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Novel Strategies to Improve DNA Vaccine Immunogenicity

Cevayir Coban[1], Kouji Kobiyama[2,3], Taiki Aoshi[2,3], Fumihiko Takeshita[2,3], Toshihiro Horii[4], Shizuo Akira[5] and Ken J. Ishii[2,3,*]

[1]*Laboratory of Malaria Immunology, Immunology Frontier Research Center, World Premier Institute for Immunology, Osaka University;* [2]*Laboratory of Vaccine Science, Immunology Frontier Research Center, World Premier Institute for Immunology, Osaka University;* [3]*Laboratory of Adjuvant Innovation, National Institute of Biomedical Innovation, Osaka;* [4]*Department of Molecular Protozoology, Research Institute for Microbial Diseases, Osaka University;* [5]*Laboratory of Host Defense, Immunology Frontier Research Center, World Premier Institute for Immunology, Osaka University*

Abstract: DNA vaccines can induce both humoral and cellular immune responses in animals. Some DNA vaccines are already licensed for infectious diseases such as West Nile virus encephalitis in horses. When used in humans, however, DNA vaccines suffer from lower immunogenicity profiles. Although the reasons for this are poorly understood, various hypotheses have been proposed. This review aims to provide better understanding of the molecular and immunological mechanisms by which DNA vaccines work and how such knowledge can be used to bring about improvements in their efficacy. Recent studies have provided evidence that the 'adjuvant effect' of plasmid DNA is mediated by its double-stranded structure. This structure activates stimulator of interferon genes/TANK-binding kinase 1 (STING/TBK1)-dependent innate immune signaling pathways in the absence of Toll-like receptors. Indeed, type-I interferons (IFNs), induced *in vivo* via the STING/TBK1 pathway, were found to be crucial for both direct- and indirect-antigen presentation via distinct cell types (i.e. dendritic cells (DC) and muscle cells, respectively). Importantly, incorporation of TBK1 into a DNA vaccine was found to enhance the antigen-specific humoral immune responses targeting the *Plasmodium falciparum* serine repeat antigen (SERA), a candidate vaccine antigen expressed in the blood-stages of human malaria parasites. Thus, the results of these studies may offer new ways to develop DNA vaccines, as well as delivering novel vaccine adjuvants against infectious diseases.

Keywords: Adjuvant, CpG motifs, DNA vaccine, innate immunity, STING, TBK1, TLR9, type I interferon.

INTRODUCTION

DNA vaccines represent a major advance in the fight against infectious diseases. Their mechanism of action enables the antigen of interest to be delivered to the host immune system in a manner that is similar to natural exposure to the pathogen. DNA vaccines comprise plasmid DNA encoding the antigen of interest whose expression is controlled by a mammalian promoter; following administration of the vaccine, the host immune system is exposed to the expressed antigen. Such *in vivo* introduction of genetic material usually elicits strong humoral and cellular (Th1 type CD4+ T cells and CD8+ cytotoxic T cells) responses, making DNA vaccines distinct from conventional protein or peptide vaccines. Indeed, DNA vaccines have shown remarkable success in most animal studies and clinical trials in humans [1-2]. For example, DNA vaccines for horses, salmonid fish and dogs have been licensed since 2005 against West Nile virus, Infectious hematopoietic necrosis virus and melanoma, respectively [3-5]. DNA vaccine clinical trials against HIV and malaria, at least showed good safety and tolerability profile in humans [6-7].

*Address correspondence to this author at the Laboratory of Adjuvant Innovation, National Institute of Biomedical Innovation, 7-6-8 Asagi, Saito, Ibaraki City, Osaka 567-0085, Japan; Tel: +81-72-641-8043; Fax: +81-72-641-8079; E-mail: kenishii@biken.osaka-u.ac.jp

However, low immunogenicity has proved a significant obstacle to efficacy for DNA vaccines, especially in higher primates and humans. To date, various approaches have been taken to improve the immunogenicity of such vaccines. For example, modifying the microenvironment of the vaccination site by co-administration of various genes (i.e. genes encoding the co-stimulatory molecules, cytokines and chemokines [8-9], and/or genes that induce apoptosis [10-12]), proteins or other immunologically active molecules is not unusual. Some other approaches that have been tried to improve DNA vaccine immunogenicity include: 'Prime-Boost' immunization with DNA followed by a viral vector encoding the antigen (or the protein antigen) [13]; various immunization techniques and DNA delivery systems such as electroporation [14-16]; and microparticles and tattoo-immunization [17-18]. However, we are a long way from a complete understanding of how DNA vaccines work.

Recent studies are starting to shed light on this subject, however. Crucial to this is the recent discovery of many innate immune system receptors such as Toll-like receptors (TLRs), Nucleotide binding oligomerization domain (NOD)-like receptors, and Retinoic acid inducible gene (RIG)-like receptors, which have revealed that the main components of successful vaccines (adjuvants) are the ligands for many innate immune system receptors. Use of such molecules has great potential to improve vaccine design and development

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[19-21]. Indeed, many of the innate immune system receptor ligands are protein, nucleic acid, lipid, and carbohydrate in nature (i.e. lipid-A and poly-IC and CpG oligodeoxynucleotides (CpG ODNs) and have been used as adjuvants in vaccine trials for many years. Similarly, recent intensive research efforts searching for a cytosolic DNA sensor has improved our understanding [22-23]. Hence, this review will discuss some of the recent developments in this field and consider some of the strategies available for improving the immunogenicity of DNA vaccines. Attention will be directed towards the use of malarial antigens as anti-malaria vaccines.

1.1. Strategies to Improve DNA Vaccine Immunogenicity; Manipulation of Intrinsic Adjuvant Properties

It was shown that bacterial DNA contains immunostimulatory CpG motifs, which comprise an unmethylated C followed by G and specific flanking sequences that are recognized by TLR9 [24-26]. These findings suggest that DNA vaccine immunogenicity might be attributed to CpG motifs present in the plasmid backbone, which could act as an intrinsic "built in adjuvant" for DNA vaccines [27-28]. Therefore, incorporation of TLR9 and related TLR pathways has been a promising approach that could improve DNA vaccine immunogenicity.

Indeed, addition of several CpG motifs into a plasmid backbone has been shown to improve the immunogenicity of DNA vaccines [29-31]. In one study, DNA plasmid backbones were modified by the addition of two distinct types of human-specific CpG motifs (D and K types ODNs), and the effects of such modifications on various types of human immune cells (and in mice) were investigated for use in humans against malaria [31]. It was found that the modified plasmid DNA could induce maturation of human monocytes into DCs via activation of plasmocytoid DC (pDC); both modified plasmids (containing as few as three to five human CpG motifs) resulted in differential DC maturation in comparison with an unmodified plasmid. In addition, although expression of the encoded antigen (*P. falciparum* surface protein 25 (Pfs25), a *P. falciparum* transmission-blocking vaccine candidate) was not affected by introduction of additional immunostimulatory CpG motifs into the plasmid backbone, at least one of the plasmids (D type ODN incorporated Pfs25) induced higher levels of Pfs25-specific IgG with subtle differences in antibody isotypes. However, later studies found that TLR9-deficient mice had comparable levels of antigen-specific IgG, IgG1 and IgG2a antibody responses (including IFNγ and cytotoxic T lymphocyte (CTL) responses) as their wild-type counterparts [32-33], although others suggest that TLR9 signaling is critical for inducing CD8+ T cell responses after DNA vaccination at least after priming [34-35].

Other approaches to improve DNA vaccine immunogenicity include targeting TLR pathway signaling molecules, which can mimic a microbial infection in the absence of any microbial components. One such study investigated an over-expression strategy using adapter molecules such as the Myeloid Differentiation Primary Response Gene (MyD88) or Toll/IL-1 receptor (TIR)-domain-containing adaptor inducing interferon-β (TRIF) and found that greater enhancement of humoral responses was achieved when the DNA vaccine incorporated the MyD88 genetic adjuvant [36]. In contrast, incorporation of the TRIF genetic adjuvant greatly enhanced cellular immune responses resulting in superior protection against influenza virus or tumor progression. Similarly, incorporation of the interferon regulatory factor (IRF) 1, 3 and 7 into DNA vaccines improved both humoral and cellular immune responses against viral infection [37-38].

Overall, these studies suggest that although the immunogenicity of DNA vaccines can be enhanced by additional CpG motifs, or over expression of TLR-mediated signaling molecules in the plasmid backbone, the 'basal' adjuvant effects of DNA vaccines are independent of TLR-mediated recognition. The results of another study using MyD88/TRIF-double deficient mice are consistent with this [39].

1.2. Double-Stranded Plasmid DNA is Recognized Via the DNA Recognition Machinery, STING and TBK1

If plasmid DNA is not recognized by TLRs (TLR9) and its adapter molecules, what is it recognized by? Recent work has shown that bacterial or synthetic CpG-DNAs are not the only molecules that can activate the innate immune system. In fact, double-stranded (ds) DNA derived from host cells in the B form (a right-handed helical structure) can also do this [40]. When transfected into the cytosol, dsDNA activated fibroblasts, macrophages and dendritic cells to produce robust amounts of type-I interferons (IFNs) independent of TLRs; but in this case, signaling is mediated through TANK-binding kinase-1 (TBK1) [40]. TBK1 is crucial for type-I IFN induction via TLR-dependent and TLR-independent pathways [21,41]. However, within the last few years, several studies have identified various cytosolic DNA-recognition molecules (recently reviewed by [23]). Briefly, STING (stimulator of interferon genes, alternatively designated MITA, MPYS and ERIS), which is localized in the endoplasmic reticulum (ER), was found to activate NF-kB and IRF3 to stimulate type I IFN production after intracellular dsDNA stimulation [42]. Upon dsDNA stimulation, STING relocalizes with TBK1 from the endoplasmic reticulum to perinuclear vesicles containing the subunit of the exocyst complex 5 (Sec5) (also called EXOC2) [43]. Quite recently, a new molecule designated the interferon-inducible tripartite-motif (TRIM) 56 was identified as an interferon-inducible E3 ubiquitin ligase that modulates STING dimerization upstream of TBK1 to confer double-stranded DNA-mediated innate immune responses [44]. At the final stage, TBK1 comprises inducible IκB kinase (IKK-i) and these two kinases directly phosphorylate interferon regulatory factor 3 (IRF3) and IRF7 [45-46], resulting the production of type I interferons (IFNs).

These components of the DNA recognition machinery (STING and TBK1) have collectively indicated that a double-stranded structure could be essential for DNA vaccine-induced immunogenicity. Accordingly, use of TBK1-deficient mice revealed that DNA vaccine immunogenicity was completely dependent on TBK1 [39]. Moreover, such immunogenicity occurs through the activation of type-I IFN-mediated innate immunity resulting in the adjuvant effect for the encoded antigen [39,41] Fig. (1). Furthermore, type I IFNs were found to be essential for optimal DNA vaccine immunogenicity (antigen-specific T and B cell induction) by

Table 1. Summary of DNA Vaccine Studies Using Mice Deficient for Innate Immune System Molecules

Knockout Mice	DNA Vaccine-Induced Antigen Specific-			Reference
	Ab Responses	CD4+ T Cells	CD8+ T Cells	
TLR9	→	→	→*	[33, 35]
MyD88/TRIF	→	→	→	[39]
IPS	→	→	→	[39]
IFNAR	↓	↓	↓	[39]
STING	↓	↓	↓	[47]
TBK1	↓	↓	↓	[39]
IRF3	→	↓	↓	[48]

*Some groups suggest that TLR9 signaling is critical for inducing CD8+ T cell responses after priming [30].

using interferon (alpha and beta) receptor 2 (IFNAR2)-deficient mice, but not attributable to TLR signaling (i.e. because MyD88/TRIF-double deficient mice normally respond to DNA vaccines [39]). Bone-marrow transfer experiments revealed that TBK1-mediated signaling in dendritic cells mainly involved the induction of both antigen-specific B cells and CD4+ T cells. Moreover, DNA-transfected non-immune cells, such as muscle cells, were found to cross-prime CD8+ T cells, which following DNA vaccination promoted adaptive immune responses Fig. (**1**).

Similar studies were performed with STING-deficient mice [47], which revealed the importance of STING for plasmid DNA immunogenicity Fig. (**1**). One possible drawback of this pathway is that RNA could be generated during DNA vaccination, which might act as an adjuvant by activating TBK1-dependent signaling [41]. Because ss- and dsRNA is recognized via TLR3/7/8, RIG-I and Melanoma differentiation-associated gene 5 (MDA5) and utilizes the adapter molecules TRIF, MyD88 and interferon-beta promoter stimulator 1 (IPS-1), respectively [21], this possibility was excluded using TRIF-, MyD88- and IPS-1-deficient mice; the results showed that DNA vaccine-induced immune responses against all of the adaptors were comparable with their WT counterparts [41].

These DNA vaccination studies in mice collectively revealed that DNA vaccination induces both antigen-specific B cells as well as CD4+ and CD8+ T cell responses entirely dependent on TBK1. However, interestingly, IRF3-deficient mice elicited strong antigen-specific humoral responses after DNA vaccinations, while CD4 and CD8 T cell responses (including the production of Th1, Th2 and Th17 cytokines) were severely impaired [48], suggesting other down-stream molecules of TBK1 leading production of antibodies, requires further investigation (see Table **1** for a summary of DNA vaccine studies as of current day by using mice deficient for immune system receptors).

1.3. Does the Inflammasome Play a Role in DNA Vaccine Immunogenicity?

In addition to TLR-independent and STING/TBK1-dependent cytosolic DNA recognition, another innate immune signaling mechanism has been suggested for cytosolic DNA recognition. That is, cytosolic DNA mediates the Apoptotic speck protein containing a caspase recruitment domain (ASC)/caspase-1-mediated secretion of IL-1β by AIM2 (absent in melanoma 2), a human IFN-inducible gene (HIN)-200 family member [49-52]. However, it should be noted that the AIM2 inflammasome is essential for caspase-1 activation, but not for type I IFN production in response to cytosolic dsDNA Fig. (**1**).

Although several studies have shown that DNA vaccine immunogenicity could be improved by incorporation of IL-1 and caspases [12,53], we do not know whether the inflammasome pathways, AIM2 and ASC, are required for DNA vaccine immunogenicity. However, recent evidence suggests that the AIM2/ASC inflammasome presumably has little impact on the immunogenicity/adjuvanticity of a DNA vaccine. We have recently shown that alum adjuvant (the only adjuvant licensed for human use) induces cell death and release of DNA; this in turn induces IL-1β release, which could be responsible for alum's adjuvanticity, independent of ASC or Caspase-1 ([1]Marichal T *et al,* in press). This hypothesis could be tested using AIM2 and/or ASC-deficient mice for DNA vaccination studies.

1.4. Can we Improve DNA Vaccine Immunogenicity by Incorporating TBK1 as a Novel adjuvant?

Having shown that plasmid DNA-induced immunogenicity was due to its adjuvant properties (mediated via the TBK1 kinase), we evaluated whether such immunogenicity could be improved by incorporating overexpressed TBK1 as an adjuvant. First, we confirmed that TBK1 protein expression was successful using *in vitro* transient transfection (*T. Aoshi, unpublished observations*). However, after many attempts to improve immunogenicity (i.e. mixing the TBK1 encoding plasmid with the antigen encoding plasmid, or using a plasmid co-expressing model of antigen plus TBK1 in a single backbone) we finally concluded that co-expressing

[1] Marichal T, Ohata K, Bedoret D, Mesnil C, Sabatel C, Kobiyama K, Lekeux P, Coban C, Akira S, Ishii KJ, Bureau F, Desmet CJ. DNA released from dying host cells mediates aluminum adjuvant activity. Nat Med In press 2011.

TBK1 in the same backbone do not enhance immunogenicity for both humoral and cellular immunity (*T. Aoshi, unpublished observations*). It is possible that the immunization route used (intramuscular electroporation) may not have been ideal (i.e. electroporation itself may release DNA) for evaluating the effect of the TBK1 molecule. Therefore, we optimized the plasmid doses and immunization routes using plasmids encoding one of the leading vaccine candidate antigens from the blood stages of *P. falciparum*, the serine repeat antigen 36 (PfSERA36) [54]. Targeting *Plasmodium* blood-stage antigens might have several benefits for humans living in malaria endemic regions [55-56]. Younger children would primarily benefit from such vaccines because they are disproportionately affected by the severity of the disease, which can result in death.

We tested intramuscular (i.m.) injection route which is milder way of introducing DNA vaccine. Equal amounts of the plasmids (50 μg) were introduced intramuscularly (i.m.)

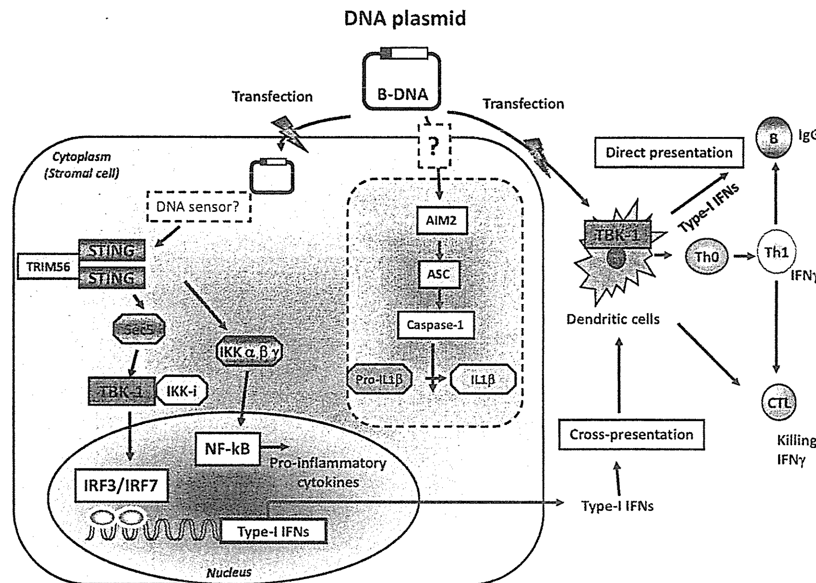

Fig. (1). Double-stranded plasmid DNA utilizes STING and TBK1 for production of type I IFN. The induction of both antigen-specific B cells and CD4+ T cells in hematopoietic cells (i.e. dendritic cells) and CD8+ T cells in non-hematopoietic cells (i.e. muscle cells) is mediated via type I IFNs controlled by STING/TBK1. Therefore, both direct priming and cross priming of the adaptive immunity occur after DNA vaccination. (Abbreviations: B-DNA; B form right-handed helical structure deoxyribonucleic acid, STING; stimulator of interferon genes, Sec5; subunit of the exocyst complex 5, TRIM56; interferon-inducible tripartite-motif 56, TBK1; TANK-binding kinase 1, IKK; IkB kinase, IKK-i; inducible IKK, IRF; interferon regulatory factor, Nf-κB; nuclear factor κB, AIM-2; absent in melanoma 2, ASC; Apoptotic speck protein containing a caspase recruitment domain).

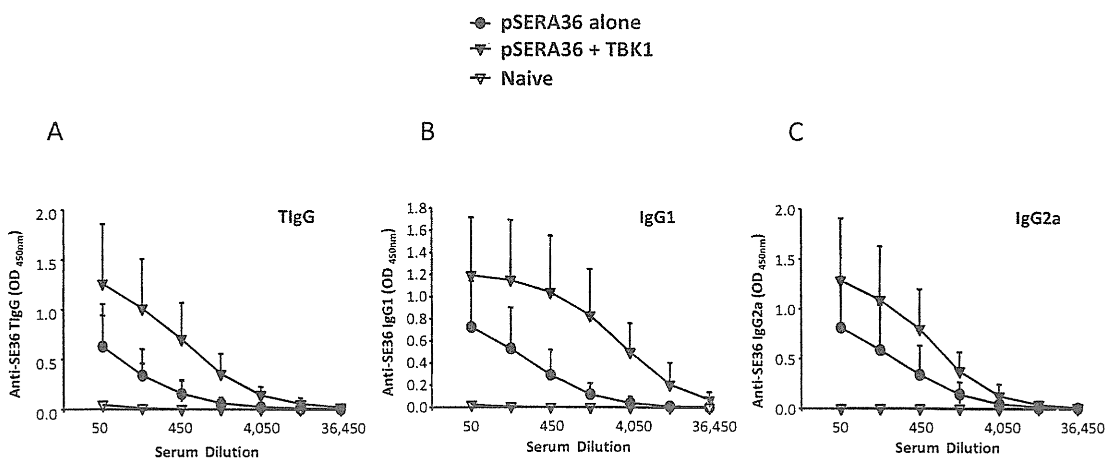

Fig. (2). Co-immunization with a TBK1-encoding plasmid improves DNA vaccine immunogenicity. Mice were immunized with 50 μg of each plasmid (encoding either the *P. falciparum* SERA36 or TBK1) via intramuscular (i.m.) immunization and boosted 4 weeks later with PfSERA36 antigen (1 μg) formulated in alum intradermally (i.d.). Total IgG (A), IgG1 (B), and IgG2a (C) responses for the *P. falciparum* SERA36 antigen were analyzed by ELISA 2 weeks after the booster immunizations.

into mice and boosted 4 weeks later with a PfSERA36 antigen intradermally (i.d.). The TBK1-encoding plasmids, while successfully improving antigen-specific antibody responses, both IgG1 and IgG2a isotypes, Fig. (**2 A-C**) in the plasmid cocktails of SERA36, failed to improve cellular immune responses (*K. Kobiyama, unpublished observations*). Our results suggested that simple injection of TBK1 expressing plasmid in DNA vaccine plasmid cocktail may improve, at least, anti-malarial humoral immunogenicity.

CONCLUSION

Recent attempts to identify the double-stranded DNA sensor have provided great insight into the molecular and cellular mechanisms contributing to DNA vaccine immunogenicity (Table **1**). Our current understanding highlights the importance of type-I IFN mediated innate immune activation via the STING/TBK1 intracellular detection machinery. Such activation confers the adjuvant effect to the encoded antigen. Evidence from our studies suggests that the TBK1-encoded DNA vaccine plasmids used for targeting intracellular signaling pathways might have enormous potential to modulate the innate immune system and increase the immunogenicity of DNA vaccines. A major goal now is to translate the findings of these studies into medical applications (i.e. develop a blood-stage malaria vaccine against *P. falciparum*). It is our hope that studies of this type may deliver new ways to develop safe and effective vaccine adjuvants.

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