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DNA Vaccines: How Much Have We Accomplished In The Last 25 Years?

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Abstract

Vaccination has been the most efficacious way to combat infectious diseases in human history. Nevertheless, there are still a variety of pathogens for which vaccines are urgently needed. In the last 25 years, DNA vaccines emerged as promising in prophylactic and therapeutic settings. However, despite all the practical advantages, DNA vaccines face challenges in inducing potent antigen specific immune responses and protection in humans. In the last years, rational approaches to improve the efficacy of DNA vaccines were developed and include: modifications of plasmid basic design, use of next-generation delivery methods, addition of adjuvants in the formulation, improvement in immunization protocols and even targeting to dendritic cells. In this review, we will explore the advances and hurdles involved in the development of more potent DNA vaccines.

Introduction

Since their development, vaccines have had an enormous impact on public health. Millions of lives were saved and pathogens that used to cause fatal diseases were controlled (or even eradicated) in many parts of the world. Vaccination is nowadays the most efficient way of controlling infectious diseases and the search for new and improved vaccines continues as a way to ameliorate human health.

Vaccines are traditionally based on immunogens delivered as inactivated (Influenza) or attenuated live (smallpox) pathogens, recombinant proteins (Hepatitis B) or virus-like particles (Human Papillomavirus). Their ability to induce protection is primarily based on antibody-dependent mechanisms that work quite well by blocking infections caused by viruses like variola, mumps, measles or polio, and bacteria like diphtheria or tetanus. However, the development of effective vaccines against other viruses (e.g. HIV, dengue or hepatitis C), bacteria (*Yersinia pestis*, *Mycobacterium leprae*, and *Staphylococcus aureus*, among others) and parasites (*Plasmodium sp*, *Toxoplasma gondii*, *Leishmania sp* or *Trypanosoma cruzi*, for example) has been hampered by the fact that humoral immune response does not seem to be the best effector arm of the immune system to provide protection [1]. Some of these are chronic diseases and it is thought that both humoral and strong cellular immunity are necessary for protection.

New vaccine modalities are being developed with the aim of generating appropriate humoral and/or cellular immunity. Among those, DNA vaccines are very promising, as they are able to elicit both humoral and cellular immune responses. The demonstration that a protein-coding gene is able to elicit a specific immune response *in vivo* was first published by Tang and Johnston [2] who showed that the direct delivery of the human growth hormone gene into the skin of mice could elicit antigen-specific antibody responses. Since then, recombinant DNA vaccines have emerged as promising tools for vaccine development against infectious agents, cancer, autoimmunity and even allergy [3].

DNA vaccines are based on the delivery of genes encoding a specific protein antigen that is transcribed and translated by host cells [4,5]. DNA can present antigens in a suitable molecular form, ranging from full-length sequence to short MHC class I- or II-binding epitopes, to optimize induction of T-cell responses [6]. This vaccination technology has shown promising results in eliciting both humoral and cell-mediated and in inducing protection against various pathogen challenges in preclinical models [3,7].

DNA vaccination provides several important advantages over current vaccine approaches. First, DNA vaccines are safer than live-attenuated vaccines or inactivated viral vaccines since they are neither infectious nor capable of replication [8]. Therefore, DNA vaccines are considered the safest vaccine platform available (Figure 1).

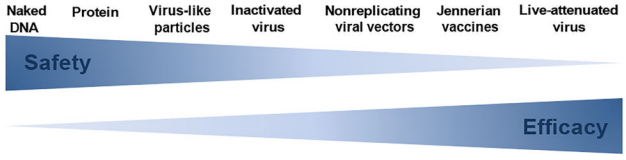

Figure 1: Safety and efficacy of different vaccine platforms.

Second, DNA vaccines can elicit both humoral and cellular immune responses against multiple defined antigens simultaneously and do not induce vector immunity in the host. Indeed, they can mimic the effects of live attenuated vaccines in their ability to induce MHC class I restricted $CD8^+$ T-cell responses. Third, DNA vaccines are easily designed manufactured at low cost and can be produced at commercial scale with a high degree of purity. Furthermore, DNA vaccines form stable formulations (they do not require a preservative in the final preparation) that facilitate storage and shipping when compared to other vaccine modalities.

Since plasmid DNA is a safe vaccine platform, the main limitation for its use in humans is due to its low immunogenicity. It has been a

challenge to transfer the success of inducing potent immunity observed in small animal models to humans. The reasons for reduced immunity presented in humans when compared to mice, for example, are still not fully understood. Hypotheses were formulated and differences in the rate of DNA uptake by target cells or in the ratio of injected DNA versus body weight are plausible. Some approaches now available to enhance the immunogenicity of DNA vaccines will be reviewed in the sections ahead. The main advantages and limitations of DNA vaccination are displayed in Table 1.

Advantages	Limitations
Easy design and production	Low immunogenicity
New molecular biology tools facilitate design and production	DNA vaccines are poorly immunogenic in humans
Easy to scale up	
Large scale production methods available	
Safe vaccine platform	
Subunit vaccine; non-infectious, non-replicative; unable to revert to virulent forms	
Stable formulation	
Easy storage and shipping; no cold chain requirement for transport	
Cost effective	
Immunogenic	
Can induce both humoral and cellular specific immune responses (including CTL)	
CTL: Cytotoxic T Cells	

Table 1: Advantages × limitations of DNA vaccines.

How DNA Vaccines Work

After *in vivo* administration, the plasmid enters the nucleus of transfected cells, initiates gene transcription and produces the corresponding protein inside them [9]. Secreted or exogenous proteins undergo endocytosis or phagocytosis by professional antigen presenting cells (APCs) to enter the MHC class II pathway of antigen processing and stimulate $CD4^+$ T cells. Endogenously produced proteins/peptides are presented to the immune system through an MHC class I dependent pathway to stimulate $CD8^+$ T cells. After DNA vaccination, the proposed three major mechanisms for antigen presentation in the context of MHC class I are: i) transfection of somatic cells (e.g. myocytes, keratinocytes, fibroblasts); ii) transfection of professional APCs (e.g. dendritic cells); iii) antigen uptake and presentation by professional APCs through cross-priming (APCs phagocytose apoptotic/necrotic transfected somatic cells).

Transfection of somatic cells

When somatic cells like myocytes and keratinocytes are transfected with DNA, the produced antigen is processed by the cell proteasome and the resulting peptides are presented to T cells via MHC class I. Although muscle cells express MHC class I molecules, they are not efficient in priming T cell responses when compared to professional APCs (e.g. dendritic cells). For other somatic cells like keratinocytes, it was shown that they constitute one of the major cell types transfected by plasmid DNA after injection into the skin [10].

Transfection of professional antigen presenting cells

Direct transfection of APCs seems to be the most efficient method of priming a T cell response, and dendritic cells are thought to play a key role. After antigen production, the endogenously synthesized protein is processed by proteasome and the resulting peptides are presented via MHC class I to $CD8^+$ T cells. DNA uptake and gene expression have been observed in dendritic cells *in vivo* following DNA immunization [11] and adoptive transfer of these in vivo transfected cells leads to induction of cytotoxic T cells (CTL) [12].

Uptake of secreted antigen and presentation by professional APCs through direct or cross-priming

Alternatively, antigens synthesized after DNA vaccination can also be released from the transfected cells (e.g. somatic cells) into the extracellular milieu, and these soluble materials are taken up by specialized APCs that express both classes of MHC molecules. Inside these APCs the antigens enter the MHC class II pathway and induce MHC class II restricted $CD4^+$ T cells, which usually secrete cytokines and provide "help" for B and $CD8^+$ T cells.

Another mechanism that has been demonstrated to occur in APCs is cross-priming. The transfected cell (e.g. myocyte) produces the protein antigen, that is phagocytized by APCs and gains entry into the MHC class I pathway that in turn activates CTL responses [13]. Cross-priming can also occur when the transfected somatic cell undergoes apoptosis/necrosis and is engulfed by APCs [14]. Furthermore, soluble antigens can encounter B-lymphocytes, be captured by specific high affinity immunoglobulins and therefore (in concert with $CD4^+$ T cell "help") induce an effective antibody response.

Independently of the APC activation mechanism (by direct transfection or through cross-priming), antigen-loaded APCs (e.g. dendritic cells) migrate to the draining lymph nodes (DLN) where they present peptide antigens to naive T cells ($CD4^+$ and $CD8^+$) via the interaction of MHC and T cell receptor (TCR) in combination with co-stimulatory molecules (e.g. CD80 and CD86). Once activated, these T cells expand and migrate out of the DLN.

Efficient antigen presentation by dendritic cells is intrinsically linked to the maturation status of these APCs. The direct contribution of the plasmid backbone for the improvement of DNA immunogenicity has been subject of research in the last decade. As DNA plasmids are normally derived from bacteria, they contain many unmethylated CpG motifs that can be recognized by mammals as a pathogen-associated molecular pattern (PAMP). They are recognized by the toll-like receptor (TLR)-9 [15] and rapidly trigger an innate immune response characterized by the production of IL-6, IL-12 and IFN-γ [16]. Rottembourg and colleagues showed that TLR9 was important for $CD8^+$ T cell priming when a DNA vaccine against lymphocytic chroriomeningitis virus (LCMV) was administered to mice [17]. However, as TLR9-deficient mice still respond to plasmid DNA immunization and are able to mount humoral and cellular responses, it seems that TLR9 signaling is not essential for the induction of immune responses following DNA immunization [18,19]. The search for the mechanisms of dsDNA sensing by mammalian cells showed that TANK-binding kinase 1 (TBK1), a non-canonical IκB kinase, is essential for immunogenicity of DNA vaccines in mice, as it

mediates the plasmid adjuvant effect [20]. An essential pathway that governs the production of type I IFN by foreign DNA was discovered and shown to be dependent on a molecule referred to as STING (stimulator of IFN genes) [21,22]. In addition, it was also shown that cytoplasmic DNA could bind to AIM2 (absent melanoma 2), triggering the formation of an inflammasome and the production of IL-1β [23,24]. Besides the molecules cited above, others were also implicated in cytosolic DNA sensing. A more detailed review on this specific topic can be found elsewhere [25].

Strategies to Enhance the Potency of DNA Vaccines

As stated previously, DNA vaccines are considered the safest vaccine platform available. They offer the promise of a molecularly defined reagent that is neither infectious nor capable of replication.

Approach	Example
Modification of plasmid design	Promoter/enhancer elements
	Polyadenylation sequence
	Kozak sequence
	Secretory leader sequence
	Consensus sequence
	Codon optimization
Delivery methods	Electroporation
	DNA tattoo
	Gene Gun
	Polyethylenimine (PEI) and Vaxfectin®
Adjuvants	Plasmid unmethylated CpG motifs
	TLR agonists
	Genetic adjuvants (e.g. cytokines/chemokines)
	Glycoprotein D of Herpes Simplex Virus (HSV)
	Chemical Compounds (e.g. bupivacaine)
Targeting to dendritic cells	DEC205 receptor
	CD11c receptor
	PD1
Heterologous prime-boost	DNA prime followed by boost with recombinant attenuated viruses (e.g. MVA)
	DNA prime followed by boost with recombinant proteins

TLR: Toll Like Receptor

PD1: Programmed Cell Death 1

MVA: Modified Vaccinia Virus Ankara

Table 2: Strategies to enhance the potency of DNA vaccines.

Usually high DNA doses in the milligram (mg) range are required in humans, and even so immunogenicity is low. Several approaches are being tested in an attempt to enhance their immunogenicity. They include modifications of plasmid basic design, use of next-generation delivery methods, inclusion of adjuvants in the formulation, improved immunization protocols and even antigen targeting directly to dendritic cells (Table 2).

Modifications of Plasmid Basic Design

In general, the higher the level of expression of the target gene is correlated to the strength of the induced immune response. The amount of plasmid that is internalized *in vivo* after intramuscular injection is usually in the picogram range [26]. Modifications in the plasmid backbone can greatly enhance the level of gene transcription. The inclusion of a strong viral-derived promoter/enhancer has provided superior gene expression *in vivo* than other eukaryotic promoters. In particular, the human cytomegalovirus (CMV) enhancer/promoter was shown to direct the highest level of transgene expression in eukaryotic tissues when compared to other promoters [27,28]. The presence of an intron in the vector backbone downstream of the promoter can enhance the stability of mRNA increasing gene expression. For example, the presence of intron A of the CMV immediate-early gene in the plasmid backbone increased the production and secretion of different proteins [29]. When a plasmid containing the CMV promoter and the human immunodeficiency virus (HIV)-1 envelope (Env) protein was used to immunize mice, anti-Env immune responses were also increased [30]. It is important to mention that high expression of the target gene may sometimes be detrimental. For example, the hepatitis C virus core protein is a good vaccine candidate but exhibits immunosuppressive properties [31]. In addition, the strong immune activation caused by the high levels of promoter-driven protein expression and secretion can induce down regulation of the viral promoter because of the inflammatory cytokine production they drive [32].

A second important modification is the inclusion of a termination site, or poly (A) signal site, that is required for proper termination of transcription and stabilization of mRNA transcripts [33]. Several DNA vaccines use the bovine growth hormone (BGH) or simian virus 40 (SV40) sequences to ensure proper transcriptional termination. The late SV40 polyadenylation signal is very efficient and increases the steady-state level of the mRNA transcript [34]. The use of the BGH polyadenylation signal in plasmids encoding Influenza A virus nucleoprotein (NP) or hemagglutinin (HA) resulted in protection from subsequent lethal challenges of influenza [35].

The optimization of the recognition start site by eukaryotic ribosomes can also improve gene expression by increasing translation efficiency. To optimize this process, a Kozak sequence must be included on the nucleotide sequence immediately upstream of the target gene´s ATG [36]. The consensus of Kozak sequence is gccRccAUGG, where R is a purine (adenine or guanine) three bases upstream of the start codon (AUG), which is followed by another 'G' [37]. Addition of a secretory leader sequence (e.g IgE leader sequence, human tissue plasminogen activator (tPA) leader sequence) is another way to enhance antigen expression by stabilizing the mRNA and contributing to translational efficiency [38].

When developing vaccines to highly mutable viruses (e.g. HIV), *in silico* tools can also be used. For example, sequences from a viral strain isolated from different patients can be analyzed and the most common amino acid used at any position can be determined and selected for further synthesis. The resulted gene is based on the consensus

sequence and has been used to design vaccines against influenza [38] and HIV [39,40].

Another important aspect to consider in the design of a DNA vaccine is codon usage. Although sharing the same genetic code, most species have preferences for the use of particular codons. This is due to the fact that not all transfer RNAs (tRNA) exist at equal levels within cells from different species. One of the most effective ways to increase the expression of the encoded protein is through the use of codon optimization, i.e. to select codons that target the more abundant tRNAs within the cell. This procedure correlates with translational efficiency in mammalian cells [41] that in turn results in increased protein production and enhanced immune responses. Plasmids encoding codon optimized bacterial genes increased humoral [42] and cellular immune responses [43]. In the case of viral proteins, codon optimization was shown to improve immunogenicity against proteins derived from human [44], HIV-1 [45,46], simian immunodeficiency virus (SIV) [47], hepatitis C virus (HCV) [48], influenza virus [49], and respiratory syncytial virus (RSV) [50], among others. Although these improvements of immunogenicity were accomplished in mouse models, such modifications hold promise for studies in humans. Taking all this information into account, the best results came from studies that optimized all components mentioned above in the vaccine design [30,51].

Other modifications such as removal of bacterial elements (necessary for plasmid replication and/or selection in bacteria) and plasmid design for long-term expression are also being evaluated and are reviewed elsewhere [52].

Delivery Methods

Nowadays, a variety of routes for gene delivery are available including intramuscular, intradermal, intravenous, intraperitoneal, oral, intranasal, and intravaginal. The amount of DNA required to elicit antigen specific immune responses by different routes (delivery methods) can vary according to the antigen expressed and the animal model used.

Although partially empirical, the type of protective immunity that one wants to achieve can influence the choice of the delivery method. For example, mucosal immunization is superior to parenteral route to induce and sustain mucosal IgA and protective immune responses to pathogens that normally infect mucosal sites [53,54].

In vivo electroporation (EP) is a method in which millisecond electrical pulses are applied to the vaccination site shortly after the administration of plasmid DNA. During this short period, the plasmid entry is facilitated and protein expression is increased [55,56]. EP enhances the overall immunogenicity of DNA vaccines in mice and in large animal models [57]. This increase in immunogenicity may be due to the induction of danger signal release (e.g. proinflammatory molecules) and APC recruitment to the site [58]. Several studies in nonhuman primates emphasized that EP enhanced the immunogenicity of DNA vaccines against monkeypox [57], malaria [59,60] and SIV [61,62]. Furthermore, EP has been shown to increase the number of vaccine specific polyfunctional T cells, a feature associated with protective immune responses against infectious agents [63]. The clinical settings mirrored to some extent these observations [64,65] and a search in the clinicaltrials.gov website revealed 33 human trials completed, active or recruiting, that involve the administration of DNA vaccines together with electroporation for the prevention of infectious diseases or cancer treatment (Table 3).

A few completed trials were already published. One of them showed the induction of specific antibody responses against the prostate-specific membrane antigen in cancer patients immunized intramuscularly with plasmid DNA followed by EP [66]. Another trial involved the electroporation of a plasmid DNA encoding IL-12 directly into the lesions of patients with metastatic melanoma. In this case, only two (out of 19) patients showed complete lesion regression [67].

Condition	Intervention	Phase	Clinical trial identifier*
Infectious Diseases			
Influenza virus	Influenza DNA vaccine/Trivalent inactivated vaccine	Phase I	NCT01609998 NCT01498718 NCT01676402 NCT00995982
	Monovalent H5 Influenza DNA vaccine (A Vietnam/1194/2004)	Phase I	NCT00347529
	Trivalent DNA vaccine (PIA0601) with or without DEI-LT (pPJV2012)	Phase I	NCT00375206
		Phase I	NCT00709800 NCT00694213
	Prime-boost with monovalent Influenza subunit virion (H5N1) vaccine followed by H5 DNA vaccine (VRC-AVIDNA036-00-VP)	Phase I	NCT01086657 NCT00776711
	H1 and H5 Influenza hemagglutinin DNA vaccines	Phase I	NCT01405885
	Trivalent (A/New Caledonia/20/99, A/Panama/2007/99, B/Jiangsu/10/20) DNA Influenza vaccine	Phase I	NCT00349037
	Recombinant H7 DNA vaccine administered alone or with monovalent Influenza subunit virion H7N9 vaccine (MIV)	Phase I	NCT02206464

	DNA-based Influenza vaccine (FVH1)	Phase I	NCT01587131
	H5 avian Influenza DNA vaccine (VGX-3400X)	Phase I	NCT01142362 NCT01184976
	H1 Influenza DNA vaccine (VRC-FLUDNA057-00-VP)	Phase I	NCT00973895
	Influenza DNA vaccine (VRC-FLUDNA047-00-VP) followed by the seasonal Influenza trivalent inactivated vaccine (TIV)	Phase I	NCT00858611
Hemorrhagic Fever With Renal Syndrome	Hantaan/Puumala Virus DNA Vaccine	Phase II Phase I	NCT02116205 NCT01502345
Venezuelan Equine Encephalitis Virus Infection	Venezuelan Equine Encephalitis Virus DNA vaccine	Phase I	NCT01984983
Dengue	Dengue-1 pre-membrane/envelope DNA vaccine	Phase I	NCT00290147
	Tetravalent Dengue vaccine formulated with Vaxfectin®	Phase I	NCT01502358
Chronic Hepatitis B	HBV envelope DNA vaccine (pCMVS2.S)	Phase I, Phase II	NCT00536627 NCT00988767
	Mixed plasmid DNA (HB-110) vaccine	Phase I	NCT00513968
	pPDPSC18 DNA vaccine	Phase I	NCT00277576
	HB110E Hepatitis B DNA vaccine in combination with Entecavir	-	NCT01813487
	DNA vaccine encoding HBsAg and HBcAg (INO-1800) and a DNA plasmid encoding human IL-12 (INO-9112)	Phase I	NCT02431312
	VGX-6150	Phase I	NCT02027116
HIV	HIV clades B and C DNA vaccine	Phase II	NCT01705223
	HIV gag, pro, RT, env, tat, vpu, and rev DNA vaccine (pGA2/JS2)	Phase I	NCT00043511 NCT00908323
	Prime-boost with pGA2/JS2 DNA vaccine followed by a modified vaccinia HIV vaccine (MVA/HIV62)	Phase I Phase II	NCT00301184 NCT00820846
	Prime-boost with pGA2/JS7 DNA vaccine followed by a modified vaccinia HIV vaccine (MVA/HIV62)	Phase I	NCT01378156
	PENNVAX®-GP (gag, pol, env) HIV-1 DNA vaccine and a DNA plasmid encoding human IL-12	Phase I	NCT02431767
	PENNVAX®-B (gag, pol, env) HIV-1 DNA vaccine and a DNA plasmid encoding human IL-12	Phase I	NCT00991354
	PENNVAX®-B (gag, pol, env) HIV-1 DNA vaccine with or without a DNA plasmid encoding human IL-12 or IL-15	Phase I	NCT00775424 NCT00528489
	PENNVAX™-B (gag, pol, env) administered by electroporation	Phase I	NCT01082692
	Prime-boost with PENNVAX-G DNA (HIV-1 env A, C, and D, and consensus gag plasmids) followed by modified vaccinia HIV vaccine (MVA-CMDR)	Phase I	NCT01260727
	Prime-boost with HIV-1 DNA Priming Regimens (Nat-B Env, CON-S Env, and Mosaic Env) followed by MVA-CMDR	Phase I	NCT02296541
	Prime-boost with HIV-1 DNA vaccine (DNA HIVIS) followed by a modified vaccinia HIV vaccine (MVA-CMDR)	Phase I	NCT01407497
	Prime-boost with env DNA vaccine followed by env recombinant adenovirus (AdV5 and AdV35)	Phase I	NCT00801697
	Prime-boost with multiclade, multigene HIV DNA vaccine followed by env recombinant adenovirus (AdV5 and AdV35)	Phase I	NCT00472719

	Prime-boost with multiclade HIV-1 DNA vaccine followed by a multiclade HIV-1 recombinant adenovirus (AdV5)	Phase II	NCT00865566
	Prime-boost with DNA-HIV-recombinant vaccine (EP-1233) followed by an HIV-recombinant viral vaccine (MVA-mBN32)	Phase I	NCT00428337
	HIV-1 gag DNA vaccine administered together a DNA plasmid encoding IL-15	Phase I	NCT00115960
	HIV-1 DNA vaccine encoding a modified Gag-Pol protein	Phase I	NCT00009685
	Prime-boost with a DNA vaccine (GEO-D03) followed by a modified vaccinia HIV vaccine (MVA/HIV62)	Phase I	NCT01571960
	HIV-1 gag DNA vaccine administered together a DNA plasmid encoding IL-12	Phase I	NCT00111605
	HIV-1 DNA vaccine encoding the env and rev genes (APL 400-003)	Phase I	NCT00001538 NCT00002232 NCT00002231 NCT00002350
	HIV-1 gag-pol DNA vaccine (APL-400-047) administered with bupivacaine	Phase I	NCT00001088
	DNA vaccines encoding HIV clades A, B and C env, clade B rev, clades A and B gag, and clade B RT	Phase I	NCT01140139
	Prime-boost DNA vaccine encoding multiple HIV-1 proteins (HIV-MAG) with or without a DNA plasmid encoding IL-12 followed by VSV-gag HIV vaccine	Phase I	NCT01578889 NCT01859325
	HIV DNA vaccine (DNA-HIV-PT123) administered with a gp120 protein vaccine (AIDSVAX®B/E) or HIV vaccine (NYVAC-HIV-PT1 and NYVAC-HIV-PT4) administered with a gp120 protein vaccine (AIDSVAX®B/E)	Phase I	NCT01799954
	HIV DNA vaccine (DNA-HIV-PT123) administered with a gp120 protein vaccine (AIDSVAX®B/E)	Phase I	NCT02376582
	Prime-boost with a HIV DNA vaccine (DNA-HIV-PT123) followed by NYVAC HIV protein vaccine (NYVAC-HIV-PT1 and NYVAC-HIV-PT4)	Phase I	NCT01783977
	DNA vaccine composed of 21 highly specific CTL epitopes (EP HIV-1090)	Phase I	NCT00054860 NCT00052182
	Prime-boost with a DNA vaccine (pSG2.HIV) followed by ChAdV63-HIV or MVA-HIV after depletion of serum amyloid P component	Phase I, Phase II	NCT02425241
	HIV-1 vaccine encoding the gag, env, pol, nef, and tat antigens (ADVAX) administered by electroporation	Phase I	NCT00545987 NCT00249106
	Prime-boost with a DNA vaccine (encoding clade B HIV-1 gag, pol and nef and HIV-1 env glycoprotein from clades A, B, and C) followed by four non-replicating AdV (encoding HIV-1 gag/pol polyproteins from clade B and HIV-1 env glycoproteins from clades A, B, and C)	Phase I	NCT00109629 NCT00321061
	Prime-boost with a DNA vaccine (encoding clades A, B, and C HIV gag, pol, nef, and env) followed by AdV (encoding gag, pol, and env).	Phase I	NCT00384787 NCT00270465
	DNA vaccine (GTU-MultiHIV B) followed by a Lipopeptide vaccine (LIPO-5)	Phase II	NCT01492985
	DNA vaccine encoding gag, pol, vpr, nef, rev, and env (EP HIV-1090) compared with recombinant protein vaccine containing the 18 HIV proteins from pol, vpu, and gag (EP HIV-1043)	Phase I	NCT00141024
	Multiclade HIV-1 DNA Vaccine (VRC-HIVDNA009-00-VP)	Phase I, Phase II	NCT00071851 NCT00125099 NCT01549470 NCT00047931
	Multiclade HIV-1 DNA vaccine (VRC-HIVDNA009-00-VP) administered with IL-2/Immunoglobulin DNA vaccine	Phase I	NCT00069030

	Multiclade HIV-1 DNA vaccine (VRC-HIVDNA016-00-VP)	Phase I	NCT00089531
	Prime-boost with a multiclade HIV-1 DNA vaccine (VRC-HIVDNA016-00-VP) followed by AdV vaccine (VRC-HIVADV014-00-VP)	Phase I	NCT01054872 NCT01386489
	Prime-boost with a multiclade HIV-1 DNA vaccine (VRC-HIVDNA009-00-VP) followed by AdV vaccine (VRC-HIVADV014-00-VP)	Phase I	NCT00270218
	HIV-1 DNA vaccine expressing gag, pol, nef, and env (VRC-HIVDNA006-00-VP)	Phase I	NCT00045838
	B clade DNA vaccine encoding a multi-HIV antigen consisting of a synthetic fusion protein built up by full-length polypeptides of Rev, Nef, Tat, p17 and p24 with more than 20 Th and CTL epitopes of protease, RT and gp160 (GTU®).	Phase I	NCT02075983
	HIV-1 multi-envelope DNA vaccine (EnvDNA)	Phase I	NCT00187148
	Prime-boost with a multigene DNA vaccine consisting of two plasmids expressing an HIV-1 subtype C polyprotein (Gag, RT, Tat, Nef) and an HIV-1 subtype C truncated Env (SAAVI DNA-C2) followed by a recombinant MVA vaccine expressing the same immunogens (SAAVI MVA-C).	Phase I	NCT00574600
	DNA vaccine encoding a gag, pol and nef polypeptide and the gp140 env followed by MVA expressing gag, pol, nef and gp120	Phase I	NCT01922284
Plasmodium falciparum Malaria	Polyepitope DNA Vaccine	Phase I	NCT01169077
	Prime-boost with a DNA vaccine followed by AdV encoding Circumsporozoite Protein and Apical Membrane Antigen 1	Phase I	NCT00870987
Herpes Simplex Virus Type 2 (genital herpes)	pPJV7630 HSV-2 DNA vaccine	Phase I	NCT00274300 NCT00310271
	Plasmid DNA vaccine encoding one or two HSV-2 proteins (VCL-HM01 and VCL-HB01)	Phase I, Phase II	NCT02030301
Cancer			
Head and Neck Squamous Cell Cancer	DNA plasmids encoding E6 and E7 proteins of HPV 16 and HPV 18 (VGX-3100) administered with DNA plasmid encoding human IL-12 (INO-9012)	Phase I	NCT02163057
Aerodigestive Malignancies	DNA plasmid encoding HPV 6 proteins (INO-3106) administered with DNA plasmid encoding human IL-12 (INO-9012)	Phase I	NCT02241369
Cervical Cancer	HPV16 E6/E7, HPV18 E6/E7 DNA Vaccine	Phase II	NCT01304524
	pNGVL4a-CRT/E7 DNA vaccine	-	NCT00988559
	DNA plasmids encoding E6 and E7 proteins of HPV 16 and HPV 18 administered with DNA plasmid encoding human IL-12	Phase I, Phase II	NCT02172911
	DNA plasmids encoding E6 and E7 proteins of HPV 16 and HPV 18 (VGX-3100)	Phase I	NCT00685412 NCT01188850
	GX-188E	Phase II	NCT02139267
Cervical Cancer, Precancerous Condition	pNGVL4a-Sig/E7(detox)/HSP70 DNA vaccine	Phase I, Phase II	NCT00121173 NCT00788164
	pNGVL4a-Sig/E7(detox)/HSP70 DNA vaccine with or without topical Imiquimod	Phase I	NCT00788164
Breast Cancer	Personalized polyepitope DNA vaccine	Phase I	NCT02348320
Metastatic Breast Cancer	Mammaglobin-A DNA vaccine	Phase I Phase I	NCT00807781 NCT02204098

Breast and Ovarian Cancers	DNA vaccine encoding the HER-2/Neu intracellular domain (pNGVL3-hICD) admixed with GM-CSF	Phase I	NCT00436254
Ovarian Epithelial Cancer	pUMVC3-hIGFBP-2 multi-epitope plasmid DNA vaccine	Phase I	NCT01322802
Melanoma	Xenogeneic Tyrosinase DNA Vaccine	Phase I	NCT00471133
	gp75 DNA vaccine	Phase I	NCT00034554
	gp100 DNA vaccine	Phase I	NCT00104845 NCT00398073
	Human tyrosinase DNA vaccine Mouse tyrosinase DNA vaccine	Phase I	NCT00698100 NCT00680589
	®MKC1106-MT)	Phase I, Phase II	NCT00033228
Lymphoma	Xenogeneic CD20 DNA vaccine	Phase I	NCT00561756
Lymphoplasmacytic Lymphoma		Phase I	NCT01209871
Prostate Cancer	DNA vaccine encoding androgen receptor ligand-binding domain (AR LBD) with or without GM-CSF	Phase I	NCT02411786
	DNA vaccine encoding the rhesus prostate specific antigen (rhPSA)	Phase I, Phase II	NCT00859729
	DNA vaccine encoding prostatic acid phosphatase (pTVG-HP) with recombinant GM-CSF	Phase II Phase I	NCT01341652 NCT00582140
	Sipuleucel-T with or without prostatic acid phosphatase DNA (pTVG-HP) Booster Vaccine	Phase II	NCT01706458
	pTVG-HP DNA vaccine with rhGM-CSF	Phase II	NCT00849121
Neuroendocrine Carcinoma	IL-12 DNA vaccine	Phase II	NCT01440816
Pancreatic Cancer	Vascular endothelial growth factor receptor 2 (VEGFR-2) DNA vaccine (VXM01)	Phase I	NCT01486329
Kidney Cancer	Human prostate-specific membrane antigen DNA vaccine	Phase I	NCT00096629
Others			
Allogeneic Hematopoietic Cell Transplant	Cytomegalovirus therapeutic DNA vaccine (ASP0113)	Phase II	NCT01903928
Allergic Rhinoconjunctivitis to Japanese red cedar	DNA vaccine encoding the CryJ2- gene fused to the lysosomal associated membrane protein (CryJ2 -DNA-LAMP)	Phase I	NCT01707069
Seasonal Allergic Rhinitis	Amb a 1 Immunostimulatory Oligodeoxyribonucleotide Conjugate (AIC)	Phase II	NCT00346086
* www.clinicaltrials.gov			
HBV: Hepatitis B Virus; HBsAg: Hepatitis B Surface Antigen; Th: T Helper; CTL: Cytotoxic T Lymphocyte; IL: Interleukin; AdV: Adenovirus; ChAdV: Reverse Transcriptase; HPV: Human Papiloma Virus.			

Table 3: Human clinical trials involving DNA vaccines.

DNA tattooing is a technique that uses a perforating needle device that oscillates at a constant high frequency and punctures the skin resulting in transfection of skin-associated cells and expression of the antigen. It was demonstrated that DNA tattooing induces stronger vaccine-specific immune responses over intramuscular immunization in mice [68,69] and in nonhuman primates [70]. More recently, DNA vaccination by tattoo induced full protection against bacterial challenge in mice in a rapid vaccination protocol [71]. A recent work developed an *ex vivo* human skin model to determine the factors that control vaccine-induced antigen expression and define the optimal parameters for the evaluation of DNA tattooing in Phase I clinical trials [72].

Another DNA delivery method tested is known as “gene gun”. In this case, plasmid DNA is coated onto high-density gold or tungsten microparticles, which are then accelerated to high velocity by a helium pulse [73,74]. This process drives the coated particles into the skin transfecting APCs like Langerhans cells and dermal dendritic cells, besides other cells in the epidermis. Induction of an immune response to the antigen of interest is then obtained [75-77]. One of the limitations of this type of technology is that it normally induces a Th2

type of response, at least in mice and in non-human primates [78]. Needle-free devices such as Biojector® have also been developed and a more comprehensive review of this technology can be found in [79].

Polyethyleneimine (PEI) is cationic polymer used as a reagent for nucleic acid transfections *in vitro* and as a delivery vehicle *in vivo* [80]. PEI has also been used to deliver genes to retinal ganglion cells [81] and to pulmonary mucosa [82]. In a murine model of influenza infection, intranasal delivery of a PEI/DNA vaccine induced strong T cell responses with polyfunctional profile and mucosal immunity. Furthermore, PEI/DNA immunization elicited full protection against the parental strain and partial cross-protection against other viral sytrains [83].

Another cationic lipid formulation developed to boost DNA vaccines is Vaxfectin® [84]. Antibody responses to a DNA vaccine administered together with Vaxfectin® against measles virus were significantly enhanced in non-human primates, but no effect on virus-specific IFN-γ producing T-cells was observed [85]. Phase I clinical studies are also being conducted using Vaxfectin® together with a tetravalent dengue virus DNA vaccine and with a therapeutic DNA vaccine for herpes simplex virus type 2 (HSV-2) (see NCT01502358 and NCT02030301, Table 3)

These findings provided important information for the further selection and optimization of DNA vaccine delivery methods for human applications.

Adjuvants

Because limited immunogenicity is the major caveat towards the use of DNA vaccines in large animal models including humans, it is clear, and even mandatory, the use of adjuvants associated with this type of vaccine. Currently, adjuvant selection brings a major breakthrough for the use of DNA vaccines for either prophylactic or therapeutic treatments. Here we will discuss only a few examples. A more detailed review on this topic can be found elsewhere [52,79].

As mentioned previously, plasmid DNA vaccines are commonly produced in bacteria and therefore contain unmethylated CpG motifs that are the natural ligands of TLR9. In this way, DNA vaccination can be improved by the addition of multiple copies of these motives in the plasmid [86]. In fact, addition of CpG sequences in a plasmid DNA encoding the HPV E7 protein enhanced IFN-γ, granzyme B, and antitumor response, especially when electroporation was used [87].

Toll like receptor (TLR) agonists have been also used as adjuvants with DNA vaccines. Adjuvanting a DNA vaccine with the TLR9 agonist, CpG oligodeoxinucleotides (ODN), at the time of priming enhanced $CD8^+$ T cell responses and control of viral load after SIV challenge in rhesus macaques [88]. A recent study showed that the TLR3 agonist poly (I:C) and the TLR7 agonist resiquimod were both able to induce significant tumor regression when administered together with a DNA vaccine against a model tumor expressing the HPV-16 in mice [89]. However, when a SIV DNA vaccine was administered together with poly (ICLC), a stabilized poly (I:C) analogue, in rhesus macaques, no improvement in immunogenicity was observed [90]. In another study, the TLR7 agonist resiquimod modestly enhanced IFN-γ production and T cell proliferation in a HIV-1 gag DNA vaccine [91]. The TLR4 ligand lipopolysaccharide (LPS) presents potent activation of the innate immune response. However, due to its toxicity, it is rather difficult to obtain approval for use in clinical trials. In addition, a few experiments showed that the dose of LPS used together with a DNA vaccine can modulate the outcome of the immune response towards a Th1 or Th2 bias [92,93].

Another common approach is to incorporate into the vaccine plasmid, genes coding for cytokines, chemokines, co-stimulatory molecules and anti-apoptotic genes as "genetic adjuvants" [94-97]. Also, these genes can be delivered as separate plasmids. It has been demonstrated that addition of such genetic adjuvants may increase the breadth and magnitude of immune response and also skew its type [8].

Plasmids encoding interleukin (IL)-2 have been used together with plasmids encoding proteins derived from different viruses. For example, both $CD4^+$ inflammatory T cell proliferative responses and $CD8^+$ CTL activity to HCV core protein were enhanced substantially after coimmunization with an IL-2 expressing plasmid [98]. In mice immunized with the nucleocapsid protein of the severe acute respiratory syndrome virus (SARS), the coadministration of an IL-2 plasmid enhanced specific cellular and humoral immunity [99]. A bicistronic plasmid expressing influenza proteins plus IL-2 was able to protect mice against a lethal challenge with this virus [100]. For HIV, progress has been also made in the induction of immune response against this virus envelope protein. In a mouse model, the anti-gp120 specific immune response was enhanced in mice when a plasmid encoding the gp120 gene was administered first and then followed by another plasmid encoding a fusion IL-2/Ig (IL-2 fused to the Fc portion of immunoglobulin G (IgG) [101]. This study was extended to monkeys using plasmids encoding SIV Gag and HIV-1 Env genes administered with the IL-2/Ig plasmid. No evidence of clinical disease was observed in the group of monkeys that received the combination of plasmids and were subsequently challenged [102]. A clinical trial was then set in place to evaluate a HIV-1 DNA vaccine with the plasmid cytokine adjuvant (IL-2/Ig) in 70 HIV-negative adults. As observed in the mouse study, IL-2/Ig plasmid increased immune responses when administered 2 days after the DNA vaccine (see NCT00069030 in Table 3) [103].

Cytokines such as interleukin (IL)-12 and IL-15 have been effective in enhancing immune responses in both murine and nonhuman primate models [104,105]. This approach was used successfully against *Mycobacterium tuberculosis* [106], *Yersinia pestis* [107,108], HIV [3,109], cytomegalovirus [110], and tumors [111]. The co-administration of plasmids encoding IL-12 and IL-15 in macaques increased the specific $CD8^+$ T cell memory populations and their ability to produce cytokines [112]. In humans, three clinical trials were completed using HIV DNA vaccines administered together with plasmids encoding IL-12 (NCT00991354, see Table 3), or IL-12 and IL-15 (NCT00528489 and NCT00775424, see Table 3). Study NCT02431767 (Table 3) is not yet recruiting, but its the goal is to evaluate the HIV DNA vaccine (composed of gag, pol and env genes) coadministered with an IL-12 plasmid delivered by intradermal or intramuscular electroporation.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) associated with DNA vaccines recruits and activates dendritic cells to the site of vaccination [113]. Promising results were observed when a plasmid encoding GM-CSF was used with DNA vaccines against HSV-2 [114] and HIV [115].

The envelope glycoprotein D (gD) of the human herpes simplex virus (HSV-1) has also an adjuvant effect [116]. The immunological effects of gD, particularly the activation of $CD8^+$ T cell responses, involve the binding of gD to herpes virus entry mediator (HVEM) and the blockage of a co-inhibitory immune mechanism involving the B-

and T lymphocyte attenuator (BTLA) cell receptor [117,118]. Immunization of mice with a bicistronic DNA vaccine expressing gD (HSV) fused with HPV-16 or HIV-1 proteins induced antigen-specific $CD8^+$ T cell responses, including in vivo cytotoxic responses. Furthermore, the vaccine conferred protective immunity against challenges showing antiviral and antitumor capacity [119].

Chemical compounds were also evaluated as adjuvants for DNA vaccines [120]. One of such compounds is bupivacaine or marcaine, a local anesthetic drug that works by blocking neuron transmission. Bupivacaine is also a myotoxin that when injected destroys myofibers, inducing the clearance of cell debris and proliferation of myoblasts. In addition, the recruitment of inflammatory cells to the site of bupivacaine injection may allow the transfection of immune cells [121]. Muscle pretreatment with bupivacaine several days prior to injection of plasmid DNA results in increased DNA uptake, as evidenced by increased expression at the injection site [122,123]. The complex formed with bupivacaine protects the plasmid DNA from nuclease degradation, and intramuscular immunization with this formulation results in higher immune responses against the encoded antigen [124]. Intranasal or intramuscular administration of a DNA vaccine encoding *Streptococcus mutants* antigens plus bupivacaine induced antibodies, IFN-γ production and significant reduction in dental caries lesions [125]. Further in support of the adjuvant effect of bupivacaine, a recent study demonstrated that the concomitant administration of bupivacaine with a DNA vaccine encoding 18 HIV-1 epitopes was able to increase the magnitude of T cell responses, cytokine production and also the longevity of specific immune responses [126].

Besides the substances discussed previously, chemokines, signaling and co-stimulatory molecules have also been tested and a very detailed review is available in [79].

Dendritic Cell Targeting

Dendritic cells (DCs) have a central role in antigen uptake and presentation to T cells. Due to their privileged localization in different tissues and their ability to sense the environment through a myriad of surface and intracellular receptors, DCs are able to rapidly detect, phagocytize and process pathogens into peptides that will be successfully presented to T cells and induce the development of adaptive immune responses. For that reason, DCs are excellent targets for the development of immunization strategies, and targeting an antigen directly to these cells constitutes a way to improve immunogenicity.

In the last decade, antigen targeting directly to DCs became a reality through the design of specific monoclonal antibodies (mAbs) against their surface receptors fused with antigens of interest. When delivered to maturing DCs, these fusion antibodies were able to elicit strong cellular and humoral responses to the fused antigen, and even protection in some experimental models [127,128]. Although promising, this approach relies on the development of an immunogen (i.e. the fusion mAb) that may be difficult to generate because the antigen has to be fused with the mAb. This can be accomplished by chemically coupling the antigen to the mAb [129] or by genetically fusing the antigen sequence with the sequence of the mAb heavy chain, and posteriorly producing the fusion mAb using transfection of eukaryotic cells [130,131]. An alternative to such a hurdle is to produce DC targeting immunogens in the form of plasmid DNAs. The addition of DC targeting capacity to a DNA vaccine has been tested as another way to help overcome their poor immunogenicity in humans [3]. Many groups have shown that this strategy can work efficiently when different antigens and DC targeting molecules are used. Demangel et al., for example, designed DNA vaccines encoding a fusion protein comprised of a mycobacterial antigen and a single-chain Fv antibody (scFv) specific for two murine DC-restricted surface receptors: CD11c and DEC205. Their results showed that antigen targeting to DCs via the DEC205 binding scFv led to enhanced immunogenicity when compared to targeting through the CD11c binding scFv [132]. To further expand these results, Nchinda et al. showed that a DNA vaccine encoding ovalbumin or the HIV gag p41-scFv DEC205 fusion protein induced higher antibody levels and increased numbers of IFN-γ-producing $CD4^+$ and $CD8^+$ T cells when compared to non-targeted constructs even when a lower dose of DNA was administered [133]. In an attempt to improve even more the induction of $CD8^+$ T cells, the ovalbumin-scFv DEC205 and HIV gag p41-scFv DEC205 DNA vaccines were used to prime mice that were subsequently boosted with adenoviruses expressing a non-targeted version of the same molecules. Surprisingly, targeting of DNA-encoded ovalbumin to DCs suppressed $CD8^+$ T-cell responses after the adenoviral booster immunization. This effect was only observed for ovalbumin-scFv DEC205 and the reasons why different outcomes were observed are not currently understood. However, when both DNA vaccines were administered together with the TLR9 ligand CpG and the TLR3 ligand poly(I:C), an increase in the $CD8^+$ T cell response was observed after the adenoviral boost [134]. This increase can be explained by the fact that DEC205-targeted protein or DNA vaccines critically depend on the activation and maturation status of the targeted DCs. scFv DEC205 DNA vaccines were also successfully used to immunize calves. In this approach, the authors constructed a multi-component DNA construct expressing the DEC205-targeted antigen fused to the CD40L minimal functional domain. Animals were then vaccinated with a low dose of the antigen-scFv DEC205-CD40L plasmid together with DNA plasmids expressing FMS-like tyrosine kinase 3 ligand (Flt3L) and GM-CSF. The DC targeted version of the plasmid elicited higher proliferation and IFN-γ production by $CD4^+$ T cells when compared to animals immunized with plasmid DNA containing the non-targeted version of the antigen [135]. Despite the good results obtained in calves, when a DNA vaccine comprising the SIV p27 capsid protein gene fused to the monkey scFv DEC205 region was administered to rhesus macaques, no improvement in antigen immunogenicity was observed even when theTLR3 ligand poly(I:C) as co-injected. On the contrary, a more robust response was detected in the monkeys immunized with SIV p27 gene fused to a non-targeted scFv [90]. scFv DEC205 DNA vaccines have also been used to target self antigens in an attempt to prevent autoimmune diseases and cancer. A recent article by Wang et al. showed that CD40 targeting directly to DCs using the CD40-scFv DEC205 DNA vaccine was able to protect rats from developing Heymann nephritis [136]. For breast cancer therapy in the mouse model, the scFv DEC205 plasmid was fused with the tumor-associated antigen HER2/neu ectodomain. HER2/neu targeting to DCs through HER2/neu-scFv DEC205 DNA immunization elicited specific cellular and humoral immune responses that were protective against challenge [137].

Even though scFv DEC205 fusion DNA constructs are the most commonly used until now, other molecules were also tested. Among them is the programmed death-1 (PD1) molecule. The PD1 ligands (PD-L1 and PD-L2) are expressed in different cell types, including DCs. The vaccination of mice with a plasmid encoding the PD1 soluble domain fused to the HIV gag p24 protein elicited high

frequencies of gag-specific polyfunctional and cytotoxic $CD8^+$ T cells. It is interesting to notice that this vaccination strategy elicited stronger responses than those elicited in mice vaccinated with the HIV gag p41-scFv DEC205 [138].

The data presented above indicates that the use of DNA constructs with the capacity to encode molecules capable of targeting antigens to DCs increases the magnitude of T cell responses and may help to overcome the low immunogenicity of DNA vaccines in humans.

Heterologous Prime-boost

Another way to increase the potency/efficacy of DNA vaccines is to use the heterologous prime-boost immunization regimen. In this approach, the immune system is primed by administering the antigen of interest by one method (e.g DNA vaccine) and then boosted by a different one that delivers the same antigen (e.g recombinant protein, recombinant virus). A vast number of experiments have been performed in mice and non-human primates [139] and these strategies are already into clinical trials [140]. Detailed reviews on heterologous prime-boost strategies have been published elsewhere [3,141-143].

Taking DNA Vaccines from the Bench to the Field

A number of safety concerns came up since the beginning of DNA vaccine utilization in the early 90´s. These include the possibility that such vaccines may (i) stimulate the production of autoantibodies against the plasmid DNA, potentially inducing or accelerating the development of systemic autoimmune diseases; (ii) integrate into the host genome, increasing the risk of carcinogenesis or other genetic abnormalities; (iii) induce the development of tolerance rather than immunity; (iv) selectively alter host´s cytokine response to infections. Many of such concerns have been addressed and elucidated in the past years. For example, cumulative data from clinical trials showed that DNA vaccines did not accelerate systemic or organ-specific autoimmune diseases. Furthermore, there is no evidence from pre-clinical or clinical trials that DNA vaccines result in the development of tolerance in adults [144].

An overview of the specific guidelines and regulatory aspects for manufacturing, accessing preclinical immunogenicity, safety, quality assurance and quality control of prophylactic DNA vaccines were developed by the World Health Organization (WHO) (http://www.who.int/biologicals/publications/ECBS%202005%20Annex%201%20DNA.pdf) and the FDA [145]. Recently, a paper described the evolution of FDA policy, the status of current regulatory guidance and several recommendations to facilitate the development of prophylactic DNA vaccines [144]. One main issue is that the production process should conform to cGMP (current Good Manufacturing Practices) guidelines and be acceptable to the FDA or other national regulatory agencies. After endorsement of the quality and pre-clinical safety of a new DNA vaccine, clinical trials should proceed through three phases. Typically, phase I trials involve a small group of healthy volunteers (20-80), who are designed primarily to determine whether the vaccine formulation is safe for human use, but also their immunogenicity can be evaluated. Phase II trials involve a larger number of healthy volunteers and are designed to further evaluate vaccine safety and potential side effects, immunogenicity, optimum dosage and schedule. Phase III trials analyze whether a vaccine provides any protection against infection or disease, and can also monitor safety and potential side effects on a large scale. These trials must be large enough (thousands of volunteers) to ensure that the vaccine works under various conditions. If Phase III results demonstrate safety and sufficient efficacy, the manufacturer applies for permission to license and market the product and submits a plan for long-term, post-licensure safety monitoring (Phase IV trials). A full set of clinical trials for a successful candidate vaccine can take 10 to 12 years, involve 50,000 to 100,000 volunteers, and cost millions of dollars. For those reasons, few vaccine candidates survive this rigorous process.

Despite the time consuming process, a large number of DNA vaccine candidates are being tested in clinical trials. A quick search in the Clinical Trials website from the U.S. National Institutes of Health (www.clinicaltrials.gov) using the key words “DNA vaccine” revealed 139 studies that are either already completed, active or recruiting (Table 3). Almost half of the studies focused on the development of an HIV-1 vaccine. Plasmids encoding different HIV-1 proteins were administered together with interleukins (such as IL-12 and IL-15), anesthetic drugs (e.g. bupivacaine) or even recombinant HIV-1 proteins. Prime-boost experiments were also conducted using normally a plasmid DNA prime followed by a boost with attenuated recombinant viruses (e.g. modified vaccinia ankara, adenovirus and vesicular stomatitis virus). Electroporation devices were also used in an attempt to improve DNA vaccine efficacy. Although HIV-1 is the most frequent target, DNA vaccines are are also being developed for Influenza, *Plasmodium falciparum* malaria, dengue, chronic hepatitis B, among others (Table 3).

Vaccine name	Species	Vaccine Target	Reference
Oncept	Dog	Melanoma	[149]
Apex-IHN	Salmon	Infectious haematopoietic necrosis virus	www.vical.com/products/infectious-disease-vaccines/Apex-IHN/default.aspx
West Nile - Innovator	Horse	West Nile virus	[150]
LifeTide SW 5	Pig	Growth hormone releasing hormone (GHRH)	www.vgxah.com/LifetideSW5.html

Table 4: Licensed DNA vaccines for animal use

The search for new treatments against different types of tumors opened the possibility for the use of DNA vaccines in therapeutic settings. Clinical trials have been set up to evaluate efficacy of DNA vaccines against melanoma, lymphoma, cervical, ovarian, prostate, pancreatic and kidney cancers, among others (Table 3). Although generally safe, no DNA vaccine for use in humans has yet met applicable efficacy requirements. On the other hand, DNA vaccines for veterinary use have shown efficacy in some trials. Potentially protective immune responses were observed against many infectious agents in different target species including fish, companion and farm animals. A veterinary DNA vaccine to protect horses against West Nile virus was first licensed in 2005 by the FDA [146]. In 2008, the Australian Pesticides and Veterinary Medicines Authority approved a DNA-based growth hormone therapy, delivered using electroporation (EP), for use in swines [147]. In total, four animal DNA vaccines were approved for the vaccination of horses, salmon, pigs and dogs (Table 4).

While the quality and safety considerations for veterinary use differ from vaccines for human use, experience with veterinary DNA vaccines can provide valuable information for the control and use of human DNA vaccines.

Recently, a web-based DNA vaccine database was developed aiming to analyze plasmid vectors and protective antigens used in DNA vaccines based on peer-reviewed articles [148].

Concluding Remarks

As described in the previous sections, a significant amount of time and effort has been spent in an attempt to improve DNA vaccines. Since the first demonstration that naked DNA was able to induce specific immune responses 25 years ago, much has been accomplished. Improvements in plasmid design and delivery methods, as well as advances in large scale DNA production under GMP conditions paved the way for human trials. Despite the disappointing initial results, especially concerning immunogenicity of DNA vaccines in humans, different groups keep working to improve this technology. New approaches and reagents are constantly being generated and many different clinical trials are set to evaluate the results in humans. The exchange of results and technologies by researchers in this field has the potential to push DNA vaccine development even further and guarantee that this promising technology is translated into effective vaccines.

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