DNA vaccines: ready for prime time?

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Abstract | Since the discovery, over a decade and a half ago, that genetically engineered DNA can be delivered in vaccine form and elicit an immune response, there has been much progress in understanding the basic biology of this platform. A large amount of data has been generated in preclinical model systems, and more sustained cellular responses and more consistent antibody responses are being observed in the clinic. Four DNA vaccine products have recently been approved, all in the area of veterinary medicine. These results suggest a productive future for this technology as more optimized constructs, better trial designs and improved platforms are being brought into the clinic.

Formulation
A mixture of one or more active ingredients is made safe and easy to store, transport, dilute or apply through the presence of other materials (for example, vehicles or solvents).

Cytotoxic T lymphocyte
(CTL. Also known as T_C, T-killer cell or killer T cell). Belongs to a sub-group of T lymphocytes that are capable of inducing the death of infected somatic or tumour cells. They target and kill cells that are infected with other pathogens or that are otherwise damaged or dysfunctional.

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DNA vaccines burst into the scientific limelight in the early 1990s. Tang and Johnston described the delivery of DNA into the skin of mice using a 'gene gun', in an attempt to deliver human growth hormone as a gene therapy[1]. The authors felt that this could be a useful technique to generate antibody responses against specific transgene products. At the same time, three presentations at the annual vaccine meeting at the Cold Spring Harbor Laboratory in 1992 reported the use of DNA vectors to drive both humoral and cellular immune responses against pathogens or tumour antigens *in vivo*. Margaret Liu and her colleagues at the pharmaceutical company Merck reported injecting 'naked' plasmids intramuscularly to deliver immunogens that would generate immune responses against influenza virus antigens in mice[2]. Similarly, Robinson described the ability of DNA plasmids to drive immune responses against influenza virus antigens[3]. A presentation by Weiner described the ability of plasmids carrying HIV antigens or tumour antigens to produce immune responses and protection from tumour challenge in mice[4]. Although none of these studies used the same delivery method, formulation or plasmid, together they provided evidence to the scientific community that this simple technique could be developed as an immunization platform.

In the past decade and a half, the DNA vaccine concept has been tested and applied against various pathogens and tumour antigens. In theory, this conceptually safe, non-live vaccine approach is a unique and technically simple means to induce immune responses. Importantly, DNA vaccines affect not only humoral immunity but also cellular immunity — the elusive target of live infection. In particular, DNA vaccines induce killer cytotoxic T lymphocytes (CTLs), suggesting that an important shift had occurred in non-live vaccine platforms. The use of the DNA approach also promised to overcome the safety concerns associated with live vaccines — their reversion risks, as was observed in a subset of primates receiving a live but attenuated simian immunodeficiency virus (SIV) vaccine, and their potential spread to unintended individuals[5]. In addition, it avoids the risks linked to the manufacture of killed vaccine, as exemplified by the tainting of a polio vaccine with live polio virus owing to a production error[6].

DNA vaccines have experienced a recent resurgence in interest. Several technical improvements have contributed to this achievement, including gene optimization strategies, improved RNA structural design, novel formulations and immune adjuvants, and more effective delivery approaches. For example, the use of species-specific codon optimization results in increased protein production on a per-cell basis, leading to enhanced T-cell responses[7–10] and antibody induction[11–14]. These methods, particularly when combined, cause augmented levels of immune responses in rodents as well as large animal models. Importantly, the recent licensing of four veterinary vaccine products — for horses, dogs, pigs and fish — have served to re-energize a field that had been hampered by poor product performance in larger animal models, in non-human primate studies and in human clinical trials.

In this Review we summarize the status of the field in animals and in human clinical trials. We describe important platform optimization strategies that improve expression, potency and immunogenicity. We also highlight creative strategies and important technical achievements that continue to drive this important platform. DNA vaccines have produced enticing results in a wide array of applications, from prophylactic vaccine

strategies that target viral, bacterial or parasitic infections to potential therapeutics used to treat infectious diseases, cancers, Alzheimer disease, allergy and autoimmune disorders.

Mechanism of action

The mechanisms by which DNA vaccines produce antigen-specific immunity *in vivo* are under intense investigation, with an idealized model presented in BOX 1. The optimized gene sequence of interest is delivered to the skin (intradermally), subcutaneum or muscle by one of several delivery methods. Using the host cellular machinery, the plasmid enters the nucleus of transfected local cells (such as myocytes or keratinocytes), including resident antigen presenting cells (APCs). Here, expression of plasmid-encoded genes is followed by generation of foreign antigens as proteins that have been converted to peptide strings. These host-synthesized antigens can become the subject of immune surveillance in the context of both major histocompatibility complex (MHC) class I and class II molecules of APCs in the vaccinated host. Antigen-loaded APCs travel to the draining lymph nodes where they 'present' antigenic peptide–MHC complexes in combination with signalling by co-stimulatory molecules to naive T cells. This interaction provides the necessary secondary signals to initiate an immune response and to activate and expand T cells or, alternatively, to activate B cell and antibody production cascades. Together, both humoral and cellular immune responses are engendered.

Preclinical studies

A proliferation of studies on small animals followed the initial reports on DNA vaccines. These approaches focused primarily on antibody induction in mice and included important targets, such as herpes simplex virus, hepatitis B virus, HIV, influenza virus and others, including viruses labelled as 'bioterroristic agents'[15–17]. The DNA platform generated a great deal of excitement preclinically as protective immunity was induced by such vaccines against a broad range of virus families. Some examples of this application include strategies that target the Rabies virus, Filovirus, Flavivirus, Togavirus and Bunyanvirus, as well as the bacterial disease agent anthrax and the malaria parasite[18,19]. In addition, owing to the ability of DNA vaccines to drive cellular immunity, cancer immune therapy agents were targeted, including those relevant to melanoma, lymphoma, and colon, prostate and breast cancers. For viruses and bacteria that have high genetic diversity and require CTLs for defence against infection, DNA platforms have been tested as immune therapy for different chronic viral infections, including human papilloma virus, hepatitis C virus and HIV. Furthermore, the CTL induction engendered by DNA approaches seems to be particularly relevant to such variable pathogens.

These results have led to numerous DNA vaccine clinical trials in humans for a variety of infectious agents and cancers, as well as for immune modulation strategies to treat asthma and allergy by targeting host production of immunoglobulin E (IgE). In addition, trials have tested the ability of the DNA vaccine platform to deliver gene therapy agents to treat specific chronic ailments.

Initial DNA vaccine studies in humans

The first DNA vaccine studies in humans, initiated almost 15 years ago, were limited in concept and high on optimism. The goals of the various clinical trials were to demonstrate the safety and tolerability of the candidate vaccines, and to and explore the limits of immune potency of specific DNA vaccines in humans (TABLE 1). The earliest Phase I clinical trial for a DNA vaccine was of an HIV-1 candidate tested in individuals infected by HIV-1, followed by studies in volunteers who were not infected by HIV-1 (REF. 20). Other prophylactic and therapeutic DNA vaccine trials followed, including trials that tested DNA vaccines against cancer, influenza, malaria, hepatitis B and other HIV-1 candidates[21–25]. These trials demonstrated that the DNA vaccine platform is well tolerated and safe, as no adverse events were reported and all studies went to completion. In this section, we focus on the results of two main applications for which prophylactic vaccines have been tested in the clinic. Owing to the wealth of studies on infectious disease targets we focus on this class first, including HIV-1, and then we discuss therapeutic vaccines, including those for cancer therapy, which merit attention owing to the relevance of these studies for many diseases that involve cell proliferation.

Overall, the first-generation DNA vaccines failed to demonstrate high levels of vaccine-specific immunity in humans, although we have learned a great deal about the safety and delivery of this platform. However, new modifications and improvements to the technology are encouraging.

DNA vaccines against HIV-1. Many current and recently completed trials for HIV-1 have used recombinant viral vector platforms or DNA vaccines in combination with other traditional vector-based vaccines. Immune responses that have been primed by the delivery of a DNA-encoded antigen can be boosted by the administration of recombinant protein or recombinant viruses, through so-called 'prime-boost' strategies. In preclinical animal models these approaches have increased the number of neutralizing antibodies and also boosted DNA-primed CTL responses.

One important viral vector platform is the modified vaccinia virus Ankara (MVA). GeoVax has used MVA in a two-pronged prime-boost approach. The DNA vaccine contains several HIV antigens as the priming immunogen, which is followed by boosting with a recombinant MVA that also contains HIV antigens; this combination approach is well tolerated and produces detectable and reproducible cellular immunity in humans[26]. A DNA prime followed by an MVA boost was also studied by the McMichael group[27]. This DNA vaccine was composed of a string of DNA epitopes for HIV and a DNA fragment encoding the subtype A HIV gag antigen as the priming immunogens; this was followed by an MVA boost that contained the same vaccine antigens. However, like other

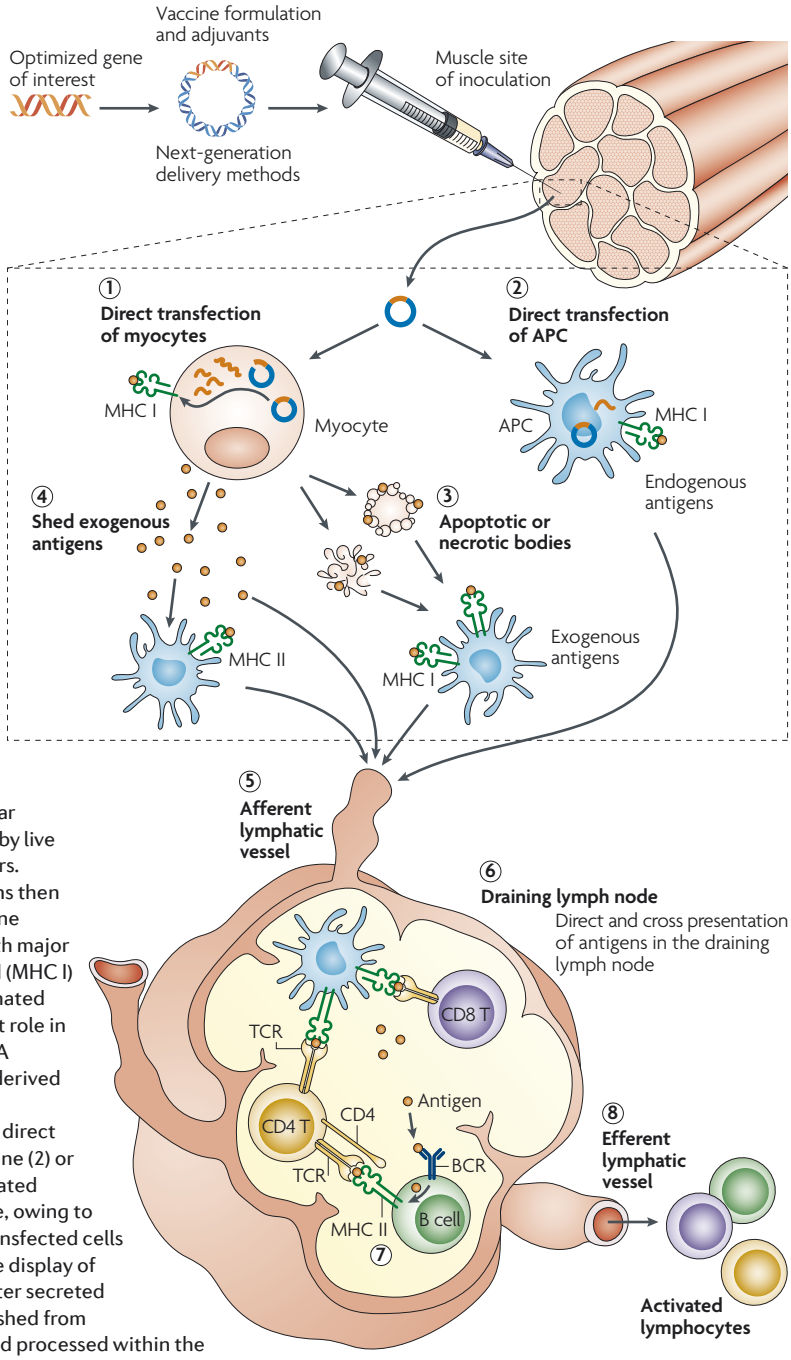
Adjuvant
An agent that can stimulate the immune system and increase the response to a vaccine, without having any specific antigenic immune response.

Codon optimization
The preference that different organisms show for one of the several codons that encode a particular amino acid. Translationally optimal codons are those that are recognized by abundant tRNAs. Within a phylogenetic group, the frequency of particular codons in a gene is highly correlated with higher translation rates and accuracy.

Subcutaneum
The layer of tissue that lies just under the surface of the skin.

Antigen presenting cell
(APC). Specialized cell that can prime naive T cells through the expression of MHC class I molecules (which are expressed by most cells and can prime $CD8^+$ cytotoxic T cells) as well as MHC class II molecules (which prime $CD4^+$ T helper cells).

Box 1 | **Induction of cellular and humoral immunity by DNA vaccines**



The optimized gene sequence of interest (for example, antigenic or immune adjuvant genes) is generated synthetically or by PCR. This sequence is enzymatically inserted into the multiple cloning region of a plasmid backbone, purified, and then delivered to the inoculation site by one of several delivery methods to either the skin, subcutaneum or muscle. Using the host cellular machinery, the plasmid enters the nucleus of transfected myocytes (1) and of resident antigen presenting cells (APCs) (2); here, the plasmid components initiate gene transcription, which is followed by protein production in the cytoplasm and the consequent formation of foreign antigens as proteins or as peptide strings. The cell provides endogenous post-translational modifications to antigens that reproduce native protein conformations and the cell customizes the antigens in a similar manner to the pathways induced by live infection with recombinant vectors.

These host-synthesized antigens then can become the subject of immune surveillance in the context of both major histocompatibility complex class I (MHC I) and MHC II proteins of the vaccinated individual. APCs have a dominant role in the induction of immunity of DNA vaccines by presenting vaccine-derived endogenous peptides on MHC I molecules. This can follow either direct transfection by the plasmid vaccine (2) or cross-presentation of cell-associated exogenous antigens; for example, owing to APC engulfment of apoptotic transfected cells (3). In addition, APCs mediate the display of peptides on MHC II molecules after secreted protein antigens that have been shed from transfected cells are captured and processed within the endocytic pathway (4). Antigen-loaded APCs travel to the draining lymph node (DLN) via the afferent lymphatic vessel (5) where they present peptide antigens to naive T cells via MHC and the T cell receptor (TCR) in combination with co-stimulatory molecules, providing the necessary secondary signals to initiate an immune response and expansion of T cells (6). In response to peptide-bound MHC molecules and co-stimulatory secondary signals, activated CD4 T helper cells secrete cytokines during cell-to-cell interaction with B cells and bind to co-stimulatory molecules that are required for B cell activation (7). In addition, shed antigen can be captured by specific high affinity immunoglobulins (B cell receptors; BCLs) expressed on the surface of B cells in the DLN; these then present processed antigen to CD4 T helper cells, thereby facilitating the induction of an effective B cell response. In theory, once migrating T cells have been primed in the DLN they could be restimulated and further expanded at the site of immunization by presentation of the peptide–MHC complexes displayed by transfected muscle cells. These processes coordinately elicit specific immunity against plasmid-encoded antigen by activating both T and B cells, which, now they are 'armed', can travel through the efferent lymphatic system (8) and provide a surveillance system. Together, the two arms of the immune system, which are induced specifically following DNA vaccination, can create a powerful defence against most infectious diseases.

Table 1 | **Current DNA product Phase I to III open clinical trial targets**

Number of open trials	Category	Diseases and conditions treated
Phase I		
54	Cancer	Melanoma, glioblastoma, lymphoma, cancer cachexia, acute lymphoblastic leukaemia, head and neck squamous cell carcinomarenal cell carcinoma, chronic lymphocytic B leukaemia, Hodgkins lymphoma, and colorectal, prostate, pancreatic, lung and breast cancer
6	Cardiovascular	Re-endothelialization, claudication, limb ischaemia, angiogenesis and Buerger disease
5	Healthy volunteers	HIV vaccine safety
2	Infectious disease	Chronic hepatitis B and HIV-1
1	Neurological	Effect of human insulin-like growth factor for cubital tunnel syndrome
1	Ocular	Retinitis pigmentosa
3	Other	Erectile dysfunction, type I diabetes mellitus and overactive bladder syndrome
Phase II		
10	Cancer	Melanoma, angioendothelioma, squamous cell carcinoma of the head and neck, lung cancer, mantle cell carcinoma and pancreatic adenocarcinoma
4	Cardiovascular	Claudication (cramp-like pains in the legs cause by poor circulation), peripheral ischaemic ulcers, limb ischaemia
3	Neurological	Relapsing remitting multiple sclerosis, diabetic neuropathy and nerve conduction velocity
3	Ocular	Atrophic macular degeneration and retinitis pigmentosa
Phase III		
1	Cancer	Lung cancer
1	Cardiovascular	Limb ischaemia

epitope vaccines delivered by different DNA approaches, only low-level T cell responses were induced by this vaccine, suggesting that the function of epitope-based vaccines in humans warrants further study.

In addition, both Novartis and the laboratory of Shan Lu at the University of Massachusetts have been exploring DNA-plasmid priming followed by homologous antigenic-protein boosting in human studies. The Novartis approach uses DNA that is formulated in polymers, for example, polylactice-coglycolides, boosted with a novel trimeric gp140 envelope protein antigen. By contrast, the University of Massachusetts group focused on a multiplasmid cocktail and a multicocktail envelope-protein boost strategy. Both studies have shown excellent safety records and strong induction of antibody responses as well as $CD4^+$ T cell responses, but only limited neutralizing antibody responses have been induced. These studies clearly support the view that this platform combination can be useful in the clinic for vaccine targets that are $CD4^+$ T cell dependent; however, new approaches that lead to the induction of more potent CTL responses continue to be a focus for HIV-1 studies.

The most potent recombinant vector platform in humans for generation of cellular immunity seems to be the adenovirus platform. The viral vector generated using adenovirus serotype 5 (Ad5) is the most potent, although vectors based on new and promising primate serotypes, as well as rare human serotypes, are also being developed. Much of the work on Ad5-based vaccines is being applied to the HIV-1 vaccine arena. Important studies from Merck and the Vaccine Research Center at the National Institutes of Health have directly compared CTL induction generated in humans by plasmid vectors versus the Ad5 recombinant vector. Both studies showed that plasmid DNA was at least four times less potent in magnitude and response rate than Ad5 vaccines that contained similar HIV antigen cassettes. However, a recent comparison of the immune responses induced by the Ad5 viral vector approach that was stopped by Merck recently[28] and that of the Vaccine Research Center, which uses a prime-Ad5 boost approach[29,30], demonstrated little difference in the immune responses elicited by the two vaccination strategies, in contrast to data from preclinical animal model testing. This study by Merck again illustrates the need to improve the immune potency of the DNA approach in priming studies. The lack of translation from animal model to humans further demonstrates the need for continued improvements in delivery technology and other optimizations of the important prime-boost strategy, and underscores the limitation of naked plasmid approaches in this format.

Cancer immune therapies. Many cancer antigens are excellent immune therapy targets for DNA vaccines. As such, cancer vaccines resemble drug therapies in that they can be re-administered on a regular repetitive schedule without vector interference concerns. In mouse models, DNA vaccines have been successfully directed against a wide variety of tumours, almost exclusively by driving strong cellular immune responses in an antigen-specific fashion. Furthermore, tumour burden has been decreased or even obliterated by novel DNA vaccine strategies that deliver cytokines as plasmids directly into tumours.

Vector interference
The observation that re-administration of the same bacterial or viral vector leads to a reduction in its potency. This occurs because the host's immune response develops neutralizing antibody responses against the vector when it is first administered. DNA does not contain protein targets so there is no vector interference or loss of potency following DNA re-administration.

Table 2 | **Current licensed DNA therapies**

Vaccine target	Product name	Company involved	Date licensed and country	Target organisms	Benefits
West Nile virus	West Nile Innovator	Centers for Disease Control and Prevention and Fort Dodge Laboratories	2005 USA	Horses	Protects against West Nile virus infection
Infectious haematopoietic necrosis virus	Apex-IHN	Novartis	2005 Canada	Salmon	Improves animal welfare, increase food quality and quantity
Growth hormone releasing hormone	LifeTide-SW5	VGX Animal Health	2007 Australia	Swine and food animals*	Increases the number of piglets weaned in breeding sows; significantly decreases perinatal mortality and morbidity
Melanoma	Canine Melanoma Vaccine	Merial, Memorial Sloan–Kettering Cancer Center and The Animal Medical Center of New York	2007 USA, conditional license	Dogs	Treats aggressive forms of cancer of the mouth, nail bed, foot pad or other areas as an alternative to radiation and surgery

*Refers to agricultural animals as opposed to pet animals — a higher level of stringency is required for approval for animals that are part of the food chain.

For cancer immune therapies that have moved into the clinic, current candidates are well tolerated. There are also indications of immune responses in patients with melanoma and prostate cancer, and in some cases suggestive clinical benefit has been reported. On the basis of meeting clinical endpoints in a Phase II study, Vical has moved forward a Phase III study of interleukin-2 cytokine delivery as a plasmid formulation for treating melanoma. This is an important milestone for the DNA vaccine field. Furthermore, studies that include the use of electroporation to deliver antigens or cytokines directly into tumours are giving interesting results. In one important Phase I clinical trial, plasmid DNA encoding the potent cytokine interleukin-12 was delivered directly into melanoma lesions, followed by electroporation. Clinical remissions of tumours were reported, even for lesions that were distant from the injection site in some patients[31]. The use of helper toxoids linked to T cell epitopes delivered by electroporation to enhance immune responses against prostate cancer is also promising[32,33]. Some interesting newer cancer studies target viral antigens directly. These new approaches are likely to promote the growth of the DNA cancer immune therapy field.

Taken together, the selected trials for DNA vaccines described here have shown that immune responses can be generated in humans, but they also highlight the need for increased potency if this vaccine technology is to be effective. Towards this end, it will be crucial to analyse the results of ongoing research in the clinic, specifically pertaining to the success or failure of certain design and delivery methodologies for these next-generation platforms. In addition, attention to successful licensure of DNA products in the veterinary arena will provide useful information for future human clinical study designs, as described in more detail below.

Electroporation
A physical process that exposes the target tissue to a brief electric-field pulse in order to induce temporary and reversible pores in the cell membrane. During the period of membrane destabilization, molecules such as plasmids can gain intracellular access.

Licensed veterinary DNA vaccines and therapies

In the past 3 years, four DNA products have been licensed for animal use. One against West Nile virus[34] in horses, one against infectious haematopoietic necrosis virus[35] in schooled salmon, one for treatment of melanoma in dogs[36] and the most recent licensure, growth hormone releasing hormone (GHRH)[37] product for foetal loss in swine (TABLE 2). These licensures are an important validation of the DNA vaccine platform because they illustrate its commercial potential. Moreover, the success of these products show that DNA vaccines can be manufactured to scale and at low cost, especially in the case of the fish vaccine for infectious haematopoietic necrosis virus, and that large animals under specific circumstances can be successfully protected or treated by specific DNA vaccine approaches.

These approvals are ground-breaking in several respects. For example, the melanoma vaccine (Canine Melanoma Vaccine) is the first licensed cellular immune therapy for cancer. GHRH (LifeTide-SW5) is the first licensed gene therapy product and the first licensed electroporation-delivered product for any application. It could be argued that the use of DNA in these animal products represents an easy target in the DNA vaccine arena, compared with potential human applications. For example, the antibody titres induced by the West Nile virus vaccine in horses are very low, suggesting a low threshold for immune protection from this specific pathogen, at least in this species. However, the canine melanoma immune therapy, a model system with clear similarity to human disease, and the electroporation delivery of GHRH in pigs, a large species, argue differently. These two clinical veterinary successes suggest that, with some adjustments, both of these products might give rise to human analogues that can be used to alleviate human disease. These product successes bode well for the sustainability and growth of the field as they are probably the tip of the iceberg regarding the practicality of DNA vaccines as formulated products for animal health.

Safety issues

Advantages of DNA vaccines. Whereas traditional vaccines rely on the production of antibodies through the injection of live attenuated virus, killed viral particles or recombinant viral proteins, DNA vaccines can be constructed to function with many encoded safety features while retaining the specificity of a subunit vaccine. As DNA vaccine plasmids are non-live, non-replicating and non-spreading, there is little risk of either reversion to a disease-causing form or secondary infection. In addition

Table 3 | **Advantages of DNA vaccination**

Commendable qualities	Attributes
Design	Synthetic and PCR methods allow simple engineering design modifications
	Optimization of plasmids through codon and RNA structure changes
	Brings the power of genomics to vaccine construction
Time to manufacture	Rapid production and formulation
	Reproducibile, large-scale production and isolation
Safety	Unable to revert into virulent forms, unlike live vaccines
	In contrast to some killed vaccines, efficiency does not require use of toxic treatments
	No significant adverse events in any clincal trial — many thousands vaccinated so far
Stability	More temperature-stable than conventional vaccines
	Long shelf life
Mobility	Ease of storage and transport
	Likely not to require a cold chain
Immunogenicity	Induction of antigen-specific T and B cell responses similar to those elicited by live attenuated platforms

Cold chain, the requirement to keep a vaccine chilled or frozen to protect its potency from degrading at room temperature over the time frame from manufacture to use.

to their safety, DNA vaccines are highly flexible, encoding several types of genes including viral or bacterial antigens, and immunological and biological proteins. DNA vaccines are stable, are easily stored and can be manufactured on a large scale. They also, for example, bypass concerns that adventitial agents might be transferred from tissue-culture lines to the vaccinated individual. Many potential advantages of DNA vaccines are summarized in TABLE 3.

Potential safety concerns. Issues have been raised with regard to the safety of DNA vaccines, these include the potential to integrate into cellular DNA, the development of autoimmunity, and the possibility of antibiotic resistance. DNA vaccines that are currently being tested do not show relevant levels of integration into host cellular DNA[38–43]. If integration is detected at all, it typically occurs at rates that are orders of magnitude below the spontaneous mutation frequency[44]. However, vectors that are modified or adjuvanted with the goal of increasing immunogenicity could increase the chances of integration. A further concern is that an integrated vaccine might cause insertional mutagenesis through the activation of oncogenes or the inactivation of tumour suppressor genes. In addition, an integrated plasmid DNA vaccine could, in theory, result in chromosomal instability through the induction of chromosomal breaks or rearrangements. However, none of these concerns have been witnessed in the preclinical or clinical evaluation of DNA products.

With regards to the development of autoimmunity induced by DNA vaccination, preclinical studies in non-human primates and early studies in humans did not detect increases in anti-nuclear or anti-DNA antibodies. Participants in human trials of DNA vaccines are followed for possible signs and symptoms of autoimmunity, and laboratory markers of autoimmunity are sometimes monitored as well. To date, there has been no convincing evidence of autoimmunity developing in association with a DNA vaccine[20,23,45–47].

A third issue regarding DNA vaccines involves antibiotic resistance. Large-scale manufacture of a DNA vaccine involves enriching cultured cells for the plasmid by virtue of its antibiotic-resistant marker. Concern has been raised that resistance to the same antibiotic might be introduced in participants and transferred into carried bacteria when the plasmid is used in clinical trials. However, the antibiotic resistance genes contained by vaccine plasmids are restricted to those antibiotics — in particular the kanamycin restriction element — that are not commonly used to treat human infections. Alternative strategies that do not use antibiotic selection at all are also important[48–50] and are being explored.

In response to these various safety concerns, summarized in TABLE 4, the European Union[51] and the US Food and Drug Association (FDA)[52–56] have developed specific advice on safety and testing of DNA vaccines. Efforts to examine integration, antibiotic resistance and the induction of autoimmunity are being followed even as an impressive unblemished record of clinical safety is continuing to be expanded upon. Based on this outstanding safety record the focus of the field has shifted to optimizing immune induction.

DNA vaccine platform: room for improvement

The principal issue regarding the future of DNA vaccines concerns improving their immunogenicity in larger animals and in humans. The DNA vaccine platform has driven significantly weaker immune responses in non-human primates and in humans compared with mice. It seems to be less immunogenic compared with recombinant viral vectors such as adenoviral vectors or recombinant protein for induction of antibody responses. However, DNA vaccine technology efforts are ongoing to optimize the platform to increase antigen expression and vaccine immunogenicity using several strategies that are discussed below.

Adventitial agents
Unknown pathogens that are present in the cell lines that are used to produce vaccines and that can become part of the final vaccine preparation. SV40 was discovered this way, as a contaminant of the polioviral vaccine. As DNA vaccines are not produced in mammalian cell culture they can not contain these agents.

Table 4 | **Potential concerns regarding DNA vaccination**

Theoretical issues	Concern	Resolution
Integration	DNA vaccines integrate into cellular DNA owing to optimized expression plasmids, resulting in insertional mutagenesis, chromosomal instability, or activation or inactivation of tumour suppressor genes	US Food and Drug Administration requires integration studies for new DNA products using accepted assays in animals before beginning human trials
Autoimmunity	Development of autoimmune disorders against patient DNA	Studies have shown this result is unlikely: no anti-nuclear or disease associated anti-DNA antibodies have been detected
	Development of autoantibodies against immune adjuvants	Examine patients for signs of autoimmunity using laboratory markers
Antibiotic resistance	Production process involves selection of bacterial cells using antibiotic resistance, which is conferred by a plasmid gene.	Antibiotic resistance in plasmid is driven by bacterial origin of replication (not mammalian)
	There is a risk that antibiotic resistance is transferred to patients receiving vaccine through the unintentional transfer of bacteria	Antibiotics used are restricted to antibiotics not commonly used to treat human infections
Low immunogenicity	First-generation DNA plasmids elicit low levels of T cell and B cell memory	Use novel formulations, immune plasmid adjuvants and delivery systems to enhance immunogenicity. Prime-boost approaches are common in clinical studies

As shown in FIG. 1, there are several ways in which antigen expression and immunogenicity can be improved for the DNA vaccine platform. These include optimization of the transcriptional elements in the plasmid backbone with the aim to improve antigen expression levels, strategies to improve protein expression of the gene of interest, inclusion of adjuvants in the formulation or as immune modulators, and the use of next-generation delivery methods.

Optimization of transcriptional elements. Several laboratories have identified methods to optimize the transcriptional elements in the plasmid backbone so that gene transcription and expression can be improved. An important component of the plasmid is the promoter that drives expression of the gene of interest.

Microbial gene promoters are not necessarily optimized for driving optimal mammalian gene expression. Early studies therefore used strong promoters from human oncogenic viruses such as Rous sarcoma virus[57] or SV40 (REF. 58). However, more recently, promoters from non-carcinogenic sources that are equally effective have been used, including one from human cytomegalovirus (CMV)[59] (reviewed in REF. 60). For most vaccine plasmids, the human CMV promoter is a common choice because it promotes high-level constitutive expression in a wide range of mammalian cells, and does not suffer from downstream read-through as might be expected from a strong promoter. Alternatively, the use of host tissue-specific promoters avoids constitutive expression of antigens in inappropriate tissues. For example, the use of the promoter of the muscle creatine kinase gene leads to the induction of antibody and T cell responses, although levels were at least tenfold lower than plasmids that contain the CMV promoter[61,62]. These data suggest that the use of host-cell promoters limits expression and, ultimately, immunogenicity, which might be an advantage for gene delivery. Moreover, promoters with significant homology to host-cell sequences might need to be optimized for improved human clinical outcomes.

A second important modification to the antigenic plasmids is the inclusion of a termination site, or poly(A) signal site, that is 11–30 nucleotides downstream from a conserved sequence (AAUAAA) at the 3′ end of the mRNA. This signal is required for proper termination of transcription and export of the mRNA from the nucleus. Many DNA vaccines use the bovine growth hormone terminator sequence[63] or endogenous terminators that are downstream from the ORF of the gene of interest to ensure proper transcriptional termination. It remains to be seen whether modifications to the polyadenylation and termination signals influence gene expression, although early reports are promising[64].

Both enhancer elements and transcriptional transactivators can enhance promoter activity when placed either upstream or downstream of the ORF. Some of the transactivator genes that have been studied are of viral origin. For example, the regulatory R region from the 5′ long terminal repeat (LTR) of human T-cell leukaemia virus type 1 acts as a transcriptional and post-transcriptional enhancer; the resulting CMV–R DNA vaccines elicit substantially higher specific cellular immune responses to HIV-1 compared with the analogous parental DNA vaccines in both mice and cynomolgus monkeys[65]. However, the use of regulatory enhancers of viral origin might not be well received in such platforms owing to their association with oncogenesis. Further experimentation with non-viral transactivators[66] or enhancer elements[17,65–68] for DNA vaccine plasmids is therefore important.

Optimization of regulatory elements would be a simple and effective strategy to augment the immunogenicity of DNA vaccines in mice and primates, and further study is needed for successful translation of this strategy in the clinic. The use of high-efficiency origins of bacterial replication relevant for the bacterial strains used for production can also markedly improve the quantity of plasmid product.

Cynomolgus monkey (*Macaca fascicularis*). A primarily arboreal macaque that is native to Southeast Asia. It is also called the long-tailed macaque.

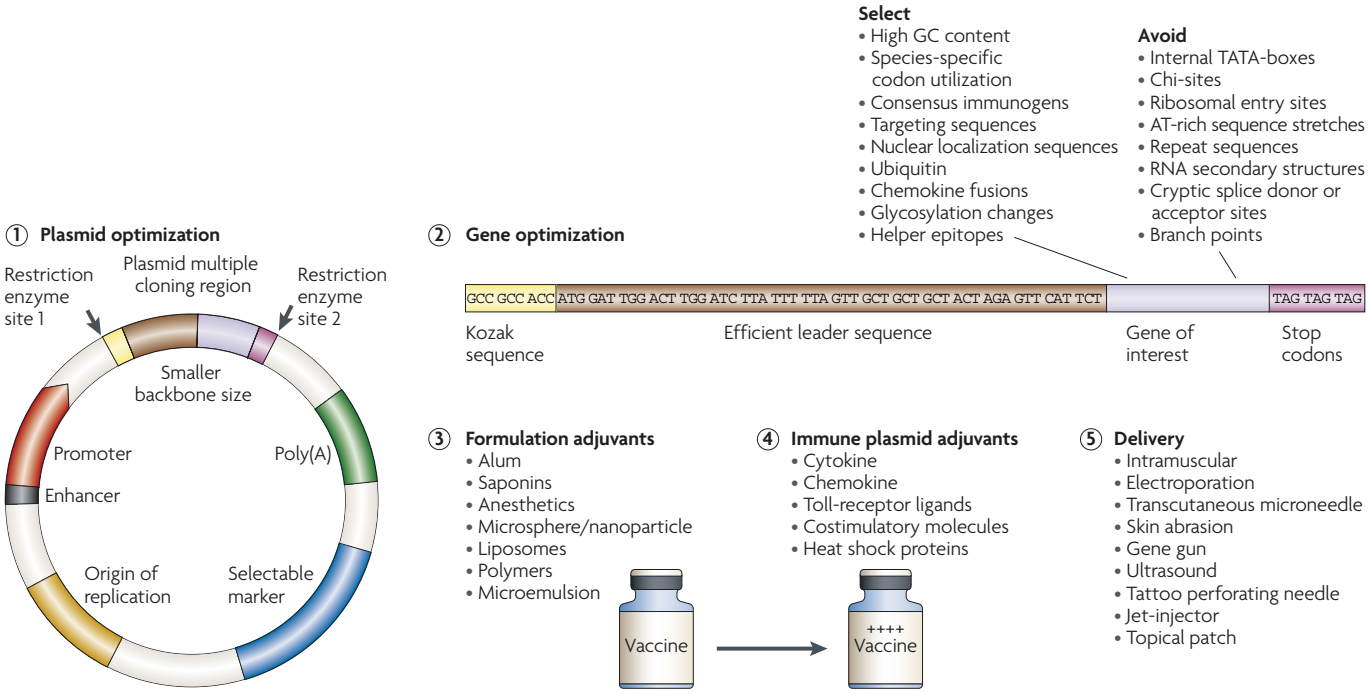


Figure 1 | **DNA vaccines: optimization strategies to enhance immunogenicity.** DNA vaccine technology has been the target of ongoing efforts to optimize the platform to increase antigen expression and vaccine immunogenicity (see main text for a detailed explanation of each step). There are currently several ways in which antigen expression and immunogenicity can be improved for the DNA vaccine platform. These include: optimization of the transcriptional elements on the plasmid backbone (1); strategies to improve protein expression of the gene of interest (2), including factors to avoid (for example, chi-sites, which are sequences that encourage crossing-over to occur at that site); inclusion of formulation adjuvants (3) or immune plasmid adjuvants (4); and the use of next-generation delivery methods (5). Several formulations have been developed and are being testing at all stages of preclinical and clinical development for their ability to enhance antigen expression and immunogenicity. These mechanisms include encapsulation and protection of DNA from extracellular degradation through to particle trapping and high-velocity delivery, with the ultimate goal of introducing the plasmid directly into the cytosol of target cells. In addition, immune adjuvant classes that encode immune modulatory molecules that target death receptors, growth factors, adhesion molecules, cytokines and chemokines as well as Toll receptor ligands exist. It should be noted that many of these plasmid optimization and formulation strategies, as well as the adjuvant systems, are used in combination with novel delivery mechanisms that result in an overall enhanced vaccine platform.

Enhancing protein production. One strategy that is used to improve the expression of the transgene product is to optimize the initiation start site for protein synthesis, because endogenous sites of viruses and bacteria might not be optimal for expression in mammalian cells. Modification of the Kozak consensus sequence[69] is a focus for this purpose. Ensuring correct termination is also important. Double stop-codons can be added to prevent read through, which could lead to oversized and incorrectly folded products and interfere with mRNA instability.

One of the most effective ways to increase protein production is through the use of codon optimization[70]. Because immunogenicity depends on the effective translation and transcription of the antigenic protein, gene expression can be greatly increased by adopting species-specific codon changes. This procedure results in increased protein production, leading to enhanced T cell[7–10] and antibody[11–14] responses.

RNA optimization can also lead to more efficient translation through several important modifications, including removal of instability elements that lower expression; these elements include secondary mRNA structures that can inhibit ribosomal loading and cryptic sequences that inhibit nuclear export of mRNA[71–73]. High antigenic expression rates and prolonged mRNA stability are not only crucial for heterologous mammalian expression, but are important for the generation of effective DNA vaccines[10].

The addition of leader sequences can enhance the stability of mRNA and contribute to translational efficiency. For example, the signal sequence for the HIV-1 envelope glycoprotein (Env) delays its own cleavage, therefore slowing down the rate of gp120 folding inside the endoplasmic reticulum[74]. A marked increase in protein production was detected when the native HIV-1 Env leader[75], or native leaders encoded by vaccinia-expressing *Mycobacterium tuberculosis* antigens[76], was replaced by the human tissue plasminogen activator leader sequence. Enhanced expression of plasmid antigens is also observed using the efficient leader from the IgE gene[77–80].

Kozak consensus sequence
A specific sequence that occurs on eukaryotic mRNA. This sequence is recognized and required by the ribosome as the translational start site.

Engineering vaccine antigens that elicit broadly cross-neutralizing antibodies has been a challenge, in particular for HIV-1. To enhance the immunogenicity of conserved neutralization epitopes on Env, elimination of glycosylation sites has been investigated[81]. This treatment can increase antibody titre, although the effect of such structural modifications on the neutralization phenotype is not as clear. Moreover, the insertion of proteolytic cleavage sites between antigenic sequences in the gene of interest to generate fused proteins or peptides might be important in CTL processing. Similarly, recombinant proteins containing fusogenic sequences provide a promising system to induce cytotoxic T cells by live-vector vaccines by destabilizing the phagosome membrane so that epitopes can reach the cytosol[82].

Recently, much attention has focused on the development of consensus immunogens as transgenes for immunization owing to their importance for targeting variable pathogens[10,16,83–86]. Several other strategies to determine consensus sequence include the analysis of ancestral sequences[87], as well as more advanced methods for epitope scanning; for example, by using T cell assays to identify reactive epitopes[10], predictive computer analysis software[88,89] or phage display[90]. In addition, the use of DNA libraries and protein sequence data for target epitopes that have the ability to be recognized by the human immune system have been used to identify novel epitopes for potential immunotherapy targets, such as viruses, bacteria, parasites, tumour-associated antigens or other self-antigens associated with the pathogenesis of disease[91–93]. Such constructs can drive improved cross-reactive immune responses, particularly at the T cell level and in mouse systems. Primate and human studies of epitope strings as DNA vaccines have not indicated that these approaches are as potent as whole antigen sequences. However, it is hoped that the potency of this strategy will be improved by new approaches to link epitopes, by the inclusion of helper T cell sequences, and by new epitope selection technologies — particularly when coupled with new delivery strategies. In the cancer model, for example, tumour-specific antigen epitopes have been linked to fragments of tetanus toxoid to improve helper T cell responses[94]. This strategy has generated strong protective immunity in model systems and is a promising clinical approach for supplying novel T cell help.

Improving immunogenicity through formulation. One current trend in DNA vaccine formulation is the use of biodegradable polymeric microparticles (reviewed in REF. 95) and liposomes. The prospects for the broad application of microparticle-based and liposome-based delivery systems for DNA vaccines are excellent — their utility for delivery and enhanced immunogenicity in several different host and antigenic vaccine platforms has been shown in mice, non-human primates and humans[96–101].

Plasmid DNA is trapped on the surface of the polymers, for example, polylactice-coglycolides or chitosan, and is delivered systemically or directly to mucosal surfaces (orally or via the respiratory tract), where the complex is taken up by denritic cells (DCs). This results in upregulation of DC activation markers and further augments systemic and mucosal immune responses[102,103]. Examples of important molecules moving forward as formulation adjuvant or delivery methods for DNA vaccines include polyethyleneimine, amine-functionalized polymethacrylates, cationic poly(β-amino esters), poloxamers and polyvinylpyrrolidone polymers. These have proven to be successful in disease models — for example, in the delivery of gene therapeutic agents for cystic fibrosis[104–108]. The poloaxmer CRL1005 has been successful in preclinical models for simian HIV vaccine strategies[109]. Importantly, Vaxfectin, a related molecule, has enhanced antibody responses to a DNA vaccine targeting influenza in human clinical studies[110]. Most of these types of formulation molecules have not shown significant clinical benefit, although recent data from the Vaxfectin trial (performed by Vical) indicate a clear improvement in immune responses in humans. Other results from trials are eagerly awaited.

In addition to polymers, ongoing studies have shown that liposome vehicles can protect DNA from degradation by serum proteins during transfer of DNA across membranes and after the release of genetic material following fusion with endosomes[111,112]. Because liposomes can be prepared with significant structural versatility, including vesicle surface charge (both cationic and anionic liposomes can be made), size, lipid content and co-delivery with other adjuvants, they offer considerable flexibility[113–115] towards vaccine optimization and have been shown to induce cellular and humoral immunity[110,116–119].

Improving immunogenicity by including immune modulatory adjuvants. In addition to optimizing the DNA antigenic plasmid construct, a separate approach is to include immune modulatory genes as cassettes as part of the plasmid vaccines cocktail. Multiple laboratories have reported that co-injection of plasmids encoding cytokines, chemokines or co-stimulatory molecules can have a substantial effect on the immune response to plasmid-encoded antigen; these include studies in multiple viral and cancer antigen systems. For example, in non-human primates interleukin-12 is a potent DNA vaccine adjuvant for cellular immunity. In addition, many other classes of immune modulatory molecule exist that target death receptors, growth factors, adhesion molecules, other cytokines[120] and chemokines as well as Toll-receptor ligands. The growing number of immune adjuvant formulations described above can be used individually or in combination to maximize effects. In addition, synthetic oligodeoxynucleotides containing unmethylated CpG motifs act as immune adjuvants in mice, as they boost the humoral and cellular response to co-administered antigens[121]. Moreover, the large number of ongoing preclinical studies underscores the importance and usefulness of these adjuvants owing to their pleiotropic effects on the growth, differentiation, maturation, survival and proliferation of many different cell types. DNA vaccination strategies that incorporate immune modulatory formulation adjuvants should improve responses in the clinic, in both prophylactic and therapeutic studies.

Cross-neutralizing antibody
An antibody that recognizes a wide range of antigenic epitopes.

Consensus immunogens
Immunogens that are designed using computer analysis and then coded in DNA vaccines. They are synthesized so that the genes represent the most common amino acid at any position in a sequence, based on a population of viral isolate sequences.

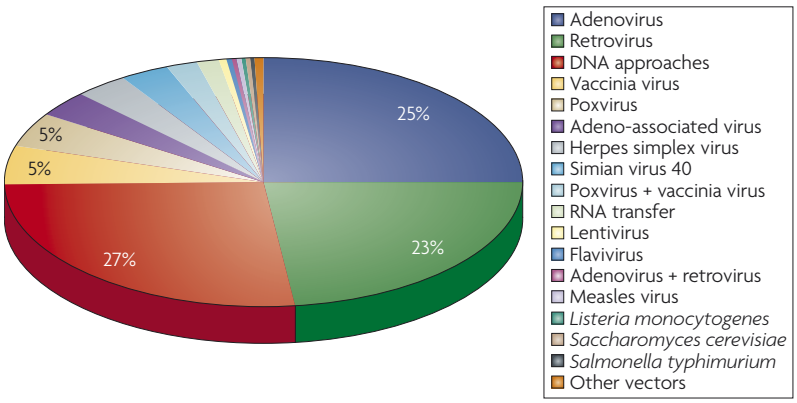
Phage display
A method that uses bacteriophage for high-throughput screening of protein interactions with other proteins, DNA or peptides.

Liposome
Made up of bilayered membranes consisting of polar and non-polar portions of phospholipids that form multilayered shells containing a charged DNA-binding region.

Improving immunogenicity by using next-generation delivery strategies. The most common route of immunization used in DNA vaccine studies is the intramuscular route. However, several studies have demonstrated the importance of direct transfection of APCs[122,123]; by contrast, following intramuscular immunizations, the predominant cell type transfected with the DNA vaccine is myocytes[124]. Therefore, increasing the transfection efficiency of target cells through various physical delivery methods is an area that is being heavily investigated.

Some of the more recent delivery methods, including the transcutaneous microneedle, have the ability to bypass the stratum corneum layer of the skin, thus reaching Langerhans cells — the APCs of the skin (reviewed in REF. 125). Further progress in microneedle array design, microneedle application apparatus and formulation will probably confirm that this methodology is a realistic clinical strategy for delivering DNA to and through skin. In addition, the use of low-frequency ultrasound as a potent physical adjuvant for successful transcutaneous immunization has been developed[126]. Another topical application method includes 'painting' a DNA vaccine with cytokine-expression plasmids onto the skin of mice after elimination of the keratinocyte layers; this method induced marked immune responses, both cellular and humoral, against the HIV-1 Env protein[127]. A second group is examining a novel vaccine for HIV called Dermavir, which is topically administered under a patch containing plasmid DNA that is chemically formulated into a nanoparticle and delivered into epidermal Langerhans cells[128,129].

Box 2 | **Growing interest in the DNA platform**



Interestingly, plasmid DNA vectors make up approximately 27% (354 out of 1,311 trials) of all gene-therapy vector platforms studied in Phase I to Phase III trials in 2007 (see figure; data modified from Gene Therapy Trials Worldwide provided by the *Journal of Gene Medicine*). These data support the overall preclinical success and Phase I safety of the platform. This platform has been growing steadily: before 1998, DNA plasmid vectors constituted an average of only 4% of all gene-therapy platform trials.

As shown in TABLE 1, when all open enrolling trials that make up the 154 Phase I to Phase III clinical trials that use the DNA platform are examined, 68% are trials testing DNA therapies for cancer, whereas 11% are for treatment of cardiovascular diseases. Infectious disease trials make up approximately 5% of the current open human clinical Phase I to Phase III trials testing the DNA vaccine platform and include vaccines against HIV-1, cytomegalovirus, and influenza virus. Most open Phase I to Phase III clinical trials for the DNA platform are being tested in the United States (68%), followed by the United Kingdom (15%) and Germany (8%).

New improvements in particle-mediated epidermal delivery (PMED) technology and vector design, including co-formulation of PMED DNA vaccines with adjuvants, are in progress to further enhance the potency of particle-mediated DNA vaccines (further reviewed in REF. 130). These efforts are expected to accelerate the commercialization of PMED as an effective approach for vaccination against infectious diseases. Jet-injection mechanical devices aim to deliver protein and DNA vaccines into the viable epidermis, thus providing potentially safer alternatives to needle injection, and this method promises increased efficacy in the prevention and/or therapy of infectious diseases, allergic disorders and cancer[131–136]. The tattoo perforating needle device has a bundle of fine metal needles that oscillate at a constant high frequency and puncture the skin, leading to DNA transfer to skin-associated cells and the expression of reporter genes in mice[137], which results in the induction of T cell responses. A recent novel approach that was reported in small animals is to target epitope-based DNA vaccines to DCs using DC-targeting ligands[138]. It will be crucial to determine how these technologies will ultimately translate to the clinic.

Among the most impressive preclinical delivery strategies is electroporation, a technique that has been studied for two decades as a method to improve delivery of chemotherapy drugs to kill specific tumour cells[139–141]. Electroporation has been extensively studied in large animal species such as dogs, pigs, cattle and non-human primates to deliver therapeutic genes that encode a variety of hormones, cytokines, enzymes or antigens[142–146]. *In vivo* expression levels improved markedly using this approach — levels increased by several fold over plasmid injection alone. This method might allow for less frequent immunizations with the DNA platform, and can improve both cellular and humoral responses. Again, this has been shown mostly in smaller animal models and, in particular, tumour systems[32,141,144,147–159]. However, recent studies have moved these delivery strategies to the non-human primate model[142,149,153,160–162]. Several different strategies of this technology are being pursued, including: those that deliver an electric current at the same time as the DNA injection; devices that use constant voltage or monitored constant current; devices fitted with penetrating probes that target the muscle or the skin; and strategies that use callipers to deliver an electric pulse through the skin. However, too little is currently known about several of these devices and much additional research in this area is warranted.

Recently, several devices have been moved into clinical evaluation, and the results are eagerly awaited. Important pressing issues include a comparison of the immune responses induced by these strategies with the best recombinant viral platforms and their tolerability in humans. As DNA vaccines delivered by electrostimulation deliver a higher bolus of antigen produced by a lower dose of plasmid, there are conceptual positive safety aspects of this technology, as well as additional concerns that arise from our unfamiliarity with this technology in humans. The strong results produced by this approach in non-human primates and large animals including pigs, horses and cattle are encouraging.

Conclusions

It has been more than 16 years since DNA vaccines stepped into the scientific limelight. During this time DNA vaccine technologies have generated great deal of excitement as well as disappointment. This situation, however, is similar to the development of a different breakthrough technology platform, that of monoclonal antibodies. More than 20 years elapsed between Kohler and Millstein's pioneering report of hybridoma technology[163] to the commercialization of the first human therapeutic antibody product. New technologies seem to be simple and straightforward but, as illustrated in this Review, they are deceptively so. An initial idea is transformed into a workable platform only through subtle and gradual improvements. In this regard it is clear that, after several years of frustration, the DNA platform is back on a productive path. In fact, as shown in BOX 2, the DNA platform represents almost one quarter of all gene therapy vector systems under clinical evaluation. This opinion is strengthened by recent licenses in the area of animal health and by the improvements in immune potency reported in the non-human primate model systems.

However, the next 2 years of clinical testing of new and more complex DNA vaccines will be pivotal for either generating a true clinical success based on immune potency, or for telling us that we still have much further to go. Previous clinical disappointments highlight the likelihood that complexity in DNA vaccine design — including better and improved formulations, better delivery technologies, enhanced plasmid and delivery approaches, and more judicious clinical implementation — will be a staple for the continued enhancement of this platform. The further advancement of the DNA platform will continue to be an exciting and highly productive adventure that illustrates the best in academic creativity and translational science. Its success will be built on a high level of cooperation between industry, the regulatory authorities, funding by non-governmental organizations, the public and academicians.

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Competing interests statement
The authors declare competing financial interests: see web version for details

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