Using DNA Immunization to Elicit Monoclonal Antibodies in Mice, Rabbits, and Humans

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In recent vaccine studies, DNA immunization was found to effectively stimulate both innate and adaptive immunities to elicit high levels of antigen-specific antibody responses. The DNA molecule itself can activate multiple pathways of innate immunity. The *in vivo* production of antigens allows for presentation by major histocompatibility complexes, so that T-cell responses are generated to help in the development of antigen-specific B cells, leading to high-affinity antibody response. By using the same process, DNA immunization should also be effective at producing functionally potent monoclonal antibodies (mAbs). Furthermore, the *in vivo* expressed proteins can maximally maintain the native structures and go through appropriate post-transcriptional modifications. By combining such advantages, DNA immunization can be expected to play more important roles in the future to elicit mAbs against difficult targets from a wide range of host systems. The current report shares our group's experience in using DNA immunization to elicit mAbs in the mouse, rabbit, and human models.

Keywords: DNA immunization, monoclonal antibody, membrane bound protein, endogenous expression

INTRODUCTION

MONOCLONAL ANTIBODIES (MABS) HAVE BEEN widely used for the diagnosis and the treatment of various diseases, including cancers, autoimmune diseases, cardiovascular diseases, and infections. Recently, the identification of new classes of molecular targets such as the T-cell-regulating immune checkpoints (e.g., cytotoxic T-lymphocyte associated protein 4 [CTLA4] and Programmed cell death-1 [PD-1]), and the subsequent development of mAbs, including ipilimumab, pembrolizumab, and nivolumab, against such targets are revolutionizing the outlook for cancer treatment. In addition, mAbs are critical components in novel therapeutic chimeric antigen receptor T-cell therapy and antibody–drug conjugate, which further demonstrate the promise and wide utility of mAb-based therapies.

Although mAbs can be derived from display platforms using nonimmunized host libraries, the majority of mAbs, including many licensed mAb drugs, were developed through traditional approaches of immunizing animals with protein or peptide antigens. While such immunization approaches

have been generally successful, they do not always work, especially when the antigens have complex structures, such as G protein–coupled receptors (GPCRs) and other membrane proteins. In the case of GPCR, although the use of synthetic peptides, larger protein fragments, and purified receptor forms have yielded some GPCR-targeting antibodies, it is common to only obtain antibodies that bind to linear peptide epitopes or certain extracellular epitopes. These antibodies have no effect on receptor function and are therefore of limited utility as therapeutic agents.¹ Various adjuvants are usually needed to enhance the immunogenicity to protein antigens, but the conformational nature of such targets remains a challenge for eliciting highly specific mAbs. Recently, DNA immunization has emerged as a new platform for eliciting mAbs against challenging targets.² DNA immunization is particularly useful to the *in vivo* expression of structurally native full-length proteins in the membrane-bound state, such as GPCRs, providing an attractive alternative for generating mAbs against membrane proteins.³

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In this review, we summarize current knowledge on how DNA immunization can contribute to the induction of high-affinity antibody responses. More significantly, our own experience in using DNA immunization to elicit mAbs in three different host systems (mouse, rabbit, and human) is presented to stimulate further interest in this exciting new application of DNA immunization.

UPDATED UNDERSTANDING ON THE MECHANISMS OF DNA IMMUNIZATION TO INDUCE ANTIGEN-SPECIFIC ANTIBODY RESPONSES

DNA immunization delivers to the hosts a plasmid coding for a specific protein antigen that will be produced *in vivo*. Such an antigen will then stimulate the B cells as in traditional vaccination using protein or peptide antigens. However, recent studies have found that endogenously expressed and presented antigens by the DNA immunization approach have a much better chance than exogenous antigens (traditional protein or peptide immunizations) to access both class I and class II major histocompatibility complex (MHC) molecules. As a result, both CD4⁺ and CD8⁺ T-cell responses are elicited.³ The original objective in developing DNA vaccines was mainly to induce improved T-cell responses because the traditional immunization approaches were unable to do so. However, it became apparent that the CD4⁺ T helper cells elicited by DNA immunization also strongly enhance the development of B-cell responses, including the activation of follicular T helper (T_{fh}) cells and an improved germinal center (GC) B-cell development, resulting in robust antibody responses.⁴ We further demonstrated that DNA immunization can use multiple innate immunity pathways to influence the development of antigen-specific B cells and high-avidity antibodies.^{4–6} Below is a brief review of key findings in this area.

DNA immunization and innate immunity

Since the discovery of DNA vaccines more than 20 years ago, it has been well documented that DNA vaccines can function as adjuvants, serving as the agonist for TLR9, a key member of the Toll-Like-Receptor (TLR) family, mainly by the CpG motifs of DNA vaccines as the ligand of TLR-9. However, our recent findings revealed the involvement of previously unrecognized innate immune response pathways, in addition to the TLR9 pathway.

The absent in melanoma (Aim2) inflammasome controls maturation of the proinflammatory cytokines interleukin (IL)-1 β and IL-18 and an in-

flammatory form of cell death called pyroptosis. Our results showed that DNA immunization elicited significantly reduced antigen-specific humoral and cellular responses in Aim2-deficient mice. The involvement of Aim2 pathway in DNA immunization is not IL-1 β /IL-18 dependent, but pyroptotic cell death was clearly involved in the immunogenicity of DNA vaccines.⁴

Our data also demonstrated the involvement of another innate immune response pathway, stimulator of interferon gene (STING), in DNA immunization. DNA vaccine induced Irf7-dependent signaling, as part of the STING pathway, which was critical for generation of both innate cytokine signaling and antigen-specific B- and T-cell responses. In contrast, the Irf3 molecule in this pathway was not as critical as expected for DNA immunization. Unexpectedly, immune responses elicited by DNA vaccines were not cGas-dependent *in vivo* in our study.⁵

The above data indicate a much broader involvement of innate immunity pathways in DNA immunization. Our work directly linked the acquired immunity (antigen-specific immune responses) with innate immunity, and we discovered unique molecular mechanisms of these innate immunity pathways for DNA immunization. More studies are needed to fully understand how innate and acquired immunities work together in developing antigen-specific responses. Table 1 summarizes three innate immunity pathways involved in DNA immunization based on our study.

DNA immunization can effectively activate follicular T helper and GC B cells

The importance of T_{fh} cells and GC B-cell reaction in the humoral response has been highlighted in recent years.⁷ In our published study, we compared the levels of T_{fh} cells and the GC B cells between mice immunized with the DNA prime-protein boost and those with the protein prime-protein boost approaches using HIV-1 gp120 as a model antigen. Priming mice with gp120 DNA and

Table 1. Innate immunity pathways involved in DNA immunization

	Innate receptor/ innate pathway	Ligand	Mechanisms/ mediators
Pathway 1	TLR-9	CpG	MyD88, IKK β , IFN α/β
Pathway 2	STING	?? (cGas independent)	TBK1, IRF3, IRF7, IFN α/β
Pathway 3	Aim2	DNA molecules	Inflammasome, pyroptosis, genomic DNA

Aim2, absent in melanoma 2; IFN α/β , interferon; α/β IKK β , inhibitor of kappa-B kinase β ; IRF, interferon regulatory transcription factor; MyD88, myeloid differentiation primary response 88; STING, stimulator of interferon gene; TBK1, TANK binding kinase 1; TLR-9, Toll-like receptor 9.

boosting with gp120 protein induced high titers of high-affinity and cross-reactive anti-gp120 antibodies, while gp120 protein immunogen alone was unable to achieve such response levels.⁸ Furthermore, priming with gp120 DNA increased Tfh cell differentiation, and induced higher numbers of GC B cells, subsequently leading to more memory B cells and higher antigen-specific antibody titers, compared to priming with gp120 protein.² This mechanism would explain why we observed high-titer anti-gp120 antibody responses in a Phase I clinical trial receiving the DNA prime-protein boost immunization.⁹ Such findings also open the possibility to using the DNA immunization approach for the induction of a high-affinity antigen-specific B-cell development for the generation of mAbs.

ADVANTAGES OF USING DNA IMMUNIZATION TO PRODUCE MABS

In addition to the above immunological mechanisms that support the use of DNA immunization, DNA immunization provides other advantages over the traditional protein-based immunization approaches. First, DNA vaccines can be more effective than the protein vaccines at inducing antigen-specific B-cell maturation with high-avidity or high-affinity antibodies as described above.^{4,8} High affinity is a much desired parameter for a therapeutic mAb. Second, DNA vaccines enable immunogen design flexibility (“antigen engineering”) in which multiple sequence variations can be tested to select antigens with improved immunogenicity. Such a process can also be used to expose hidden epitopes to increase epitope diversity.⁸ Third, DNA immunization does not need *in vitro* production of protein antigens, which is time-consuming, potentially costly, and sometimes difficult to accomplish, especially for multi-pass membrane proteins (GPCRs and ion channels). Finally, *in vivo* expressed proteins can maximally maintain the native conformation and unique structures that are formed by various posttranslational modifications, such as glycosylation. The combination of these features contributes to the final induction of high-affinity antibodies against the natural conformation of the target antigens and establishes the basis for isolating desired high-quality and functional mAbs.

KEY CONSIDERATIONS IN DNA IMMUNIZATION

Construction of DNA vaccines

DNA vaccines are constructed to express desired proteins in a mammalian system. Both the selection of expression vector and the design of antigen

inserts are important for the final antibody responses as we previously described.¹⁰ The following text highlights key technical considerations for the vector and the inserts.

Choice of expression vectors. In the last two decades many research groups were involved in optimizing the design of commonly used DNA vaccine vectors. The promoter of a DNA vaccine vector has been established as the most critical component for driving the overall expression of the immunogens. The cytomegalovirus (CMV) promoter drives transient antigen expression very efficiently and has been widely used as part of many different DNA vectors. However, other promoters that drive constitutive antigen expression may have the potential to induce better immune responses than the CMV promoter.¹¹ The function of promoters can be enhanced by other regulatory components in the vector. The CMV intron A sequence can significantly increase the efficacy of a CMV promoter.¹⁰ Selection of a poly A tail may affect the stability of transcripts and the final amount of expressed antigens. Codon optimization is extremely important for DNA vaccines expressing antigens from various pathogens that often have different codon usage from mammalian hosts.

Design of immunogen inserts. The DNA immunization approach provides a wide range of options to produce designed antigen inserts for the induction of mAbs against even the most difficult targets. Immunogen inserts can be the full-length sequences of target proteins, or only the extracellular domain of a membrane protein, or even “designer proteins,” which are modified from the original coding sequences. For example, in the case of a bacterial toxin, a truncated toxin antigen can be used for immunization instead of a potentially lethal full-length toxin protein, thereby eliminating unwanted biological activity during DNA vaccine production and animal immunization.^{12,13}

Delivery approach

Since the early 1990s, a wide range of DNA immunization delivery approaches have been evaluated. These approaches can be divided into two major categories. One is the conventional needle injection of DNA plasmids dissolved in various buffers. Optional facilitating agents such as lipids and nanoparticles can be added to the solution to improve delivery efficacy. The formulation of the chemical solution determines the delivery efficacy of the DNA vaccines. The other major delivery approach is based on the external physical forces. The

most well-known approach is the gene gun, which uses a “ballistic” force to deliver DNA plasmids. The DNA plasmids are coated on gold particles, which are then delivered by the ballistic force to penetrate the cells of the targeted tissues.^{14,15} Another physical approach of DNA plasmid delivery is electroporation. With this approach, DNA vaccine plasmids are first delivered by the regular needle injection, followed by an electrical current at the injection site to enhance the entry of DNA vaccines into host cells.

We conducted a side-by-side study comparing the efficacy of DNA vaccine delivery among intramuscular (IM) needle injection, electroporation (EP) following IM injection, and gene gun (GG) alone.¹⁶ Our results showed that GG and EP delivery methods were more effective than IM injection at eliciting higher antibody responses.¹⁶ Table 2 summarizes various DNA vaccine delivery approaches.

DNA prime and protein boost strategy

The heterologous prime-boost approach promoted by our group presents a great advancement for the induction of high-titer and highly functional antibodies. With this approach, the DNA plasmids are first delivered as the priming vaccination, followed by a protein boost, which can be in the form of proteins, peptides, or inactivated or live attenuated vaccines. Our studies have shown that DNA priming is more effective than protein priming in activating GC B cells.⁴ Higher levels of antigen-specific B cells induced by DNA vaccines establish the basis for more robust antibody responses upon a boost using the matching antigens but in a different vaccine modality. This prime-boost approach was highly effective in generating high-titer and high-avidity mAbs in mouse, rabbit, and human.

Table 2. DNA vaccine delivery approaches

<i>Delivery mechanisms</i>		<i>Dose ranges, μg</i>	<i>Efficacy</i>
Chemical delivery			
Needle injection	In regular aqueous solutions such as PBS and saline (may mix with lipids, polymer, nanoparticles, etc.)	≥ 100	Low immunogenicity
Physical delivery			
Electroporation	Electrical field to increase cell permeability	40–100	High immunogenicity (high individual variation)
Gene gun	High-power shockwave (gold particle penetration)	1–10	High immunogenicity (low individual variation)

The following sections provide an example of a mAb that was induced by this approach from each of three types of hosts.

DNA IMMUNIZATION TO INDUCE MABS IN MICE, RABBITS, AND HUMANS

Mouse mAbs

Mouse is the most commonly used animal model for the generation of mAbs. The first report to demonstrate the utility of DNA immunization as a method to induce mAbs was also demonstrated in mice in the year 1994.¹⁷ Over the past two decades, different research groups have accumulated significant experience in optimizing key steps to improve the induction of mAbs, such as promotor selection, insert design, delivery approach and schedule, and application of molecular adjuvants, which we recently reviewed with a primary focus on mouse studies.²

By using the DNA immunization approach, our group has produced a long list of mouse mAbs. These mAbs were developed against a wide range of targets; several of them were designed against *Clostridium difficile* (*C. difficile*) toxins. *Clostridium difficile* is one of the most common causes of nosocomial infections among hospitalized patients, especially those with malignancy or aging.¹⁸ *Clostridium difficile* Toxin A and Toxin B are key virulence factors. The use of antibodies to detect the presence of both Toxin A and Toxin B in patient stool samples is a common diagnostic method. Among commercially available *C. difficile* toxin detection assays, either goat or rabbit polyclonal antibodies (pAbs) have been predominantly used to achieve enough detection sensitivity. Polyclonal antibodies in general have a high avidity by binding to the multiple epitopes of the target, but they are prone to batch-to-batch variability and non-specific background binding as compared to mAbs. A great need exists for generating high-affinity mAbs for the diagnosis of *C. difficile* infection.

Using the DNA immunization approach, we produced a panel of mouse mAbs against Toxin A¹³ and Toxin B (Table 3). Those mAbs targeted different epitopes on Toxin A and Toxin B with high binding affinity. After an extensive testing to pair those mAbs for detection of the toxins, we successfully identified a mAb-mAb pair for the detection of Toxin A and a mAb-pAb pair for the detection of Toxin B. This mAb-based detection assay showed equal or better sensitivity compared with the three major detection kits on the market (Table 3). Moreover, several mAbs specific to Toxin A showed functional activity on both *in vitro* cell

Table 3. The antibody pairs for detection of *Clostridium difficile* toxin A and toxin B

Manufacturer	Toxin A			Toxin B		
	Capture Ab	Detector Ab	Detecting sensitivity, ng/mL	Capture Ab	Detector Ab	Detecting sensitivity, ng/mL
Vendor A	Mouse mAb	Goat pAb	≥1.4	Goat pAb	Goat pAb	≥2.4
Vendor B	Goat pAb	Mouse mAb	≥0.8	Goat pAb	Goat pAb	≥2.5
Vendor C	Mouse mAb	Goat pAb	≥0.2	Rabbit pAb	Rabbit pAb	≥0.6
Authors' data	mAb 5D8	mAb 2C7	≤0.78	mAb 7E2	Rabbit pAb	≤0.75

mAb, monoclonal antibody; pAb, polyclonal antibody.

protection and *in vivo* protection against a lethal challenge.¹³ They can serve as the candidates for therapeutic applications.

Rabbit mAbs

Rabbit pAbs have been widely used in biomedical research, such as immunohistochemistry, Western blotting, and flow cytometry. Although no therapeutic rabbit mAbs have been approved by the U.S. Food and Drug Administration (FDA) thus far, rabbit mAbs are FDA-approved for *in vitro* diagnostic uses in the clinic.¹⁹ Most of these rabbit mAbs are used to assess the expression of tumor-associated antigens, such as PD-L1, progesterone receptors, estrogen receptors, and HER2. In addition, several therapeutic mAbs from rabbits are currently at various stages of clinical trials, including APX005M, a humanized rabbit anti-human CD40 mAb (NCT02482168); sevacizumab, a humanized rabbit anti-human vascular endothelial growth factor mAb (NCT02453464); and YYB101, a humanized rabbit anti-human HGF mAb.

Compared to producing mAbs by DNA immunization in the mouse, far fewer studies have been reported using this approach for the induction of rabbit mAbs. Our group has conducted pioneering work in this area. Currently, the majority of published mAbs in the HIV vaccine field are isolated from HIV-1-infected people. The availability of a rabbit mAb technology platform will greatly enhance the ability to test various designs of Env immunogens to gain a better understanding of the structural features and evolution of HIV-1 Env-specific antibodies induced by AIDS vaccines before they can be tested in nonhuman primates or in actual human studies.

We produced several panels of unique mAbs from the rabbit model. The first panel of 12 rabbit mAbs was produced from one single rabbit immunized with the DNA prime and protein boost immunization regimen expressing the HIV-1 clade B JR-FL gp120 immunogen.²⁰ These rabbit mAbs covered a diverse repertoire of Env epitopes ranging from the well-characterized V3 domain to several previously less known epitopes in the C1, C4,

and C5 domains. Nine mAbs had cross-reactivity to gp120 from non-clade B isolates. At least three rabbit mAbs had neutralizing activities with different potency and breadth.^{20,21}

We published structure analysis of rabbit mAb R20 and R56; both are specific to V3 region of gp120 protein. R56 binds to the well-studied V3 crown, while R20 is against a less known epitope at the C terminus of V3 loop. By comparing the crystal structures of these two rabbit mAbs, in complex with their respective epitopes, with that of the corresponding human mAbs isolated from chronically infected HIV patients, rabbit mAbs can mimic the binding modes of human mAbs in recognizing the same immunogenic regions.²²

Another rabbit mAb R53 specifically binds to C4, the fourth conserved region of the HIV-1 Env protein. Our R53 crystal structure result suggested a masking mechanism used by HIV-1 to protect this critical domain from the human immune system and explained how this family of mAbs can block the binding of gp120 to CD4.²¹

Human mAbs

Human-derived mAbs can come from infected patients or vaccinees. In the HIV vaccine research field, most available human mAbs were generated from patients infected with HIV. Limited human mAbs have been generated from human vaccine studies. We are the only group who has used DNA immunization as part of the DNA prime-protein boost immunization regimen to generate human mAbs. In a phase I HIV vaccine clinical trial, named DP6-001,⁹ participants received priming vaccination with a polyvalent HIV-1 DNA vaccine, including six DNA plasmids (five expressing different primary gp120 antigens from clades A, B, C, and E and one expressing a clade C Gag antigen), followed by boosts with a polyvalent recombinant gp120 protein formulation (five primary gp120 antigens matching those used in the DNA prime). Using single B-cell cloning technology, we produced a panel of human HIV-1 Env-specific mAbs from four DP6-001 volunteers.²³ These mAbs recognized a broad range of primary HIV-1 Env gly-

Figure 1. Breadth of antibody-dependent cell-mediated cytotoxicity (ADCC) activity for Env-specific mAbs isolated from either DP6-001 (open bar) or RV144 (solid bar) clinical trials against different major subtypes of HIV-1. The percentage breadth was calculated using published ADCC data from DP6-001²² and RV144²⁴ trials.

coproteins across multiple major subtypes. Although these mAbs did not exhibit high neutralizing activity, they showed potent and cross clade Fc-mediated effector functions, including antibody-dependent cell-mediated cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis activities.²³

The roles of Fc receptor-mediated protective antibody responses are gaining more attention because of their potential contribution to the protection against HIV-1 infection as observed in the RV144 trial, which gave the first supporting evidence of any vaccine being effective in lowering the risk of contracting HIV.²⁴ When we compared our mAbs with the ones generated from RV144 trial in which a pox vector was used to prime the host, we observed some key differences between the two panels of mAbs. The mAbs from DP6-001 recognized more diverse epitopes, including linear and possible conformational epitopes. More signifi-

cantly, mAbs from DP6-001 displayed a greater breadth of gp120 binding and ADCC activity (Fig. 1) as compared with the mAbs isolated from RV144.²⁵ These characteristics are possibly due to the DP6-001 vaccine formulation, which included a five-valent gp120 prime and boost, while, only two Env proteins were included in the RV144 trial.

In summary, DNA immunization can effectively stimulate both innate and adaptive immunity. *In vivo* production of antigen allows for presentation by both class I and class II MHC molecules, so both CD4⁺ and CD8⁺ T-cell responses are generated to facilitate high-quality antibody responses. DNA immunization offers several unique advantages: it produces high-affinity antibody, permits *in vivo* antigen production, and bypasses the time-consuming and sometimes difficult processes of immunogen purification, especially for multi-pass membrane proteins. The proteins expressed *in vivo* can maximally maintain their native structures and go through appropriate posttranscriptional modifications. DNA immunization can be expected to play increasingly more important roles in the future with regard to the generation of high-quality mAbs from a wide range of host systems as we have demonstrated in the mouse, rabbit, and human models.

ACKNOWLEDGMENTS

This study was supported in part by National Institutes of Health grants U19AI082676, P01AI082274, and 5R21/R33AI087191.

AUTHOR DISCLOSURE

No competing financial interests exist.

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Received for publication April 18, 2018;
accepted after revision July 19, 2018.

Published online: July 19, 2018.