DNA vaccines
A simple DNA sensing matter?

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Since the introduction of DNA vaccines two decades ago, this attractive strategy has been hampered by its low immunogenicity in humans. Studies conducted to improve the immunogenicity of DNA vaccines have shown that understanding the mechanism of action of DNA vaccines might be the key to successfully improving their immunogenicity. Our current understanding is that DNA vaccines induce innate and adaptive immune responses in two ways: (1) encoded protein (or polypeptide) antigen(s) by the DNA plasmid can be expressed in stromal cells (i.e., muscle cells) as well as DCs, where these antigens are processed and presented to naïve CD4 or CD8 T cells either by direct or cross presentation, respectively; and (2) the transfected DNA plasmid itself may bind to an un-identified cytosolic DNA sensor and activate the TBK1-STING pathway and the production of type I interferons (IFNs) which function as an adjuvant. Recent studies investigating double-stranded cytosolic DNA sensor(s) have highlighted new mechanisms in which cytosolic DNA may release secondary metabolites, which are in turn recognized by a novel DNA sensing machinery. Here, we discuss these new metabolites and the possibilities of translating this knowledge into improved immunogenicity for DNA vaccines.

There have been dozens of human clinical trials of DNA vaccines against infectious and non-infectious diseases such as influenza, hepatitis B, HIV, malaria and cancer; however, with disappointing outcomes (reviewed in refs. 1–3), suffering from lower immunogenicity than that had been observed in other mammals. Safety issues such as integration of plasmid DNA into genomic DNA, the risk of autoimmunity or antigen tolerance were successfully addressed in those studies. It was concluded that despite its low immunogenicity, DNA vaccination is a safe form of immunization. Therefore, it still has a future for use in humans if its immunogenicity can be strengthened. Moreover, successful DNA vaccines have been licensed in veterinary applications since 2005, including canine melanoma, West Nile viruses in

horses and fish hematopoietic necrosis viruses.[4-6] The successful veterinary applications indicate that selecting an appropriate, strongly immunogenic antigen is very important and should be considered for improving DNA vaccine immunogenicity in humans.

Given such clinical results, understanding the underlying mechanism(s) of how DNA vaccines function is remains as a key step for its success. Recent studies investigating the immunological role of DNA-sensing machinery have brought new interest to the DNA vaccine field. It was demonstrated that the presence of double-stranded DNA in the cytoplasm of mammalian cells can trigger host immune responses by a mechanism involving as yet unidentified cytosolic DNA sensor(s).[7] This is expected to have an important impact on future researches on the cytosolic DNA-sensing machinery and on technological advance of delivery and processing of DNA plasmid.[8] In this review, we discuss research developments in the understanding of cytosolic DNA sensing and its implications for development of DNA vaccines.

Immune Responses Initiated by DNA Vaccination

A DNA vaccine is composed of a bacterial plasmid that encodes the protein of interest (an antigen) under a mammalian promoter enabling it to function in the transfected mammalian cells. DNA vaccination could trigger immune responses through (1) the antigen(s) encoded by the DNA plasmid, which is the main component of the vaccine and (2) the transfected DNA plasmid itself (which has possible unwanted or unexpected, but surprising helper [adjuvant] activity of vaccines—to be discussed later).

Antigen(s) encoded by DNA plasmid. As soon as the plasmid DNA is administered in vivo, the encoded protein is expressed in the host cells under the control of the mammalian promoter. In the general understanding, expressed proteins can be processed as peptides, bind to MHC class I or class II molecules and be presented by antigen presenting cells (APCs) such as dendritic cells (DCs) to activate (prime) naïve T cells. Alternatively, the expressed, secreted proteins can be processed to activate B cells for antibody production. The plasmid DNA, administered by various methods, is directly transfected into resident stromal cells (e.g., muscle cells) at the injected site,

and can be directly delivered and transfected into APCs such as DCs.[9] If plasmid DNA is taken up and the antigen directly expressed and processed in, and/or secreted by, DCs, then that would lead to direct presentation of the encoded antigen(s) to CD8+ and/or CD4+ T cells, respectively. On the other hand, if plasmid DNA is introduced into stromal cells at the injected site such as muscle cells, the antigen may be indirectly captured by DCs after release from transfected stromal cells and then cross-presented to CD8+ T cells. It is also well accepted that the expressed antigen can be captured by B cells to become antigen-specific B cells with the help of CD4 Th1 or possibly follicular helper T (Tfh) cells. Therefore, it is obvious that the mode and the amount of expression of the encoded antigen(s) are critical factors for the DNA vaccine immunogenicity. In this regard, various transfection modalities have been introduced, a promising one of which was the electroporation of plasmid DNA in vivo.[10,11] Immune responses initiated by DNA vaccination are summarized in the figure.

The transfected DNA plasmid. Recognition of foreign DNA (i.e., of bacterial origin) has been well-studied by now that foreign DNA can bind to TLR9 in the endosome and stimulate the production of type I IFNs in DCs. However, recent studies have additionally shown that transfected double-stranded DNA into cytosol (and/or nucleus) is also immunogenic.[7] If introduced into the cytosol, double-stranded (ds)DNA derived from host cells (mainly in the B-form, a right-handed helical structure), can induce immune responses in fibroblasts, macrophages and DCs to produce robust amounts of type I IFNs. This effect is independent of the CpG motifs and TLR9, but completely dependent on TRAF-family-member-associated NF-κB activator (TANK) binding kinase 1 (TBK1).[7] Interestingly, similar to the dsDNA sensing pathway, DNA plasmids, although of bacterial origin, also interact with an un-identified cytosolic DNA sensor and induce the activation of TBK1 and IκB kinase-ε (IKKε) through STING, (stimulator of interferon genes, also called MITA, ERIS or TMEM173).[9,12] Hence, it was proposed that the recognition of the double-stranded backbone of cytosolic plasmid DNA by as yet un-identified cytosolic DNA sensors could contribute (i.e., have adjuvant activity) to enhanced adaptive immune responses induced by DNA plasmids.

Recent studies, on the other hand, have addressed the recognition of cytosolic DNA and the roles of DNA-mediated secondary products. In the light of these new studies of the mechanisms by which cytosolic dsDNA and possibly DNA plasmid are sensed, we will look into the details of cytosolic DNA sensors.

Update on Cytosolic Double Stranded (ds)-DNA-Recognition Sensors

Nucleic acids, namely DNA and RNA, are composed of nucleotide chains that convey genetic information important for all living organisms. Normally, these genetic molecules are metabolized or recycled and are subsequently re-used. Several endonucleases, phosphodiesterases and nucleoside phosphorylases work together to metabolize nucleic acids into polynucleotides, oligonucleotides and free nucleosides and finally into ribose-1-P and free bases to be re-utilized.[13] Nucleic acid-derived purine bases are further catabolized into uric acid. Malfunctioning of nucleic acid utility/recycling may occur for various reasons. Therefore, even though nucleic acids are known to be immunologically inert during homeostasis, it should be noted that once released from microbes or damaged host cells, nucleic acids, together with their excess metabolites, could be detected by the immune system and result in disease manifestations.[13] Recent research has been heavily focused on the sensors and mechanisms involved in recognition of DNA abnormally present in cytoplasm. Below is a summary of recently identified "potential" DNA sensors.

ZBP1/DAI. Z-DNA binding protein 1 (ZBP1), also called DNA-dependent activator of IFN-regulatory factors (DAI) or DLM-1, was found to bind directly to dsDNA and enhance its association with IRF3 and TBK1, resulting in the DNA-mediated activation of innate immune responses in vitro.[14] However, because the role of DAI in DNA-induced IFN production is very cell-type specific and DAI-deficient mice induced normal type I IFN responses to dsDNA as well as plasmid DNA vaccinations, DAI's role in innate signaling in humans has been questioned and remains to be further clarified.[9,15]

AIM2. Absence in melanoma 2 (AIM2), an IFN-inducible gene (HIN)-200 family member, was identified as a cytosolic DNA sensor for activation of inflammasomes by inducing the apoptotic speck protein containing a caspase recruitment domain (ASC)/caspase-1-mediated secretion of IL-1β. However, the AIM2 inflammasome is only essential for caspase-1 activation, but not for type I IFN production in response to cytosolic dsDNA.[16-19]

IFI16. Interferon-gamma inducible protein 16 (also called p204) is a member of the PYHIN protein family that contains a pyrin domain and two DNA-binding HIN domains. Reports have suggested that IFI16 depletion by RNA interference reduces the induction of type I IFN by synthetic DNA as well as DNA.[20]

RNA polymerase III. RNA polymerase III is an enzyme responsible for the transcription of DNA, and synthesizes various ribosomal and small RNAs in eukaryotes. It was recently shown that cytosolic poly(dA-dT) DNA are converted into RNA species (namely 5'-triphosphate RNA) by RNA polymerase III and in turn induce RIG-I-mediated type I IFN production. Therefore, although RIG-I is a cytosolic RNA receptor, RIG-I knockdown was associated with reduction in dsDNA-induced type I IFN production.[21]

HMGB. High-mobility group protein B1 (HMGB1) is normally localized in the nucleus. It has high DNA-binding capacity and recruits other proteins to the DNA-HMGB1 complex, functioning as a transcription regulator. Once HMGB1 is released from the nucleus and is present extracellularly, it can activate the immune system and might be responsible for various autoimmune diseases.[22] It was shown that HMGBs (HMGB1, HMGB2, and HMGB3) can bind to pathogen-derived nucleic acids, and that knockdown of HMGBs in mice is responsible for defective type I IFN and inflammatory cytokine induction by cytosolic DNA.[23]

Histone H2B. Histones are essential basic proteins that help to form chromatin structure by association with DNA in the

nucleus. Histone H2B, which mainly localizes to the nucleus, recognizes aberrant genomic DNA from damaged cells or cytosolic dsDNA generated by DNA viruses and activates TBK1/IRF3 axis to produce type I IFN.[24]

STING. Upon stimulation by various cytoplasmic dsDNAs, an endoplasmic reticulum (ER)-localized molecule, STING re-localizes with TBK1 from ER to perinuclear vesicles and activates IRF3/IRF7 to stimulate type I IFN production and NF-κB activation.[25] Recent studies have reported that STING could also sense bacterial secondary messenger molecules such as c-di-GMP or c-di-AMP.[26] This raises the important possibility that cytosolic dsDNA stimulation might produce secondary products such as c-di-GMP/c-di-AMP that might be recognized by STING.

TRIM56. Another interferon-inducible molecule, tripartite-motif 56 (TRIM56), an interferon-inducible E3 ubiquitin ligase, was recently identified as a modulator of STING dimerization upstream of TBK1 and to confer dsDNA-mediated type I IFN responses.[27]

DDX41. DDX41 is a member of the DEXDc family of helicases. It was found to sense intracellular DNA in myeloid dendritic cells (mDCs) and to be responsible for type I IFN and cytokine responses to cytosolic DNA and DNA viruses with direct co-localization with STING in the cytosol.[28] In addition, dsDNA from bacteria (small bacterial nucleic acids called cyclic dinucleotides) together with cyclic di-GMP and cyclic di-AMP were found to bind to central DEAD-box domain (DEADc), albeit not directly to STING.[28,29] Further studies are needed to understand the interactions between DDX41, dsDNA, cyclic dinucleotides and STING.[30]

cGAS. Recently, an enzyme in mammalian cells called cGAS was found to directly bind to transfected dsDNA and viral DNA. cGAS is a cyclic guanosine monophosphate-adenosine monophosphate (cGAMP) synthase that functions in the cytoplasm by direct binding to dsDNA. As a result, cGAMP is synthesized, binds directly to STING and induces transcription factor IRF3 and the induction of type I IFN in a STING-dependent manner, suggesting that cGAS is a cytosolic DNA sensor.[31]

DNA Sensing Machinery vs. DNA Plasmid-Mediated Immunogenicity

To clarify the role of various dsDNA-sensing/signaling molecules on the immunogenicity of DNA vaccines, several groups have taken advantage of knockout mice to investigate the innate as well as adaptive responses evoked by DNA plasmids (**Table 1** summarizes the current knowledge on the dsDNA-sensing machinery and its known impact on the mechanism of DNA vaccination). The main discovery was that transmembrane-localized foreign DNA receptor, TLR9, had minimal involvement in the activity of plasmid DNA in vivo.[32-34] Rather, optimal DNA vaccine immunogenicity (antigen-specific T and B cell induction) required type I IFNs, with clear evidence of little or no activity of such immunogenicity in IFNαR2-deficient mice.[9] Overall, TLR signaling may play little or no role, as MyD88/
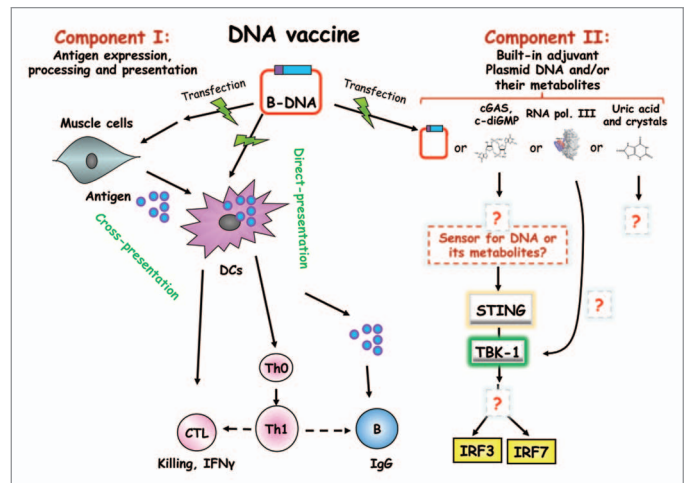

Figure 1. Current understanding of the mechanism of action of DNA vaccines. DNA plasmid transfection into the cytosol (i.e., by electroporation) induces innate and adaptive immune responses by two components; Component I: Encoded protein (or polypeptide) antigen(s) by the DNA plasmid can be expressed in stromal cells (i.e., muscle cells) as well as DCs, where these antigens are processed and presented to naïve CD4 or CD8 T cells either by direct or cross presentation, respectively. Component II: the transfected DNA plasmid and its metabolites (such as cGAS, c-diGMP, RNA polymerase III, and uric acid) interact with an unidentified cytosolic DNA receptor and activate the STING-TBK1-IRF pathway, with production of type I IFNs or some other unknown functions that act as a built-in adjuvant.

TRIF-double deficient mice showed normal responses to DNA vaccines. However, TBK1 was the major player in DNA vaccine immunogenicity.[9] Importantly, studies with STING-deficient mice confirmed that STING was a critical molecule for immunogenicity of DNA plasmids.[12] Collectively, these studies strongly suggest that STING and TBK1, the critical components of the DNA recognition machinery, are also important for DNA vaccine-induced immunogenicity. DNA vaccination requires TBK1 activation in hematopoietic cells (i.e., DCs) for Th1 and B cell responses to the encoded antigen, while TBK1 activity in non-hematopoietic cells (i.e., muscle cells), not hematopoietic cells, is essential for CD8+ T cell activation.[9] Direct presentation and cross-presentation of the encoded antigens via hematopoietic and non-hematopoietic cells differentially contribute to the generation of adaptive immune responses to DNA vaccines, and that STING-TBK1-dependent type I IFN is required to promote both responses (**Fig. 1**).[8,9,34]

ZBP1/DAI and its function as an intracellular DNA-sensor is controversial because of the differing cell type-dependent requirements in mouse and human cells. Nevertheless, DNA vaccine immunizations of ZBP1/DAI-deficient mice revealed a minimal role for ZBP-1 in innate or adaptive immune responses to DNA vaccine in vivo.[9]

Earlier reports successfully showed that DNA vaccine immunogenicity could be improved by incorporation of IL-1 and caspases into the plasmid backbone.[35,36] However, it is less likely that the inflammasome components AIM2 and ASC, which are important components for cytosolic DNA-mediated

Table 1. Current known signaling molecules in the DNA vaccine-induced immune responses

Knockout mice	DNA vaccine-					Reference
	Antigen	Route	Ab responses	CD4+ T cells	CD8+ T cells	
TLR9	LacZ	i.m e.p.	→	→	→	[9]
MyD88/TRIF	LacZ	i.m e.p.	→	→	→	[9]
ZBP-1 (DAI)	LacZ	i.m e.p.	→	→	→	[9]
STING	OVA	i.m e.p.	↓	↓	↓	[12]
TBK1	LacZ NP	i.m e.p.	↓	↓	↓	[9]
IRF3	OVA-Luc	i.m.*	→	↓	↓	[37]
IFNαR2	LacZ	i.m. e.p.	↓	↓	↓	[9]

*This data is available only by i.m. route of immunization which might be different than i.m. e.p. immunization.

IL1β responses, are required for DNA vaccine immunogenicity (unpublished observations).

Currently no data are available on the possible involvement of IFI16, HMGBs, TRIM56 or DDX41 in the mechanism of DNA vaccines, which should be explored in the future by using available mice that are deficient for those molecules.

In the down-stream signaling of the STING-TBK1 complex, IRF3-deficient mice elicited strong antigen-specific humoral responses after DNA vaccination, while CD4+ and CD8+ T cell responses (including the production of Th1, Th2 and Th17 cytokines) were severely impaired (**Table 1**).[37]

Cytosolic DNA Sensing Machinery as a Genetic Adjuvant

The discoveries in which cytosolic plasmid DNA-induced immunogenicity was attributed to its adjuvant properties (mediated via STING and TBK1 kinase) have led researchers to evaluate whether such immunogenicity could be improved by incorporating overexpression of these signaling molecules as an external adjuvant to DNA vaccines. Several incorporation techniques (i.e., in the plasmid backbone or by co-immunization) have been challenged. The pioneering studies have shown that although dispensable for the immune signaling of DNA vaccines, adaptor molecules such as the Myeloid Differentiation Primary Response Gene (MyD88) or Toll/IL-1 receptor (TIR)-domain-containing adaptor inducing interferon-β (TRIF), if incorporated into DNA vaccines as dual-promoter plasmids and overexpressed, greatly enhance humoral as well as cellular responses to given antigen.[38]

A similar approach was taken with RIG-I and MDA5, although IPS-1-deficiency has not played a role in the immunogenicity of DNA vaccines.[9] RIG-I is upstream of IPS-1, and can also recognize dsDNA by involving DNA-dependent RNA polymerase III.[21] Based on this, Luke et al. have generated DNA vaccines co-expressing antigen and potent RIG-I ligands from RNA polymerase III promoters as RIG-I agonists.[39] The resultant vectors potently induced type I IFN production and increased influenza-specific serum antibody binding avidity of DNA vaccines in mice. Co-administration of influenza-HA plasmid and plasmid DNA for MDA5 expression resulted in enhanced immunogenicity as well as protection against a lethal H5N1 challenge infection in chickens.[40]

Incorporation of HMGB1 into DNA plasmids as a genetic adjuvant successfully enhanced adaptive immune responses against HIV-1 as well as influenza viruses with greater neutralizing antibody responses against lethal influenza virus challenge.[41,42]

Although ZBP1/DAI has no role in the immunogenicity of DNA vaccines, it has been shown to have a role as a genetic adjuvant.[43] A recent study has evaluated transcription factors of the IRF family (IRF-1, IRF-3, and IRF-7) as adjuvants to DNA vaccines. Plasmid DNA encoding IRF-1, but not IRF-3 or IRF-7, enhanced antigen-specific immune responses.[44] A cocktail of TBK1-encoding plasmids with plasmids encoding Plasmodium antigens might be able to improve humoral responses to malarial antigens.[34]

DNA Plasmid Metabolites: An Adjuvant?

Several studies have independently shown that synthetic non-coding dsDNA as well as genetic material from dying cells could improve the immunogenicity of protein-based vaccines and act mainly as adjuvants.[8,45,46] However, the studies also suggest that other by-products resulting from DNA metabolism could be involved in increasing the immunogenicity of the vaccines.[13] Currently there is no clear evidence as to whether in vivo transfection of plasmid DNA could release metabolites that are capable of inducing type I IFN production, however, recent discoveries have revealed that several DNA metabolites such as cGAMP or cyclic-diGMP (c-diGMP) can be immunogenic and can interact with DNA sensing machinery such as STING.

Cyclic-di-GMP (c-diGMP) is a small nucleotide second messenger synthesized by bacteria. When c-diGMP is transfected into mammalian cells, it strongly induces high levels of type I IFN,[47] utilizing the same signaling pathway as dsDNA by direct binding to DDX41 and STING and activating downstream TBK1-IRF3 signaling.[28,29,48] c-diGMP was studied for this immunogenic property, and shown to be an effective adjuvant for vaccines targeting the mucosal route.[49,50]

As mentioned earlier, not only bacterial DNA metabolites, but transfected dsDNA or viral DNA can be catalyzed in the cytoplasm by a directly-bound enzyme called cGAS.[31] As a result, cGAMP is synthesized and directly binds to STING. Although currently no study has reported it, cGAMP is expected to have adjuvant properties.

Uric acid is a nucleic acid metabolite of degraded purine nucleotides. However, aberrant cell death under certain conditions could lead increased uric acid levels and DC activation.[51] Un-excreted crystalized uric acid results in disease like gout. Uric acid crystals, but not soluble uric acid, could function as adjuvant (unpublished observation). However, it is unclear whether DNA plasmids produce uric acid and/or crystals in the transfected cells.

These studies have revealed that not only DNA, but its metabolites could target DNA sensing machinery, intrigued a hypothesis that synthetic compounds that target the DNA sensing pathway might be capable of inducing type I IFN responses and may function as an adjuvant. 5,6-dimethylxanthenone-4-acetic acid (DMXAA) is a good example. DMXAA is a small synthetic molecule and a potent type I IFN inducer that resembles viral infections and dsDNA in the inflammatory signaling events it triggers.[52] Furthermore, DMXAA utilizes the TBK1-IRF3 signaling pathway without the involvement of TLRs or RNA helicases in its mechanism of type I IFN induction. Experience with DMXAA as adjuvant in protein immunizations suggest that it could indeed act as a vaccine adjuvant. Its unique property as a soluble innate immune activator and its adjuvant effect are directly dependent on the IRF3-mediated production of type I IFNs.[53] Of note, very recently another known anti-viral compound,

10-carboxymethyl-9-acridanone (CMA) was highlighted as potent type I IFN inducer via the STING-TBK1-IRF3 axis.[54] Interestingly, both DMXAA and CMA have a species-specific mode of action in binding to the STING pocket where c-diGMP also binds. Perhaps that is a clue to future understanding of the immunogenicity of DNA vaccines in different species.[54,55]

Overall, our understanding of how DNA vaccines might function is continuously improving along with recent developments in the investigation of the DNA sensors. Discovery of adjuvant properties of DMXAA, a compound with high similarity to DNA-mediated metabolites, is a promising advance in the field of DNA vaccines and adjuvants and for the generation of successful vaccines.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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