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The future of human DNA vaccines

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Abstract

DNA vaccines have evolved greatly over the last 20 years since their invention, but have yet to become a competitive alternative to conventional protein or carbohydrate based human vaccines. Whilst safety concerns were an initial barrier, the Achilles heel of DNA vaccines remains their poor immunogenicity when compared to protein vaccines. A wide variety of strategies have been developed to optimize DNA vaccine immunogenicity, including codon optimization, genetic adjuvants, electroporation and sophisticated prime-boost regimens, with each of these methods having its advantages and limitations. Whilst each of these methods has contributed to incremental improvements in DNA vaccine efficacy, more is still needed if human DNA vaccines are to succeed commercially. This review foresees a final breakthrough in human DNA vaccines will come from application of the latest cutting-edge technologies, including “epigenetics” and “omics” approaches, alongside traditional techniques to improve immunogenicity such as adjuvants and electroporation, thereby overcoming the current limitations of DNA vaccines in humans

Keywords

DNA vaccine; immunogenicity; adjuvant; epigenetics; RNAi; omics

INTRODUCTION

Unlike conventional protein or polysaccharide based vaccines, DNA vaccines comprise plasmids encoding the vaccine antigen along with a strong eukaryotic promoter used to drive protein expression (Rajcani et al., 2005). Such nucleic acid based vaccines can be delivered intramuscularly, subcutaneously or mucosally with the aim that they will gain access to the cell cytoplasm and thereby induce antigen expression *in vivo* that, like the protein vaccine they mimic, will then elicit an desired immune response. DNA vaccines have been successfully applied to animal models to variously prevent or treat infectious diseases, cancer, autoimmunity and allergy (Ulmer et al., 1996). On the positive side, the straightforward plasmid structure of DNA vaccines gives them inherent advantages over

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traditional protein or carbohydrate based vaccines. The one-step cloning of target coding sequence into plasmid vectors offers more convenient development and production when compared to culture and inactivation of whole infectious pathogens or expression and purification of recombinant proteins. Furthermore, by inducing expression of proteins *in vivo*, antigenic structure is more likely to resemble the native protein structure and include any essential post-translational modifications. From a safety perspective, amplification of the nucleic acids encoding a potential antigen avoids the need to directly handle dangerous pathogens. The convenient manipulation of plasmid DNA *in vitro* allows easy introduction of beneficial mutations into the antigen coding sequence. I*n vitro* mutation also enables modification of antigen coding sequences to counter rapidly drifting virus strains. Plasmid DNA is stable at room temperature allowing for convenient storage and shipping. In addition to these physical properties, DNA vaccines enable expressed antigens to be presented by both MHC class I and class II complexes, thereby stimulating Th1 and Th2 CD4 and CD8 T cells in addition to B cells (Liu, 2011). To date, veterinary DNA vaccines have been approved for use in fish (infectious haematopoietic necrosis virus), dogs (melanoma), swine (growth hormone releasing hormone) and horses (West Nile virus) (Kutzler and Weiner, 2008). However, success in veterinary approvals has not translated into successful human DNA vaccine applications, with low immunogenicity remaining the Achilles heel of human DNA vaccines. In recent years, many clinical trials have been undertaken on DNA vaccines covering the full range of prophylactic through to therapeutic vaccines vaccines against infections, cancers and a range of other disorders, with details of these studies available through a range of websites including http://www.cancer.gov/clinicaltrials; http://clinicaltrials.gov; http://clinicaltrialsfeeds.org/; http://www.dnavaccine.com/; http://www.niaid.nih.gov/volunteer/vrc/Pages/default.aspx. However, despite more than 100 such clinical trials, more work is still clearly required on design and delivery to lift the immunogenicity of DNA vaccines to the levels required for human regulatory approval and commercial exploitation.

MECHANISM OF ACTION OF DNA VACCINES

In 1990, Wolff et al showed that injection of DNA encoding lactase reporter genes into mouse quadriceps muscle induced sustained protein expression (Wolff et al., 1990). Tang et al. subsequently showed that introducing a plasmid encoding human growth hormone (hGH) into mouse skin induced an antibody response against the expressed protein (Tang et al., 1992), thereby directly mimicking a protein vaccine. Final proof that a DNA encoded antigen could provide effective vaccine protection came from the demonstration that injection of plasmid encoding influenza nuclear protein into mouse muscle generated influenza-specific $CD8^+$ cytotoxic T lymphocytes (CTL)s that then protected the mice from a subsequent influenza challenge (Ulmer et al., 1993).

Whilst these studies confirmed the theoretical utility of DNA vaccines, practical considerations remained. For example, DNA inoculation results in antigen expression in the low picogram to nanogram range and most transfected somatic cells are not professional antigen presenting cells (APC). A potential offset is that the sustained low level antigen expression achieved with injected DNA may better prime adaptive immune responses when compared with the short half-life of injected protein antigens. At least three different mechanisms have been proposed to contribute to the immunogenicity of DNA vaccines: 1) DNA-encoded antigens are presented by somatic cells (myocytes or keratinocytes) through their MHC class I pathway to CD8 T cells; 2) DNA immunization results in direct transfection of professional antigen presenting cells (APC) (e.g. dendritic cells); and 3) cross-priming results from transfected somatic cells being phagocytosed by professional APCs which then present the antigens to T cells. Muscle cells are not efficient at presenting

antigens via MHC class I, so the latter two mechanisms may be more important to DNA vaccines.

Immunostimulatory elements of plasmid DNA such as unmethylated CpG motifs may also make a contribution to DNA immunogenicity. CpG dinucleotide motifs have a low frequency and are mostly methylated in the mammalian genome. By contrast, bacterial DNA contains many unmethylated CpG motifs enabling this motif to be recognised by mammals as a pathogen associated molecular pattern (PAMP). Unmethylated CpG activates innate immune cells through binding to toll-like receptor (TLR)-9 (Hemmi et al., 2000; Klinman et al., 1997). TLR9 was shown to be important for the effectiveness of a DNA vaccine against lymphocytic choriomeningitis virus (LCMV) in a prime but not in a boost context (Rottembourg et al., 2010). TLR9 on dendritic cells (DCs) was required for efficient priming of CD8+ T cells upon plasmid exposure, *in vitro*, or single-dose vaccination, *in vivo*. However, TLR9-deficient mice still respond to DNA, suggesting that CpG motifs are not essential to DNA vaccine action (Babiuk et al., 2004; Tudor et al., 2005). Another study showed that TANK-binding kinase 1 (TBK1), a non-canonical IκB kinase, mediated the adjuvant effect of DNA vaccines in mice (Ishii et al., 2008). It is known that TBK1 can phosphorylate IRF3 and IRF7 to activate the type I interferon genes transcription (Sharma et al., 2003). Furthermore, cytoplasmic DNA can also activate AIM2 (absent in melanoma 2), STING (stimulator of IFN genes) and IRF3-dependent, innate immune pathways (Barber, 2011; Rathinam et al., 2010; Stetson and Medzhitov, 2006) and these may, therefore, also contribute to DNA vaccine action. The current proposed mechanisms of DNA vaccines are summarized in Figure 1.

TRADITIONAL STRATEGIES FOR DNA VACCINE DESIGN

Over the last twenty years different methods have been developed, as listed in Table 1, to increase the efficacy of DNA vaccines including, as discussed below, codon optimization, alternative promoters, plasmid backbone refinements, inclusion of antigen subcellular targeting systems and genetic adjuvants.

CODON OPTIMIZATION

Because codon usage by pathogens is often quite different to mammalian genomes, antigen coding sequences may need optimization for efficient expression in host cells. An early study of a DNA vaccine encoding an H-2Kd-restricted epitope of listeriolysin O (LLO) of L. monocytogenes, showed that in BALB/c mice codon optimization improved CD8 T cell responses against this intracellular bacterium (Uchijima et al., 1998). Codon optimization of DNA encoding bacterial botulinum neurotoxins markedly increased neutralizing antibody titers in mouse models (Trollet et al., 2009). Likewise, codon optimization has been shown to increase immunogenicity and provide better protection against challenge in DNA vaccine against Schistosoma japonicum, influenza virus, HIV, HPV, RSV, and SIV in mouse models (Cheung et al., 2004; Lin et al., 2006; Megati et al., 2008; Ngumbela et al., 2008; Siegismund et al., 2009; Tenbusch et al., 2010; Ternette et al., 2007). Automated algorithms for calculating codon optimization are now available to assist vaccine developers (Harish et al., 2006; Sandhu et al., 2008).

While codon optimization increases antigen expression, this may not, per se, always increase vaccine efficacy. For example, a malaria DNA vaccine study showed more robust CD4+ and CD8+ T cell responses and protection against P. yoelii sporozoite challenge with native, rather than codon-optimized, plasmids in mice (Dobano et al., 2009). Another study in mice using a codon-optimized plasmid encoding the Sm14 antigen of Schistosoma mansoni found that while codon optimization increased Sm14 expression it did not enhance immunity or

protection against S. mansoni challenge (Varaldo et al., 2006). The reason for these discrepancies is not known.

PROMOTER DESIGN

The coding sequences in DNA vaccines are driven by polymerase II type promoters. As endogenous mammalian promoters are generally not strong enough for driving high level antigen expression strong virus-derived promoters like cytomegalovirus (CMV) or SV40 promoters (vectors used include pcDNA3.1, pVAX1, pVIVO2, pCI, pCMV and pSV2). Some early studies showed that CMV immediate early enhancer/promoter activity was consistently the strongest explaining its widespread usage in DNA vaccines (Cheng et al., 1993; Manthorpe et al., 1993). The intron A of the CMV immediate-early gene is often included in the complete CMV promoter, as studies showed that secretion of glycoproteins was significantly higher when cells were transfected with intron A-containing plasmids (Chapman et al., 1991). Studies of HIV-1 Env DNA vaccines showed that the strong promoter plays an important role in increasing antigen expression and thereby vaccine immunogenicity in mice (Wang et al., 2006). However, in some cases strong promoter activity can be a problem for DNA vaccine design. For example, the hepatitis C virus (HCV) core protein is a candidate vaccine antigen but has immuno-suppressive properties. To overcome this problem, a hepatitis C virus (HCV) DNA vaccine utilized an inducible *in vivo* activated Salmonella promoter to drive core protein expression along with a CMV promoter to drive envelope protein 2 (E2) expression. In this way immune responses were generated against both the induced HCV core protein and the E2-protein in BALB/c mice (Cao et al., 2011). However, some studies have shown that the potency of viral promoters does not necessarily correlate with DNA vaccine efficacy. This may be because induction of TNF-α and INF-γ by potent DNA vaccines can paradoxically down-regulate viral promoters and thereby reduce immune responses to the vaccine (Gribaudo et al., 1993; Kerr and Stark, 1992; Vanniasinkam et al., 2006; Xiang and Ertl, 1995). Non-viral promoters such as the MHC class II promoter have also been shown to be effective in DNA vaccines in mice (Vanniasinkam et al., 2006). Thus, although the CMV promoter remains the first choice, in some cases alternative promoters may achieve better immunogenicity for DNA vaccines.

REMOVAL OF BACTERIAL ELEMENTS

Currently used DNA plasmids are composed of bacteria-derived sequences providing replication signals and selection markers necessary for propagation in different *E. coli* strains. This material may pose safety issues and could have a negative impact on mammalian gene expression by these bacterial elements. For example, the expression vector pcDNA3.1, which has been widely used in DNA vaccines, was modified to generate a new generation of pVAX1 vector, in order to reduce redundant sequences and to change the ampicillin selection marker to kanamycin, given the potential for ampicillin to trigger allergy. The reduced size of the pVAX1 vector permitted cloning of larger DNA fragments and yet retained comparable antigen expression level to the antecedent pcDNA3.1 vector. The advantage of pVAX1 over pcDNA3.1 was demonstrated by the fact that pcDNA3-ANXB1 (pcDNA3-b1) but not pVAX-ANXB1 (pVAX-b1) induced autoimmunity in inoculated mice (Zhou et al., 2011).

The sucrose selection system has recently been developed to remove the need for an antibiotic selection marker. Vectors expressing a 150bp RNA-OUT antisense RNA repressed expression of a chromosomally-integrated, constitutively-expressed, counter-selectable marker (sacB), allowing plasmid selection on sucrose (Luke et al., 2009). Using this antibiotic-free system, a SV40 72 base pair enhancer was further incorporated upstream of the CMV promoter to increase the extra-chromosomal transgene expression or the human T-lymphotropic virus type I (HTLV-I) R region downstream of CMV promoter to increase

mRNA translation efficiency. Increased HIV-1 gp120 DNA vaccine-induced neutralizing antibody titers were demonstrated in rabbits using this vector system (Luke et al., 2011b).

Minicircle DNA (mcDNA) technology was developed to completely remove the bacterial backbone by using site-specific recombination based on the ParA resolvase to generate mcDNA (Jechlinger et al., 2004). Another method for mcDNA production used genetically modified *E. coli* to construct a producer strain that stably expresses a set of inducible minicircle-assembly enzymes, PhiC31 integrase and I-SceI homing endonuclease (Kay et al., 2010). mcDNA technology has been successfully used in gene therapy experiments in mouse models (Osborn et al., 2011; Zuo et al., 2011) and in generation of adult human induced pluripotent stem cells (Narsinh et al., 2011), with potential for this technology to similarly be applied to DNA vaccine design.

SCAFFOLD/MATRIX ATTACHMENT REGION (S/MAR) VECTOR

For some vaccine applications it may be important to have long-term tissue antigen expression. This can be achieved using retrovirus systems that have high transduction efficiency and integration rate. Unfortunately, cases of leukemia (Dave et al., 2004) have resulted from the random integration of therapeutic retrovirus vectors. Episomally-maintained vectors utilizing scaffold/matrix attachment region (S/MAR) have been developed as a potential substitute (Conese et al., 2004). Such vectors include Epstein-Barr virus (EBV) oriP or EBNA1 elements to maintain self-replication. After a single administration, such transgenes have been shown to be expressed in mouse tissues for at least 6 months (Argyros et al., 2011). When minicircle DNA technology was also applied to the episomal vector, higher and more sustained *in vitro* and *in vivo* (mouse model) transgene expression was achieved for several months in the absence of selection (Argyros et al., 2011). This suggests that such systems could be applied to vaccine design to prolong antigen expression.

DNA VACCINE TARGETING TECHNOLOGIES

As discussed above, the immune responses induced by DNA vaccines may variously be mediated by antigen presentation by transfected somatic cells, by transfected professional APCs or cross-presentation of antigens by APCs that have ingested apoptotic transfected cells. The ability of non-professional APCs to present antigens on the MHC class II pathway and thereby induce CD4 T helper cells is very limited. Strategies have therefore been developed to target DNA vaccines to professional APCs, such as DCs. Skin, mucosal tissues and lymph nodes contain more DCs than muscle, and thus are useful tissues in which to target DCs. Lymph node targeting vectors were created by designing plasmids encoding human IgG fused to either L-selectin or cytotoxic T-lymphocyte antigen 4 (CTLA4) (Boyle et al., 1998). L-selectin facilitates targeting to lymph nodes by binding to CD34 on endothelial cells, while CTLA4 targets expressed antigen to APCs expressing B7 and both targeting strategies enhanced immune responses. In a recent study, the Hantaan virus (HTNV) nucleocapsid protein was fused with CTLA4 to produce a HTNV DNA vaccine in C57BL/6 mouse model (Liu et al., 2011). In a pig study, increased vaccine immunogenicity was obtained by fusion of ASFV antigen with a single chain antibody variable region against swine HLA-II (Argilaguet et al., 2011). Many more DC targeting methods have been successfully used in mouse models with other targeting molecules, FIRE (F4/80-like receptor) or CIRE (C-type lectin receptor), Cle9A, Flt3, DEC205, or synthetic MHC class II-targeting peptides (Corbett et al., 2005; Daftarian et al., 2011; Kataoka et al., 2011; Lahoud et al., 2011; Njongmeta et al., 2012). These targeting methods differ in regards to their effects. For example, Cle9A and DEC205 but not Cle12A are effective targets for induction of cytotoxic T lymphocyte (CTL) responses with Cle9A shown to enhance antibody production (Lahoud et al., 2011). When DNA vaccines are targeted to Cle9A,

FIRE or CIRE, they appear not to need other “danger” signals or adjuvants to elicit a robust immune response (Corbett et al., 2005; Lahoud et al., 2011).

Some specially designed synthetic materials help target plasmids to professional APCs. For example, pDNA-lipoplexes engrafted with flagellin-derived peptides were able to target plasmids to DCs and other APCs in mouse (Faham et al., 2011).

Subcellular targeting is another strategy for enhancing plasmid-encoded antigen processing and/or presentation. This strategy uses host protein trafficking mechanisms to target expressed proteins to particular cellular compartments or mark them for secretion, thereby facilitating antigen processing and presentation. One of the most widely used signal sequence for subcellular targeting is lysosome associated membrane protein 1 (LAMP1). An early study fused the human papillomavirus type 16E7 DNA with LAMP1 to generate a chimeric DNA vaccine targeting the HPV 16E7 antigen to the endosomal compartment, thereby enhancing the immunogenicity of the DNA vaccine (Ji et al., 1999). The LAMP targeting system has subsequently been shown effective in many different model systems (Anwar et al., 2005; de Arruda et al., 2004; Lu et al., 2003; Marques et al., 2003; Rigato et al., 2010; Yang et al., 2009). The LAMP sequence can also be combined with other signal sequences. For example, the protective antigen (PA63) of Bacillus anthracis was fused with tissue plasminogen activator and LAMP1 to generate the pTPA-PA63-LAMP1construct, which was shown to induce high neutralizing antibody titers against anthrax (Midha and Bhatnagar, 2009). Ubiquitin and the human CD1 tail sequence have also been tested as DNA vaccine targeting sequences (Chen et al., 2011; Niazi et al., 2007; Wang et al., 2011b). Such targeting strategies are not infallible, however, as a hepatitis C virus core protein vaccine was not enhanced by fusion with ubiquitin or LAMP1 (Vidalin et al., 1999). A rabies DNA vaccine similarly showed no benefits of MHC class I and II targeting sequences (Kaur et al., 2009). A recent study compared different targeting sequences including a secretion signal, LAMP1 or endoplasmic reticulum (adenovirus e1a) signal which were fused with the green fluorescent protein (GFP)-tagged model genes invariant surface glycoprotein or trans-sialidase from Trypanosoma brucei. For different expressed genes, the effects of cellular targeting varied (Carvalho et al., 2010). A study on intracellular transport and fate of plasmid DNA in mammalian cells showed that plasmid endocytosis can alter the pH value of the late endosome and thereby interfere with antigen processing (Trombone et al., 2007). Thus, when different antigens are fused with lysosome or other targeting sequences, the physical and chemical properties of such non-host proteins may interfere with normal endosomal function. To address this problem, further understanding of the molecular pathways of plasmid-expressed antigens is needed. Recent progress has shown the importance of autophagy pathways in this process (Munz, 2009). Rapamycin-induced autophagy was shown to enhance the presentation of mycobacterial antigen Ag85B, while inhibition of autophagy by 3-methyladenine, or RNAi against beclin-1, attenuated presentation (Jagannath et al., 2009). Similarly, a short polypeptide from the herpes simplex virus type 2 ICP10 gene that can induce antigen aggregation and autophagosomal degradation, enhanced T cell responses when it was co-expressed with chicken ovalbumin (Fu et al., 2010).

Another subcellular targeting method involves inclusion of leader sequence or other localization signals in DNA to allow direct localization of antigens to membranes or secretion outside the cells. (Forns et al., 2000) found that membrane-bound and secreted forms of HCV E2 DNA vaccine induced more E2-specific antibodies compared to the intracellular form (Ma et al., 2002). As DNA vaccines need to get into the nucleus for transcription, strategies of nuclear targeting including liposomes, small peptides and use of a nuclear localization signal (NLS) have also been used to increase antigen expression (Wang et al., 2011a).

DNA VACCINE ADJUVANTS

Vaccine adjuvants function through a range of mechanisms including innate immune activation, antigen depot formation, chemotaxis, antigen uptake and presentation by professional APC and upregulation of co-stimulatory molecules. Conventional vaccine adjuvants including, alum particles (Khosroshahi et al., 2012) and MF59 emulsions (Ott et al., 2002) when mixed with plasmid have also been shown to modesty improve the immunogenicity of DNA vaccines. CpG oligonucleotides that activate TLR9 have also been used as DNA vaccine adjuvants (Bode et al., 2011). Another place in which adjuvants may be relevant is in the DNA prime/protein boost context where the adjuvant is combined with the protein boost to magnify the immune response induced by DNA priming. For example, Advax™, a polysaccharide nanoparticle adjuvant,was successfully combined with a HIV envelope protein boost to enhance the immunogenicity of an *env*-encoding DNA vaccine (Cristillo et al., 2011; Lobigs et al., 2010; Petrovsky, 2008, 2011; Petrovsky and Cooper, 2011).

Given that DNA vaccines can easily be designed to express cytokines or co-stimulatory molecules, and the nucleic acid sequence *per se* may serve as an agonist for TLR-9 or other cellular DNA sensors, DNA vaccines can be designed to co-express so called ‘genetic adjuvant’ molecules. Such genetic adjuvants include cytokines, chemokines or immune stimulatory molecules that are expressed from plasmid DNA in *cis* or *trans*. Use of genetic adjuvants to enhance DNA vaccine immunogenicity has recently been extensively reviewed and hence won’t be covered in detail here (Liu, 2011; Saade and Petrovsky, 2012; Tovey and Lallemand, 2010).

In addition to these well characterized genetic adjuvant molecules, other strategies for enhancement of DNA vaccines by manipulation at the molecular level could also provide adjuvant effects. For example, the MHC CIITA is a critical regulator of MHC class II expression and co-administration of HPV16 E6 DNA and CIITA DNA resulted in enhanced antigen-specific CD8(+) T cell responses in mouse models (Kim et al., 2008). Retinoic acid-inducible gene I (RIG-I) is an important cellular receptor for dsRNA and RIG-I agonist expressed from the RNA polymerase III promoter enhanced hemagglutinin-specific antibody avidity after intramuscular injection of influenza DNA vaccine in mice (Luke et al., 2011a). A recent study found that upon dsRNA viral infection, the mitochondrial protein MAVS forms prion-like aggregates, which then activate IRF3 (Hou et al., 2011). This suggests that manipulation of MAVS conformation changes during delivery of DNA vaccines or development of new aggregate forming polypeptides may successfully increase DNA vaccine immunogenicity. Other studies have also used a decoy system by fusing antigen to a viral DnaJ-like sequence (J domain) associated with the constitutively expressed host cell stress protein, heat shock protein HSP73. This system supported efficient protein expression including of some unstable and/or toxic antigens (Riedl et al., 2006; Schirmbeck et al., 2002).

DNA VACCINE DELIVERY AND ELECTROPORATION

Another issue in DNA vaccine efficacy is the mode of its delivery. Standard intramuscular injection of naked DNA is very inefficient, with only a tiny fraction of injected DNA being taken up by cells and expressed. An alternative method is to inject DNA vaccines in coated nano- or microparticles, which protect plasmids from degradation and increase phagocytic uptake by professional APCs (Xiang et al., 2010). Plasmid DNA can also be coated on colloidal gold particles and delivered by the “gene gun” method. Although one advantage of “gene gun” is its targeting to Langerhans cells and other professional APCs (Porgador et al., 1998; Stoecklinger et al., 2007), it is limited in dose capacity, which means multiple “shots” at multiple sites are needed for effective immunization. However, the most significant

improvement in delivery of DNA vaccines has been electroporation (EP). EP efficiently transfects somatic cells *in vivo* and its induction of local inflammation also enhances the immune response. Clinical trials have confirmed the efficacy of EP (van Drunen Littel-van den Hurk and Hannaman, 2010). For example, EP of a hepatitis B virus (HBV) DNA, induced potent CTL responses in mice and rabbits (Luxembourg et al., 2006). EP similarly enhanced humoral responses to a DHBV DNA vaccine in ducks (Khawaja et al., 2012). EP was also shown to enhance the immunogenicity in mice of DNA coated microparticles (Barbon et al., 2010). EP therefore remains a promising approach to improve the immunogenicity of DNA vaccines, although the poor tolerability of EP remains a concern in the prophylactic vaccine setting (Sardesai and Weiner, 2011)

USE OF EPIGENETICS IN DNA VACCINE DISIGN

Epigenetics is the study of heritable mechanisms that affect the transcriptional state of a gene, not due to changes in DNA sequence. Epigenetic mechanisms include histone modifications and variants, DNA methylation, chromatin remodeling, RNAi and noncoding RNAs. Epigenetics has been shown to be involved in a wide variety of biological processes including immune system function (Cuddapah et al., 2010; Goldberg et al., 2007). Epigenetic tools may be useful to unravel DNA vaccine mechanisms and to design more potent DNA vaccines. In a study of adenovirus-vector mediated gene delivery, rats were given an intramuscular injection of virus expressing human fibroblast growth factor 4 driven by the CMV promoter, with the ratio of copies of hFGF-4 mRNA per copy of viral DNA decreasing 385-fold between 6 hours and 28 days after the injection, with extensive methylation of the CMV being shown to be responsible for the gene silencing (Brooks et al., 2004). Gene expression using plasmid DNA faces similar silencing effects, suggesting that enhanced antigen expression could be achieved if silencing effects are avoided. Sequences in episomal vectors that originate from bacterial sources play a critical role in transcriptional silencing of transgenes *in vivo* (Riu et al., 2007). Furthermore, episomal vectors undergo chromatinization *in vivo* and both persistence and silencing of transgene expression is associated with specific histone modifications. Removal of the bacterial elements by using minicircle DNA technology enabled higher transgene expression, manifested by active histone marks detected by ChIP assays (Riu et al., 2007). This indicates that when plasmids are located within the nucleus, epigenetic regulation of transcription takes place. A study of cancer vaccines confirmed that modulatory agents, including DNA methyltransferase (DNMT) and histone deacetylase (HDAC) inhibitors, enhanced antigen and MHC class I expression (Karpf, 2006). Another study found that active histone modification by HDAC inhibitor enhanced the effectiveness of IL-13 receptor targeted immunotoxin in murine models of human pancreatic cancer (Fujisawa et al., 2011). A study on parasite infection found that a DNA-protein complex was required for the entry of parasite DNA into cells for recognition by TLR9. The nucleosome (histone-DNA complex) was confirmed as the TLR9-binding immunostimulatory component of Plasmodium falciparum that activated DCs (Gowda et al., 2011). Another study found that histones released during tissue injuries can mediate cell death by activating TLR2 and TLR4 (Xu et al., 2011). Thus, plasmid DNA may be reconstituted with histones bearing active modifications to achieve higher antigen expression and increase DNA vaccine immunogenicity. As CD8+ T cells are critical for control of viral infections, epigenetic mechanisms involved in activation of naïve T cells and maintenance of the memory T cell identity (Pearce and Shen, 2006; Youngblood et al., 2010) may also be utilized for DNA vaccine design (Fernandez-Morera et al., 2010).

RNAi TECHNOLOGIES IN DNA VACCINE DESIGN

RNA interference (RNAi) is a post-transcriptional gene silencing process triggered by double-stranded short hairpin RNA (shRNA) structures. Since its discovery, RNAi has

mainly been used as a research tool for loss of function studies of target genes but also as a therapeutic method for human diseases (Lares et al., 2010). Due to its ease of production and flexibility in delivery, RNAi technology has potential applications in DNA vaccine design. One way to use RNAi for DNA vaccines would be to use it to block genes that suppress vaccine action. For example, immune responses induced by DNA vaccines are attenuated due to the limited duration of antigen expression *in vivo*. Due to death of transfected cells, use of shRNA to knock down caspase 12 (Casp12), a cell death mediator that is upregulated after DNA vaccination resulted in increased plasmid luciferase and HIV-gp120 Env antigen expression and higher CD8 T cell and antibody production (Geiben-Lynn et al., 2011). Similarly, RNAi-mediated depletion of the pro-apoptotic proteins Bak and Bax at the time of immunization of HPV16 E7 vaccine prolonged the life of antigen-expressing DCs and increased antitumor effects against E7-expressing tumors (Kim et al., 2005). Fas-mediated apoptosis limits DNA vaccine-induced immune responses (Greenland et al., 2007), and co-delivery of HPV-16 E7 DNA vaccine with DNA expressing shRNA against Fas ligand significantly enhanced CTL responses against E7 (Huang et al., 2008). RNAi may also be used to block immune-suppressive genes that otherwise inhibit vaccine responses. Depletion of Foxo3, a critical suppressive regulator of T cell proliferation, by RNAi increased the efficacy of a HER-2/neu DNA cancer vaccine (Wang et al., 2011c). Similarly knockdown of the IL10 receptor enhanced the potency of a DC vaccine (Kim et al., 2011). Furthermore, blockade of the programmed cell death-1 (PD-1) ligand B7-H1 (PD-L1) by RNAi augmented DC-mediated T cell responses and antiviral immunity in HBV transgenic mice (Jiang, 2012). Thus, use of RNAi against target genes limiting plasmid expression such as apoptosis genes or mediating immune suppressive effects is a powerful new strategy for DNA vaccine enhancement. However, safety issues of use of RNAi to enhance human DNA vaccines still need to be addressed and hence such technologies are most likely to first be applied to therapeutic cancer vaccines rather than more typical prophylactic vaccines.

SYSTEMS OR “OMICS”APPROACHES TO DNA VACCINE DESIGN

Accelerating advances in next-generation sequencing, microarrays, and high throughput proteomics approaches, provide the opportunity to apply these new techniques to DNA vaccine design. One recent proteomics study screened proteins for interaction with plasmid DNA and found that human serum amyloid P (SAP) inhibited plasmid transfection and enhanced plasmid clearance. SAP may contribute to the low efficacy of DNA vaccines in humans, as in other species suppressive effects of SAP are much weaker (Wang et al., 2011d). Hence SAP could, for example, serve as a new siRNA target for enhancing DNA vaccine efficacy, although the feasibility, effectiveness and safety of such an approach would first need to be tested in suitable animal models.

Systems biology approaches have also been used to analyze the molecular signatures that correlate with a positive immunization response. For example, expression levels of CaMKIV kinase at day 3 were negatively correlated with subsequent influenza antibody titers (Nakaya et al., 2011). This provides a successful example of the applications of systems biology to identify biomarkers that predict vaccine effectiveness (Trautmann and Sekaly, 2011). A study of Leptospira interrogans used bioinformatics, comparative genomic hybridization and transcription analysis to screen for candidate antigens from the pathogen’s genome and found 226 candidate genes out of 4727 open reading frames (ORFs) (Yang et al., 2006). This is the concept of reverse vaccinology (Sette and Rappuoli, 2010). A ribosome display of Cryptosporidium parvum cDNA library enabled identification of a new adhesion protein named Cp20, which when included in a pVAX1-Cp20 DNA vaccine, induced antibody and cellular responses and protection (Xiao et al., 2011). Since DNA vaccines are quick and easy to prepare, they are particularly useful for screening potential antigens identified through reverse vaccinology approaches. Thus the development of new DNA vaccines will in future

be assisted by next generation sequencing, advanced bioinformatics analysis and other cutting-edge “omics” technologies (Kennedy and Poland, 2011; Poland et al., 2011).

PRIME/BOOST DNA VACCINE STRATEGIES

Whilst DNA vaccines by themselves suffer from low immunogenicity, this is not necessarily true when they are combined with other vaccine modalities in prime-boost type approaches. Regimens like DNA prime/protein boost, DNA prime/viral vector boost (e.g. using adenovirus) have shown major success. An early study in mouse models has shown that a DNA prime followed by a single protein boost of the same modified vaccinia virus Ankara (MVA) antigen induced complete protection in challenges, which was correlated with induction of very high levels of CD8+ T cells (Schneider et al., 1998). A mouse study of Leishmania donovani gp63 vaccine comparing different prime/boost combinations, found that the DNA prime/protein boost regimen was better than DNA/DNA or protein/protein regimens for long-term protection in mouse models (Mazumder et al., 2011). Human studies have also shown superior immune responses of mixed modality prime-boost compared to pure DNA vaccine regimens (Lu et al., 2008). Recent studies have used DNA/protein or DNA/Ad-vector regimens for HIV immunization (Churchyard et al., 2011; De Rosa et al., 2011; Jaoko et al., 2010; Koblin et al., 2011; Ledgerwood et al., 2011). The underlying mechanisms behind the effectiveness of heterogeneous prime-boost regimens are not well understood but DNA priming results in much lower antigen expression compared to protein vaccines, and this may preferentially prime T-helper cell responses with the humoral response subsequently being boosted by the high dose protein or viral vector boost.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The immunogenicity of DNA vaccines in humans is limited by low levels of antigen expression, when compared to conventional protein vaccines. DNA vaccines may be able to make up for this limitation by altering plasmid construction to maximize protein expression, targeting of the expressed antigen to professional APC thereby ensuring efficient MHC-I and MHC-II compartment loading, inclusion of a genetic adjuvant, use of electroporation and, where suitable, use of a DNA prime/protein or vector boost approach. As summarized in Figure 2, the development of new technologies provides even greater opportunities to further enhance the efficacy of DNA vaccines. The most likely scenario for the first successful human DNA vaccines is that they will be part of a DNA prime/protein boost vaccine strategy where the initial DNA prime is used to ensure efficient CD8 and CD4 T-cell priming whereas the protein boost is used to maximize antibody production. Notably, more than twenty years from their initial discovery, and after many disappointing human clinical trials of first generation vaccines, DNA vaccines are currently undergoing somewhat of a revival thanks to introduction of more efficient designs and better delivery technologies including electroporation. While many outside the field may still be rightfully skeptical, given the failure to meet early promise, this is not an uncommon phase in new technology introduction and often heralds, final success. This is very reminiscent of the history of monoclonal antibody therapeutics that similarly went through a highly negative phase before all the initial technology problems were solved and they emerged as pharmaceutical blockbusters. DNA vaccine may similarly be just moving past their darkest hour and thereby be soon ready to re-emerge as commercially viable products, most likely initially in the area of therapeutic cancer vaccines.

Literature searching method

The literature referenced in this review was searched by using the PubMed database for literature published prior to 1 March 2012 with keywords (“DNA vaccine” OR “DNA vaccines” OR “DNA vaccination” OR “DNA vaccinations” OR “DNA Immunization” OR

"DNA immunizations" OR "gene vaccination" OR "gene vaccinations" OR "genetic vaccine") alone or along with other related topics (e.g. epigenetics, RNAi. Studies published in the most recent two years were paid more attention, as they represented the most up to date development in the area.

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Highlights

- Overview of strengths and weaknesses of previous DNA vaccine optimization strategies
- Identifies new technologies (S/MAR vector, mcDNA, RNAi) relevant to DNA vaccine design
- “Epigenetics” and “omics” technologies provide exciting new opportunities
- Imminent breakthrough in human DNA vaccines incorporating latest technologies

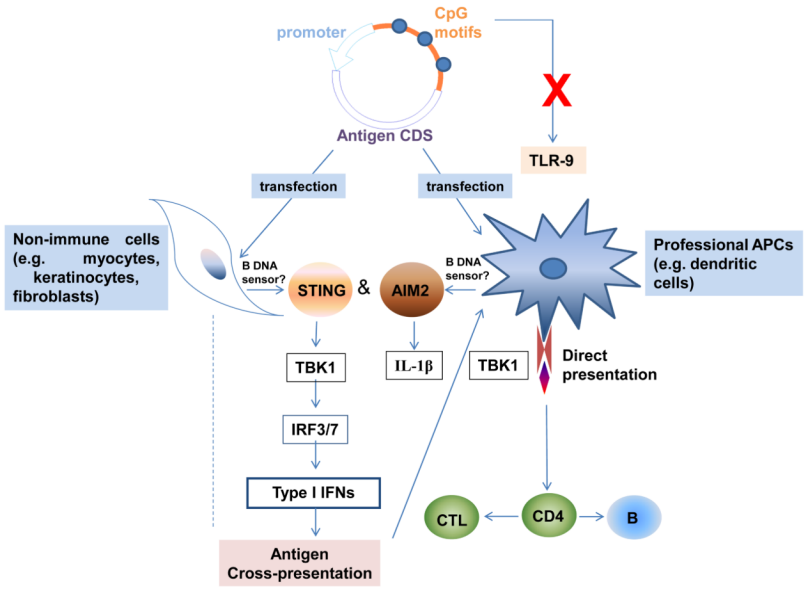

Figure 1. Mechanisms of DNA vaccines

Recent studies found that TLR-9 is dispensable for DNA vaccines, while TBK1 and AIM2 pathways were shown to be critical for plasmid DNA induced innate and adaptive immune responses. However, the upstream bona fide B DNA sensor still remains unknown.

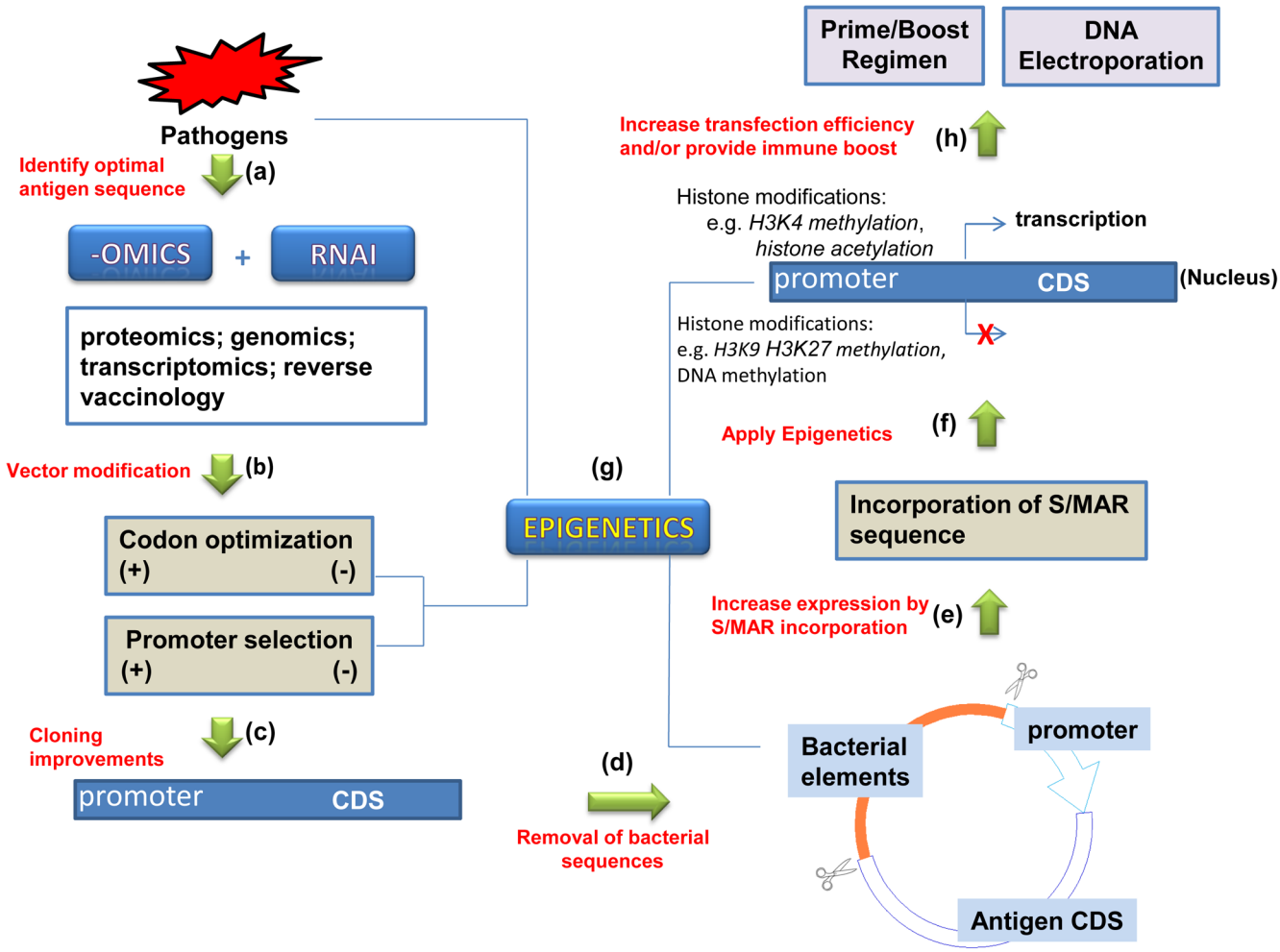

Figure 2. Potential design strategies and technologies for future DNA vaccine development

(a) With the ease of DNA synthesis and manipulation, a lot of high throughput technologies, e.g. gene expression arrays, proteomics, genomics, transcriptomics, reverse vaccinology and RNAi screening platforms, which were accompanied by comprehensive bioinformatics tools, can efficiently map and find the DNA sequences encoding optimal antigens for vaccine development. (b) After selecting the candidate pathogen DNA sequences, codon optimization and promoter design are the main two steps before cloning into expression vectors. Simple codon conversion and using the strong viral promoter will most likely result in higher gene expression, but controversial effects were also reported. The more precise algorithm is anticipated for this purpose in the field of plasmid DNA based therapy or vaccination. (c) The optimized DNA fragments are then cloned into expression vector to test expression. To further optimize the DNA vaccine, minicircle DNA technology (d) will be used to completely remove the bacterial elements and incorporate S/MAR sequence (e) to enhance expression and safety. (f) and (g) Epigenetics mechanisms are closely related with most of the above steps and will be applied in DNA vaccine design. (h) The immunogenicity of the DNA vaccine construction could be further increased by injection of the optimized construction using new delivery devices (e.g. EP) and/or by using prime/boost regimen. See the article for details.

Table 1

Optimization strategies for DNA vaccines

Categories	Approaches	Effects		Safety	Practical considerations
		Pros	Cons		
Codon optimization	Use of host codons to maximize antigen expression	Most studies have shown increased antigenicity and immune responses (Cheung et al., 2004; Lin et al., 2006; Megati et al., 2008; Ngumbela et al., 2008; Siegismund et al., 2009; Tenbusch et al., 2010; Ternette et al., 2007; Trollet et al., 2009; Zhu et al., 2010). (These studies were carried out in mice)	In some cases no enhancement was observed or codon optimized construct was inferior to native sequence (Dobano et al., 2009; Varaldo et al., 2006). (These studies were carried out in mice)	No safety concerns	When antigen expression level is a problem, codon optimization should be considered
Promoter optimization	Use of strong viral promoters (CMV, SV40).	High expression and antigenicity in most cases (Chapman et al., 1991, monkey cells; Cheng et al., 1993, mouse, rabbit and rhesus monkey; Manthorpe et al., 1993, mouse; Wang et al., 2006, mouse).	Some promoters may have suppressive effects on antigenicity or may be inhibited by endogenous cytokines (Cao et al., 2011, mouse; Gribaudo et al., 1993, mouse fibroblast cells; Kerr and Stark, 1992; Vanniasinkam et al., 2006, mouse; Xiang and Ertl, 1995, mouse).	No safety concerns	Strong viral promoters are the first choice to achieve high antigen expression
	Inducible or endogenous promoters.	Showed greater efficacy for specific DNA vaccines (Cao et al., 2011, mouse; Vanniasinkam et al., 2006, mouse).	Not broadly effective.		
Reduction of bacterial elements	Delete redundant bacterial sequences	e.g. pcDNA3.1 is upgraded to pVAX1	In some cases, may be time consuming.	Increased safety	More collaboration is needed to standardize new technologies and achieve consistent evaluation.
	Sucrose induction system	Replace the bacterial selection marker (Luke et al., 2009, mouse; Luke et al., 2011b, rabbit).			
	Mini-circle technology	Maintain the minimum antigen expressing cassette (Jechlinger et al., 2004; Kay et al., 2010, mcDNA preparation technology; Narsinh et al., 2011, stem cells; Osborn et al., 2011, mouse; Zuo et al., 2011, cultured cancer cells and mouse).			
S/MAR vectors	Generation of episomal vectors	Decrease the integration rate and increase long-term expression (Argyros et al., 2011, cultured cells and mouse; Conese et al., 2004, review).	No commercial standard. Need more evaluation.	Increased safety	This method can be combined with mini-circle technology to achieve maximum safety and efficiency

Categories	Approaches	Effects		Safety	Practical considerations
		Pros	Cons		
Targeting technologies	Targeting to APCs (ligand fusion and other methods)	Enhanced antigen processing and presentation (Anwar et al., 2005; Argilaguet et al., 2011; Boyle et al., 1998; de Arruda et al., 2004; Faham et al., 2011; Ji et al., 1999; Liu et al., 2011; Lu et al., 2003; Marques et al., 2003; Midha and Bhatnagar, 2009; Niazi et al., 2007; Palumbo et al., 2011; Rigato et al., 2010; Wang et al., 2011b; Yang et al., 2009). (These studies were carried out in mice)	Some studies showed no enhanced effects (Carvalho et al., 2010, cultured cells; Kaur et al., 2009, mouse; Vidalin et al., 1999, mouse).	Potential for interference with the endogenous immune system needs to be evaluated	Targeting effects need to be tested individually. More suitable for anti-cancer DNA vaccines
	Subcellular targeting (use endogenous trafficking system)				
Adjuvants	Genetic adjuvants, e.g. cytokines and chemokines	Enhanced immune responses were observed in many studies (Bode et al., 2011, review; Kim et al., 2008, mouse; Kolka et al., 2005, mouse; Liu, 2011, review; Luke et al., 2011a, mouse; Riedl et al., 2006, cultured cells and mouse; Schirmbeck et al., 2002, mouse; Tovey and Lallemand, 2010, review).	Co-expression of inflammatory cytokines or chemokines has the potential for increased side effects	Potential interference with the endogenous immune system needs to be evaluated.	Considering uncertain safety issues may be more suitable for use in therapeutic DNA vaccines, e.g. cancer vaccines
	Modulation of the pattern recognition receptors, e.g. TLRs, RLRs and NLRs				
	Other adjuvants				
Epigenetics	Drugs regulating DNA methylation or histone modifications	Some initial promising potential for DNA vaccines (Brooks et al., 2004, rat; Fujisawa et al., 2011, mouse model; Gowda et al., 2011, mouse; Karpf, 2006, review; Pearce and Shen, 2006, review; Riu et al., 2007, mouse; Xu et al., 2011, mouse; Youngblood et al., 2010, review).	Both mechanistic and applied studies are in early stages	Safety issues not known	Need extensive studies, which will provide basis for designing new DNA vaccine strategies.
	Avoiding epigenetic silencing				
	Epigenetics mechanisms of immune responses				
RNAi technology	Targeting potential suppressive factors.	Enhanced immune responses were observed in some studies (Geiben-Lynn et al., 2011, mouse; Greenland et al., 2007, mouse; Huang et al., 2008, mouse; Kim et al., 2011, mouse; Kim et al., 2005, mouse; Wang et al., 2011c, mouse).	More studies are needed to identify the appropriate factors to be targeted.	No safety issues have been identified to date but still early days	This technology will serve as a powerful tool when appropriate targets are identified.
Systems or “omics” technology	Systems biology	These studies provide valuable guides for DNA vaccine design (Nakaya et al., 2011, human, mouse model; Rappuoli and Aderem, 2011, review; Sette and Rappuoli, 2010, review; Trautmann and Sekaly, 2011, review; Wang et al., 2011d (human and mouse; Xiao et al., 2011, mouse; Yang et al., 2006, in silico and arry study).	Requirement of advanced technology and expensive experiments currently limit use to large companies	No safety issues	These technologies will greatly enhance the rational design of future DNA vaccines.
	Library technology				
	Proteomics				
	Genomics (reverse vaccinology)				
	Other “omics” and combinations				