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Molecular Adjuvants for DNA Vaccines

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Abstract
Poor immunogenicity remains the single biggest obstacle to human DNA vaccines achieving their potential. Strategies to improve DNA vaccine efficacy include codon optimization, transfection reagents, electroporation, vaccine adjuvants or combination with a protein or vector boost. Increased understanding of molecular events driving innate and adaptive immune responses has assisted development of molecular adjuvants for DNA vaccine use. Such adjuvants comprise plasmid-encoded signalling molecules including cytokines, chemokines, immune co-stimulatory molecules, toll-like receptor agonists or inhibitors of immune suppressive pathways. New approaches including gene knockdown, epigenetics and systems biology have also contributed to an increased range of molecular adjuvant options. This review explores current and future trends in vaccine design including the latest molecular adjuvants for enhanced DNA vaccine efficacy.

Introduction
Unlike conventional protein-based vaccines, DNA vaccines are composed of bacterial or synthetic plasmids that encode the vaccine antigen together with a strong eukaryotic promoter to help drive protein expression (Rajcani et al., 2005). DNA vaccines have already been approved for use in fish (infectious haematopoietic necrosis virus), dogs (melanoma), pigs (growth hormone releasing hormone) and horses (West Nile virus) (Kutzler and Weiner, 2008). More than 150 human clinical trials of DNA vaccines have been conducted, ranging in scope from prophylactic to therapeutic vaccines against infection, cancer, allergy, Alzheimer's and other diseases (refer to Web Resources listed at end of this review for more information), but this has yet to translate into approval of a human DNA vaccine application. The single biggest problem for DNA vaccine development has been insufficient immunogenicity of this approach when applied to humans.

Vaccine adjuvants have long been used in conventional protein vaccines to enhance vaccine immunogenicity. Adjuvants comprise a very broad group of heterologous materials that share the common feature that boost vaccine responses through a wide variety of different mechanisms including enhanced chemotaxis, dendritic cell maturation, antigen presentation, T-cell activation, B-cell receptor affinity maturation, and immunoglobulin isotype switching. Adjuvants can be used to reduce the dose of antigen required to elicit an immune response, which they do by antigen depot formation, enhanced phagocytosis, enhanced antigen processing and presentation or enhanced expression of surface expressed, e.g. CD40 or CD86, or secreted, e.g. cytokines and chemokines, co-stimulatory molecules. Very different substances have been shown to work as adjuvants including bacterial products, mineral salts, oil emulsions, microparticles, nucleic acids, saponins and liposomes. Not surprisingly, many of these compounds, in either identical or modified forms, can also be used to enhance the immunogenicity of DNA vaccines, as discussed in more detail below.

Mechanism of action of DNA vaccines
Since the first demonstrations that nucleic acids could be used for immunization (Tang et al., 1992; Ulmer et al., 1993; Wolff et al., 1990), DNA

vaccine approaches have become increasingly sophisticated and refined. Safety issues have been addressed and, for example, the risk of DNA integration into human chromosomes, thereby activating oncogenes or increasing chromosome instability, has been shown to be minimal, lower even than spontaneous genomic mutations (Faurez et al., 2010). Many human clinical trial studies have further confirmed the safety and tolerability of DNA vaccines. The biggest ongoing issue, therefore, is immunogenicity. This largely reflects the fact that plasmid injection induces only pico- to nano-gram amounts of antigen expression *in vivo*, in contrast to the microgram doses of traditional protein vaccines. However, compared to the short half-life of injected protein antigens, plasmids induce long-lasting antigen expression and immune stimulation that may help compensate for the low levels of plasmid protein expression. In regards to antigen presentation induced by DNA vaccines, various pathways are possible including (1) plasmid antigens are expressed by transfected somatic cells, e.g. myocytes, and presented on MHC class I complexes to CD8 T cells; (2) professional antigen presenting cells (APC) are transfected by the plasmids and expressed antigens presented on MHC class II complexes to CD4 helper T cells; and (3) apoptotic plasmid-transfected somatic cells are phagocytosed by APC and then the antigens presented on MHC class I and II to both CD8 and CD4 T cells. Because muscle cells are not very efficient in antigen presentation, direct or indirect presentation of DNA-encoded antigens by professional APC is likely to be most important for DNA vaccines administered by intramuscular injection. By contrast, newer approaches such as transdermal skin delivery or intrapulmonary immunization take advantage of the high abundance of APC in both the skin and lung, thereby allowing for higher efficiencies of direct transfection and antigen expression by APC rather than somatic cells.

Traditional adjuvants for DNA vaccines

Adjuvants have been in use for almost a hundred years to increase the immunogenicity of traditional vaccines. These adjuvants function through various mechanisms including activation of the innate immune system, formation of antigen depots, induction of chemotaxis, enhanced antigen uptake and presentation by professional APC and upregulation of co-stimulatory surface molecules on immune cells. Alum is the most widely used vaccine adjuvant with its action being mediated by cell death and subsequent release of host cell DNA that provides an endogenous innate immune signal (Marichal et al., 2011). Addition of alum to DNA vaccines has been shown to increase antibody responses in mice, guinea pigs and nonhuman primates (Ulmer et al., 1999). For example, a DNA vaccine against *Toxoplasma gondii,* when formulated with alum, provided increased survival (Khosroshahi et al., 2012). However, alum activates the inflammasome and favours a Th2-type immune responses (Awate et al., 2013) and hence may not be suited to DNA vaccines where a cellular immune response is desired. Polysaccharides are polymeric carbohydrate molecules expressed by plants and microorganisms, e.g., fungi and bacteria. Delta-inulin polysaccharide adjuvant (Advax™, Vaxine Pty Ltd, Adelaide, Australia) has shown promise as an adjuvant in traditional protein vaccines (Bielefeldt-Ohmann et al., 2014; Gordon et al., 2014; Honda-Okubo et al., 2014) (Petrovsky, 2011) and in a DNA prime-protein boost HIV vaccine study, significantly increased humoral and cellular immune responses when given with an intramuscular or intranasal gp120 protein boost following a DNA *env* prime (Cristillo et al., 2011). Zymosan has similarly been successfully used as an DNA vaccine adjuvant (Ara et al., 2001). Another traditional adjuvant class is oil emulsions, e.g. MF59. Oil emulsion adjuvants are thought to act via activation of local inflammation together with creation of a tissue antigen depot. Injection of MF59 emulsion activates monocytes, neutrophils and eosinophils and when mixed with plasmids, modestly improved the immunogenicity of a HIV-1 DNA vaccine (O'Hagan et al., 2012). Hence, traditional adjuvants may be beneficial to enhance the efficacy of otherwise poorly immunogenic DNA vaccines.

Liposomal and nanoparticle adjuvants

Liposomes are spherical vesicles composed of a lipid bilayer made up of phospholipids and cholesterol that can be used to deliver traditional or plasmid-encoded antigens. Liposomes entrap or bind plasmid DNA and facilitate DNA entry into cells by penetrating the lipid bilayer of the cell membrane (Karkada et al., 2010). Liposomes also help protect DNA from degradation by serum and cytosolic enzymes (Nakanishi and Noguchi, 2001). Plasmid

formulation into liposomes has been shown to enhance cellular and humoral immunity (Schwendener et al., 2010; Wang et al., 2007). This can be further enhanced by targeting liposomes directly to APCs using scavenger or other receptors (Foged et al., 2004; van Broekhoven et al., 2004). Liposomes have the drawback that they increase the reactogenicity of intramuscular DNA injections, but are particularly promising for mucosal immunisation. In a recent study, mice immunized orally with cationic liposome-encapsulated influenza vaccine showed enhanced humoral and cellular immunity and influenza protection (Liu et al., 2014b). Liposomes are similarly effective with intranasal DNA vaccines (Xu et al., 2014).

Nanoparticles made of biodegradable and biocompatible synthetic polymers such as polyvinylpyridine, polylactide-co-glycolides (PLG) and polylactide-co-glycolide acid (PLGA) have been extensively used for vaccine delivery and as adjuvants. Like liposomes, nanoparticles protect plasmids from degradation and increase cellular uptake (Xiang et al., 2010). For example, in a rabbit study, a *Treponema pallidum* DNA vaccine formulated with chitosan nanoparticles showed enhanced immune responses and protective efficacy (Zhao et al., 2011). Similarly, a peptide-based gene delivery system called MPG that forms stable non-covalent nanoparticles with DNA was shown to enhance Th1 cellular immune responses in mouse tumour model (Saleh et al., 2015). Furthermore, a multifunctional envelope-type nanoparticle modified with KALA, a peptide that forms an α-helical structure at physiological pH, induced robust cytotoxic T lymphocyte activity (Miura et al., 2015). Hence, liposomes and other polymer nanoparticles show considerable promise as DNA vaccine adjuvants.

Molecular adjuvants for DNA vaccines

Molecular adjuvants differ from traditional adjuvants or liposomes and nanoparticles in that they are plasmid-encoded proteins that act as adjuvants by targeting innate immune receptors or regulating molecular signalling events. Molecular adjuvants include pathogen-recognition receptor (PRR) agonists, cytokines, chemokines and immune-targeting genes. Compared to traditional adjuvants, more is known about the mechanism of action of these molecular adjuvants, thanks to extensive studies on PRR activation pathways. Molecular adjuvants take advantage of recombinant DNA technology and are compatible with all nucleic acid-based vaccines.

PRR agonist-based molecular adjuvants

Toll-like receptor (TLR) ligands

TLRs play a key role in innate immune system activation. They are usually expressed by sentinel immune cells including macrophages and dendritic cells and recognize structurally conserved molecules derived from microbes. Once microbes breach physical barriers such as the skin or intestinal tract mucosa they are recognized by TLRs, resulting in innate immune activation as a first line of defence. To date, 13 related TLR genes (TLR1–TLR13) have been identified (Oldenburg et al., 2012). Among them, TLR3 and TLR9 can recognize dsRNA and ssDNA, respectively, thus these ligands can be used as molecular adjuvants. A mucosal vaccine formulated with the TLR3 agonist, poly(I:C), enhanced protection against influenza infection (Ichinohe et al., 2005). CD8+ T cells responses were also improved when a DNA vaccine was adjuvanted with poly(I:C) (Grossmann et al., 2009). Poly(I:C) also enhanced CTL immunity and tumour destruction by a DNA cancer vaccine in mice (Hansen et al., 2012), enhanced immune responses to a HPV-16 E7 DNA vaccine (Sajadian et al., 2014) and when combined with the TLR9 agonist, CpG oligonucleotide, enhanced the immunogenicity of a DNA vaccine against eastern equine encephalitis virus (Ma et al., 2014). CpG oligonucleotides have similarly been used to increase the immunogenicity of a broad range of DNA vaccines (Jiang et al., 2014; Lu et al., 2013b; Ma et al., 2014; Yu et al., 2014).

Other PRR ligands

The discovery of TLRs and their roles in innate immune signalling has led to their being exploited as vaccine adjuvants (Dempsey and Bowie, 2015). RIG-I and MDA5 are receptors for viral RNA, replication intermediates and/or transcription products. Therefore, RIG-I agonists have been tested as potential molecular adjuvants. A RIG-I agonist, eRNA41H, was shown to enhance humoral immunity induced by a DNA vaccine against influenza (Luke et al., 2011a). Similarly, a Sendai virus-derived 546 nucleotide-long RNA agonist of RIG-I enhanced influenza vaccine immunogenicity (Martinez-Gil et al., 2013). Another cytosolic dsDNA sensor, DAI, was shown to be an efficient molecular adjuvant for a DNA cancer vaccine, boosting

CTL antitumor immunity (Lladser et al., 2011). TLRs, RIG-I-like receptors (RLRs), inflammasomes and STING-dependent cytosolic DNA sensors in T cells can all initiate Th2 T-cell differentiation (Imanishi et al., 2014), indicating the possibility of using the corresponding ligands to boost DNA vaccine antibody responses. Hence inclusion of innate immune receptor ligands in vaccine plasmids offers a promising direction for enhancing DNA vaccine activity.

Plasmid-encoded genetic adjuvants

Genetic adjuvants based on cytokines

Cytokines are small regulatory proteins secreted by leukocytes and other cell types, which mediate immune signalling. Type I interferons produced in response to TLR signalling activate innate immunity, while other cytokines produced by antigen-specific T cells enhance adaptive immune responses. Given their fast and efficient action, plasmid-encoded cytokines provide the opportunity to boost DNA vaccine immunogenicity. Cytokine-encoding plasmids can be designed and prepared along with antigen-expressing plasmid, which has the advantage of simplicity and low cost. Cytokines are not typically stored as pre-synthesized proteins, and their mRNAs turnover time is very short, providing tight control over their actions. Furthermore, the local expression of cytokines at the DNA vaccine injection site may help avoid the side effects of systematically administered cytokines, such as the fever, myalgia and acute phase responses resulting from actions of circulating cytokines on organs such as the brain and liver.

Interleukin (IL)-2 is produced by T cells and in addition to being a major autologous growth factor for T cells, IL-2 promotes B-cell proliferation and activates NK cells and monocytes. Plasmids expressing IL-2 have been shown to enhance immune responses against viral antigens including hepatitis C core antigen (Geissler et al., 1997), glycoprotein E2 of bovine diarrheal virus (Nobiron et al., 2000), and the S glycoprotein and nucleocapsid of SARS-coronavirus (Hu et al., 2009; Nobiron et al., 2000). IL-2 expression vectors also increased the immunogenicity of two different HIV DNA vaccines expressing Nef or gp120 (Kim et al., 1998; Kim et al., 1999; Moore et al., 2002). Bicistronic plasmids sequentially expressing IL-2, and influenza hemagglutinin and neuraminidase genes provided better protection against influenza than immunization with DNA encoding either IL-2 or GM-CSF in trans (Henke et al., 2006). Furthermore, the adjuvant effects were much greater for a DNA HIV vaccine when IL-2 was expressed as a fusion protein with a IgFc fragment (Barouch et al., 1998) (Barouch et al., 2004). Fusion of IL-2 to a *Mycoplasma pneumoniae* p1 gene region enhanced DNA vaccine effectiveness (Zhu et al., 2013). IL-2 plasmids similarly increased immunogenicity and protection of DNA vaccines encoding Tp92 DNA vaccine for *Treponema pallidum* (Zhao et al., 2011), an alphavirus replicon-based DNA vaccine pSFV1CS-E2 against classical swine fever in a pig model (Tian et al., 2012), a Haemonchus contortus H11 DNA vaccine in a goat model (Zhao et al., 2012), and a therapeutic vaccine against chronic myeloid leukaemia expressing BCR/ABL-pIRES-hIL-2 (Qin et al., 2013).

IL-12 is a pro-inflammatory cytokine secreted by DCs and monocytes that induces Th1 responses by stimulating IFN-γ production (O'Hagan et al., 2001). IL-12's ability to enhance cellular immunity makes it a good candidate as a genetic adjuvant. Plasmid encoding IL-12 enhanced the ability of a DNA vaccine encoding influenza hemagglutinin to induce a Th1 response (Bhaumik et al., 2009). Bicistronic plasmids expressing IL-12 with *Yersinia pestis* epitopes increased mucosal IgA and serum IgG and protected mice against challenge (Yamanaka et al., 2008). IL-12 expression plasmids were also used in an early human clinical trial of a hepatitis B DNA vaccine (Yang et al., 2006). IL-12 plasmid enhanced immunogenicity of hepatitis C virus DNA vaccine, increasing both IL-4 and IFN-γ production (Naderi et al., 2013). A *Toxoplasma gondii* DNA vaccine with IL-12 plasmid enhanced survival (Zhao et al., 2013). IL-12 plasmid also successfully enhanced a HIV DNA prime/protein boost vaccine (Li et al., 2013a). A mixture of three expression plasmids encoding HIV-1 Clade B Env, Gag, and Pol, adjuvanted by a plasmid expressing human IL-12 p35 and p40 when administered with electroporation had a significant dose-sparing effect with 88.9% of immunized individuals developing either a CD4+ or CD8+ T-cell response after the third vaccination (Kalams et al., 2013).

Granulocyte-macrophage colony stimulatory factor (GM-CSF) is a major growth factor

cytokine produced by CD4 T cells and other immune cells that stimulates DC maturation and recruits APCs to the vaccination site thereby promoting antigen presentation. GM-CSF has been extensively studied for its adjuvant potential. A gB-encoding DNA vaccine against pseudorabies virus when adjuvanted by GM-CSF expression plasmids created a Th1-type bias and provided enhanced protection against virus challenge (Yoon et al., 2006). Increased humoral and cellular responses against SIV were observed in Rhesus macaques co-immunized with plasmids for SIV and GM-CSF (Lena et al., 2002; O'Neill et al., 2002). In another Rhesus macaque study, co-immunization with plasmids for influenza hemagglutinin and GM-CSF enhanced both systematic and mucosal immunogenicity (Loudon et al., 2010). DNA vaccine encoding dengue serotype 2 (DENV-2) premembrane and envelope proteins and non-structural 1 protein with GM-CSF enhanced protection in mice (Lu et al., 2013a). Co-immunization with plasmids for GM-CSF and HER2 induced protective immunity against HER2-positive tumours (Lindencrona et al., 2004). Similarly, breast cancer patients co-immunized with HER2, GM-CSF and IL-2 plasmids exhibited long-term immune responses against HER2 (Norell et al., 2010) and the combination of gp100, tyrosinase and GM-CSF plasmids induced memory CD8+ T cells in 42% of stage III/IV melanoma patients (Perales et al., 2008).

IL-15 induces proliferation of NK and T cells (Bergamaschi et al., 2014). IL-15 expressing plasmids were shown to enhance immunogenicity of DNA vaccines against HIV-1 Gag and gp120 (Li et al., 2008), Trypanosoma cruzi (Eickhoff et al., 2011), Eimeria acevulina (Ma et al., 2011), hepatitis B (Kwissa et al., 2003) (Zhang et al., 2006), influenza (Kutzler et al., 2005) and foot and mouth disease virus (Wang et al., 2008). Studies using IL-15 of other species, for example chicken, also achieved increased immune responses (Lim et al., 2012; Ma et al., 2012). Recent studies showed a synergistic effect of murine IL-21 and IL-15 in enhancing efficacy of a DNA vaccine against *Toxoplasma gondii* (Chen et al., 2014b; Li et al., 2014). Furthermore, sequential administration of IL-6, IL-7 and IL-15 plasmids enhanced cellular immune responses and CD4 T memory cells to DNA vaccination with a foot and mouth disease VP1 capsid protein (Su et al., 2012).

Plasmid-encoded cytokines provide long-term protein expression thereby avoiding the problem of very short half-lives of many cytokines. In addition, the low level of plasmid-expressed cytokines helps avoid potential cytokine toxicity. Cytokines exhibit redundancy and synergism and several cytokines may synergistically modulate immune responses. It is easy to incorporate multiple cytokines into a DNA vaccine and combinations of multiple cytokines may provide stronger adjuvant effects. In particular, the combination of IL-15 and IL-21 showed significant synergy (Chen et al., 2014b; Li et al., 2014). Based on current knowledge, there is no one cytokine that stands out as the top DNA vaccine adjuvant, as this is likely to be vaccine dependent. For instance, a study of GM-CSF adjuvant found enhancement of immune responses to hepatitis virus C vaccine, but significant suppression of a dengue virus DNA vaccine (Chen et al., 2014a). Therefore, selection of candidate cytokine adjuvants for each antigen should be based on careful evaluation.

Genetic chemokine adjuvants

Chemokines are a structurally-related family of polypeptides that bind to G-protein coupled surface receptors and regulate leukocyte trafficking. Transfection of chemokine expression vectors along with DNA vaccines helps recruit APCs to the injection site where they uptake expressed antigen and enhance T-cell activation. Macrophage inflammatory protein (MIP)-1 alpha, MIP-3 alpha, and MIP-3beta plasmids when co-formulated with HIV Gag DNA vaccine all increased inflammatory cells infiltration, activated DCs and induced a Th1 response (Song et al., 2007).

RANTES is an inflammatory chemokine that promotes the accumulation and activation of CD4 and CD8 T cells, and DCs (Kim et al., 2003; Ma et al., 2007). Co-immunization of hepatitis B vaccine with RANTES plasmid enhanced Th1 responses (Kim et al., 2003), with the Th1 effect only seen when RANTES was expressed as a fusion protein (Williman et al., 2008).

IP-10, another Th1-polarizing chemokine, has been used as an adjuvant for vaccines against cancer (Kang et al., 2011; Lu et al., 2008) and autoimmune disease (Salomon et al., 2002; Wildbaum et al., 2002). HPV E7 cancer vaccine, when fused with IP-10 and administered by

intradermal vaccination, induced a higher survival rate (Kang et al., 2011).

CCR7 is the receptor for CCL19 and CCL21 and is able to facilitate interactions between mature DC and T cells. CCR7 ligand encoding plasmids were shown to improve DNA vaccine protection (Han et al., 2009). Plasmids encoding CCL19 (Westermann et al., 2007) and CCL21 (Yamano et al., 2006) also increased immune responses including in a prime–boost study targeting herpes simplex virus gB protein (Toka et al., 2003). CCR7 expression plasmids also increased the generation of antigen-specific CD4+ T cells in response to DNA vaccination (Han et al., 2009).

Overall, different chemokines can be easily engineered into DNA vaccine constructs, or administered as separate plasmids and thereby used to help shape the desired direction of the immune response. Compared to cytokines, chemokines are more stable and have less potential for inflammatory toxicity and may therefore serve as better DNA vaccine adjuvant candidates.

Genetic adjuvants based on co-stimulatory molecules

Co-stimulatory molecules on APCs stimulate T-cell activation and thereby have the potential to be used as genetic adjuvants. CD28 is a ligand for CD80 and CD86 and induces T-cell proliferation and IL-2 secretion. CD80 and CD86, which belong to the TNF ligand/receptor superfamily, have been studied as DNA vaccine adjuvants. Co-aadministration of CD86 plasmid with DNA vaccines enhanced both CD4 T-cell and CTL responses, while CD80 was less effective (Flo et al., 2000; Kim et al., 1997; Kim et al., 1998). Studies using bone marrow chimeras showed that CD86 co-transfection allowed non-immune cell types, e.g. muscle cells, to behave as APC (Agadjanyan et al., 1999). CD80 co-transfection with HSV plasmid enhanced T-cell responses only when injected intradermally indicating that the delivery route is critical for adjuvant effect (Flo et al., 2000). This may be because of the higher density of APC in the dermis, with CD80 transfection of dermal dendritic cells being expected to generate more co-stimulation of T cells than myocyte CD80 expression. A prime–boost vaccine study using CD80 and CD86 co-transfection enhanced CD4 T-cell activation and suppressed Visna/Maevi virus infection (de Andres et al., 2009). CD86 co-expression also enhanced a therapeutic vaccine against rheumatoid arthritis (Xue et al., 2011). The extracellular domain of CTLA-4 when used to target antigen B7 on APCs increased antibody responses (Boyle et al., 1998; Deliyannis et al., 2000). CTLA-4 expression plasmids also increased immunity of a HER2-based breast cancer vaccine (Sloots et al., 2008). CTLA-4 fusion constructs with Streptococcus mutans antigen similarly promoted antibody responses in rabbit and monkey studies (Jia et al., 2006).

CD40, a TNF superfamily member expressed on B cells and DC, is involved in B and T cell activation, differentiation and proliferation. CD40 interacts with CD40 ligand (CD40L) expressed on the T-cell surface. CD40 is critical for maturation of B cells, driving them to differentiate into plasma cells. Furthermore, CD40-CD40L interactions stimulate DC maturation, enabling them to then prime CD8 memory T cells. Co-transfection of CD40L increased antibody responses and imparted a Th1 bias (Gurunathan et al., 1998; Mendoza et al., 1997). Co-transfection of CD40 plasmid with foot and mouth disease antigens enhanced antibody responses (Xu et al., 2010) and soluble CD40L multimers increased cellular responses to HIV plasmids (Gomez et al., 2009; Stone et al., 2006a), with multimers more efficient than monomers (Stone et al., 2006b). Other studies have also tested RANK/RANKL or 4-1BBL co-stimulatory molecules with DNA cancer vaccine but failed to show an increase in antigen-specific CTL responses or cancer protection (Herd et al., 2007).

Overall, because co-stimulatory molecules play a key role in the interactions between innate and adaptive immune cells they represent highly promising DNA vaccine adjuvants. These molecules are usually membrane bound and this helps ensure that their adjuvant activity is restricted to the site of injection, thereby helping reduce any potential for toxicity due to excess systemic immune activation.

Genetic adjuvants based on immune-signalling molecules

As discussed above, different PRR ligands induce innate immune responses followed by signal transduction through TRIF or MyD88 dependent pathways. This leads to activation of critical transcription factors that then initiate

Table 1. Comparison of signalling molecules as DNA vaccine adjuvants.

Class	Name	Protein function	Adjuvant effect
Pattern Recognition Receptors	MDA5 and RIG-I	Recognise viral RNA genome; replication intermediates and transcription products	Enhanced antigen-specific antibody responses
Adaptor proteins	MyD88	Adaptor protein for most TLRs	Enhanced humoral immune responses
	TRIF	Adaptor protein for TLR3	Enhanced cellular immune responses
Inflammation signalling protein	HMGB1	Inflammatory mediator	Enhanced IFN-γ and antibody response; enhanced CD8+ T cell response
	HSP70	Anti-inflammatory protein	Enhanced CTL activity
Transcription factor	NF-κB	Induce type I interferon genes; important for both B and T cell signalling	Enhanced IFN-γ and antibody responses
	IRF1	Induce type I interferon gene expression; activate innate immunity	Enhanced CTL and IFN-γ response
	IRF3		Enhanced antibody response and CTL activity for model OVA DNA vaccine
	IRF7		
	T-bet	Imparts Th1 polarisation to T cell response	Enhanced IFN-γ and antibody responses
Immune regulator	Programmed cell death protein 1 (PD-1)	Regulates Th cells; binds to ligand on DC	Enhanced CD8+ T cell response by enhancing antigen binding and uptake of DC

transcription of target immune genes, for example, IRF3 and IRF7 that drive the expression of type I interferon genes, NF-κB which induces IL-6, TNF expression, and MAPKs which activate CD80 and CD86 expression (Gilliet et al., 2008). In fact, these downstream signalling molecules can also be used as genetic adjuvants (Table 1). For example, a plasmid encoding TRIF enhanced immunogenicity of a DNA vaccine encoding classical swine fever virus E2 protein (Wan et al., 2010). TRIF plasmids have also been shown to be effective adjuvants for DNA vaccines encoding influenza hemagglutinin or HPV E7 (Takeshita et al., 2006). A dual-promoter construct expressing LacZ followed by TRIF or MyD88 showed that the MyD88–LacZ construct induced higher antibody responses while the TRIF–LacZ construct favoured cellular immune responses (Takeshita et al., 2006). This bias may be because MyD88 promotes DC maturation and increases antigen presentation, while TRIF promotes CTL activity.

HMGB1 is an important chromatin protein that binds and bends DNA that is secreted by immune cells as an inflammatory mediator. Co-transfection of HMGB1 plasmids along with

HIV-1 Gag and Env DNA vaccine increased IFN-γ and antibody responses (Muthumani et al., 2009). Likewise, co-transfection of HMGB1 plasmids along with an influenza antigen increased CD8+ T cell responses and survival of mice against lethal challenge (Fagone et al., 2011). Co-transfection of HSP70 also induced better CTL responses to DNA vaccines (Chu et al., 2014). PD-1 based plasmids have also been shown to enhance CD8+ T cell responses against HIV DNA vaccination (Zhou et al., 2013). Some PRR may be used as genetic adjuvants as well. A recent study showed that MDA5, a RIG-I like dsRNA receptor, can be used as a genetic adjuvant for a DNA vaccine against H5N1 influenza in chickens (Liniger et al., 2012).

Plasmids encoding transcription factors can potentially be used as DNA vaccine adjuvants. An DNA vaccine against ovalbumin when co-expressed with IRF3 and IRF7 significantly enhanced antigen-specific antibody response and CTL activity (Bramson et al., 2003). Conversely, IRF1 but not IRF3 or IRF7 plasmids enhanced CTL responses and IFN-γ responses induced by a HIV-1 DNA vaccine (Castaldello et al., 2010). This suggests that different IRF proteins may have different adjuvant effects. Te transcription factor, NFκB, is another master regulator of innate immune responses. Co-administration of plasmid encoding the NFκB subunit p65/RelA enhanced vaccine immunity (Shedlock et al., 2014). Similarly, a plasmid expressing another key T-cell transcription factor, T-bet, was shown to enhance Th1 immunity against a tuberculosis DNA vaccine encoding Ag85B (Chen et al., 2012; Hu et al., 2012). Hence, use of signalling molecules as genetic adjuvants has promise, but more studies are required to make sure such approaches do not lead to any excess toxicity that may limit their use.

RNA knockdown for molecular adjuvant action

RNA interference (RNAi) is a post-transcriptional gene silencing process triggered by double-stranded short hairpin RNA (shRNA) structures. RNAi is mainly used as a research tool for loss-of-function studies of target genes (Lares et al., 2010). RNAi can be used to down-regulate anti-inflammatory genes that suppress DNA vaccine action. For example, 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 Env protein expression, and higher T cell and antibody responses (Geiben-Lynn et al., 2011). Depletion of Foxo3, a critical inhibitor of T-cell proliferation, by RNAi increased the efficacy of a HER-2/neu DNA cancer vaccine (Wang et al., 2011a). Similarly, knockdown of the IL10 receptor enhanced the potency of a DC vaccine (Kim et al., 2011) and blockade of programmed cell death-1 ligand (PD-L1) by RNAi augmented DC-mediated T cell responses and antiviral immunity in hepatitis B transgenic mice (Jiang, 2012). Furthermore, increased protective immunity against B cell lymphoma was obtained by a DNA vaccine combined with IL10 siRNA plus CpG oligonucleotide (Pradhan et al., 2014). shRNA against furin plus GM-CSF enhanced the efficacy of a cancer DNA vaccine (Nemunaitis et al., 2014). Hence, use of RNAi to know down target genes that inhibit DNA vaccine expression represents a powerful new adjuvant strategy, especially for cancer vaccines, although the safety issues with blocking important anti-inflammatory pathways and thereby triggering autoimmune and other inflammatory diseases could be a major concern.

Targeting technologies as molecular adjuvants

Parenteral DNA vaccines mainly transfect muscle cells and result in poor antigen presentation. Therefore, technologies have been developed to better target DNA gene expression to professional APC, as discussed extensively elsewhere in this book. Targeting antigen expression to lymph nodes enhances immunogenicity of DNA vaccines (Chen et al., 2013; Liu et al., 2014a; Toke et al., 2014) and other targeting methods have used FIRE (F4/80-like receptor) or CIRE (C-type lectin receptor), Cle9A, Flt3, DEC205, xrc1, or MHC class II-targeting peptides or DC specific promoters (Cao et al., 2013; Chen et al., 2013; Corbett et al., 2005; Daftarian et al., 2011; Fossum et al., 2014; Kataoka et al., 2011; Lahoud et al., 2011; Moulin et al., 2012; Njongmeta et al., 2012; Toke et al., 2014). Subcellular targeting is another strategy for enhancing plasmid-encoded antigen processing and/or presentation. The endoplasmic reticulum can be targeted using E1A or lysosomes using LAMP (Freitas et al., 2014; Godinho et al., 2014), and autophagy pathways can also be targeted (Hu et al., 2014; Meerak et al., 2013; Saiga et al., 2014) with, for example, a short polypeptide from the herpes simplex virus ICP10 gene that induced antigen aggregation and autophagosomal degradation enhancing T-

cell responses when co-expressed with chicken ovalbumin (Fu et al., 2010). Similarly a plasmid, pATRex, expressing the aggregation domain of TEM8 induced autophagy and caspase activation and increased IgG1 responses against a malaria DNA vaccine (Capitani et al., 2014). Thus, targeting DNA vaccines to specific cells or subcellular compartments can greatly increase antigen processing and presentation and promote desired immune responses, making this a promising field for molecular adjuvants.

High-throughput methods for identification of new molecular adjuvants

Development of next-generation sequencing, microarrays, and high throughput proteomics approaches, provides the opportunity to apply these new techniques to identification of new molecular adjuvants for DNA vaccines. A 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, contributing to the low efficacy of DNA vaccines in humans (Wang et al., 2011b). Thus, for example, it might be possible to target SAP using siRNA to improve DNA vaccine effectiveness in humans. Systems biology approaches have also been used to analyse 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 titres (Nakaya et al., 2011). This provides a successful example of the application of systems biology to identify biomarkers that predict vaccine effectiveness (Trautmann and Sekaly, 2011), and the identified molecules may in turn warrant testing as potential new molecular adjuvants. Thus the development of new molecular adjuvants will in future be facilitated by next generation sequencing, advanced bioinformatics analysis and other cutting-edge “omics” technologies (Kennedy and Poland, 2011; Li et al., 2013c; Poland et al., 2011) as discussed more extensively in other chapters of this book.

Optimal design of DNA vaccines

The immunogenicity of DNA vaccines has been helped by use of traditional or molecular adjuvants. However, DNA vaccine design is equally important to maximization of DNA vaccine efficacy. For example, intrinsic elements of plasmid DNA can also be used to activate the innate immune system, thereby contributing to the enhancement of adaptive immune responses to DNA-expressed antigens. The innate immune system uses pattern-recognition receptors (PRR) to sense invasion of pathogens and induce downstream signalling pathways including type I interferon and other pro-inflammatory cytokine production. In both mice and humans, toll-like receptor (TLR)-9 is a cytosolic PRR that binds unmethylated CpG DNA leading to activation of MyD88-dependent signalling pathways (Hemmi et al., 2000). In mammalian genomes, CpG dinucleotide motifs have a very low frequency and most of them are methylated, while in bacteria such CpG motifs are common. Hence, built-in CpG motifs in the backbone of plasmid DNA could be used to activate TLR9 after transfection. A study showed that TLR9 was important in plasmid DNA prime but not prime-boost vaccines to activate dendritic cells and enhance vaccine protection (Rottembourg et al., 2010). In addition, TLR9 is important for dendritic cells to prime CD8 T cells although gene knockout studies suggest TLR9 is not essential for DNA vaccines action (Babiuk et al., 2004; Tudor et al., 2005).

Thus a multiplicity of redundant cytosolic DNA sensors likely contribute to DNA vaccine immunogenicity. One such PRR is cyclic-GMP-AMP (cGAMP) synthase (cGAS) that, after recognition of dsDNA induces the production of cGAMP to activate the stimulator of interferon genes (STING) (Gao et al., 2013; Sun et al., 2013; Zhang et al., 2014). Yet another PRR that recognises dsDNA is DAI (DLM-1/ZBP1), which also activates STING and induces type I interferon expression (Takaoka et al., 2007). Indeed, TBK1, the common downstream of cGAS and DAI, was shown to contribute to the adjuvant effect of DNA vaccines (Ishii et al., 2008).

Another cytosolic DNA sensor AIM2 induces pro-inflammatory cytokine production through inflammasome activation and may thereby enhance DNA vaccine immunogenicity (Fernandes-Alnemri et al., 2010; Schroder et al., 2009). The helicase proteins DHX29 and RIG-I both sense cytosolic nucleic acids in the human airway system (Sugimoto et al., 2014) and may contribute to the immunogenicity of mucosally-delivered DNA vaccines. There are many other DNA sensors, for example DDX41, IFI16, DNA-PK and MRE11 (Ferguson et al., 2012; Jakobsen

and Paludan, 2014; Kondo et al., 2013; Parvatiyar et al., 2012; Unterholzner et al., 2010; Zhang et al., 2011), that may similarly act as potential DNA vaccine adjuvants. Studies on these PRR and downstream signalling pathways will not only help researchers understand the mechanisms of action of DNA vaccines but also provide valuable information for design of more immunogenic DNA vaccines.

Codon optimization for enhanced DNA vaccine immunogenicity

Codon usage of pathogens is often very different to that of mammalian species. Thus codon optimization is required when using human DNA vaccines to express pathogen antigens. Codon optimization enhanced CD8 T-cell responses against a *Listeria monocytogenes* DNA vaccine encoding an epitope of listeriolysin O protein (Uchijima et al., 1998). Codon optimization enhanced immunogenicity of a DNA vaccine encoding bacterial botulinum neurotoxins (Trollet et al., 2009), and similarly for other DNA vaccines (Li et al., 2013b; Seo et al., 2013; Spatz et al., 2013; Williams, 2014; Zhu et al., 2010). Various algorithms for codon optimization are available to assist DNA vaccine development (Jacobs et al., 2014; Liu et al., 2014c). Although codon optimization increases protein expression, it does not always correlate positively with DNA vaccine efficacy. For example, a study on a malaria DNA vaccine showed that the native nucleic acid sequence provided more robust CD4+ and CD8+ T cell responses and protection against *Plasmodium yoelii* sporozoite challenge (Dobano et al., 2009). Another study using codon-optimized plasmids expressing Sm14 from *Schistosoma mansoni* showed no increase in immunity or protection against *S. mansoni* challenge in mice (Varaldo et al., 2006). Hence, while it can be helpful, codon optimisation is not guaranteed to enhance DNA vaccine immunogenicity.

Promoter design for enhanced DNA vaccine immunogenicity

DNA vaccine expression is normally driven by a polymerase II type promoter. The endogenous mammalian Pol II promoters are not as strong as promoters derived from virus origin, such as cytomegalovirus (CMV) or SV40 promoters (vectors include pcDNA3.1, pVAX1, pVIVO2, pCI, pCMV and pSV2). The CMV immediate early enhancer/promoter has the strongest activity in most cell types and thus was widely used for DNA vaccine constructs (Cheng et al., 1993; Manthorpe et al., 1993). Studies using HIV-1 Env DNA vaccines have shown that use of a strong promoter resulted in higher expression levels and higher immune responses (Wang et al., 2006). But in some cases, strong promoters may result in inferior immune responses. For example, hepatitis C virus core protein when driven by the strong CMV promoter showed immuno-suppressive effects (Cao et al., 2011). To address this problem, a new construct was designed to express the core protein from an *in vivo* inducible *Salmonella* promoter while keeping expression of envelope protein 2 driven from a CMV promoter, and this resulted in higher immune responses to both HCV core and E2 proteins (Cao et al., 2011). Furthermore, some viral promoters that drive high antigen expression may result in activation of cytokines, for example TNFα or IFN-γ by the viral element, which in return may suppress the viral promoter. To overcome this kind of problem, some non-viral promoters such as the MHC class II promoter have been tested as alternative DNA vaccine promoters (Vanniasinkam et al., 2006). Hence while the CMV promoter remains the most commonly used, other promoters including non-viral medium level promoters may ultimately represent better choices for human DNA vaccines.

Optimization of the DNA backbone

Plasmid vectors used for DNA vaccine usually contain some bacterial elements, such as replication signals and selection markers for propagation in *E. coli.* However, these elements may pose safety issues and reduce expression of DNA vaccines. An example is expression vector pcDNA3.1, which needed to be modified by replacing the Ampicillin selection marker that was reported to cause autoimmunity with a Kanamycin selection marker (Zhou et al., 2011). Removal of redundant vector sequences also makes it possible to clone larger DNA vaccine fragments. Traditional selection markers can also be replaced using the sucrose selection system. Translation efficiency and immunogenicity of a HIV-1 gp120 DNA vaccine was increased using a sucrose selection plasmid combined with a 72 base pair SV40 enhancer at the 5' of CMV promoter to increase the extra-chromosomal transgene expression (Luke et al., 2011b). To completely remove bacterial elements, minicircle DNA (mcDNA) technology uses site-specific recombination based on the ParA resolvase to

generate mcDNA (Jechlinger et al., 2004). Alternatively, this can be done using 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). A recent study showed minicircle DNA is superior to plasmid DNA in eliciting antigen-specific CD8+ T-cell responses (Dietz et al., 2013). A modified mini-intronic plasmid system was used to express a transgene *in vivo* and *in vitro* for use as a more optimal DNA vaccine backbone (Lu et al., 2013c). Minicircle DNA technology can also be combined with electroporation delivery, with enhanced immunogenicity seen with an electroporated HIV-1 gag minicircle DNA vaccine in mice (Wang et al., 2014).

Prime-boost strategies for optimal DNA vaccine efficacy

With the help of the above-described vaccine design strategies, the immunogenicity of DNA vaccines has been greatly enhanced. However, human efficacy remains suboptimal. Prime-boost approaches such as DNA prime/protein boost, DNA prime/viral vector boost (e.g. using adenovirus(Ad)) or even protein prime/DNA boost regimens can dramatically improve immunogenicity without losing the benefits of a DNA vaccine approach. Much effort has gone into DNA prime /Ad-vector boost approaches for HIV vaccination, where these studies have shown good tolerance and safety in human clinical trials. The DNA priming in a prime/boost regimen results in enhanced T-cell and antibody responses when compared to boost vaccine alone, even when the DNA prime does not induce detectable antibody responses (Churchyard et al., 2011; De Rosa et al., 2011; Jaoko et al., 2010; Koblin et al., 2011). A DNA prime/protein boost study showed that influenza H5 DNA priming followed by inactivated H5N1 virus boost enhanced vaccine efficacy (Ledgerwood et al., 2011). A heterologous prime/boost therapeutic hepatitis C virus vaccine strategy similarly showed enhanced immunogenicity and improved survival in a challenge model (Fournillier et al., 2013). A DNA prime/adenovirus boost malaria vaccine induced cell-mediated immunity and complete malaria protection (Chuang et al., 2013). Similarly, peptide prime/DNA boost or live Bacillus Calmette-Guérin (BCG) prime/DNA boost, delivered enhanced immunogenicity (Cervantes-Villagrana et al., 2013; Lambracht-Washington et al., 2013). The DNA prime/alternative boost strategy takes advantage of the efficient DNA priming effect on memory B cells and T cells that may be undetectable until after boost immunization, so normally adjuvant has not been formulated with the DNA prime. Recent studies on prime/boost strategies showed that the interval between prime and boost may be critical to optimal vaccine efficacy (Khurana et al., 2013; Ledgerwood et al., 2013). Hence currently prime-boost strategies are the most promising in terms of optimization of DNA vaccine efficacy, but come at the price of requiring two separate vaccine formulations thereby increasing the cost of vaccine development and manufacture. The underlying mechanisms to explain the increased effectiveness of prime-boost strategies remain poorly understood, but the lower protein expression from DNA immunization may preferentially prime T-helper cell responses, with the humoral response subsequently being stimulated by the high dose protein or viral vector boost in combination with this pre-existing T-cell help.

Concluding remarks and future trends

The immunogenicity of DNA vaccines in humans remains limited by their low level of antigen expression compared to protein vaccines. To overcome this, various physical or molecular adjuvants can be incorporated into DNA vaccine design. Advances in related research areas including genomics and systems biology have increased the number of immune genes that could be used as potential genetic adjuvants. Additional strategies include optimization of DNA construct design to maximize protein expression, targeting of expressed antigens to professional APC thereby ensuring efficient MHC-I and MHC-II compartment loading, use of electroporation or other transfection tools and use of DNA prime/protein or vector boost approaches. The most likely scenario for a successful human DNA vaccine is as part of a DNA prime/protein boost strategy where the DNA prime is used to ensure efficient CD8 and CD4 T-cell priming and the protein boost is used to maximize antibody production. Not to be forgotten, recent years have witnessed very fast development in the area of RNA vaccines with the potential to overcome problems of low antigen expression. Most molecular adjuvants discussed above in the context of DNA vaccines can equally be applied to RNA vaccines. It remains likely that

the first human DNA vaccines will be in the area of therapeutic vaccines against cancer given the large number of trials being conducted in this area, but infectious disease applications such as in the area of HIV also look promising.

Web resources

Refer to the following websites for more information about DNA vaccine clinical trials.

- 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
- http://www.clinicaltrials.gov/ct2/show/NCT00005916

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