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Invited Review

DNA Vaccines against Infectious Diseases and Cancer

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Abstract – Progress in the development of DNA vaccines and their delivery strategies has been made since their initial concept as a next generation vaccine. Since DNA vaccine includes non-infectious DNA parts of pathogens, it can't cause disease yet it closely mimic the natural process of infection and immune responses. Despite their early promising results of controlling infectious diseases and cancer in small animal models, DNA vaccines failed to display a level of immunogenicity required for combating these diseases in humans, possibly due to their lower protein expression levels. However, increasing evidence has shown that DNA vaccines are clinically well-tolerated and safe. Furthermore, one notable advantage of DNA vaccines includes convenient utilities of plasmid DNAs coding for antigens. For instance, any emerging pathogens could be prevented easily and timely by allowing the simple exchange of antigen-encoding genes. In this review, newly developed DNA vaccine strategies, including electroporation, which has emerged as a potent method for DNA delivery, targeting infectious diseases and cancer will be discussed with a focus on any on-going DNA vaccine trials or progress made pre-clinically and in clinics.

Keywords: Cancer, DNA vaccines, Infectious diseases

INTRODUCTION

DNA immunization may afford several potential advantages both for basic research as well as for clinical applications over conventional vaccination strategies. DNA vaccines are non-replicating and the vaccine components are produced within the host cells. They can be constructed to function and mimic the safety and specificity of a subunit vaccine. Due to the production of immunogenic proteins within host cells, DNA vaccine cassettes should produce immunological responses that are more similar to live vaccine preparations. By directly introducing DNA into the host cell, the host cell is essentially directed to produce the antigenic protein, mimicking pathogen's replication and protein production in the host. This results in host customized antigen (Ag) production mimicking antigen structure of the native pathogen. This process has been reported to generate both antibody (Ab) and cell mediated, particularly cytotoxic T lymphocyte (CTL)-mediated, immunity (Cohen *et al.,* 1998). DNA vaccines are also likely to be cost-effective in their application and are easily constructed to express multivalent antigenic proteins through molecular gene manipulation. The flexibility and power of molecular biology is an advantage for developing vaccines directed at multiple target antigens. This technique has the potential to augment Ag-specific immune responses through the modification of the biological properties of antigens, and coinjection of immunologically important molecules including cytokines and chemokines and of others targeting co-stimulatory or co-inhibitory signaling. Moreover, electroporation (EP) as a DNA delivery method has the high potential to augment Ag-specific immune responses in humans (Luxembourg *et al.,* 2007; Bodles-Brakhop *et al.,* 2009). Below we review important features of this vaccine technology and its applications to control a number of infectious diseases and cancer.

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Mechanism of DNA vaccines

The nature of immune responses is in general influenced by delivery routes of plasmid DNA. For delivery of DNA vaccines, a variety of methods including intradermal,

intramuscular (*i.m.*), mucosal, biojector injections and EP have been reported (Fynan *et al.,* 1993; Pertmer *et al.,* 1995; Kuklin *et al.,* 1997; Ray *et al.,* 1997; Chen *et al.,* 1998; Luckay *et al.,* 2007; Rosati *et al.,* 2009). To date, the majority of DNA vaccine studies have utilized skin or muscle as an immunization target. In *i.m.* injection, DNA is taken up into myofibers and antigen presenting cells (APCs) with subsequent endogenous expression, leading to presentation of a more natural form of antigen to the immune system in the context with Class I molecules (Levy *et al.,* 1996; McDonnell and Askari, 1996; Chattergoon *et al.,* 1997). Secreted antigens are ingested by phagocytosis and then presented as a peptide-major histocompatibility complex (MHC) II complex on APCs which can provide the primary activation signal, co-stimulatory ligands and cytokines necessary for stimulation of naive T cells (Wells, 1993). For antigen-MHC Class I presentation to prime naïve CD8+ T cells to become effector cells, only bone marrow-derived APCs, not myocytes are able to present antigen to T cells (Corr *et al.,* 1996; Casares *et al.,* 1997; Iwasaki *et al.,* 1997). This suggests that normally bone marrow-derived cells are responsible for antigen presentation to prime the immune response while muscle can serve as an antigen depo to expand activated effector cells. When DNA was delivered by gene gun to skin, the gene expression was observed in Langerhans cells and/or dermal dendritic cells (Condon *et al.,* 1996). However, DNA vaccines have been considered less immunogenic in humans than in animals (MacGregor *et al.,* 1998; Wang *et al.,* 1998; Barouch, 2006). It has been suggested that the main reason for this might be resulting from a lack of APCs at the DNA injection site in humans as there are very few professional APCs in muscles after DNA immunization (Barouch *et al.,* 2002). However, EP, the administration of electrical pulses to muscle or dermal tissue following DNA injection, has been shown to enhance the immunogenicity of DNA vaccines in a wide variety of small and large animal models (Buchan *et al.,* 2005; Folgori *et al.,* 2006; Ahlén *et al.,* 2007; Hooper *et al.,* 2007; Luckay *et al.,* 2007; Hirao *et al.,* 2008b; Rosati *et al.,* 2009). EP functions partially by increasing myocyte permeability and thus facilitating plasmid uptake and antigen expression by host cells (Aihara and Miyazaki, 1998; Rizzuto *et al.,* 1999; Widera *et al.,* 2000; Gronevik *et al.,* 2005). EP also recruited dendritic cells (DCs) and macrophages to the site of inoculation (McKay *et al.,* 2004; Sumida *et al.,* 2004; Liu *et al.,* 2008). For example, EP recruited large mixed cellular inflammatory infiltrates to the site of inoculation and these infiltrates contained 45-fold-increased numbers of macrophages and 77-fold-increased numbers of DCs as well as 2- to 6-fold-increased numbers of B and T cells, as compared to infiltrates following DNA vaccination alone (Liu *et al.,* 2008). It is thought that danger signals caused by EP's needle probes and the electric pulses might be possibly responsible for this immune cell infiltration. These localized effects likely help to insure that the strength and duration of the responses are maintained when the vaccine is tested in larger animals, including rabbits and humans (Ahlén *et al.,* 2007). Thus, it seems likely that EP can induce a more potent immune response through both more uptake of DNA to host cells, leading to more antigen expression, and more recruitment of APCs to the injection site (Fig. 1). Similarly, skin EP has also been tested to have importance as an immunization approach in larger animal models including pigs and rhesus macaques (Hirao *et al.,* 2008b). In an influenza DNA vaccine study, more Ab production was induced by EP following intradermal DNA injection (ID-EP) than by EP following DNA injection at the muscle (IM-EP) (Laddy *et al.,* 2009). This data is somewhat consistent with previous other studies without EP that intradermal injection of DNA vaccine drives immune response to both Th1 type (Feltquate *et al.,* 1997; Leitner *et al.,* 1997) and Th2 type (Okada *et al.,* 1997; Prayaga *et al.,* 1997) responses depending on the specifics. However, *i.m.* delivery of DNA vaccines drives immune responses mainly towards the Th1 type responses (Raz *et al.,* 1996; Kuklin *et al.,* 1997). The mechanisms of selective Th1 and Th2 type immune induction by these different DNA delivery methods are presently not known, but suggest that selection of EP sites is an additional factor for obtaining immunity required for controlling infectious diseases and cancer.

Immune characteristics

A variety of studies have demonstrated that DNA vaccines preferentially induce Th1 type immune responses (Raz *et al.,* 1996; Sin *et al.,* 1999c). In particular, such vaccines can induce Ag-specific interferon (IFN)-γ production and isotype switch to an increased IgG2a subtype. Th1 type immune responses have been reported to be correlated with protective immunity in certain viral challenge models (Chow *et al.,* 1998; Sin *et al.,* 1999a, b), as well as parasitic challenge models (Scott *et al.,* 1988; Heinzel *et al.,* 1989). Raz *et al.* (1996) reported that plasmid DNA expressing β-galactosidase elicits Ag-specific Th1 type immune responses while the protein immunization induces Th2 type responses. The mechanisms of differentiating Th1 and Th2 types are presently not known, but it was suggested that CpG motif of bacterial plasmid might be responsible for driving immune responses towards Th1 type responses (Chu *et al.,* 1997; Klinman *et al.,* 1997; Leclerc

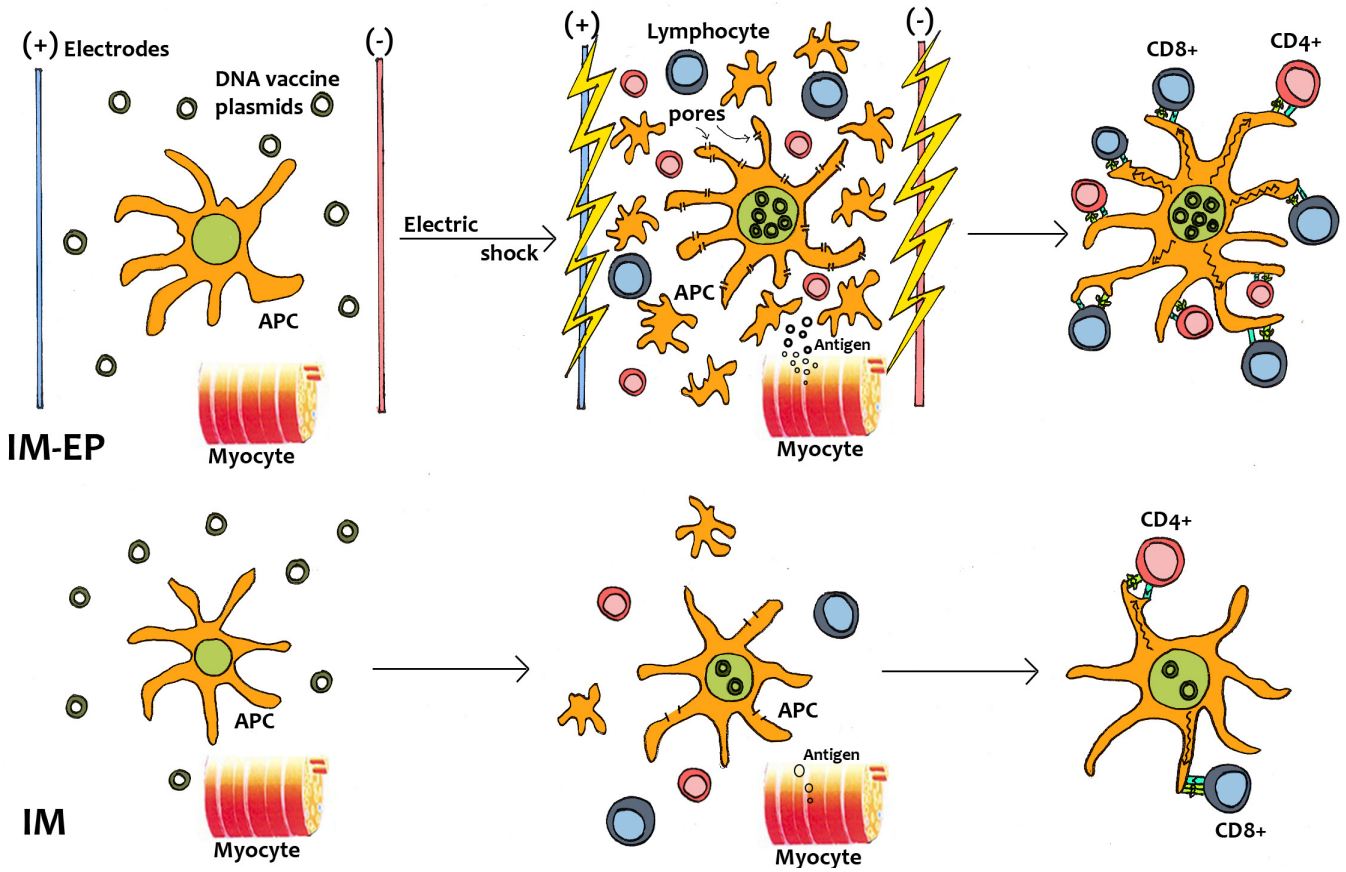

Fig. 1. Illustration of more antigen presenting cells (APCs) and lymphocytes attracted to the DNA vaccine injection site, and more uptake of DNA vaccines by APCs and myocytes, followed by more Ag expression and presentation, in particular, by APCs to CD4+ and CD8+ T cells by IM-EP (electroporation following DNA injection at the muscle) delivery, as opposed to IM (DNA injection alone at the muscle) delivery alone.

et al., 1997; Jakob *et al.,* 1998). For example, CpG plasmid sequences drive macrophages to secrete interleukin (IL)-12, a potent inducer of IFN-γ production *in vivo* from natural killer (NK) cells. IFN-γ production drives Th1 type immune responses through inducing differentiation of type 1 T helper cells which see an antigen in the presence of IFN-γ from the uncommitted T cell pool (Germann *et al.,* 1993; Chu *et al.,* 1997; Roman *et al.,* 1997). This might be supported by our finding that vaccination of human papillomavirus (HPV) 16 E7 proteins with CpG-oligodeoxynucleotide (ODN) as an adjuvant induced Ag-specific Th1 type and CD8+ T cells immune responses, as compared to E7 protein vaccine alone (Kim *et al.,* 2002). Krieg *et al.* also reported that intraperitoneal injection of CpG ODN reduced the number of infectious Listeria organisms in spleen and liver, as compared to control mice (Krieg *et al.,* 1998). The protective effect of CpG ODN was lost when animals had defects in IFN-γ gene, suggesting that IFN-γ induced by injection of CpG DNA sequence plays an important role in providing resistance to the bacterial infection. This is in line with our unpublished findings that myeloid differentiation primary response gene (MyD) 88 knockout animals are less immunogenic to E7 DNA vaccines in induction of Ab and CD8+ T cell-mediated antitumor responses. It is likely that CpG sequences of DNA vaccines interact with toll like receptor-9 (CpG DNA sequence-specific cell surface receptor) expressed on APCs and then activate MyD88-mediated signaling pathway for APC activation. Of note, this signaling pathway can be negatively regulated by TANK (tumor necrosis factor receptor-associated factor [TRAF] family member-associated NF-κB activator) through TANK's decreasing the ubiquitation of TRAF6 downstream of the MyD88 adaptor proteins (Kawagoe *et al.,* 2009). Taken together, CpG sequences of DNA vaccines appear to be involved in directing immune responses to Th1 type and CD8+ CTL responses *in vivo*.

PROMISING STRATEGIES FOR AUGMENTING IMMUNOGENICITY

It has been well known that direction and magnitude of DNA vaccine-mediated immune responses could be modulated by the modification of the biological properties of antigens, and by coinjection of immunologically important molecules coding for cytokines or chemokines and of other molecules targeting co-stimulatory and co-inhibitory signaling. Fig. 2 illustrates the basis of Ag-specific immune induction by regulating these immune molecules. This is in particular important in designing a vaccine towards controlling intracellular infection and cancer as Th1 type and CD8+ CTL responses are known to play a critical role in controlling pathogen's replication and its spread to other cells, as well as cancer cell growth and metastasis.

Modification of the biological properties of antigens

Modification of antigen's biological properties has been utilized to enhance Ag-specific immune responses to infectious diseases and cancer. Wu's group first reported that conjugation of HPV 16 E7 genes to the targeting sequence of lysosome-associated membrane proteins-1 (Ji *et al.,* 1999; Smahel *et al.,* 2001) showed both lysosomal targeting of E7 proteins in the cells and enhanced E7- specific protective immunity against E7-expressing tumor cell challenge. Similarly, conjugation of E7 genes to either signal or transmembrane sequences of herpes simplex virus (HSV)-2 glycoprotein B (gB) or glycoprotein D (gD) was tested by our group, based upon the previous finding that

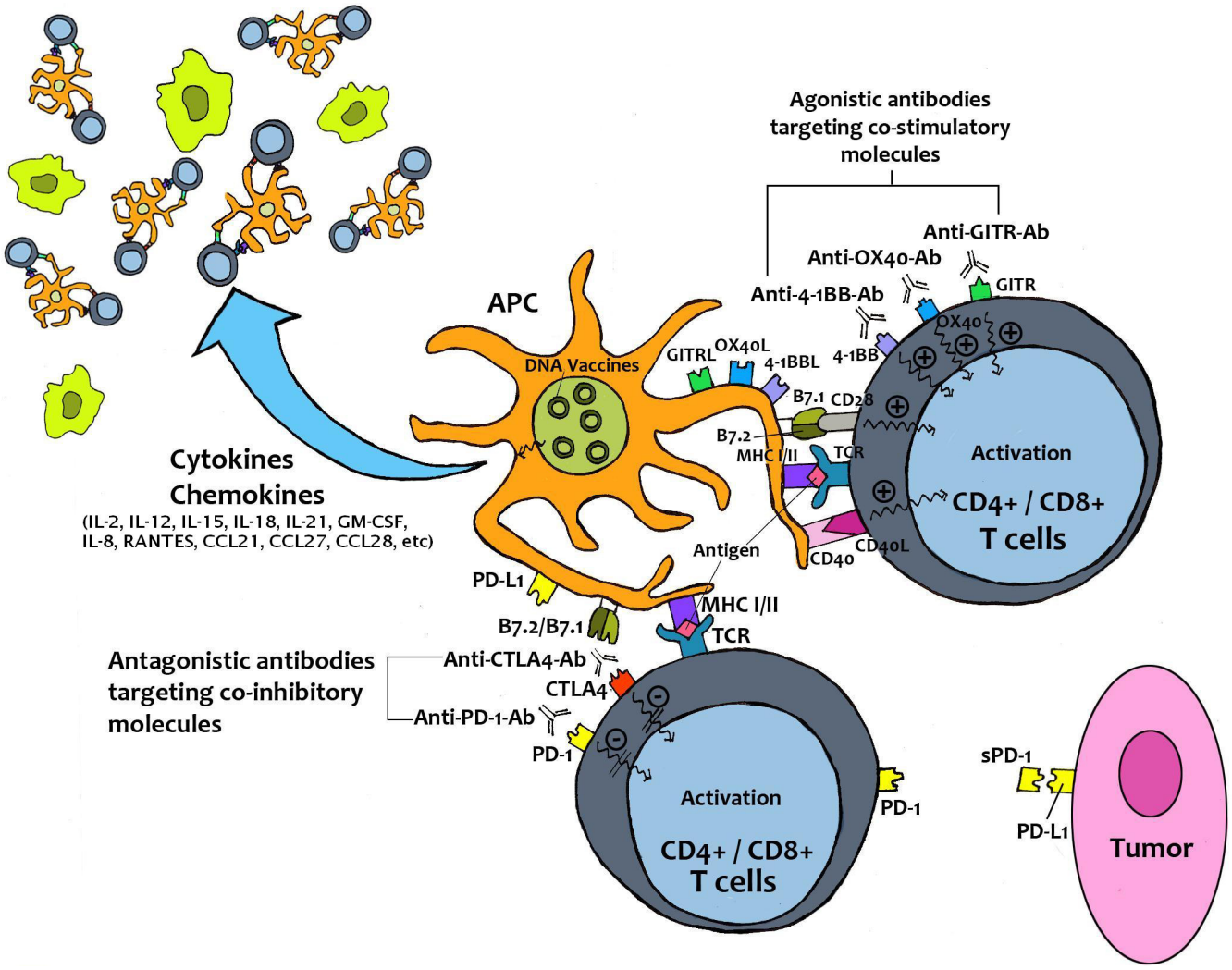

Fig. 2. Strategies to augment Ag-specific immune responses in DNA vaccination. These include co-delivery of plasmid DNAs expressing cytokines, chemokines and co-stimulatory molecules as an adjuvant, and regulation of the signaling of co-stimulatory or co-inhibitory molecules expressed on T cells using agnostic or antagonistic antibodies.

the cellular locations (secreted, cytosolic or transmembrane types) of an antigen expressed by HSV-2 gD DNA vaccines play a critical role in induction of immune responses as well as protection from HSV-2 challenge (Higgins *et al.,* 2000; Strasser *et al.,* 2000). In the study, we observed an increased antitumor immunity by this modification, but the increased level was far below that of lysosomal targeting delivery (Kim and Sin, 2005). To further increase the vaccine efficacy, E7 codon was optimized in the lysosomal targeting system, showing more production of E7 proteins *in vitro* and more dramatically enhanced antitumor protective immunity through augmentation of CTL responses (Kim and Sin, 2005). The effectiveness of antigen codon optimization strategy was also reported by another group that codon-optimized NS3/4A genes of hepatitis C virus (HCV) work better as a DNA vaccine at inducing Ab responses and CTL-mediated protective immunity from NS3/4A-expressing tumor cells (Frelin *et al.,* 2004). More recently, DNA vaccines containing a conserved HA5 sequence from different HA5 subtype sequences displayed neutralizing Ab responses to and protection from divergent clades of H5N1 influenza virus (Chen *et al.,* 2008; Laddy *et al.,* 2008), suggesting that usage of a conserved antigen sequence might allow us to generate a potential vaccine against rapidly emerging viruses with antigenic drift and shift, such as influenza virus. Besides the lysosomal targeting strategy, many additional targeting methods have been reported. For instance, targeting of E7 antigens by conjugating to bacterial toxin (cellular targeting), calreticulin (endoplasmic reticulum targeting), viral protein 22 (intercellular antigen spreading), and γ-tubulin (centromal compartment targeting) results in enhancing Ag-specific immune responses (Cheng *et al.,* 2001; Hung *et al.,* 2001, 2002, 2003). Furthermore, conjugation of heat shock protein (HSP) 70 genes to E7 genes also augmented DNA vaccine's immunogenicity to tumor, highlighting importance of making a hybrid DNA vaccine encoding both immunostimulatory molecules and antigens for Ag-specific immune induction (Hsu *et al.,* 2001). Thus, these collective studies clearly show that modification of the biological properties of antigens might be a promising approach to augmenting Ag-specific immune responses *in vivo*.

Cytokines and chemokines as adjuvants

To date, a variety of cytokine and chemokine genes have been tested as an adjuvant in an effort to improve immunity as well as to explore important immune correlates for protection from infectious diseases or cancer. In this regard, co-delivery of immunostimulatory molecules as an adjuvant has been a main focus in DNA vaccination techniques and immunotherapeutic strategies. For example, when granulocyte macrophage-colony stimulating factor (GM-CSF) plasmid DNA was tested as an adjuvant, Ag-specific immune responses and protective immunity against viral infections were dramatically augmented (Sin *et al.,* 1997, 1998). Furthermore, coinjection of HSV-2 gD DNA vaccine with plasmid DNA expressing Th1 (IL-2, IL-12, IL-15, IL-18) vs Th2 (IL-4, IL-10) cytokines drove Ag-specific immune responses to Th1 vs Th2 phenotypes (Sin *et al.,* 1999b). Moreover, IL-12 cDNA delivered with human immunodeficiency virus (HIV) or hepatitis B virus (HBV) DNA vaccines enhanced production of Ag-specific Th1 cell type, IgG2a isotype, and CTL responses (Kim *et al.,* 1997b; Chow *et al.,* 1998). Similarly, coinjection with IL-12 genes plus HSV-2 gD DNA vaccines significantly increased Ag-specific cellular immune responses, resulting in reducing both mortality and morbidity from HSV-2 challenge (Sin *et al.,* 1999a). IL-12 gene coinjection also induced much higher production of gD-specific IgG2a, as opposed to IgG1. In this study, we also observed that IL-12 coinjection increased production of Th1 type cytokine (IL-2 and IFN-γ), but inhibited production of Th2 type cytokines (IL-4 and IL-10), suggesting that driving immune responses to Th1 type cellular immune responses could be achieved by co-delivering IL-12 plasmid in DNA vaccination. In simian immunodeficiency virus (SIV)-infected macaques, furthermore, codelivery of SIV DNA vaccines plus IL-12 or IL-15 cDNA improved Ag-specific CD8+ effector memory T cell responses (Halwani *et al.,* 2008). Another Th1 type cytokine, IL-21 was also demonstrated to be effective as equally as IL-12, IL-15, and IL-18 for augmenting HSV-1 gB-based DNA vaccine potency (Cui *et al.,* 2005). More recently, EP delivery of IL-12 cDNA showed a similar result. For example, EP codelivery with IL-12 cDNA induced more potent Ag-specific humoral and cellular responses in a HIV DNA vaccine macaque model, as compared to EP alone (Hirao *et al.,* 2008a). Our group also demonstrated possible *in vivo* effects of coinjection with DNA vaccines and chemokine expression vectors in HIV and HSV vaccine models (Kim *et al.,* 1998a; Sin *et al.,* 2000). In particular, coinjection with IL-8 or RANTES either modulated Ag-specific cellular responses or increased protective immunity from HSV-2 infection. Intranasal codelivery of HSV-gB DNA vaccines with plasmid DNA expressing another chemokine, CC chemokine ligand (CCL) 21 also enhanced Ag-specific Ab and CTL responses, resulting in protecting animals from HSV challenge (Toka *et al.,* 2003). The antitumor adjuvant effect of CCL21 was also demonstrated in a melanoma animal model (Yamano *et al.,* 2006). In an influenza virus DNA vaccine study, we al-

so observed that coinjection with chemokines, CCL27 and CCL28 induces long-lived viral neutralizing antibodies, leading to protection from morbidity and mortality associated from a lethal influenza virus challenge (Kutzler *et al.,* 2009). Along with this, coinjection of HBsAg DNA vaccines with plasmid DNAs expressing CCL20 and a cytokine, Flt3 (fms-related tyrosine kinase 3) ligand induced Ag-specific immune responses in HBsAg transgenic mice by recovering DC functions (personal communication with Sidong Xiong), suggesting an important role of coinjection of certain chemokines and cytokines in overcoming Ag-specific immune tolerance. Taken together, these studies show that both cytokines and chemokines could be utilized to attract more immune cells to the DNA injection site and then direct Ag-specific immune response to the required effector phenotypes in DNA vaccination.

Regulating co-inhibitory molecules and negative factors

Immune regulation has been known to be mediated by two types of cell surface molecules expressed on T cells, co-inhibitory and co-stimulatory molecules (Peggs *et al.,* 2009; Pentcheva-Hoang *et al.,* 2009). These molecules are associated with either induction or inhibition of T cell responses *in vivo*. In particular, co-inhibitory molecules include CTLA (cytotoxic T lymphocyte-associated antigen)-4, PD (programmed cell death)-1, and B and T lymphocyte attenuator. As these molecules serve as a negative factor for T cell functionality, blocking these appears promising for up-regulating Ag-specific effector T cell function. For example, the antitumor effectiveness of anti-CTLA-4 antibodies was demonstrated in melanoma cell vaccine models (van Elsas *et al.,* 1999; Quezada *et al.,* 2006). Furthermore, Blockade of PD-1 by anti-PD-1 Abs enhanced adoptive T cell therapy against squamous cell carcinoma and potentiated cancer therapeutic immunity (Strome *et al.,* 2003; Hirano *et al.,* 2005). In DNA studies, codelivery of plasmid DNA expressing murine secondary lymphoid tissue chemokine (SLC) with plasmid DNA expressing a soluble form of PD-1 (sPD-1) resulted in enhanced protection against hepatoma cell challenge in mice (He *et al.,* 2004). In the same way, metastasis of melanoma cells was suppressed more dramatically when animals were treated with HSP vaccines in combination with plasmid DNA expressing sPD-1 (Geng *et al.,* 2006). This was resultant from blocking the interaction between PD-1 ligand-expressing tumor cells and PD-1-expressing tumor infiltrating lymphocyte (TIL) by sPD-1, and then preventing the apoptosis of TIL. Thus, blocking the interaction of PD-1 ligand with PD-1, which induces T cell apoptosis (Dong *et al.,* 2002), appears to be an additional approach for vaccine-driven immune induction as well as inhibition of tumor-driven immune cell apoptosis. Along with this, blocking or removal of Treg cells (known to inhibit T cell responses) has also been reported to increase vaccine-induced immune responses (van Elsas *et al.,* 1999; Prasad *et al.,* 2005; Comes *et al.,* 2006; Quezada *et al.,* 2006; Viehl *et al.,* 2006), highlighting importance of deleting a negative immune factor for enhancing vaccine-driven immunity. As some of these strategies using antibodies (e.g., Ipilumumab) are currently in clinical trials, they are highly expected to contribute to augmenting Ag-specific effector and memory T cell functions for controlling infectious diseases and cancer in humans.

Stimulating co-stimulatory and adhesion molecules

Contrary to co-inhibitory molecules, co-stimulatory molecules (such as CD28, ICOS [inducible T cell co-stimulator], OX40, CD27, 4-1BB, CD30, GITR [glucocorticoid-induced tumor necrosis factor receptor family-related gene] and HVEM [herpes-virus entry mediator]) have been known to be involved in stimulating T cell functions (Peggs *et al.,* 2009). In particular, 4-1BB is effective for inducing CT8+ T cell responses (Myers *et al.,* 2006) and superior to CD28 for activating CD8+ T cell responses (Zhang *et al.,* 2007). In this context, vaccination of DCs pulsed with apoptotic tumors in combination with anti-OX40 and anti-4-1BB Abs increased T cell-mediated antitumor protective immunity in Her-2/neu transgenic mice (Cuadros *et al.,* 2005). Moreover, co-delivery of SIV gag DNA vaccines with anti-4-1BB Abs resulted in induction of an enhanced and long-lasting cellular immune response in a macaque model (Calarota *et al.,* 2008). The beneficial role of anti-GITR in association with melanoma DNA vaccines in antitumor protection was also reported (Cohen *et al.,* 2006). To further increase the CD28 co-stimulatory pathway, we previously tested plasmid DNA encoding B7.1 or B7.2 (a ligand of CD28) in HIV-1 or HSV-2 DNA vaccine models, showing a significant induction of Ag-specific cellular responses to HIV-1 antigens or protective responses to HSV-2 challenge (Kim *et al.,* 1997a; Weiner and Sin, 2005). In addition, CD40 and CD40 ligand (L) are well known adhesion molecules and their coinjection in a DNA form with a DNA immunogen (β-galactosidase) has also been reported to augment Ab and CTL responses (Mendoza *et al.,* 1997). This effect was also confirmed in HSV-2 gD DNA vaccine models by co-delivering with plasmid DNA coding for CD40L (Sin *et al.,* 2001). Thus, the magnitude and nature of the immune response to a DNA vaccine and protective immunity to viral infection and cancer could be greatly regulated by co-delivery of these co-stimulatory

Table I. Infectious diseases and types of cancer in DNA vaccine preclinical or clinical trials using EP delivery

Types	Targeting molecules	Locations	Phase
Influenza virus	HA, N, M2e-NP, M1	Inovio, VGXI	Pre-clinical[a]
HIV	Env, Gag, Pol	HIV Vaccine Trial Network National Institutes of Health Uni. of Pennsylvania	I
HBV	HBsAg, HBcAg	Genexine	Pre-clinical[a]
HCV	NS3/4A	Tripep/Inovio	I/II
Cervical cancer	HPV 16/18; E6, E7	Inovio	I
Prostate cancer	PSMA	Inovio	I/II
Melanoma	IL-2, IL-12, tyrosinase	Vical, H. Lee Moffitt Cancer Center, MSKCC	I I
Breast/Lung/Ovarian/colorectal	Her-2, CEA, hTERT	Merck	I

CEA: carcinoembryonic antigen, HA: hemagglutinin, HBV: hepatitis B virus, HCV: hepatitis C virus, HIV: human immunodeficiency virus, HPV: human papillomavirus, hTERT: human telomerase reverse transcriptase, M: matrix protein, MSKCC: Memorial Sloan-Kettering Cancer Center, N: neuraminidase, NP: nucleoprotein, NS: non-structural protein, PSMA: prostate specific membrane antigen. [a]Now preparing for a clinical study.

and adhesion molecules as well as co-delivery of agonistic Abs targeting these molecules. Finally, these studies strongly support the notion that stimulating co-stimulatory signal pathways is also another potential approach to enhancing DNA vaccine-driven immune responses.

DNA VACCINES TARGETING AGAINST INFECTIOUS DISEASES AND CANCER

Some detrimental infections caused by influenza virus, HIV, HBV, and HCV, as well as cancers including HPV-associated cervical cancer, prostate cancer, melanoma and others will be mainly reviewed here as these are already entering or in a clinical study using EP delivery (Table I).

Influenza virus

Presently, swine (H1N1) influenza viruses have spread extensively worldwide, exposing the majority of people at high risk of viral infection. There is also great concern that an H1N1 virus may generate mutant strains which can be resistant to an antiviral drug, Tamiflu. Furthermore, presently available vaccines might not induce neutralizing antibodies against divergent clades of H1N1 strains. To counterattack this, a consensus viral antigen sequence was selected from different clades of influenza viruses and then tested as DNA vaccines. For instance, IM-EP of DNA vaccines containing a codon-optimized consensus hemagglutinin (HA) 5 sequence elicited antibodies that neutralized divergent clades of H5N1 viruses (Chen *et al.,* 2008). At the same time, our group also demonstrated that DNA vaccines encoding a consensus HA5 sequence induce both protective cellular and humoral immune responses to different clades of influenza virus infection in mice, ferrets or non-human primates (Laddy *et al.,* 2008). In particular, EP delivery of DNA vaccines resulted in protection from both morbidity and mortality in a ferret challenge model, in both the presence and absence of neutralizing Ab, suggesting that besides neutralizing Abs, cellular responses are also associated with prevention from influenza virus infection. More recently, EP delivery of the DNA vaccine also induced cross-reactive cellular and humoral immune responses individually capable of providing protection from influenza virus infection in the rhesus macaque (Laddy *et al.,* 2009). Taken together, these data illustrate that EP delivery of DNA vaccines containing a consensus antigen sequence of influenza virus might induce cross-protective immunity to any emerging subtypes of H5N1 or H1N1 influenza viruses in humans.

HIV

It has been reported that neutralizing antibodies, and CD4+ and CD8+ (CTL) T cell immunity play an important role in preventing HIV infection (Barrett *et al.,* 1991; Rowland-Jones *et al.,* 1993, 1995; Rosenberg *et al.,* 1997; Manato *et al.,* 1998). For developing DNA vaccine against HIV infection, HIV-1 env, gag, pol, rev, tat, nef, p24 have been cloned to an expression vector. Previously, our group along with others has shown that HIV-specific neutralizing Ab and cellular immune responses (Th proliferation and CTL) were induced by HIV DNA vaccination in animals (Wang, 1993a, b, Shiver *et al.,* 1996). In one study, in particular, vaccination with five HIV gene constructs (rev, tat, nef, gp160, p24) in combination induced strong immune reactivity to all of these antigens (Hinkula *et al.,* 1997). In primate studies, HIV-1 DNA vaccine successfully protected macaques from challenge with chimeric simian human immunodeficiency virus (Boyer *et al.,* 1996). In addition, immunization with DNA expressing HIV-1 env, rev,

and gag/pol induced Ag-specific immune responses and then provided protection from following challenge with a high dose heterologous strain of HIV-1 (Boyer *et al.,* 1997a). HIV-1 viral load was also declined by immunizing chimpanzees with HIV-1 env/rev plasmid construct, followed by booster injection with env plasmid DNA vaccine (Boyer *et al.,* 1997b). Recently, EP delivery has been tested. IM-EP delivery of HIV-1 env DNA vaccines augmented Ag-specific cellular and humoral immune responses in mice and allowed a 10-fold reduction in vaccine dose, as compared to IM alone without EP (Liu *et al.,* 2008). EP delivery of optimized plasmid DNAs encoding the majority of SIVmac239 proteins also elicited strong immune responses in rhesus macaques and decreased viremia in both the acute and chronic phases of infection following challenge with pathogenic SIVmac251 (Rosati *et al.,* 2009). At the same time, our group also demonstrated the availability of skin + EP delivery using pig and macaque models, showing that high plasmid concentrations resulted in improved gene expression for plasmid green fluorescent protein delivered by the intradermal/subcutaneous route and higher cellular and humoral responses to an HIV DNA vaccine, suggesting that ID-EP may have importance as an immunization approach in larger animals (Hirao *et al.,* 2008b). Finally, recent failure of STEP clinical trials using adenoviral vector delivery of HIV antigens due to preexisting immunity to the viral vector highlights importance of naked HIV DNA vaccines and their EP delivery in vaccination studies for preventing HIV infection. In a recent RV-144 clinical trial using 4 priming injections of a recombinant canarypox vector (ALVAC-HIV) and 2 booster injections of recombinant gp120 proteins, the regimen reduced the rate of HIV-1 infection by 31.2% (Rerks-Ngarm *et al.,* 2009). This accomplishment will move the vaccine field forward and allow more clinical research of this platform. At this time, however, the first goal of any HIV DNA vaccine study is to generate improved CTL responses and some Ab responses. Presently, HIV DNA vaccines and their EP delivery strategies are under evaluation in a phase I clinical trial.

Hepatitis virus

Chronic hepatitis virus infection is associated with cirrhosis and liver carcinoma formation. Various therapeutic approaches to control acute and chronic HBV and HCV infection have not been successful. It has been known that high-titer neutralizing antibodies against HBsAg are correlated to protective immunity against HBV (Purcell and Gerin, 1975; Dreesman *et al.,* 1981). However, another group showed that adoptive transfer of HBsAg-specific CTL results in inhibiting HBV replication by releasing IFN-γ and tumor necrosis factor-α (Guidotti *et al.,* 1994). These data suggest that both neutralizing Ab and cellular responses are likely associated with controlling HBV infection. In the case of HBV infection, HBsAg subunit vaccine has been successful for preventing HBV infection through induction of sterilizing immunity against HBV. However, present studies have focused at developing a therapeutic vaccine against chronic HBV infection. Mancini *et al.* reported that HBV replication was inhibited by HBV DNA vaccination without any detectable liver cytopathy in mice (Mancini *et al.,* 1996). DNA vaccines expressing HBV antigens also induced Ag-specific Ab responses in primates (Davis *et al.,* 1996; Gramzinski *et al.,* 1998). Furthermore, IFN-γ secreting memory T cells responses is induced by HBV DNA vaccination even in chronic HBV carriers under lamivudine treatment (Yang *et al.,* 2006). Recently, Ag-specific humoral and cellular responses have been reported to be induced more significantly by EP delivery of DNA expressing HBV antigens (Kim *et al.,* 2008). In the case of HCV, chimpanzees have been protected from viral challenge after vaccination with E1 and E2 subunit vaccines, in which 5 out of 7 chimpanzees showed complete protection from primary infection and hepatitis while 2 chimpanzees displayed delayed incidence of viral infection (Choo *et al.,* 1994). This suggests that Ab responses could be an important protective correlate for protection against HCV infection. Previously, DNA vaccines expressing HCV core, E1, and E2 proteins have been shown to induce Ag-specific Ab and Th cell proliferative responses, as well as CTL in animals (Lagging *et al.,* 1995; Major *et al.,* 1995; Encke *et al.,* 1998; Fournillier *et al.,* 1998). Moreover, gene gun delivery of DNA vaccine containing both a codon-optimized synthetic HCV NS3/4A sequence and Semliki forest virus replicon resulted in an improved immunogenicity as evidenced by higher levels of NS3-specific antibodies (Frelin *et al.,* 2004). This improved immunogenicity also showed a more rapid priming of CTL as demonstrated by an NS3/4A-specific tumor-inhibiting immunity. The same group further tested this vaccine in EP delivery, showing that DNA vaccines induce functional CD8+ responses *in vivo*, resulting in elimination of HCV NS3/4A-expressing liver cells in transiently transgenic mice (Ahlén *et al.,* 2007). It was also reported that EP of a novel candidate DNA vaccine encoding an optimized version of the nonstructural region of HCV (from NS3 to NS5B) induced substantially more potent, broad, and long-lasting CD4+ and CD8+ cellular immunity than naked DNA injection in mice and in rhesus macaques (Capone *et al.,* 2006). Taken together, EP delivery of HBV and HCV DNA vaccines is thought to be promising in eliciting immune responses to

viral Ag-expressing liver cells and is presently under evaluation in a phase I/II clinical trial.

Cervical cancer

HPV infection has been known to be a main cause of cervical cancer incidence. Presently, Gardasil™ (Merck) and Cervarix™ (GSK) have been licensed as a prophylactic vaccine. These vaccines are estimated to reduce the incidence of cervical cancer but are not effective for treating cervical cancer or its precancerous diseases (Sin, 2006a, 2009b). In both animal and human studies, HPV E6 or E7-specific CTL responses have been found to be critical in therapeutically controlling HPV-associated cervical intraepithelial neoplasm and cervical cancer. To date, numerous DNA vaccine approaches (such as codon optimization, antigen targeting modification, etc) have been tested to augment Ag-specific CTL responses as mentioned previously. However, we observed that antitumor therapeutic efficacy of E7 DNA vaccines are far lower than that of E7 subunit vaccines in mice (Sin *et al.,* 2006b). In the study, there detected approximately a 100 fold difference in vaccine dose displaying equivalent antitumor activity between E7 DNA and subunit vaccines. Using IM-EP, we observed that E7 DNA vaccines induced 29 times higher IFN-γ production from CD8+ T cells than IM alone, thus displaying more potent antitumor therapeutic efficacy (our unpublished data). It can be further speculated that antitumor therapeutic activity of EP-delivered E7 DNA vaccines could be enhanced greatly by combining DNA vaccination with chemotherapy or radiation, based upon our preclinical data showing therapeutic synergy between chemotherapy/radiotherapy with E7 subunit vaccine-based immune therapy (Bae *et al.,* 2007; Ye *et al.,* 2007; Sin *et al.,* 2009a).

Prostate cancer

Prostate cancer is one of the most common cancer types in men. It is treated mainly by surgery and radiation. Prostate specific antigen (PSA) has been tested as a target for immunotherapy against prostate cancer as it is expressed high in prostate cancer cells. It was previously reported that a DNA vaccine encoding PSA induces Ag-specific Ab and cellular immunity, in particular CTL in mice (Kim *et al.,* 1998b). Further co-injection of PSA DNA vaccines with DNA expressing IL-2 and GM-CSF induced a significant level of Ag-specific CTL and antitumor responses to PSA-expressing tumors (Roos *et al.,* 2005). More recently, ID-EP has been demonstrated to be a more effective way to induce PSA-specific immune responses (Roos *et al.,* 2006). In the study, ID-EP delivery increased PSA gene expression (100- to 1,000-fold) and induced higher levels of Ag-specific T cells, compared to DNA delivery without EP. Thus, EP delivery targeting PSA appears to be promising in generating CTL responses to prostate cancer, and is now in a phase I clinical study.

Melanoma

Melanoma is a malignant tumor of melanocytes which is found mainly in skin. Due to its location, melanoma has been tested first for antitumor therapeutic activity of EP delivery. In this regard, EP of plasmid DNA expressing IL-2 and IL-12 into tumor sites was reported to induce gene transduction and significant tumor growth delay in a mouse melanoma model (Lohr *et al.,* 2001). Heller's group also demonstrated that intratumoral EP of IL-12 cDNA showed an increased level of IL-12 and IFN-γ within the tumors, and showed the influx of lymphocytes into the tumors and reduction in vascularity (Lucas *et al.,* 2002). Aside from cytokine delivery, melanoma-associated antigens have also been tested by EP delivery. For instance, IM-EP of plasmid constructs containing the relevant H-2K(b)-restricted epitope (SVYDFFVWL) of tyrosinase-related protein-2 (melanoma-associated antigen) resulted in a significant induction of CD8+ T cells and delay in tumor growth in mice (Kalat *et al.,* 2002). In accordance with these preclinical studies, EP of plasmid vectors expressing IL-2, IL-12 or tyrosinase-related proteins is presently under clinical evaluation.

Other cancer types

Her-2/neu, carcinoembryonic antigen (CEA), and hTERT (human telomerase reverse transcriptase) have been tested as a cancer-associated target for immune therapy against breast, colorectal, ovarian and lung cancers. To this end, EP delivery of DNA vaccines coding for these antigens has been under clinical evaluation at a phase I stage.

CONCLUSION

Numerous strategies have been applied to improve the effectiveness of DNA vaccines in generating immunity required for controlling many infectious diseases and cancer. The advent of EP delivery likely contributes to accomplishing this goal. Presently, application of EP has been in clinical trials targeting a number of viral infection and cancer. The clinical outcome of these vaccination schemes is yet unknown. However, there is obviously no limit in future developments of more effective DNA vaccines and the delivery strategies for preventing and controlling these intractable diseases.

CONFLICT OF INTEREST

Jeong-Im Sin serves as a consultant to VGXI. David B. Weiner serves as a consultant to Inovio.

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