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Molecular mechanisms for enhanced DNA vaccine immunogenicity

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Summary

In the two decades since their initial discovery, DNA vaccines technologies have come a long way. Unfortunately, when applied to human subjects inadequate immunogenicity is still the biggest challenge for practical DNA vaccine use. Many different strategies have been tested in preclinical models to address this problem, including novel plasmid vectors and codon optimization to enhance antigen expression, new gene transfection systems or electroporation to increase delivery efficiency, protein or live virus vector boosting regimens to maximise immune stimulation, and formulation of DNA vaccines with traditional or molecular adjuvants. Better understanding of the mechanisms of action of DNA vaccines has also enabled better use of the intrinsic host response to DNA to improve vaccine immunogenicity. This review summarizes recent advances in DNA vaccine technologies and related intracellular events and how these might impact on future directions of DNA vaccine development.

Keywords

DNA vaccine; immunogenicity; molecular adjuvant; plasmid; vaccine delivery

Introduction

Unlike conventional protein-based vaccines, DNA vaccines are based on bacterial plasmids that encode vaccine antigens driven by efficient eukaryotic promoters [1]. Like protein vaccines, DNA vaccines can be delivered through a variety of different routes, including intramuscular, subcutaneous, mucosal, or transdermal delivery. However, unlike protein antigens, the DNA vaccine to be effective must gain entry to the cytoplasm of cells at the injection site in order to induce antigen expression *in vivo*, thereby enabling antigen presentation on major histocompatibility molecules (MHC) and T-cell recognition. DNA vaccines have proved successful in a variety of animal models in preventing or treating

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infectious diseases, cancer, autoimmunity or allergy [2]. DNA vaccines have many advantages over traditional vaccines with vaccine design being straightforward, generally only requiring one-step cloning into plasmid vector thereby reducing cost and production time. Furthermore, the *in vivo* expression of an antigen gene driven by a eukaryotic promoter and endogenous post-translational modification results in native protein structures ensuring appropriate processing and immune presentation. From a safety aspect, cloning or synthesis of nucleic acids rather than having to purify proteins from pathogens avoids the need for use of pathogenic microorganisms in vaccine manufacture. Recombinant DNA technology allows almost any kind of molecular manipulation on plasmid DNA, including *in vitro* mutation, enabling rapid redesign of antigens for pathogens such as influenza that exhibit constant antigenic drift. Plasmid DNA has a good safety record in humans, with the most common side effects being mild inflammation at the injection site. Plasmid DNA is stable at room temperature, avoiding the need for a cold chain during transportation. DNA vaccines enable expressed antigens to be presented by both MHC class I and class II complexes, thereby enabling stimulation of both CD4 and CD8 T cells [3]. Already, several veterinary DNA vaccines have been approved for use, including in fish (infectious haematopoietic necrosis virus), dogs (melanoma), swine (growth hormone releasing hormone) and horses (West Nile virus) [4]. Human applications of the technology have lagged, largely due to the sub-optimal immunogenicity when compared to traditional vaccine approaches. To address these obstacles, a large number of different strategies to enhance DNA vaccine immunogenicity have been tested including vector design improvement, antigen codon optimization, use of traditional adjuvant and molecular adjuvants, electroporation, co-expression of molecular adjuvants and prime-boost strategies. In this review, we have specifically focused on promoter selection for DNA vaccine vectors, potential shortcomings of codon optimization, potential new insights from 'omics' studies and RNAi technologies, molecular adjuvants, targeting strategies and technologies for delivery of DNA vaccines. Although many of the described advances have so far only been tested in preclinical models, an increasing number have advanced to the stage of human clinical trial testing.

Mechanism of action of DNA vaccine

The first proof-of-concept DNA vaccine was tested in 1990 and involved injecting RNA or DNA vectors expressing chloramphenicol acetyltransferase, luciferase, and beta-galactosidase into mouse skeletal muscle [5]. The expression of reporter genes *in vivo* was readily detectable and lasted for two months. Subsequent gene gun studies showed that DNA-coated gold micro-projectiles when propelled into mouse skin were highly efficient in inducing antibody responses to the expressed antigen [6]. Finally it was shown that injecting plasmid expressing influenza nucleoprotein into the quadriceps of BALB/c mice could induce cytotoxic T lymphocytes with the immunized mice being protected from challenge with heterologous influenza A virus strains [7]. Similar studies in chickens also demonstrated protection against H7N7 influenza virus challenge after two doses of H7-HA DNA vaccine [8]. Although, these early studies confirmed the potential of using nucleic acids as vaccines, many practical questions still needed to be addressed. The first was the safety of plasmid DNA and the risk that it would integrate into chromosomes and induce

activation of oncogenes or mutation of tumour suppressor genes or increase chromosome instability. Subsequent studies confirmed that DNA vaccines have an extremely low probability of human genome integration, at a level lower even than that of spontaneous mutations [9]. Another important question was how DNA vaccines, given their extremely low level of expression, are able to induce immune responses. Compared to the short half-life of injected protein antigens, plasmid DNA can provide tissue expression of antigens over much longer periods of time, thereby potentially priming the immune system better. In regard to antigen presentation, three possible mechanisms have been proposed: (1) the plasmid DNA is internalised and expressed by somatic cells (e.g. myocytes) and presented by their MHC class I complexes to CD8 T cells; (2) professional antigen presenting cells (APC), e.g. dendritic cells, attracted to the injection site are transfected by the plasmid DNA and the expressed antigens are presented to T cells through MHC class I and II complexes; (3) phagocytosis of plasmid-transfected somatic cells by professional APC resulting in cross-priming and presentation of antigen to both CD4 and CD8 T cells. Because muscle cells are not able to present antigen through MHC class II as needed to induce CD4 helper T cells, direct or indirect presentation by professional APC is the most likely route.

Intrinsic elements of plasmid DNA can also activate innate immune responses, thereby enhancing adaptive immune responses against the expressed antigens. The innate immune system uses pattern-recognition receptors (PRR) to sense invasion of pathogens and induce downstream production of type I interferons and pro-inflammatory cytokines. In both mouse and human, toll-like receptor-9 (TLR9) is a cytosolic PRR that binds DNA sequences containing unmethylated cytosine-guanine (CpG) motifs leading to activation of MyD88-dependent signalling pathways [10]. CpG motifs are rare in mammalian genomes, in which they are generally methylated, whereas in bacteria unmethylated CpG motifs are common. Inclusion of built-in CpG motifs in DNA vaccine backbones acts to activate TLR9 after transfection. Ability of DNA vaccines to activate TLR9 has been suggested to be important in prime but not prime-boost contexts [11]. Although CpG motifs can play important roles in vaccine action, TLR9 knockout mice show that TLR9 is not essential for DNA vaccines to work [12,13], suggesting other cytosolic DNA sensors may also contribute to DNA vaccine immunogenicity. One such PRR is cyclic-GMP-AMP (cGAMP) synthase (cGAS) which, after recognition of dsDNA, induces cGAMP to activate the stimulator of interferon genes (STING) [14-16]. Another DNA PRR is DAI (DLM-1/ZBP1), which also activates STING and induces type I interferon expression [17]. TBK1, downstream of cGAS and DAI, is important to enhancement of DNA vaccine action [18]. Another important cytosolic DNA sensor is AIM2, which induces inflammasome activation and inflammatory cytokine production [19,20]. A recent study showed that both the humoral and cellular antigen-specific adaptive responses to DNA vaccines were significantly reduced in AIM2-deficient mice [21]. The helicase proteins, DHX29 and RIG-I sense cytosolic nucleic acids and may contribute to DNA vaccine action [22]. Other potential DNA sensors include DDX41, IFI16, DNA-PK and MRE11 [23-28]. Hence, these PRR and downstream signalling pathways may provide valuable means to enhance DNA vaccine action (Figure 1).

Research models of DNA vaccines

As in traditional vaccine research, small rodent laboratory animals are principally used for DNA vaccine research. For example, the query for “human” as the host from DNAVaxDB [29], a database collecting verified DNA vaccine study data returns only 6 records, while a query for “mouse” returns 168. Currently, this database has a total of 421 records of verified DNA vaccine studies including other animal models and some that are not clearly labelled. Although most of the studies cited in this review have been undertaken in mouse models rather than humans, they have been of critical importance in understanding the mechanisms by which DNA vaccines work, have helped in improving vector design and delivery methods and been useful for testing of adjuvants and for valuation of safety issues. While vaccine success in small animal models does not guarantee translation to humans, such studies remain an important part of the vaccine development pipeline. An increasing number of these advances from preclinical models have advanced into human clinical trials. As shown in Figure 2, we retrieved a total number of 162 records of DNA vaccines from a leading human clinical trial database (ClinicalTrials.gov) with more than half of the entries being from after 2011 with only 9% being from before 2005, indicating the relative infancy of human DNA vaccine research. Eighty-two percent of these registered DNA vaccine trials are listed as Phase 1, 13% as Phase 2 and only one trial is listed as Phase 3. Looking at the distribution of diseases DNA vaccines are currently being used to target, ~ 60% are directed at viruses like HIV, influenza or hepatitis and with ~ 27% targeting cancer. This is consistent with the need for new types of vaccines for viruses or diseases that are not well addressed using traditional vaccine approaches. Reassuringly, to date no major safety problems have emerged from human DNA vaccine trials.

DNA vaccine constructs design

Codon optimization

Codon usage of pathogens is often different to mammalian species, hence codon optimization is generally required to achieve efficient mammalian expression of pathogen proteins. Early studies in mouse models showed successful codon optimization results in enhanced CD8 T cell responses [30] and neutralizing antibody titres [31] and this has been supported by more recent studies [32-36]. Currently, different academic or commercial algorithms for codon optimization are available to assist DNA vaccine development [37,38]. However, codon optimization does not always positively correlate with DNA vaccine efficacy. For example, a malaria DNA vaccine study in mice showed that native nucleic acid sequence provided more robust T-cell responses and protection against *P. yoelii* sporozoite challenge [39]. Another murine study using codon-optimized plasmids that expressed *Schistosoma mansoni* Sm14 protein showed no increase in immunity or protection against *S. mansoni* challenge [40]. These discrepancies suggest that assumptions about codon optimization may not always hold true. Many studies have shown that rare-codons may not always be a speed-limiting step and frequently used codons do not guarantee increased protein production. Furthermore, synonymous codons could potentially change protein conformation and function as recently reviewed by Mauro et al [41]. Furthermore, the original pathogen sequences may provide better adjuvant effects through PRR interaction

than codon optimized sequences. Therefore, it is best that both original and codon-optimized sequences be always compared in the early phase of developing a new DNA vaccine.

Promoter selection

DNA vaccine gene expression is normally driven by a polymerase II type promoter. The endogenous mammalian Pol II promoters are not as strong as virus-derived promoters, such as cytomegalovirus (CMV) or SV40 promoters (e.g. pcDNA3.1, pVAX1, pVIVO2, pCI, pCMV and pSV2). Early studies showed the CMV immediate early enhancer/promoter had the strongest activity in most cell types and it was thus widely used for DNA vaccine design [42,43]. Studies using HIV-1 Env DNA vaccines have shown that stronger promoters induced higher protein expression and immune responses [44]. While some viral promoters can efficiently drive antigen expression, this has not always correlated with vaccine efficacy, an effect that may be explained by the viral promoters being sensitive to inhibition by inflammatory cytokines such as TNF α or IFN- γ . Non-viral promoters such as the MHC class II promoter have been shown in mice to overcome this issue [45]. Hence, while the CMV promoter, given its ability to drive a high level of protein expression, remains the first choice for most DNA vaccines, alternative promoters may result in better vaccine outcomes.

Optimization of plasmid vector backbone

Plasmid vectors used for DNA vaccine contain bacterial elements, such as replication signal and selection markers for propagation of *E. coli*. These elements can pose safety issues and result in poor antigen expression. For example, autoimmunity issues occurred when the Ampicillin selection marker in expression vector pcDNA3.1 was replaced with a Kanamycin selection marker [46]. Removing redundant vector sequences also makes it possible to clone larger DNA vaccine fragments. By using sucrose selection systems, traditional selection marker can be replaced. A sucrose selection construct was designed with incorporation of 72 base pair SV40 enhancer at the 5' of CMV promoter to increase the extra-chromosomal transgene expression of the human T-lymphotropic virus type I (HTLV-I) R region at the 3' of a CMV promoter to increase translation efficiency. Using this system, increased neutralizing antibody titers to HIV-1 gp120 DNA vaccination was achieved in rabbits [47].

To completely remove bacterial elements, a minicircle DNA (mcDNA) technology has been developed using site-specific recombination based on the ParA resolvase to generate mcDNA [48] or using inducible minicircle-assembly enzymes, PhiC31 integrase and I-SceI homing endonuclease [49]. mcDNA technology has been successfully used in gene therapy experiments in mouse models [50,51]. A recent study has shown that minicircle dna is superior to plasmid DNA in eliciting antigen-specific CD8⁺ T-cell responses [52]. Another study showed a modified novel Mini-Intronic Plasmid (MIP) system was robustly expressed *in vivo* and *in vitro* [53] and may thereby serve as a better vaccine backbone. Another study combined electroporation delivery along with minicircle DNA technology resulting in enhanced immunogenicity of a HIV-1 gag DNA vaccine in mice [54].

Traditional adjuvants for DNA vaccines

Vaccine adjuvants can be used to increase the immunogenicity of otherwise weakly immunogenic antigens. Vaccine adjuvants function through a series of mechanisms including activation of innate immune systems, formation of depot for efficient antigen delivery, induction of different chemokine expression, enhancing antigen uptake and presentation by professional APC and upregulation of co-stimulatory surface molecules [55]. Some such adjuvants have been shown to enhance the immunogenicity of DNA vaccines. Alum has been widely used as a vaccine adjuvant since 1926 [55], and is thought to work via induction of phagocytic cell death resulting in an immune danger signal [56]. Addition of alum adjuvant to a DNA vaccine encoding HBsAg increased antibody responses in mice, guinea pigs and nonhuman primates [57]. Similarly, formulation of a *Toxoplasma gondii* DNA vaccine with an alum adjuvant lead to increased survival in BALB/c mice [58]. As an alternative to alum, a polysaccharide adjuvant based on delta-inulin particles when used in a DNA prime-protein boost vaccine strategy, significantly increased humoral and cellular responses anti-Env-responses in mice [59]. Other approaches to DNA vaccines have used liposomes, which are spherical vesicles composed of lipid bilayer including phospholipids and cholesterol. Liposomes entrap or bind plasmid DNA and facilitate DNA entry into cells by penetrating the lipid bilayer of the cell membrane [60]. Although use of liposomes for intramuscular antigen delivery needs further improvement to overcome injection-site reactogenicity, they remain promising candidates for mucosal vaccination. A recent study in C57BL/6 mice showed that oral vaccination with cationic liposome-encapsulated pcDNA3.1-based influenza A virus M1 gene induced both humoral and cellular immune responses and protected the mice against respiratory infection [61]. Liposomes have also shown to be effective with intranasal DNA vaccination [62]. However, on the whole, traditional adjuvants have at best only modest effects on DNA vaccine immunogenicity, leading to attempts to develop more potent molecular adjuvant approaches.

Molecular adjuvants for DNA vaccines

Many vaccine plasmid-encoded immune-stimulatory molecules including various cytokine genes or PRR ligands have been tested as 'genetic adjuvants'. These genetic or molecular adjuvants take advantage of recombinant DNA technology, allowing them to be encoded in the same plasmid as the vaccine or a co-administered plasmid.

Ligands of pattern recognition receptors

Toll-like receptors (TLRs) are a class of membrane-bound PRR that play key roles in the innate immune system. So far, 13 related human TLR genes (TLR1–TLR13) have been identified [63]. TLR3 and TLR9 recognize dsRNA and ssDNA, respectively, and their ligands have been shown to act as molecular adjuvants. Poly (I:C) is a classical TLR3 ligand. Poly (I:C) adjuvant enhanced inducing CTL immunity and decreased tumour burden in mice given a DNA cancer vaccine [64]. Poly (I:C) similarly enhanced responses to a HPV-16 E7 DNA vaccine [65]. A combined CpG/Poly (I:C) adjuvant enhanced the immunogenicity in mice of a DNA vaccine against eastern equine encephalitis virus [62]. Similarly, the TLR9 ligand, CpG, has been successfully used to enhance immunogenicity of

DNA vaccines [62,66-68]. RIG-I-like receptors are important intracellular proteins that sense double stranded RNA. The RIG-I ligand, eRNA41H, enhanced the humoral immune response to an influenza DNA vaccine [69]. Similarly, a Sendai virus-derived 546-nucleotide ligand of RIG-I enhanced immunogenicity of influenza DNA vaccine [70]. TLRs, RIG-I-like receptor (RLRs), inflammasome and STING-dependent cytosolic DNA sensor ligands can all initiate Th2 cell differentiation [71]. Ligands of the cytosolic DNA sensor DAI have also been shown to be efficient molecular adjuvant for DNA cancer vaccines [72].

Plasmid-encoded cytokines

Cytokines are naturally produced small proteins critical for immune cell signalling. Cytokine-encoding plasmids can be prepared along with the antigen-expressing plasmid and, local expression of cytokines at the injection site avoids the potential toxicity of systemically administered cytokines. Interleukin (IL)-2 induces the proliferation of T and NK cells, and has been extensively studied as a genetic adjuvant [73-82]. A fusion construct of *Mycoplasma pneumoniae* p1 gene carboxy terminal region with IL-2 resulted in enhanced vaccine responses in mice [83]. A therapeutic vaccine for chronic myeloid leukemia expressing BCR/ABL-pIRES together with IL-2 also showed enhanced immune responses [84].

IL-12 is another proinflammatory cytokine secreted by DCs and monocytes. IL12 expression plasmids have been shown to enhance Th1 immune responses [85]. A bicistronic plasmid expressing *Yersinia pestis* epitopes and IL-12 increased mucosal IgA and serum IgG and protected mice from challenge [86]. IL-12 expression plasmids have also been used for a clinical trial of a weakly immunogenic hepatitis B DNA vaccine [87]. A recent study showed that IL12 genetic adjuvant enhanced hepatitis C DNA vaccine immunogenicity through stimulation of IL-4 and IFN- γ production [88]. A study of a *Toxoplasma gondii* DNA vaccine showed that the immune responses and survival rate were increased by inclusion of an IL-12 genetic adjuvant compared [89]. An IL-12-adjuvanted HIV/SIV DNA vaccine was also successful in a DNA prime /protein boost study [90]. Evaluation of the PENNVAX-B HIV1 DNA vaccine that is a mixture of 3 expression plasmids encoding HIV-1 Clade B Env, Gag, and Pol, adjuvanted by the IL-12 DNA plasmid showed the vaccine to be safe. Administration of PENNVAX-B with IL-12 plus electroporation had a significant dose-sparing effect and provided superior CD4+ and CD8+ T-cell immunogenicity [91].

GM-CSF is known to recruit APC to immunization site and promote DC maturation. It has been successfully used in DNA vaccines, including with a Pseudorabies virus gB-encoding, SIV encoding and DENV serotype 2 prM/E encoding, vaccines [92-95]. However, a recent study showed that co-administration of GM-CSF plasmid can have deleterious effects, causing suppression to a DNA vaccine against dengue virus type 1 and type 2 and failing to improve the response induced by HCV vaccine [96]. Furthermore, excessive GM-CSF can expand myeloid suppressor cells and suppress adaptive immune responses. Notably, in preclinical macaque studies GM-CSF expressed in recombinant SIV and MAV vaccines did not enhance protection [97]. Therefore, when used as a molecular adjuvant fine-tuning of GM-CSF expression levels must also be considered.

IL-15 is a cytokine that induces NK and T cell proliferation [98]. A synergistic effect of IL-15 and IL-21 was seen in a DNA vaccine against *Toxoplasma gondii* infection [99,100]. Sequential administration of IL-6, IL-7 and IL-15 genes enhanced CD4+ T cell memory to a DNA vaccine against foot and mouth disease [101]. Hence combinations of multiple cytokines in a DNA vaccine formulation or sequential vaccination with cytokines may enhance vaccine efficacy.

Easy cloning of cytokine genes makes them promising candidates as DNA vaccine adjuvants. The moderate but more durable expression at the injection site of plasmid-expressed cytokines helps overcome the problem of the short half-life of many cytokines while minimizing the risk of a systemic cytokine “storm” by restricting cytokine expression to the injection site. Although human data on use of cytokine-encoding plasmids as vaccine adjuvants is limited, this appears a promising direction for fine-tuning immune responses to DNA vaccines.

Plasmid-encoded signalling molecules

In the past ten years, understanding of immune signalling pathways has greatly progressed, making it possible to test such signalling molecules as vaccine adjuvants. Signalling molecules, TRIF and HMGB1, have been successfully tested as genetic adjuvants for DNA vaccines [102]. Similarly, co-transfection with HSP70 enhanced better CTL responses to DNA vaccines [103]. PD-1 based plasmids were shown to enhance DNA-vaccine-induced CD8+ T cell responses against HIV [104]. MDA5, a RIG-I like dsRNA receptor, enhanced DNA vaccination against H5N1 influenza virus infection in chickens [105]. Recent studies showed that a plasmid expressing interferon regulatory transcription factor (IRF), enhanced CTL and IFN- γ responses against an HIV-1 Tat vaccine [106], whereas no effects were observed with IRF3 or IRF7 plasmids [106]. NF- κ B is a master innate immune regulator. A recent study showed that co-administration of a plasmid expressing NF- κ B subunit p65/RelA enhanced DNA vaccine immunogenicity [107]. A T-cell transcription factor, Tbet was effective in driving a Th1 response against an Ag85B DNA-based tuberculosis vaccine [108,109]. DNA, itself, through binding to cellular receptors can activate innate immune pathways. A recent study found that a short noncoding DNA fragment of ~300bp improved electroporation-mediated gene transfer *in vivo* [110], and the immune potency of a HBV vaccine [111].

shRNA or siRNA as molecular adjuvants

RNA interference (RNAi) is a post-transcriptional gene silencing process triggered by double-stranded short hairpin RNA (shRNA) structures. Since its discovery, RNAi has mainly been used as a research tool for loss of function studies of target genes [112]. RNAi can be used to down-regulate genes that suppress DNA vaccine action. For example, use of shRNA to knock down caspase 12, a cell death mediator that is upregulated after DNA vaccination, increased plasmid gene expression and T-cell and antibody responses [113]. Similarly, depletion by RNAi of Foxo3, a critical suppressive regulator of T cell proliferation, increased the efficacy of a HER-2/neu cancer vaccine [114]. Knockdown of the IL10 receptor was also shown to enhance vaccine potency [115]. Furthermore, blockade of the PD-1 ligand (PD-L1) by RNAi augmented DC-mediated T cell responses and antiviral

immunity in HBV transgenic mice [116]. A recent cancer vaccine study that combined IL-10 siRNA and CpG showed increased protective immunity against B cell lymphoma [117]. Another cancer therapeutic vaccine showed that GM-CSF combined with shRNA knockdown of furin augmented vaccine efficacy [118]. Knockdown of APOBEC expression by RNAi also enhanced DNA vaccine immunogenicity [119]. Thus use of RNAi against target genes that limit plasmid expression might be a powerful new strategy for DNA vaccine enhancement, especially for tumour vaccines, but with the safety of this approach still needing to be fully evaluated in animal studies.

DNA vaccine targeting technologies

DNA vaccines mainly transfect muscle cells resulting in poor antigen presentation due to lack of co-stimulation. Instead gene expression can be targeted to professional APC to enhance vaccine action. One strategy is direct transfection of DC. A conventional approach is to use ex vivo-engineered DC vaccines, where enriched DC from blood are transfected with DNA before being given back to the patient. One recent mouse study showed that DC transfected with Spermine-dextran/CCR7 plasmid/gp100 plasmid migrated to lymph nodes when re-administered to mice [120]. Given the high cost of DC vaccines this approach is only likely to be used for therapeutic cancer vaccines [121]. More recent studies improved the efficacy of plasmid DNA by adding targeting protein, polymer or peptides. Liu et al. translated the “albumin hitchhiking” approach to DNA vaccines by using the lipophilic albumin-binding tail to target the cargo to DC [122]. Skin delivery of DNA loaded into polymer forming nanoparticles has been used to target Langerhans cells [123]. The barrier of direct targeting DNA to DC was overcome by incubating DNA vaccines with a small peptide derived from the rabies virus glycoprotein fused to protamine residues [124]. In addition to direct targeting of DNA to DC, another strategy is to co-express DC-targeting molecules in the DNA vaccine construct. Many such targeting approaches have been successful in mouse models, utilising a wide range of targeting molecules including FIRE (F4/80-like receptor), CIRE (C-type lectin receptor), Cle9A, Flt3, DEC205, xrc1 or synthetic MHC class II-targeting peptides [125-132].

Subcellular targeting is another strategy for enhancing plasmid-encoded antigen processing and/or presentation. Using E1A targeting to the endoplasmic reticulum or LAMP targeting to lysosome can greatly enhance DNA vaccine efficacy [133,134]. Targeting of the autophagy pathway enhanced the efficacy of tuberculosis DNA vaccines [135-137]. Similarly, a short polypeptide from herpes simplex virus that induces antigen aggregation and autophagosomal degradation enhanced T-cell-responses when co-expressed with chicken ovalbumin [138]. The plasmid pATREx expressing the aggregation domain of TEM8 induces intracellular protein aggregation, autophagy and caspase activation and this translated to an increased IgG1 response by a DNA vaccine encoding *Plasmodium yoelii* merozoite surface protein 4/5 [139]. Fusion of the PADRE MHC class II epitope to a cancer DNA vaccine was also able to induce stronger antigen-specific responses [140].

Targeting DNA vaccine to specific cells or subcellular compartments can greatly increase antigen processing and presentation and promote the desired immune responses, while minimizing systemic toxicity. It is thereby a very promising area of molecular adjuvant

development. Among different ways of targeting DC, skin targeting methods (intradermal injection or micro-needles) may be superior to traditional intramuscular or subcutaneous injection routes [141] and this will be further discussed in the section on vaccine delivery devices.

High-throughput screening technologies

Developments in next-generation sequencing, microarrays, and high throughput proteomics approaches now provide opportunity to identify additional potential molecular adjuvants for DNA vaccines. One recent proteomics study screened proteins for interaction with plasmid DNA and found that human serum amyloid P (SAP) inhibited plasmid transfection and enhanced clearance. SAP may contribute to the low efficacy of DNA vaccines in humans, with its suppressive effects being much weaker in other species [142]. Therefore, it might be possible to include shRNA against SAP as a molecular adjuvant for human DNA vaccines. Systems biology approaches have also been used to analyze the molecular signatures that correlate with a positive immunization response. For example, CaMKIV kinase expression levels at day 3 were negatively correlated with subsequent influenza antibody titers [143]. This provides an example of the application of systems biology to identify biomarkers that predict vaccine effectiveness [144-147] with identified molecules serving as potential new candidate molecular adjuvants.

Prime-boost strategies

The immunogenicity of DNA vaccines is limited in humans. Prime-boost approaches like DNA prime/protein boost, DNA prime/viral vector boost (e.g. using adenovirus) and even protein prime/DNA boost provide opportunities to develop more effective human DNA vaccine regimens. Previous studies have used DNA/protein or DNA/Ad-vector approaches for HIV vaccination [148-152]. A recent study using a heterologous prime/boost approach showed enhanced immunogenicity of therapeutic vaccines for hepatitis C virus, with higher protection in a recombinant *Listeria*-NS3 1a –based surrogate challenge model [153]. Another study showed that a DNA prime/adenovirus boost malaria vaccine encoding P. falciparum CSP and AMA1 induced cell-mediated immunity and sterile protection [154]. Similar studies also showed greatly enhanced immunogenicity with a peptide prime/DNA boost or BCG prime/DNA boost, [155,156]. The interval between prime and boost was also important for best vaccine efficacy [157,158]. The underlying mechanisms of the effectiveness of prime-boost strategy still remain poorly understood, but we hypothesize that the lower antigen expression from DNA vaccines may preferentially prime T-helper cell responses with the humoral response being subsequently boosted by the protein or viral-vector boost.

Vaccine delivery

To overcome the main issue of low DNA vaccine immunogenicity, efficient delivery to plasmid to preferred tissues or cells is a critical research area. Just as in the case of conventional protein vaccines, DNA vaccines can be administered by many different methods, including conventional syringe injection, gene gun, electroporation (EP) [159,160],

nanoparticles [161], microneedles [162] and liposomes [163]. Based on animal studies, EP combined with a prime-boost strategy may give the highest immunogenicity. However, even though clinical trials of EP have been successfully undertaken [164,165] and shown dose-sparing effects [166], EP is not guaranteed to increase vaccine immunogenicity [165], and may not be practicable for many human vaccines. Hence simpler but more efficient methods are still needed for DNA vaccine delivery. Potential candidates include needle-free skin delivery or pulmonary delivery. Needle-free skin gene delivery systems use high-pressure fine streams to target different skin depths. The delivered DNA is then able to transfect Langerhans cells in the skin. Commercial devices such as Biojector or Pharmajet have been shown to be able to deliver DNA vaccines in animal models and human trials. A recent study using a rabbit model showed that an influenza DNA vaccine delivered by needle-free IDAL vaccinators elicited a similar antibody response to intradermal electroporation [167]. Another clinical trial used Biojector to deliver an HIV-1 DNA vaccine (VRC-HIVDNA016-00-VP) with good tolerance and priming for CD8+ T cell IFN- γ production and antibody responses after rAd5 boosting [168]. Considering its safety, low-cost and ease of use and the ability to deliver dry-coated plasmids [169,170], needle-free injection devices show much promise for DNA vaccine delivery.

Intranasal or intrapulmonary delivery of DNA vaccines are additional alternatives although the number of human studies of such administration routes is very limited. In theory, pulmonary vaccination should expose the delivered DNA and expressed antigens to a large surface of epithelial and immune cells while avoiding the need for needle injection [171-174]. A recent study showed that cation-complexed HIV-1 plasmid DNA vaccines applied topically to the murine pulmonary mucosa induced both mucosal and systemic immune responses models [175]. Furthermore, when complexed with PEI a pulmonary DNA vaccine induced significant influenza protection [175]. Another study showed that pulmonary DNA immunization mediated protective anti-viral immunity through enhancement of airway CD8+ T cell responses [176]. The biggest challenge for pulmonary delivery is the need for specialized delivery methods. Conventional jet or ultrasonic nebulizers fail to deliver large intact biomolecules like plasmid DNA to the lung because of the high shear and cavitation stresses present during nebulization. However, recent progress in the area of delivery methods may change this, with surface acoustic wave nebulization being shown to efficiently deliver an aerosolized plasmid DNA vaccine in rats or sheep with induction of systemic and mucosal antibody responses [177].

Expert commentary and 5-year view

Progress in overcoming the low immunogenicity problem of DNA vaccines has been steadily made. Recent advances are summarized in Table 1. Along with better understanding of immune pathways and vaccine action, a large array of immune receptors, signalling molecules, cytokines and transcription factors are in the process of being tested as DNA vaccine adjuvants. It is likely that at least some of these will turn out to be potent adjuvants, although each will need to be carefully assessed for human safety and tolerability. The ability to present native conformational immunogens and be able to prime both humoral and cellular immune responses are the hallmarks of DNA vaccines. Considering the suitability

and convenience of needle-free injection and pulmonary delivery, these represent an exciting area for human DNA vaccine development over coming years.

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Reference annotations

* *Of interest*

** *Of considerable interest*

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Key issues

- DNA vaccines have the benefits of being able to express antigens in their native conformation inside cells and thereby induce CD8+ T cell responses.
- Currently more than 150 human DNA vaccine trials are ongoing or completed.
- Animal and human trial data has showed good tolerance and safety of DNA vaccines.
- Codon optimization and strong viral promoters do not always enhance DNA vaccine immunogenicity.
- Minicircle DNA or Mini-Intronic Plasmid systems are promising for bacterial element-free DNA vaccine production.
- Molecular adjuvants including plasmid-encoded cytokines and signalling molecules and RNA knockdown strategy can be used to enhance DNA vaccine immunogenicity.
- Needle-free skin delivery or pulmonary delivery methods may be promising directions for future use.

Figure 1. Mechanisms of DNA vaccine sensing in transfected immune or non-immune cells
 (A) DNA vaccines transfect myocytes, keratinocytes or dendritic cells (DC). DCs are able to present antigens directly while myocytes or keratinocytes rely on cross-presentation pathways. (B) Molecular events are summarized for DC vaccines. TLR9 is not critical for DNA vaccine action, although it may still play a contributory role given the CpG motifs in most DNA vector backbones. Recent studies showed that STING/TBK1/IRF3 pathways and the AIM2 inflammasome are important to DNA vaccine action. Other innate immune receptors playing a role in DNA vaccine sensing include cyclic-GMP-AMP (cGAMP) synthase (cGAS), AIM2, DHX29 and RIG-I. Additional potential DNA sensors are now under study, including DDX41, IFI16, DNA-PK and MRE11. Molecular adjuvants that represent ligands of the above sensors and signalling proteins are currently being tested for their ability to improve DNA vaccine immunogenicity.

Year of completion

Phases

Type of disease

Figure 2. Summary of human clinical trials involving DNA vaccines

Number of clinical trials of DNA vaccines carried out in different time periods, clinical trial phases and the diseases being targeted are summarized for all 162 DNA vaccine trials registered in the ClinicalTrial.gov database.

Table 1
Summary of recent key advances in DNA vaccine design

Development of DNA vaccine			
		Knowledge or technologies from before	Recent updates and comments
Mechanism of action of DNA vaccine	Critical dsDNA sensor and signalling pathways	TLR9 as sensor of CpG DNA bacterial origin [10].	TLR9 is now known not to be critical for DNA vaccine action [12, 13].
		MAVS, TBK1, IRF3 and STING are key signalling proteins.	TBK1-STING signalling shown important for DNA vaccine action and STING shown to be a dsDNA sensor [18].
		DAI, AIM2 and RNA pol III are DNA sensors [19-21].	DAI is now known not to be critical for DNA vaccine action [17]. More dsDNA sensors were found, including LRRFIP1, DHX9, DHX36, IFI16, Ku70, DDX41, DNA-PK, MRE11, cGAS and Rad50 [22-28]. cGAS could be another key sensor of DNA vaccines [14-16].
DNA vaccine design	Antigen sequence design	Based on experience or back-translation of protein antigens.	High-throughput 'omics' technologies will allow rapid identification and design of optimal antigen sequences [143-147].
	Codon optimization	Codon optimization thought important for all DNA vaccines [30-36].	Evidence showed codon optimization may not always have positive effect on vaccine immunogenicity [39-41]. It is recommended to always include the original gene version for comparison with any new codon-optimized antigen sequence.
	Promoter selection	CMV promoter considered optimal [42, 43]. DNAVaxDB records show the CMV promoter was used in 109 out of 181 total DNA vaccine trials. The dominant vectors were pcDNA3 or pVAX based [29].	CMV promoter is still the first choice. But non-viral promoters, e.g. MHC-II promoter, have also been shown to be good alternatives if CMV promoter not suitable [45].
	Removal of bacterial element	Hard to achieve using conventional vectors such as pcDNA or pVAX.	Minicircle DNA (mcDNA) [49-52] and Mini-Intronic Plasmid (MIP) systems [53, 54] are promising for future development.
Molecular adjuvants		TLR ligands, e.g. CpG ODN or poly(I:C) as DNA vaccine adjuvants [64, 65].	Greater range of molecular adjuvants. RIG-I ligand, e.g. eRNA41H, shown to enhance DNA vaccine action [69]. STING ligand can initiate Th2 cell differentiation in mouse model [71]. Ligands to dsDNA sensor, DAI, shown to be efficient adjuvants for cancer DNA vaccines [72].
		Plasmid-encoded cytokines or chemokine shown to have adjuvant effects for DNA vaccines in animal studies [102].	Effects of genetic cytokine adjuvants have been supported by more recent studies. But studies also showed that in some cases GM-CSF is negative [96, 97]. Cytokine expression needs to be tuned to avoid "cytokine storm". Safety aspects need to be carefully considered for such adjuvants.
		Plasmid-encoded signalling molecules such as TRIF or HMGB1 shown to act as DNA vaccine adjuvants [102].	MDA5 (a RIG-I like dsRNA receptor) [105], NF-kB [107] and Tbet [108, 109] shown to be effective adjuvants. A large range of signaling molecules are now available as candidate adjuvants for DNA vaccines.
		Immune regulatory genes impeding DNA vaccine action not considered.	shRNA or siRNA targeting certain regulatory genes, e.g. caspase 12 [113], Foxo3 [114], PD-L1 [116] and IL-10 [117] shown to have adjuvant effects. RNAi against APOBEC enhanced DNA vaccine immunogenicity [119].

Development of DNA vaccine			
		Knowledge or technologies from before	Recent updates and comments
Targeting technologies	Direct DC targeting	DC vaccines loaded with antigen, <i>ex vivo</i> .	DCs can now be targeted directly <i>in vivo</i> . Lipophilic albumin-binding tail approach directly targets DC <i>in vivo</i> [122]. Polymer forming nanoparticles shown to target Langerhans cells after skin delivery [123].
	Co-expression of DC targeting molecules	DC targeting <i>ex vivo</i> .	Broader range of molecules targeting DC <i>in vivo</i> identified, e.g. FIRE, CIRE, Cle9a, Flt3, DEC205, xrc1 or synthetic MHC-II [125-132]. Now being used in combination with skin-delivery methods [141].
Prime-boost strategy		DNA vaccines largely used by themselves but with poor immunogenicity despite repeated doses.	Increased awareness of the importance of prime boost strategies to boost DNA vaccine immunogenicity [153-158].
DNA vaccine delivery		Syringe injection, gene gun [159, 160], nanoparticle [161], micro-needle [162] and liposomes [163] extensively studied. Limitations in regard of ease of use and efficiency of delivery.	Although new methods are being tested, conventional intramuscular injection still dominates DNA vaccine delivery.
		Electroporation shown to be efficient in animal models.	Electroporation (EP) is now being combined with intradermal vaccine delivery. Tolerability and cost remains an issue and EP is simply not practical for most human vaccine applications.