved specifically to deliver DNA into cells and are the most common gene delivery tools used in gene therapy. However, there are limitations including their limited DNA carrying capacity, toxicity, immunogenicity, the possibility of random integration of the vector DNA into the host genome and their high cost [61, 62].

Non-viral or synthetic vectors have many advantages over their viral counterparts as they are simpler, easier to manufacture on a large scale, their flexibility in the size of the transgene to be delivered and are potentially safer for clinical use. Non-viral vectors include naked DNA, DNA-liposome complexes and DNA-polymer complexes [63,64]. At present, non-viral vectors are under intense investigation as a safer alternative for gene therapy. For successful delivery, the non-viral vector must be able to overcome many barriers to protect DNA and specifically deliver it for efficient gene expression in target cells [57]. Some of the most common non-viral vectors include polyethylenimine, dendrimers, chitosan, polylysine and many types of peptides which are generally cationic in nature and able to interact with plasmid DNA through electrostatic interactions [57].

In our laboratory, protective efficiency of dendrosomes (e.g. Den123) as novel nano-sized adjuvants for DNA vaccination has been studied against birch pollen allergy [65]. Higher and increasing ratios of IgG2a/IgG1 were seen in mice which received DNA plasmids in combination with Den123. Den123 and DNA vaccine synergistically enhanced the IFN-γ released from splenocytes. In the presence of Den123, IgE inhibition was independent of the dose and type of the injected DNA. All DNA-pre-immunized mice demonstrated low basophil degranulation. Indeed, administration of the DNA entrapped in Den123 nanoparticles has resulted in sustained release of plasmids, Th1/ Th2 balanced immune response with promising IgE inhibition. Also higher amounts of DNA contributed to stronger Th1 response [65].

Polyethylenimine (PEI) has recently been used successfully for transfection both *in vitro* and *in vivo* [66, 67]. As a polycation, the PEI will spontaneously adhere to and condense DNA to form toroidal complexes that are readily endocytosed by cells. The presence of multiple unprotonated amines in the complexes is thought to buffer endolysosomal pH, thus allowing cytoplasmic release of the PEI/DNA before lysosomal degradation can occur. The PEI/DNA complexes are eventually translocated into cell nuclei, but it remains to be seen what effects this has on host cell transcription. Future non-viral vectors could also be designed based on data obtained for the PEI mediated transfection [66, 67]. Increasing transfection efficiency while reducing toxicity must be accomplished before the PEI can ultimately be used for efficacious gene therapies [68].

The use of peptides as gene delivery vectors is advantageous as non-viral agents. Cell-Penetrating Peptides (CPPs) are a novel class of membrane translocating agents. Interaction of CPP and cargo is either achieved by covalent attachment or by non-covalent complex formation through mainly ionic interactions [69]. In the case of non-covalent complexes a further assembly of cargo/carrier complexes occurs, leading to the formation of nanoparticles. The complexes are taken up by directly penetrating the cell membrane or by an endocytotic pathway. Recent data suggests that the main uptake route is endocytosis. The CPP must be able to tightly compact and protect DNA, target specific cell-surface receptors, disrupt the endosomal membrane and deliver the DNA cargo to the nucleus (Figure 2) [57, 69].

Different cationic peptides have been

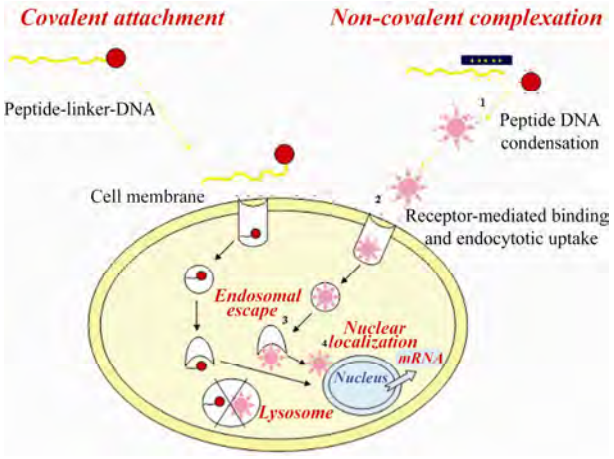


Figure 2. Peptide-based nucleic acid delivery systems must be able to: 1) tightly condense DNA into small, compact particles; 2) target the condensate to specific cell surface receptors; 3) induce endosomal escape and 4) target the DNA cargo to the nucleus for target gene expression

designed that each construct is more effective in part of delivery pathway than the others. For example, cationic peptides rich in basic residues such as lysine and/or arginine are able to efficiently condense DNA into small compact particles that can be stabilized in serum [57]. Attachment of a peptide ligand to the polyplex will allow targeting to specific receptors and/or specific cell types [57]. Peptides sequences derived from protein transduction domains are able to selectively lyse the endosomal membrane in its acidic environment leading to cytoplasmic release of the polyplex [57]. And finally, short peptide sequences taken from longer viral proteins can provide nuclear localization of condensates when they are in the cytoplasm [57].

Furthermore, it has been shown that Nuclear Localization Signal (NLS) peptides conjugated to DNA can increase transfection efficiency *in vitro* [57]. Also, conjugation of NLS peptides to DNA vaccines enhances their immunogenicity after intramuscular injection or gene gun mediated intradermal delivery [70, 71].

One of the peptides derived from a viral sequence is the Tat (48-60) sequence from the *Human immunodeficiency virus* 1 (*HIV*-1) protein [72]. The entire Tat protein is 86 aminoacids in length and contains a highly basic region required for translocation activity. The peptide fragment from residues 37 to 72 contains the basic region along with an α-helical structure that is capable of internalization into several cell lines. It was later discovered that the α-helix (residues 27-47) is not necessary for activity of the peptide [57]. The minimally active sequence of the Tat (48-60) peptide contains a 9 amino acid stretch of basic residues that are required for the membrane lytic activity. It has been shown that multimers of the Tat peptide can efficiently condense DNA and produce a 6- to 8-fold increase in transfection over control peptides [57, 72].

In our recent study, two delivery systems including polymer PEI 25 *kDa* and polymer-peptide hybrid as PEI600-Tat conjugate were used to compare their efficiency for HPV16 E7 DNA transfection *in vitro*. Our data indicated that both delivery systems including the PEI 25 *kDa* and PEI600-Tat conjugate are efficient tools for E7 gene transfection. In fact, the PEI potency for E7 gene transfection is higher than PEI600-Tat *in vitro*, but as it is expected, its toxicity is obstacle *in vivo* [73]. Also, the effect of the PEI600-Tat conjugate was evaluated on the potency of HPV16 E7–specific immunity in C57BL/6 mice model. Assessment of lymphoproliferative and cytokine responses against recombinant E7 protein (rE7) showed that the PEI600-Tat/E7DNA could induce Th1 response. This study has demonstrated that the PEI600-Tat conjugate in certain ratio was efficient to improve immune responses *in vivo* [74].

Moreover, the tegument protein VP22 has been identified as being a so-called "ferry protein", since it has the ability to translocate from *HSV*-infected cells to uninfected cells. Fusion of the HPV16 E7 to the *Herpes simplex virus* type 1 VP22 protein and to other protein export/import signals can improve its ability to induce a cellular immune response (CTL response) upon DNA vaccination [75].

Overall, various strategies have been developed to enhance the potency of DNA

vaccines and to augment vaccine-elicited T cell immune responses. Although, some important current strategies have been described as above, however, in general the action mechanisms are divided into a number of groups as follows: 1) increasing the number of antigen-expressing DCs such as a) intradermal administration of DNA vaccines via gene gun as an efficient route for the delivery of DNA to DCs; b) intercellular antigen spreading as a strategy to increase the number of antigen-expressing DCs; c) linkage of antigen to molecules capable of binding to DCs as a method to target antigen to DCs (e.g. HSPs); d) employment of chemotherapy-induced apoptotic cell death to increase the number of antigen-loaded DCs [76], 2) improving antigen expression, processing, and presentation in DCs including a) codon optimization as a strategy to enhance antigen expression in DCs; b) employment of intracellular targeting strategies to enhance MHC class I and class II antigen presentation in DCs (e.g. HSPs); c) by passing antigen processing as a method for generating stable antigen presentation in DCs [76], 3) enhancing DC and T cell interaction including a) prolonging DC survival to enhance T cell interaction; b) induction of CD4+ T cell help as a strategy for augmenting CD8+ T cell responses [76]. These strategies can potentially be combined to further enhance DNA vaccine potency.

## Why the DNA Vaccine Strategy is Important for Challenging against Different Diseases?

Difficulties in developing vaccines against chronic infections with viral agents such as the *Human immunodeficiency virus*, *Herpes Simplex virus*, *Hepatitis C virus* and *Human papilloma virus* are partly due to the poor immunogenicity of standard vaccines. These problems have stimulated the development of new vaccine strategies that use DNA instead of protein. The DNA vaccines contain the gene or genes for an antigenic portion of a virus such as the core protein or the envelope protein. Host cells take up the foreign DNA, express the viral gene and make the corresponding viral protein inside the cell. An important advantage of this system is that the viral protein enters the MHC class I pathway. The MHC class I molecules carry peptide fragments of the viral protein to the cell surface where by stimulating CD8 cytotoxic T cells they evoke cell mediated immunity (Figure 1) [77].

In contrast, standard vaccine antigens are taken up into cells by phagocytosis or endocytosis and are processed through the MHC class II system which primarily stimulates antibody responses (Figure 1). It should be considered that the preferential stimulation of cytotoxic T cells is a desirable property of a vaccine against a virus or parasite [77]. Viral infections are primarily intracellular. Within an infected cell the virus finds shelter from antibodies. A cell-mediated immunity is needed to detect and destroy virus-infected cells. Antibodies play an important part in neutralizing extracellular virus especially when the virus is released into the bloodstream or the extracellular space after an infected cell has been killed by the virus itself or by cytotoxic T cells [77].

Effective neutralizing antibodies are often directed against viral surface antigens, because these envelope proteins are exposed on intact virions. By mutating the genes for its envelope protein, the virus can evade the humoral system. This phenomenon is striking in chronic infections with agents such as *Hepatitis C virus* and *HIV* in which many mutations in the envelope genes are often found in the viruses from a single infected patient. Influenza is not a chronic infection, but, influenza viruses can acquire many mutations in their envelope genes that are known as genetic drift. These mutations are an important reason for current vaccines containing influenza subunits that are directed at the envelope glycoprotein and fail to prevent re-infection with a different strain of influenza in the following year [77].

Researchers have developed a prototypical

naked-DNA vaccine against influenza that uses the nucleoprotein gene of the virus. The nucleoproteins in many strains of influenza are similar. They are internal viral proteins and thus are less subject to antibody-induced antigenic drift than the surface (envelope) glycoproteins. This naked-DNA vaccine has protected mice against lethal doses of a heterologous strain of influenza which current vaccines cannot do [77]. It is therefore critical that cell-mediated immunity is responsible for protection against more than one viral strain [77].

The studies on bacterial diseases have shown that a number of DNA vaccines encoding various mycobacterial antigens are protective in prophylactic models [78]. Also, the DNA vaccination in mice has elicited significant levels of the cell-mediated immune responses with a broader T cell repertoire compared with subunit vaccination characterized by CD4+ and CD8+ T cells [43]. Sometimes, the utilization of DNA vaccine cocktails or immunization with constructs expressing two or more antigens has provided improved levels of protection compared to monovalent DNA vaccines [43].

However, a lot of suspicion exists about DNA vaccines when they were used in therapeutic models. For example, a DNA vaccine coding bacterial HSP65 administered in a post-exposure regimen was effective in reducing the bacterial load in mice infected with *M. tuberculosis*, while a DNA vaccine cocktail that induced protection in a prophylactic regimen did not show any effect when administered in a therapeutic regimen. Hence, a broad evaluation in pre-clinical animal models is required in order to predict post-exposure vaccination strategies against *M. tuberculosis* infections [43].

But some questions remained to be determined in this connection. For example, why do some DNA vaccines that effectively protect naïve animals against *M. tuberculosis* infection have no protective effect in post-exposure models? Why do DNA vaccines used in therapeutic models have different or even opposite effects in different laboratories? These differences may be related to the complex nature of stimulated immune cells in the host, the specific animal species, bacterial strains of infection and even the routes and dose of infection. Other factors such as the vaccination route, frequency of vaccination and the time of observation may also impact the therapeutic and prophylactic effects of DNA vaccines [78].

## Conclusion

The DNA vaccination has presented a new and exciting approach to vaccine technology. The numerous animal models have demonstrated the efficacy of these vaccines against viral, bacterial and parasitic diseases. Future DNA vaccination studies using animal models must provide valuable information regarding safety and efficacy, before large scale human DNA vaccination studies are conducted. The feasibility of using DNA as a treatment has been demonstrated in animal models, but clinical applications of this form of technology remain elusive. Clearly, the mechanisms of tolerance and immunoescape have also limited the clinical outcome of DNA vaccination. In order to overcome these drawbacks and augment the immune response, several strategies are involved such as identification of the target antigens, definition of the desired immune response and design of the optimal structure for DNA vaccine, and efficient delivery of the vaccine.

At present, researchers have developed the increased understanding of antigen-processing mechanisms and activation of the innate immune system to design new DNA vaccines. The recent improvements in the optimization of the plasmid DNA and the development of the delivery methods such as EP and viral/non-viral vectors, and different adjuvants have been a major progress for gene therapy and DNA vaccine. In our opinion, for the application of DNA-based vaccines in humans, novel adjuvants or delivery systems are needed to be developed. However, the confirmation of the success of these and other

optimization methods will only be realized with the completion of successful clinical trials.

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