

Li Liu, Matthew P. Morrow, and Mark Bagarazzi

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### Abstract

Owing to their unique advantages in simplicity, safety, scalability, and possibility of repeated administrations, DNA vaccines represent an appealing and competitive immunization approach for a wide array of conditions, including but not limited to infectious diseases and cancer immunotherapy. Despite the exciting efficacy observed in preclinical studies, DNA vaccines have faced challenges in inducing strong immune responses in humans. This unexpected poor immunogenicity has severely hampered the translation of DNA vaccines from investigational medications to licensed products. To overcome this obstacle, tremendous efforts have been made to improve antigen expression and enhance immunogenicity. Among these endeavors, *in vivo* DNA electroporation (EP) has proved to be a breakthrough technology capable of mediating efficient DNA uptake and resulting in enhanced antigen expression and vaccine immunogenicity. EP-mediated DNA delivery has become one of the major platforms used in clinical trials to evaluate DNA vaccines in humans. In this chapter, in addition to EP delivery, other progress made in DNA vaccine development including plasmid optimization, antigen design, and immunologic adjuvants is also reviewed. Finally, the use of DNA vaccines in the context of clinical trials for infectious diseases and cancer immunotherapy is summarized. Specifically, the strategies that allow DNA vaccines to overcome antigenic diversity for viral infection and break immune tolerance for cancer therapy are explored. Based on the advantages of DNA vaccines and the immense progress, led by the electroporation-mediated vaccine delivery, DNA vaccines appear to have the potential to fundamentally transform the vaccine field, providing important benefits for preventing and curing diseases.

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L. Liu • M.P. Morrow • M. Bagarazzi (✉)  
Inovio Pharmaceuticals, Inc., Plymouth Meeting, PA, USA  
e-mail: [lliu@inovio.com](mailto:lliu@inovio.com); [mmorrow@inovio.com](mailto:mmorrow@inovio.com); [mbagarazzi@inovio.com](mailto:mbagarazzi@inovio.com)

## Keywords

DNA vaccines • Plasmid • Electroporation (EP) • Infectious diseases • Cancer immunotherapy

## Contents

Introduction .....	1934
Mechanisms of Action .....	1936
Antigen Presentation and Immune System Activation .....	1936
Route of Administration .....	1937
Optimization of DNA Vaccines .....	1937
Optimization of DNA Vaccine Delivery .....	1938
Optimization of Plasmid Design .....	1939
Use of Adjuvants .....	1939
Clinical Use in Infectious Diseases .....	1940
Application in Influenza Virus Infection .....	1940
Application in Human Immunodeficiency Virus (HIV) Infection .....	1942
Application in other Viral Diseases .....	1943
Clinical Use in Cancers .....	1945
Cancer Antigens .....	1945
Antigen Design for Breaking Immune Tolerance .....	1946
Cancer Clinical Trials .....	1947
Conclusions .....	1950
Cross-References .....	1951
References .....	1951

## Introduction

In 1990, Wolff and colleagues first demonstrated that long-term gene expression could be achieved simply by direct injection of plasmid DNA into mouse skeletal muscle (Wolff et al. 1990). Two years later, Tang et al. reported that *in vivo* delivery of plasmid DNA encoding human growth hormone (hGH) resulted in the production of detectable levels of hGH in host mice. Importantly, the inoculated mice developed anti-hGH antibodies, suggesting the immunological use of DNA (Tang et al. 1992). These pioneering observations engendered great enthusiasm for using DNA immunization against infectious diseases, as well as in the applications of gene therapy. The immunological potential of DNA was confirmed by several independent pre-clinical studies demonstrating that DNA immunization could result in protection from infectious diseases in immunized animals (Moss 2009).

The action of DNA immunization involves intramuscular or subcutaneous injection of a DNA plasmid encoding the antigen of interest expressed under the control of a eukaryotic promoter. Upon administration of the plasmid DNA, the *in vivo* expressed antigen can stimulate the host immune system to elicit a response. As a third generation platform, DNA vaccination has distinct advantages over other vaccine approaches. DNA vaccines can induce both humoral and cellular immune responses making them distinct from conventional inactivated vaccines and subunit vaccines. In addition, plasmid DNA has a number of unmethylated CpG motifs, due

to its bacterial origin, that are capable of stimulating innate immune responses via Toll-like receptor 9 (TLR9). The ability to activate all arms of the immune system (B cells, T<sub>H</sub> cells, and cytotoxic T cells) means the DNA vaccine platform has the potential for not only prophylactic but also therapeutic applications. Unlike the traditional prophylactic vaccines that prevent infections from occurring, therapeutic DNA vaccines can be used as an immunotherapy to treat chronic viral infections and cancers by direct killing of latently infected cells and mutated tumor cells. DNA vaccines have also been shown to have a benign safety profile. The injected plasmid only encodes a selected gene from a pathogen, eliminating risk of pathogenic infection or reversion to virulence, a safety concern that is associated with live attenuated vaccines. Importantly, as plasmid DNA vaccines are noninfectious, the elicited immune responses are solely specific to the antigen encoded by the transgene rather than to the plasmid itself. As a result, no anti-DNA vector responses have been observed, thereby allowing for repeated vaccine administration without weakening the specific immune response. This feature is critical for the prime and boost regimen, a commonly used approach for enhancing vaccine immunogenicity. Another advantage that makes DNA vaccines a competitive option is its ease of design and manufacture. The advancement of gene synthesis and recombinant DNA technology allows for flexible and rapid alterations of the expressed immunogen. Large-scale production of plasmids can be completed within a short period of time, making DNA vaccines particularly suitable for responding to pandemic outbreaks. Finally, DNA vaccines are more stable and resistant to temperature extremes than other vaccine forms which are of great benefit for storage, transport, and distribution especially in resource-limited regions.

Despite all the positive characteristics and the superb efficacy observed in preclinical studies, DNA vaccines have been slow to demonstrate similar success in humans. The unforeseen poor immunogenicity observed in most early clinical trials was disappointing and poses a formidable challenge for DNA vaccine licensure. However, several DNA vaccines have been approved for animal health. The first one, developed by Fort Dodge laboratories against West Nile virus infection in horses was approved by the US Department of Agriculture (USDA) in 2005. A second DNA vaccine (Apex-IHN, Novartis Animal Health) was licensed in Canada for prevention of hematopoietic necrosis virus infection in farmed salmon and trout. In 2010, a therapeutic cancer vaccine (Oncept, Merial) designed to treat dogs with melanoma was approved by the USDA. Finally, in 2008, an injectable DNA plasmid (LifeTide SW 5, VGX Animal Health) encoding porcine growth hormone releasing hormone (GHRH) was licensed in Australia as a gene therapy for use in breeding age sows to increase the number of piglets in litters.

The approval of these plasmids as commercial products in veterinary practice is very encouraging and sheds lights on human DNA vaccine development. For example, the licensed therapeutic vaccine for canine malignant melanoma was developed by using human tyrosinase (a melanoma associated antigen) as the immunogen to immunize dogs. The human tyrosinase elicited a strong immune response against dog melanoma and significantly prolonged the lives of dogs with advanced disease (Bergman et al. 2003). The success of this vaccine supported the

concept of using a xenogeneic protein to break central immune tolerance for cancer vaccine development (Liu 2011). Additional insight gained from the success of DNA vaccines in veterinary practice is that the DNA scaling-up might not be a potential hindrance for DNA vaccine being effective in humans. It has been extrapolated that an impractically high dose of DNA vaccine would be required for use in human subjects in order to reproduce the vaccine efficacy obtained in mice (Tregoning and Kinnear 2014). However, the clinical benefits observed in large animals, including horses and pigs, suggest that the size of humans may not be the reason for the various disappointing clinical results (Liu 2011).

In light of the aforementioned advantages of DNA vaccines and encouraged by their success in veterinary medicine, great efforts have been made to translate DNA immunization into the clinic, with novel strategies being explored to enhance immunogenicity. Since the first human study in 1998 (Calarota et al. 1998), numerous clinical trials with DNA vaccines targeting a wide spectrum of diseases have been performed or are currently underway. In the following sections, various aspects related to DNA vaccine development including its working mechanisms, route of delivery, advances being made in optimizing DNA vaccine immunogenicity, and their usage in the applications of infectious diseases and cancer treatment will be addressed.

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## Mechanisms of Action

### Antigen Presentation and Immune System Activation

Upon intramuscular or intradermal injection, plasmid DNA is internalized by myocytes or keratinocytes located at the site of vaccine administration. The plasmid encoded antigen is then expressed intracellularly using host cell machinery. However, since myocytes and keratinocytes are not professional antigen presenting cells (APC) and do not express co-stimulatory molecules, they are unable to productively stimulate cytotoxic T lymphocytes (CTL) activation. In order to prime a cellular response, the intracellularly produced antigens must be released into extracellular matrix either by secretion or by cell lysis. The released antigens are then engulfed by APCs and loaded onto major histocompatibility complex (MHC) class I molecules leading to activation of CD8<sup>+</sup> T cell. This uptake mechanism is different from that which occurs with exogenous proteins and is thus termed as cross-priming (Liu 2011). DNA plasmids can also directly transfect APCs that have migrated to the injection site. After being expressed in APCs, the antigens are cleaved to peptides and uploaded onto MHC I or II molecules, leading to activation of both CD8<sup>+</sup> CTLs and CD4<sup>+</sup> T<sub>H</sub> cells. Additionally, antigens released from myocytes and keratinocytes can be captured by B lymphocytes leading to activation of humoral immunity.

In addition to the stimulation of the adaptive immune system, DNA plasmids can also trigger activation of innate immunity. Plasmids derived from bacteria contain hypomethylated CpG dinucleotides motifs, which are immunostimulatory and rarely seen in eukaryotes. The CpG motifs bind to Toll-like receptor 9 (TLR9), a trans-membrane protein found on a number of immune cells including dendritic cells

(DC), B cells, and natural killer (NK) cells. Activation of TLR9 leads to a cascade of pro-inflammatory responses and results in the production of various cytokines including type I interferon (IFN), Interleukin (IL)-12, IL-18, and tumor necrosis factor-alpha (TNF- $\alpha$ ). In addition to CpG-TLR9 pathway, the double-stranded structure of plasmids can be detected by DNA sensors in cytosol, which triggers activation of the stimulator of interferon genes (STING)/Tank-binding kinase 1 (TBK1) pathway, leading to type I interferon (IFN) production.

## Route of Administration

Historically, various administration routes including intramuscular, intradermal, subcutaneous, intranasal, vaginal, oral, and intravenous route have been tested (Liu 2011; Tregoning and Kinnear 2014) in order to elicit a desired immune response from DNA immunization. The selection of the appropriate delivery method is dependent on multiple factors including optimum expression, immunogenicity, feasibility, and tolerability. Among the considered delivery approaches, intramuscular (i.m.) injection is the principal route of choice for DNA vaccination due to the fact that skeletal muscle is readily accessible and highly effective in taking up DNA. Furthermore, myocytes are energy rich and postmitotic cells capable of sustaining long-term transgene expression for eliciting durable immunity (Aurisicchio et al. 2013; Tregoning and Kinnear 2014). In addition to i.m. injection, intradermal (i.d.) delivery is also considered to be an effective pathway for vaccination not only because of its greater ease of accessibility but also the prominent presence of large amount of immune cells in the dermis including dendritic cells, Langerhans cells, and migrating lymphocytes. Both i.m. and i.d. are two major administration routes used in preclinical studies and clinical trials for DNA delivery. Some studies suggested that the nature of the immune response could be driven by the vaccine administration route. For example, due to the enrichment of antigen presenting cells in skin, the i.d. route is believed to preferentially induce cellular immune responses as a result of direct transfection of APCs. On the other hand, i.m. injection equally induces both humoral and cellular immunity via direct transfection and by the cross-priming mechanism. However, in a recent clinical trial where an HIV-specific DNA vaccine was delivered via either an i.m., or i.d. route, no differences in induced immune responses were observed (Enama et al. 2014).

## Optimization of DNA Vaccines

After administration, the plasmid DNA must successfully overcome a series of barriers before mounting the desired immune response. Compared to viral vectors, uptake of naked DNA by somatic cells is inefficient; most of the i.m.-injected DNA does not actually transfect cells (Liu 2011). After entering the cell, plasmid DNA needs to translocate to the nucleus where DNA transcription occurs. However, the efficiency of intracellular movement is very low, with less than 0.1% of plasmid

DNA that enters the cytosol is eventually transcribed (Tregoning and Kinnear 2014). Moreover, prior to arrival at the nucleus, DNA in the cytosol is subject to degradation by nuclease digestion. Finally, during the process of protein synthesis, mRNA translation efficiency can also be modulated by factors such as the presence of a Kozak sequence and codon usage. To overcome these obstacles, several approaches focusing on augmenting DNA uptake, maximizing protein expression, and enhancing antigen immunogenicity have been developed and tested in clinical trials.

## Optimization of DNA Vaccine Delivery

Several DNA delivery methods including gene gun, jet injection, electroporation (EP), cationic polymers, and liposomes have been used in clinical trials. Given that the inclusion of extra chemical components may delay the licensure of the vaccine due to concerns over the inflammatory profile of the agents, the device-mediated delivery is considered to be a more universal and preferred approach for enhancing DNA uptake (Tregoning and Kinnear 2014). Among the device-mediated approaches, EP has the highest transfection efficiency and has achieved the most remarkable progress in DNA vaccine delivery (Aurisicchio et al. 2013; Best et al. 2009).

The EP technology was initially devised for *in vitro* transfection (see also ► Chap. 23, “Gene Delivery by Electroporation *In Vitro*: Mechanisms”). The mechanism of EP involves using controlled electric pulses to open transient pores on cell membrane through which the negatively charged DNA molecules gain access to the cytoplasm. The cell membrane is permeabilized for only a short period of time and rapidly closes after electrical treatment terminates, returning the cell to its original state. EP has been proven to be a very powerful technology, resulting in 100–1000-fold increases in plasmid uptake and subsequent antigen expression compared to naked DNA vaccines (Aurisicchio et al. 2013). Furthermore, EP has the additional benefit of causing tissue damage that can lead to local inflammation and release of cytokines (Fioretti et al. 2010). This adjuvant effect of EP consequently promotes the induction of innate and adaptive immune responses, potentiating the immunogenicity of the delivered DNA. The EP technology has been safe and well tolerated (Bagarazzi et al. 2012) with the most common minor side effects observed in clinical trials being local injection-site pain and discomfort associated with short muscle contraction triggered by electric pulses. It is believed that EP represents the most promising technology in DNA delivery (Aurisicchio et al. 2013). Recently, the application of *i.m.* injection followed by EP delivery has become one of the major platforms used in clinical trials to evaluate DNA vaccines in humans. However, it should be noted that EP technology has intrinsic limitations. The EP approach requires penetration with several sharp needle-like electrodes into the subject’s body followed by introduction of transient electric pulses. As a result, the EP procedure is less convenient, more invasive, and sometimes intimidating, potentially reducing patient compliance compared to simple

i.m. injection. Furthermore, the EP procedure demands developing practical and affordable devices and providing adequately trained health professionals to administer the vaccine.

## Optimization of Plasmid Design

The immune responses to DNA vaccines have been shown to be dependent on antigen expression (Tregoning and Kinnear 2014). Therefore, optimizing antigen expression is key to improving vaccine performance. Plasmid codon optimization is one such approach to augment expression. It refers to changing the antigen coding sequence, via synonymous substitutions, from the wild type to a selected sequence that has the most abundant tRNAs in the cytosol of transfected cells. Through codon optimization, adverse rare codons are avoided and secondary structures in the mRNA are minimized, therefore, protein expression can be increased severalfold. In addition to codon optimization, various manipulations of the DNA sequence can be performed to modulate antigen intracellular processing and immune response priming. For example, adding nuclear localization signals to the plasmid helps direct DNA to the nucleus for transcription. For the purpose of introducing cellular immunity, ubiquitin coding sequence can be incorporated in the DNA vaccine to enhance protein degradation and antigen peptide production in the proteasome (Leifert et al. 2004). If humoral immunity is desired, the antigen coding region can be modified by adding a leader sequence capable of guiding the expressed antigen so that it undergoes proper folding in the endoplasmic reticulum (ER), export through the secretory pathway, and release into extracellular matrix for further presentation to B lymphocytes and APCs (Fioretti et al. 2010).

## Use of Adjuvants

Due to its bacterial origin, plasmid DNA carries a naturally occurring adjuvant. Co-injection of antigen-encoding plasmid with an empty vector carrying no insert has been shown to induce stronger immune responses than using the antigen-encoding plasmid alone (Liu 2011). The enhanced immunogenicity was attributed to the adjuvant effect of the hypomethylated CpG oligodinucleotides that can bind to TLR9, thereby stimulating innate immunity and creating an inflammatory milieu for boosting the adaptive immune response against the encoded antigen (Liu 2011). In an attempt to mimic this adjuvant effect, additional CpG motifs have been used in DNA vaccine platforms resulting in enhanced immune responses. Alternatively, instead of using incorporated CpG sequence to trigger TLR activation, co-administration of additional plasmid expressing ligands for TLR7/8/9 or their signaling molecules has been shown to improve the immunogenicity of DNA vaccines (Kwissa et al. 2007).

One disadvantage of using the inflammatory adjuvants is that they boost the vaccine immunogenicity in a sequential fashion, i.e., innate immunity activation followed by adaptive immunity activation. It is possible that the acute inflammation

induced by the adjuvant may result in clearance of the transfected cells before sufficient antigen is expressed (Tregoning and Kinnear 2014). To avoid this timing issue, genetic adjuvants have been investigated to enhance vaccine immunogenicity. Genetic adjuvants refer to immunoregulatory cytokines and proteins encoded by additional plasmids. The most commonly used genetic adjuvants include IL-2, IL-12, IL-15, granulocyte-macrophage colony-stimulating factor (GM-CSF), Intercellular Adhesion Molecule 1 (ICAM-I), CD86, and Heat Shock Protein 70 (HSP70) (Fioretti et al. 2010; Tregoning and Kinnear 2014). The plasmid encoding the genetic adjuvant is normally delivered by co-injection with the plasmid encoding the vaccine. Alternatively, the adjuvant transgene can be tailored and encoded in *cis* with the antigen coding sequence in the same DNA vector as well (Fioretti et al. 2010). In this way, the peak expression of the genetic adjuvant will match antigen expression, leading to a synergistic response. Moreover, the effect tends to be more specific than TLR-type adjuvants, which can be reactogenic (Tregoning and Kinnear 2014).

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## Clinical Use in Infectious Diseases

### Application in Influenza Virus Infection

Conventional licensed influenza (flu) vaccines have traditionally been made by growing the recommended seasonal vaccine strains in eggs followed by inactivation or attenuation. The inactivated or attenuated virus in the vaccine promotes an immune response that can prevent infection from identical or very similar viruses. However, due to antigenic diversity, the annually recommended flu vaccine can become ineffective if new strains emerge. In order to avoid frequent composition adjustment for emerging influenza strains, new vaccine platforms capable of inducing a universal immune response against a broad spectrum of influenza strains are greatly needed.

Viral surface proteins are the primary targets for prophylactic vaccine development, because of their crucial role in initiation of infection and their prominent display on the virion surface. However, due to host immune pressure, these surface proteins are constantly evolving and have high degrees of variability, making it difficult to develop a universal vaccine. Thanks to the versatility and ease of rapid alteration of the expressed immunogen, a DNA vaccine strategy has great potential in overcoming influenza antigenic diversity. For example, a novel synthetic consensus sequence technology has been developed to broaden the effectiveness of the vaccine to cover variant strains of influenza displaying antigenically altered hemagglutinin (HA). Specifically, using the combination of extensive genetic data and a computer algorithm, the gene sequences encoding the HA protein of a large number of circulating influenza strains were analyzed. Subsequently, a new arrangement of the DNA sequence is created resulting in an average gene sequence for the HA protein. The synthetic consensus DNA sequence is substantially similar to but different from those encoded by naturally occurring circulating variants. Upon delivery of the synthetic consensus plasmid DNA, the *in vivo* expressed, artificial, and unmatched HA led to generation of neutralizing antibodies capable of



recognizing a wide variety of related strains (Choo et al. 2010). The ability of synthetic consensus plasmid-generated H5N1 DNA vaccine in cross-protection has been tested in ferrets, in which the sera derived from immunized animals exhibited protective neutralization against all four subclades of H5N1; this protection level had not been attained previously with strain-matched influenza vaccine candidates (Choo et al. 2010). A synthetic consensus DNA vaccine targeting H5 and H1 variant strains of influenza has been tested in a phase I clinical trial (NCT01405885).

It is well known that the vaccine-induced influenza neutralizing antibodies invariably target the globular head region of influenza hemagglutinin (HA) and are largely strain specific. On the other hand, a localized region in the HA stem was found to be structurally conserved among type A influenza viruses; antibodies specific to this region are broadly neutralizing. Although the HA stem-specific antibodies have been identified in humans, it has not been possible to specifically elicit them through vaccination. A prime-boost strategy involving DNA vaccination has been developed and shown to be able to induce broadly neutralizing antibodies (Wei et al. 2010). Specifically, vaccination with plasmid DNA encoding H1N1 influenza hemagglutinin and boosting with seasonal vaccine or replication-defective adenovirus 5 vector encoding HA stimulated the production of broadly neutralizing antibodies specific to the conserved stem region. This prime-boost combination increased the neutralization of diverse H1N1 strains dating from 1934 to 2007 and offered cross-protection against divergent H1N1 viruses in mice and ferrets (Wei et al. 2010). Based on these results, the prime-boost strategy was further tested in a human study (NCT00776711) to evaluate its ability in stimulating broadly neutralizing antibodies (Ledgerwood et al. 2011). It was found that H5 DNA priming with a 24-week Monovalent Inactivated Vaccine (MIV) boost interval induced protective hemagglutination inhibition (HAI) titers in 81% of individuals. Moreover, as compared to another phase I trial (NCT01086657), where an H5N1 MIV vaccine was used as the priming immunization (homologous prime-boost), DNA-MIV heterologous prime boost approach demonstrated a stronger ability in eliciting humoral immunity as evidenced by a more than fourfold increase in HAI titers (Ledgerwood et al. 2011). The results obtained from these studies suggested that DNA vaccine prime followed by a MIV or viral vector boost is a promising approach for the development of a universal influenza vaccine for humans.

In addition to surface proteins, viral antigens that are inaccessible to antibodies, such as nucleocapsid protein (NP) and matrix protein (M), can also be included as the immunogen in DNA vaccines. Generally, these internal structural proteins are highly conserved among variant strains. CTLs raised against these conserved internal proteins can confer cross-protection against diverse related strains. For example, in a preclinical study, a DNA vaccine targeting the NP from influenza H1N1 strain elicited broadly effective CTLs that provided cross-protection against challenge from a H3N2 strain arising three decades later. These findings suggest that immune responses to both surface and internal viral proteins will provide optimal protection. Therefore, in order to obtain the greatest efficacy, it is conceptually reasonable to include multiple plasmids in an influenza DNA vaccine cocktail to induce antibody to the HA protein and cytotoxic immunity to the viral NP and M protein.

In the case of influenza, the traditional manufacturing process for the licensed flu vaccines is labor intensive and time consuming, which impedes an imperative response against outbreak crisis. This occurred in 2009, when a swine-origin H1N1 influenza caused a global pandemic. The licensed pandemic vaccine in the form of killed viruses could not be released until a half year after the World Health Organization (WHO) announcement of the global outbreak. In contrast, by virtue of its simplicity in manufacturing, an investigational pandemic H1 DNA vaccine was quickly made available 3 months earlier than the licensed pandemic vaccine. When tested in a phase I clinical trial (NCT00973895), the H1 DNA vaccine elicited both humoral and cellular immunity against the pandemic strain. The immune response could be further boosted by the licensed pandemic vaccine when it became available (Crank et al. 2015). The results derived from this phase I clinical trial suggests that DNA vaccines are of particular value in a pandemic setting to control the outbreak of emerging infectious disease.

## Application in Human Immunodeficiency Virus (HIV) Infection

Since its initial identification in early 1980s, the scientific community has worked diligently towards a safe and efficacious vaccine that renders sterilizing immunity against HIV infection. However, despite the tremendous efforts made during the past more than three decades, a successful HIV vaccine remains elusive. Historically, for a large number of infectious diseases, the live attenuated or killed whole virus proved to be the first and second best approach in successful vaccine development (Gallo 2005). However, neither of them can be included in HIV vaccines due to the safety concerns that attenuated HIV would cause AIDS and one cannot be certain that all virus particles would be completely inactivated. To date, only two subunit vaccines, namely, the Hepatitis B Virus (HBV) vaccine and the Human papillomavirus (HPV) vaccines are approved for clinical use. However, similar success has not been reproduced for other viral diseases including HIV infection. It was noted that the HBV recombinant protein is different from many other viral recombinant proteins in regards to its ability to form particles, a feature that may render HBV recombinant protein super immunogenic (Liu 2011). Nevertheless, the field remains far less experienced with the recombinant subunit vaccine approach. Hence, it has been reasoned that for a long period of time, the formulation of HIV vaccines might be limited to HIV proteins or their DNA form (Gallo 2005).

The first clinical trial of DNA vaccination against HIV was performed in 1998, in which the infected patients were immunized with plasmid constructs encoding the *nef*, *rev*, or *tat* regulatory protein. The immunization resulted in detectable but transient-specific cytotoxicity in eight of nine patients immunized (Calarota et al. 1998). Since then, clinical studies using improved formulation and delivery strategies have been conducted. In several phase I trials, in an effort to improve the immunogenicity, additional plasmids encoding IL12 or IL15 were given together with DNA vaccine encoding HIV *env*, *gag*, and *pol* proteins. The inclusion of these molecular adjuvants led to an increase in magnitude and breath of cellular and humoral immunity (Felber et al. 2014). Recently, several vaccines using DNA as a

prime in combination with different boosts have also been evaluated. The boosts used in these trials were either viral vectors (recombinant Modified Vaccinia Ankara (rMVA), recombinant adenovirus (rAde), or recombinant Vesicular Stomatitis virus (rVSV)) expressing *env* or in the form of a purified recombinant gp120 protein (Felber et al. 2014). The rationale of using a DNA prime and protein boost approach is based on the observations that DNA vaccination is suited to eliciting strong cellular immunity, whereas protein immunization preferentially induces antibody response (Felber et al. 2014). From 2003 to 2006, a large-scale efficacy trial carried out in Thailand (RV144 trial) used a similar prime-boost schedule, in which the priming DNA (HIV *env*, *gag*, and *pro* gene) was introduced by a canarypox vector rather than by a naked DNA vaccine. Although the DNA uptake efficiency mediated by viral vectors could be higher than naked DNA, one potential disadvantage of using viral vector is that the preexisting anti-vector immunity may dampen the effectiveness of the priming immunization, an issue that can be perceived from the failed STEP (also referred to as Merck V520-023) trial. Nonetheless, until now the RV144 trial is the only one to demonstrate a transient and modest protection benefit. Notably, the limited protection appeared to be correlated with the humoral responses. Nevertheless, a consensus agreement has been reached in the field that an effective HIV vaccine should afford both humoral and cellular immunity whereby neutralizing antibody blocks virus entry at the sites of infection, and CTLs eliminates any infiltrating infection (Felber et al. 2014).

An additional application of DNA vaccines for HIV is the potential for use as therapy. Using the Simian immunodeficiency virus (SIV)/macaque model, a therapeutic DNA vaccine induced potent cellular responses so that the viral rebound was not observed for many months after cessation of the anti-retroviral therapy (ART) (Felber et al. 2014). Similarly, up to tenfold reduction in viremia and delay in the kinetics of viral rebound has been observed in some human studies testing the therapeutic DNA vaccines (Mylvaganam et al. 2015). However, the clinical benefit of these positive results is still unclear. It has been hypothesized that to optimally boost the preexisting HIV-specific immune responses, therapeutic vaccines need to be combined with other therapeutic approaches such as check point inhibitors (Mylvaganam et al. 2015).

## Application in other Viral Diseases

The application of DNA vaccination is not limited to influenza and HIV infection. To date, there are more than 10 viral diseases for which DNA vaccines have entered clinical trials. A list of clinical trials targeting other viral diseases is summarized in Table 1. Among these trials, a therapeutic DNA vaccine targeting cytomegalovirus (CMV) reactivation in CMV-seropositive hematopoietic stem cell transplant recipients has entered the pivotal phase III study (NCT01877655) in 2013. This vaccine consists of two plasmids expressing CMV antigens glycoprotein B (gB) and phosphoprotein 65 (pp65). In a previous phase II trial (NCT01903928), this CMV DNA vaccine provided initial evidence of the safety and revealed significant reduction in viral load and extended period of time before the onset of viremia (Smith et al. 2013).

**Table 1** Clinical trials of DNA vaccines in infectious diseases

Pathogen	Encoded antigen	Character	Additional protocol	Trial phase	Identification number
Influenza	H1N1 HA	Preventive		I	<sup>i</sup> NCT01587131
	H5N1 HA	Preventive		I	NCT01142362
	H5N1 HA	Preventive	H5N1 MIV boost	I	NCT00776711
HIV <sup>a</sup>	Gag, Pol, Env	Therapeutic	IL12 DNA adjuvant	I	NCT02431767
	Rev, Nef, Tat, p17, p24, RT, gp160	Preventive	i.m., i.d., s.c., delivery	I	NCT02075983
	Multi-Ag cocktail	Preventive	IL12 adjuvant, rVSV-gag boost	I	NCT01578889
	gag, env, pol, nef, and tat	Preventive		I	NCT00545987
	env, gag	Preventive	MVA-env, MVA-gag/pol boost	I	NCT01260727
	Nef/tat/vif, env	Preventive	rSVS-env boost	I	NCT02654080
	Multi-Ag cocktail	Preventive	IL12 adjuvant, Ad35-env boost	I	NCT01496989
	Multi-Ag cocktail	Therapeutic	IL12 adjuvant, rVSV-gag, boost	I	NCT01859325
HCV <sup>b</sup>	NS3/4A, NS4B, and NS5A	Therapeutic		Ila	NCT00563173
		Therapeutic		I	NCT02027116
HBV <sup>c</sup>	S, L, C	Therapeutic	IL12 adjuvant	I	NCT01641536
	S, C	Therapeutic	IL12 adjuvant	I	NCT02431312
	PreS2/S	Therapeutic		II	NCT01487876
EEV <sup>d</sup>	C-E3-E2-6 K-E1	Preventive		I	NCT01984983
HNTV <sup>e</sup> and PUUV <sup>f</sup>	M	Preventive		Ila	NCT02116205
MERS CoV <sup>g</sup>	S	Preventive		I	NCT02670187
CMV <sup>h</sup>	gB, pp65	Therapeutic		III	NCT01877655
Malaria	CS, SSP2, LSA-1, Exp-1	Preventive		I	NCT01169077

<sup>a</sup>HIV: human immunodeficiency virus<sup>b</sup>HCV: human hepatitis virus C<sup>c</sup>HBV: human hepatitis virus B<sup>d</sup>EEV: Equine Encephalitis virus<sup>e</sup>HNTV: Hantaan virus<sup>f</sup>PUUV: Puumala virus<sup>g</sup>MERS Cov: Middle East Respiratory Syndrome Coronavirus<sup>h</sup>CMV: Cytomegalovirus<sup>i</sup>NCT: National Clinical Trial

## Clinical Use in Cancers

It has been thought for some time that T lymphocytes act as sentinels in recognizing and eliminating continuously arising neoplastic cells. Indirect evidence supporting the notion that immunotherapy could be a useful alternate treatment for cancers comes from the observations that the incidence of some malignancies, such as Kaposi's sarcoma, non-Hodgkin's lymphoma, and invasive cervical cancer, was increased significantly in immunocompromised patients. The presence of CD8<sup>+</sup> and T<sub>H</sub> cells in the tumor microenvironment was also found to be a favorable prognostic factor for many different cancers. Moreover, tumor infiltrating T lymphocytes (TILs) have been successfully used in adoptive T cell transfer for treatment of metastatic melanoma and resulted in tumor regression. Thus, one effective approach for developing cancer immunotherapies is to activate the immune system through vaccination to generate cytotoxic T lymphocytes capable of eliminating tumor cells.

DNA immunization is of particular interest for developing therapeutic cancer vaccines based on the generation of T cells. Ideally, the vaccination-activated T cells should be able to recognize the tumor-associated antigens (TAAs) and initiate targeted killing of malignant cells. However, most tumors are caused by loss of growth control in normal tissues and thus express self-antigens that are either not recognized by or are only weakly reactive to the immune system. This phenomenon is described as immune tolerance, an inherent mechanism to prevent autoimmunity in which the immune system attacks the host's own cells and tissues. Therefore, one key element to improve DNA vaccine efficacy is to formulate a vaccine with an immunogenic cancer antigen so that it can prime T cells for immune responses.

## Cancer Antigens

To date, a large number of cancer antigens have been identified. Based on their expression pattern, cancer antigens can be classified into four categories. The first group consists of antigens that are known as cancer-testis (CT) antigens. These antigens are only expressed on particular tumor cells and immune privileged germ line tissues but not on normal adult cells. Melanoma-associated antigen (MAGE) and New York Esophageal Squamous Cell Carcinoma 1 (NY-ESO-1) are examples of this group. The second class of antigens is a large and diverse group that includes any protein found at increased levels in tumors compared with normal healthy cells and tissues. These antigens are termed as overexpressed self-proteins. Representative members for this family are prostate-specific membrane antigen (PSMA), human telomerase reverse transcriptase (hTERT), and human epidermal growth factor receptor 2 (HER2/neu). Antigens that are specific to a certain type of tissue and shared between tumors and normal tissue of origin are called differentiation antigens. Prototypes in this class include gp100, tyrosinase, and Melan-A/melanoma antigen recognized by T cells 1 (MART-1), which are molecules expressed by both melanoma and normal melanocytes. Finally, antigens that are found exclusively on tumor cells but not on any normal tissues are called tumor-specific antigens (TSA).

The unique tumor antigens are generated by somatic mutations as a result of aberrant gene expression. The mutated proteins usually play an important role during the process of cancer development and thus are selected to survive (Yang et al. 2014). Because tumor-specific antigens are only present on tumor cells, they are readily recognized by the host immune system and are ideal targets for cancer vaccine development. However, incorporating tumor-specific antigens in the vaccine design is complicated by the fact that the same tumor type can be induced by various distinct point mutations, making it difficult to identify a broadly applicable tumor-specific antigen for a vaccine (Yang et al. 2014).

Viral antigens carried by oncogenic viruses such as HPV and HBV represent another class of tumor-specific antigen. For example, the two HPV viral oncoproteins, E6 and E7, are required for the induction and maintenance of cellular transformation and are consistently co-expressed in HPV-associated cancers. Their exclusive presence on virus-infected cells and their nature of foreign origin renders vaccines targeting these proteins less vulnerable to the central immune tolerance and the undesired “on-target off-tumor” cytotoxicity.

Although cancer antigens have the potential to activate the immune system resulting in tumor regression, their application in human cancer vaccination is hampered by their inherent poor immunogenicity. Most cancer antigens are tumor related rather than tumor specific. The unresponsiveness to self-antigens caused by immune tolerance is a major roadblock for cancer vaccine development. Thus, there is a pressing need for innovative cancer vaccine design so that immune tolerance can be circumvented.

## Antigen Design for Breaking Immune Tolerance

One way to induce immunity against a tissue specific differentiation antigen on cancer cells is to vaccinate with a xenogeneic version of antigen. As the name suggests, a xenogeneic antigen is homologous to the cancer antigen but originates from a species foreign to the host. Xenogeneic DNA vaccines incorporate plasmid DNA encoding for a protein homologous between the two species. Ideally, xenoantigens should be sufficiently different from self-antigens in order to be immunogenic; they must, on the other hand, also preserve an optimal homology range with self-proteins to secure the cross-reactivity of the induced immune responses. The most striking example of using xenogeneic antigen to break immune tolerance is evidenced by the licensed canine melanoma DNA vaccine, a therapeutic agent for advanced melanoma (Bergman et al. 2003). This vaccine consists of DNA encoding a human version of tyrosinase, which enabled the dog’s immune system to break tolerance to the dog tyrosinase and mount an effective response. The concept of using xenoantigens to enhance vaccine immunogenicity is also supported by a recent preclinical study in which mice immunized with human p53 (hp53) DNA vaccine protected against challenge with murine colon cancer MC38 while those immunized with mouse p53 (mp53 DNA) were not (Soong et al. 2013). In a therapeutic model, established MC38 tumors were also well controlled by treatment

with hp53 DNA therapy in tumor bearing mice compared to mp53 DNA. Mice vaccinated with hp53 DNA plasmid exhibited an increase in mp53-specific CD8<sup>+</sup> T-cells compared to vaccination with mp53 DNA (Soong et al. 2013).

Associative recognition is another strategic antigen design aimed at breaking central immune tolerance. The rationale of this approach is to introduce additional gene coding sequences to a DNA vaccine by which a fusion protein containing the cancer antigen and the associated peptide is expressed. The peptide moiety has epitopes that are highly immunogenic to T<sub>H</sub> cells, which help induce CD8<sup>+</sup> cytotoxic T cell and CD4<sup>+</sup> helper T cell responses against the weakly immunogenic self-antigen. For example, tetanus toxin (TT) contains epitopes that avidly bind to T helper cells. In several preclinical studies, TT-fused antigen has been used in DNA vaccines to significantly enhance the immunogenicity of various cancer antigens including c-Myb, PAS Domain Containing 1(PASD1), and NY-ESO-1. These studies demonstrate the potential of DNA vaccines incorporating TT epitopes in order to enhance immunogenicity (Yang et al. 2014).

Breaking central immune tolerance is critical for cancer vaccine efficacy. However, breaking tolerance may raise the risk of autoimmunity. Many cancer antigens are also expressed on normal cells, albeit at very low level. Enhancing antigen immunogenicity should not be at the expense of damaging normal tissues. The detrimental “on-target off-tumor” side effect has been reported in cancer treatment using adoptive transfer of genetically engineered T cells. Until now, strategies used to break immune tolerance have not been shown to trigger harmful autoimmune attacks against normal tissues. However, more studies are needed to further evaluate the safety of breaking immune tolerance. It also should be noted that breaking immune tolerance represents only one of the greatest obstacles faced by scientists developing effective cancer vaccines. Other factors in the peripheral immunosuppressive networks including, but not limited to, myeloid-derived suppressor cells (MDSC), regulatory T cells (T<sub>reg</sub>), inhibitory cytokines, and immune exhaustion also profoundly affect the efficacy of DNA vaccination. Therefore, in agreement with the notion that most effective cancer therapies are multimodal, clinical development of cancer vaccines may ultimately be part of polytherapy (e.g., vaccine + checkpoint blockade mAbs) to counteract central and local immunosuppressive mechanisms.

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## Cancer Clinical Trials

Numerous therapeutic DNA vaccines are currently being evaluated in clinical trials to assess their translational potential. These vaccines target a wide variety of malignancies including cervical cancer, colorectal cancer, prostate cancer, breast cancer, head and neck cancer, melanoma, Merkel cell carcinoma, and leukemia. A summary of these trials including their targeted cancer antigens, strategies used to break the immune tolerance, and trial stages is shown in Table 2.

**Table 2** Clinical Trials of DNA vaccines in cancer immunotherapy

NCT Number	Indication	Encoded protein	Adjuvant/Additional intervention	Strategy for breaking immune tolerance	Trial phase
<sup>a</sup> NCT02241369	Aerodigestive Malignancies (e.g., Squamous Cell Carcinoma)	HPV6 E6 and E7	IL-12 DNA	Tumor-specific viral antigen	I
NCT02204098	Breast cancer	Mammaglobin-A	Neoadjuvant Endocrine Therapy	Tumor-specific antigen	I
NCT02348320	Triple negative breast cancer	Personalized Antigenic Peptide	N/A	Tumor-specific antigen	I
NCT02172911	Cervical cancer	HPV16/18 E6 and E7	IL-12 DNA	Tumor-specific viral antigen	I
NCT01304524	Cervical intraepithelial neoplasia	HPV16/18 E6 and E7	N/A	Tumor-specific viral antigen	II
NCT02596243	Cervical intraepithelial neoplasia	HPV16/18 E6/E7-FLT3L fusion	Built-in genetic adjuvant FLT3L	Tumor-specific viral antigen	II
NCT01064375	Colorectal cancer	CEA-tetanus toxoid fusion	GM-CSF adjuvant	Associative recognition	I/II
NCT01493154	Head and neck cancer	HPV E7-Calreticulin (CRT) fusion	Built-in genetic adjuvant CRT	Tumor-specific viral antigen	I



NCT02163057	Head and Neck squamous cell cancer	HPV16/18 E6/E7	IL12 DNA	Tumor-specific viral antigen	I/II
NCT01334060	Acute and chronic Myeloid Leukemia (AML, CML)	WT1-DOM fusion protein	N/A	Associative recognition	II
NCT01138410	Malignant melanoma (Stage III/IV)	SCB1	N/A	Associative recognition	I/II
NCT00471133	Skin and intraocular melanoma (Stage IIB, IIC, III or IV)	Mouse tyrosinase	N/A	Xenogeneic antigen	
NCT01440816	Merkel cell carcinoma	IL12	N/A	None	II
NCT00859729	Prostate cancer	rhesus PSA	N/A	Xenogeneic antigen	I/II
NCT02514213	Prostate cancer	PSA and PSMA	IL12 DNA	SynCon™ antigen	I
NCT02327468	Multiple solid tumors	Human TERT	IL12 DNA	Antigen with modifications <sup>b</sup>	I

<sup>a</sup>NCT: National Clinical Trial

<sup>b</sup>The TERT antigen encoded by the DNA vaccine was synthesized and codon-optimized. Two immunogenic mutations (R589Y and D1005Y) were incorporated into the hTERT sequence to assist in breaking tolerance

To date, the most successful and encouraging outcomes of using DNA vaccine in the clinical setting were obtained from treatment of malignant diseases where the etiological agent is of foreign viral origin, such as the human papillomavirus (HPV), as these viral agents can readily induce a strong immune response against cancerous cells harboring viral antigens. In the case of HPV, more than 100 strains have been identified with most of them causing asymptomatic infections. However, oncogenic high-risk HPV types (e.g., HPV16, 18, 31, 33, and 45) have been shown to be the etiological agents for cervical, penile, vulvar, vaginal, anal, and oropharyngeal cancers. Particularly, HPV16 and 18 have been regarded as the genotypes most closely associated with cervical intraepithelial neoplasia (CIN) and cervical cancers.

The HPV genome encodes two classes of proteins including the early (E) and late (L) proteins. The late proteins (L1 and L2) are structural components of viral capsid, whereas, the early proteins (E1, E2, E4, E5, E6, and E7) are regulatory proteins participating in DNA replication, transcriptional regulation, cell transformation, and viral assembly. Among these viral proteins, E6 and E7 can inactivate tumor suppressor p53 and Retinoblastoma (Rb) protein, respectively, thereby preventing cellular apoptosis, promoting cell proliferation, and contributing to progression to intraepithelial neoplasia and carcinoma. Moreover, E6 and E7 expression in HPV-infected cells is constitutive and irrespective of infection stage. In contrast, after primary infection, the expression of several early (E2, E4, and E5) and late (L1 and L2) proteins becomes invisible as a result of the deletion of the viral genes upon viral integration into host genome (Lin et al. 2010). Therefore, owing to their constitutive expression in HPV-associated malignancies and their crucial role in tumor pathogenesis, the E6 and E7 proteins are ideal choices of HPV antigens for developing therapeutic vaccines against HPV-associated malignancies. Indeed, several DNA vaccines targeting the HPV16/18 E6 and E7 proteins have been tested in clinical trials and demonstrated impressive efficacy as evidenced by regression of high grade CIN to normal tissue and clearance of viral infection.

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## Conclusions

During the last few years, great progress has been made in the field of DNA vaccination. The advancements in plasmid construction, antigen design, delivery device optimization, and use of immunologic adjuvants have greatly enhanced the immunogenicity and efficacy of DNA vaccines. Among these developments, electroporation represents the most remarkable progress, which exerts the greatest impact on DNA vaccine efficacy. Recently, a HPV16/18-specific DNA vaccine delivered by EP demonstrated clinical benefit in a phase IIb clinical trial in the form of regression of cervical intraepithelial neoplasia (CIN2/3). This encouraging result has led to resurgence of the platform. It is promising that with further improvement, DNA immunization will bring revolutionary transformation to the vaccine field. To that end, scientists are working diligently towards the first licensure of DNA vaccines.

## Cross-References

### ► Gene Delivery by Electroporation In Vitro: Mechanisms

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