Published in final edited form as:

Vaccine. 2008 April 16; 26(17): 2100–2110. doi:10.1016/j.vaccine.2008.02.033.

THE RELATIVE IMMUNOGENICITY OF DNA VACCINES DELIVERED BY THE INTRAMUSCULAR NEEDLE INJECTION, ELECTROPORATION AND GENE GUN METHODS

Shixia Wang^{3,4}, Chunghua Zhang^{1,3}, Lu Zhang^{1,3}, Jun Li^{1,2,3}, Zuhu Huang^{1,2,3,#}, and Shan Lu^{1,3,4,#}

¹ Jiangsu Province Key Laboratory in Infectious Diseases, The First Affiliated Hospital of Nanjing Medical University, Nanjing, China 210029

² Department of Infectious Diseases, The First Affiliated Hospital of Nanjing Medical University, Nanjing, China 210029

³ China-US Vaccine Research Center, The First Affiliated Hospital of Nanjing Medical University, Nanjing, China 210029

⁴ Laboratory of Nucleic Acid Vaccines, Department of Medicine, University of Massachusetts Medical School, Worcester, MA 01605

Abstract

Immunogenicity of DNA vaccines varies significantly due to many factors including the inherent immunogenicity of the protein antigen encoded in the DNA vaccine, the optimal immune responses that can be achieved in different animal models and in humans with different genetic backgrounds and, to a great degree, the delivery methods used to administer the DNA vaccines. Based on published results, only the gene gun-mediated delivery approach has been able to elicit protective levels of immune responses in healthy, adult volunteers by DNA immunization alone without the use of another vaccine modality as a boost [1,2]. Recent results from animal studies suggest that electroporation is also effective in eliciting high level immune responses. However, there have been no reports to identify the similarities and differences between these two leading physical delivery methods for DNA vaccines against infectious disease targets. In the current study, we compared the relative immunogenicity of a DNA vaccine expressing a hemagglutinin (HA) antigen from an H5N1 influenza virus in two animal models (rabbit and mouse) when delivered by either intramuscular needle immunization (IM), gene gun (GG) or electroporation (EP). HA-specific antibody, T cell and B cell responses were analyzed. Our results indicate that, overall, both the GG and EP methods are more immunogenic than the IM method. However, EP and IM stimulated a Th-1 type antibody response and the antibody response to GG was Th-2 dominated. These findings provide important information for the further selection and optimization of DNA vaccine delivery methods for human applications.

#Correspondence should be addressed to, S, Lu Rm 304, Lazare Research Building,, University of Massachusetts Medical School, 364 Plantation Street, Worcester, MA 01605, USA, Phone: 1-508-856-6791, Fax: 1-508-856-6751, E-mail: shan.lu@umassmed.edu. Or Z. Huang, Department of Infectious Diseases, The First Affiliated Hospital of Nanjing Medical University, 300 Guangzhou Road, Nanjing, China 210029, Phone: 86-025-83781217, E-mail: zhhuang@public1.ptt.js.cn.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

DNA vaccine; antibody; T cell; B cell; influenza

Introduction

DNA immunization was introduced as a new method of immunization in the early 1990s. Several research groups were able to demonstrate that immunization with plasmid DNA encoding for a specific protein antigen could elicit an antigen-specific immune response [3–8]. While this vaccination technology showed promising results in eliciting both humoral and cell-mediated immune responses and generating protection against various pathogen challenges, DNA immunization using conventional needle methodologies appeared less successful in non-human primates and humans. Although more recent human studies have showed that DNA immunization via needle injection is effective in priming human immune responses [9], high DNA doses in the milligram (mg) range are usually required. On the other hand, physical methods of delivering DNA plasmids, such as a gene gun (GG) and electroporation (EP) have been shown to produce an immune response in large animals and humans using microgram (μ g) amounts of DNA [10–13].

Particle Mediated Epidermal Delivery (PMED), the commercial name for the current gene gun technology, is a needle free device which delivers gold beads coated with DNA vaccine plasmids into the epidermal layer of skin. Preclinical studies have shown that multiple plasmids can be successfully combined into one formulation [14], and that a single vaccination, in certain cases, can protect against multiple potential pathogens. This vaccine delivery method has been used in small animal studies with strong immunogenicity results [15], and it has also shown promise in larger animals, such as pigs [16] and non-human primates [17,18], and in humans against hepatitis B virus [19–22], influenza [2] and malaria [23].

Electroporation was reported for *in vitro* gene transfer (also named electroporomeabilization) as early as in 1982 [24] and has been used for optimization of *in vitro* and *ex vivo* gene transfer [25–29]. In the last decade, *in vivo* EP has been proven to be an efficient approach for delivering genes into muscle tissue [30–33]. The local (*in vivo*) delivery of multiple long square-wave electric pulses (20–30 ms) of low voltage (50–200 V/cm) shortly after the administration of naked DNA in various tissues improves transfection efficiency, resulting in a 100–1000-fold increase in protein expression [34–38]. Indeed, *in vivo* electroporation markedly enhances the effectiveness of DNA vaccination in eliciting both humoral and cellular immune responses in various animal models including mice [11,34,39,40], goats and cattle [41], and non-human primates [42]. Furthermore, DNA immunization by EP has been shown to induce prolonged primary immune responses and maintained immune memory for up to 6 months following just one DNA immunization [43,44].

The goal of the current study was to compare the relative immunogenicity between these two leading physical delivery methods of DNA vaccines, gene gun (GG) or electroporation (EP), by using a prototypic DNA vaccine which expresses a highly immunogenic antigen, the hemagglutinin (HA) antigen from an H5N1 influenza virus. The more conventional intramuscular needle immunization (IM) was included as a control. HA-specific antibody, T cell and B cell responses were analyzed in two animal models (rabbit and mouse) in order to determine the relative strength and types of immune responses produced by these various immunization methods.

RESULTS

HA-specific antibody responses in NZW rabbits

Groups of New Zealand White (NZW) rabbits were immunized with either H5 HA DNA vaccine or empty DNA vaccine vector as outlined in Table 1. The encoded HA antigen has the same amino acid sequence as that in an avian H5N1 virus isolate A/VietNam/1203/04 but the gene sequence included in this H5 HA DNA vaccine was codon optimized as previously reported [45]. Nine rabbits (3/group) received the H5 HA DNA vaccine by IM, EP or GG delivery. The dose range used in the current study was based on the reported optimal doses for each delivery method so the immune responses will not be biased due to inadequate doses of DNA vaccines. In addition, 4 immunizations were given so the peak level antibody responses, as well as the kinetics of antibody responses can be evaluated. A total of 5 rabbits received the empty DNA vector as the negative controls.

Rabbits that received the HA DNA vaccine via IM inoculation were slower to produce HA-specific antibody responses and the peak level antibodies were also lower when compared to rabbits that received the HA DNA vaccine via EP or GG inoculations (Fig. 1 and Fig. 2). The temporal HA-specific antibody responses measured by ELISA at 1:500 serum dilution showed that HA-specific antibody responses started to appear in rabbits from the IM group only after three immunizations while rabbits in the EP and GG groups produced detectable antibody responses after one to two DNA immunizations (Fig 1). GG immunization was significantly more effective than EP method in eliciting higher antibody responses ($p < 0.01$) (Fig. 2), particularly when the fact that ~ 5 times more HA DNA vaccine was used in the EP group (200 μ g) than the GG group (36 μ g) at each immunization is taken into account. As expected, rabbits that received the empty DNA vector did not have any H5 HA-specific antibody responses after 4 immunizations (Fig 1A).

HA-specific antibody responses in Balb/C mice

Four groups of Balb/C mice (5 mice/group) were immunized with either H5 DNA vaccine or empty DNA vector as shown in Table 2. Mice in Groups A, B, and C received the H5 HA DNA vaccine by IM, EP or GG immunizations, respectively. For the empty vector control group (Group D), we only included the gene gun method because it is the most immunogenic delivery approach based on the above rabbit study and thus, may be the most likely approach to elicit any non-specific immune responses. Again the doses used for different delivery approaches were based on the optimal doses reported in literature. The mice in the experimental and control GG groups received 6 μ g of DNA plasmid while those in the EP and IM groups received 100 μ g at each immunization.

As with the rabbit immunizations, mice that received the empty DNA vector did not have any H5 HA-specific responses after 4 inoculations (Fig 3). The mice that received the HA DNA vaccine via IM inoculation were slower to produce HA-specific antibody responses and produced lower responses when compared to mice that received the H5 DNA immunization via EP or GG inoculation (Fig 3). The temporal HA-specific antibody responses measured by ELISA at 1:500 serum dilution showed that HA-specific antibody responses started to appear in mice from the IM group after three (3) immunizations while the mice in the EP and GG groups produced detectable antibody responses after only 2 DNA immunizations (Fig 3). Temporal antibody curves showed that antibody responses in the GG immunized group rose earlier and higher when compared to mice in the EP group (Fig 3), although ~15 times more HA DNA vaccine was used in the EP group (100 μ g) compared to the GG group (6 μ g). However, after four immunizations, the end titers at peak level between these two approaches were very similar (Fig. 3). Both EP and GG DNA immunization induced significantly higher

HA-specific antibody titers when compared to IM DNA immunization ($p < 0.05$, for both comparisons) (Fig 3).

HA-specific antibody secreting cells (ASC) in Balb/C mice

In addition to analyzing serum HA-specific IgG responses induced by various DNA vaccination approaches, we also examined HA-specific antibody secreting cells (ASC) from splenocytes of immunized mice two weeks after the 4th DNA immunization. ELISPOT plates were coated with recombinant H5 HA proteins. Mice that received HA DNA vaccines displayed HA-specific ASC in splenocytes and the number of HA positive ASC in each group (i.e., IM, GG, EP) were in general consistent with their respective HA-specific antibody responses (Fig 4). Mice that received empty DNA vector did not display any HA-specific ASC in the splenocytes and that background levels against a mock antigen coding (PBS) on the ELISPOT plate for HA DNA vaccine groups were very low (Fig. 4). Overall, mice that received an IM immunization of the HA DNA vaccine generated significantly lower numbers of ASC in splenocytes following the 4th DNA immunization when compared to mice in the EP and GG groups ($p < 0.05$, both comparisons) (Fig 4).

HA-specific T-cell responses in Balb/C mice

In addition to the antibody responses, HA-specific T cell responses were also evaluated following HA DNA vaccination using the three different DNA delivery methods in mice. Mouse splenocytes were collected 2 weeks after the 4th DNA immunization and stimulated with the peptide IYSTVASSL, a previously reported HA-specific T cell epitope. HA peptide-specific IFN- γ secreting cells were measured by IFN- γ ELISPOT. The mice that received the HA DNA vaccine generated HA peptide-specific IFN- γ secreting cells following HA peptide stimulation (Fig. 5). Mice that received the empty DNA vector via GG immunization did not have HA peptide-specific IFN- γ spots in the splenocytes, and the background level spots by stimulation with an unrelated mock peptide were very low (Fig. 5). In general, mice in all three groups that received the HA DNA vaccine produced a high level of IFN- γ T cell responses (200~400 spot forming cells/million splenocytes) after the 4th DNA immunization. Although mice in EP and GG groups had slightly higher IFN- γ T cell responses than those in IM group, the difference was not significant (Fig. 5).

Because the above results demonstrate that all three DNA vaccination approaches induced HA peptide-specific T cell responses, we further examined if there is any difference in the types of T-helper responses among these approaches by using the IgG1/IgG2a ratio as a surrogate marker. Interestingly, the ratio of IgG1/IgG2a in mice receiving either IM or EP immunizations was much less than 1, indicating that these DNA immunization methods induced predominantly a Th1-type antibody response. However, the ratio of IgG1/IgG2a in mice receiving GG immunizations was clearly greater than 1, indicating that this DNA immunization method induced predominantly a Th2-type antibody response.

Materials and Methods

Codon optimized H5 HA DNA vaccine

The codon usage of the HA gene from influenza A human viruses H5N1 A/VietNam/1203/04 was analyzed using MacVector software 7.2 against codon preference of *Homo sapiens*. The less optimal codons in the HA genes were replaced by the preferred codons of mammalian systems in order to produce a higher expression of the HA proteins. Sequence optimization was also performed to make the mRNA more stable and the gene more favorable for transcriptional and translational processes. During the sequence optimization, the following cis-acting sequence motifs were avoided: internal TATA-boxes, chi-sites and ribosomal entry sites; AT-rich or GC-rich sequence stretches; ARE, INS, CRS sequence elements; cryptic

splice donor and acceptor sites; and branch points. Despite these DNA level sequence changes, the final codon optimized HA DNA sequences still produce the same HA amino acid sequences as in the original H5N1 virus A/VietNam/1203/04. The codon optimized HA gene was chemically synthesized by Geneart (Regensburg, Germany). With a human tissue plasminogen activator (tPA) leader sequence substituting the natural HA leader sequence, the tPA-HA gene insert was cloned into the pSW3891 vector. The DNA vaccine plasmid was produced from *Escherichia coli* (HB101 strain) with a Mega purification kit (Qiagen, Valencia, CA) for both *in vitro* transfection and *in vivo* animal immunization studies. H5 HA expression was verified by *in vitro* transfection of 293T cells and Western blot analysis, as previously described [45].

DNA immunization of New Zealand White (NZW) rabbits and BALB/c mice

NZW rabbits (~ 2 kg body weight) and BALB/c mice (6–8 weeks old) were purchased from Shanghai Animal Center, Chinese Academy of Science. Both rabbits and mice were housed in the Animal Research Center at the Nanjing Medical University in accordance with approved protocol. Three DNA vaccine immunization systems were used for rabbit and mouse immunizations as follows: 1) Intramuscular injection (IM): 200 µg (rabbits) or 100 µg (mice) of HA DNA vaccine plasmid or vector control plasmid was delivered at 2 different sites in the quadriceps muscle at each immunization. 2) Electroporation (EP): a Caliper Electrodes style electroporator (SCIENTZ-2C) from Scientz Co., Ltd (Ningbo, China) was used. Following IM injection of DNA vaccines as described above, the injection sites were electroporated once with the following parameters: 100 V, 60 ms and 60 Hz for rabbits and 50 V, 60 ms and 60 Hz for mice. 3) Gene gun immunization (GG): a Helios gene gun (Bio-Rad) was used to deliver the DNA vaccine at the shaved abdominal skin [46]. In gene gun groups, a total of 36 µg (rabbits) or 6 µg (mice) of HA DNA vaccine plasmid or vector control plasmid was delivered at each immunization. Each shot delivers 1 µg of DNA coated to gold beads (1 micron in size) at a ratio of 2 µg plasmid/mg gold.

All immunizations were given at Weeks 0, 2, 4, 6. Serum samples were taken prior to the first immunization and 2 weeks after each immunization for the study of HA-specific antibody responses. Mouse splenocytes were collected 1 week after the last DNA immunization to measure the H5 HA-specific ASC and HA peptide-specific T cell responses.

ELISA (Enzyme-linked immunosorbent assay)

An ELISA was conducted to measure the H5 HA-specific antibody (IgG) responses in immunized rabbits and mice, as previously described [45]. The 96-well flat-bottom plates were coated with 100 µl/well of transiently expressed HA antigen at 1 µg/ml from 293T cells transfected with the H5 HA DNA vaccine plasmids. After being washed 5 times, as described above, the plates were then blocked with 200 µl/well of blocking buffer (5% non-fat dry milk, 4% Whey, 0.5% Tween-20 in PBS at pH7.2) for 1 hour. After five washes, 100 µl of serially diluted rabbit or mouse serum in Whey buffer (4% Whey, 0.5% Tween-20 in PBS) was added in duplicate wells and incubated for 1 hour. To detect rabbit antibody, the plates were incubated for 1 hour at 37°C with 100 µl of biotinylated anti-rabbit (Vector Laboratories, Burlingame, CA) diluted at 1:2000 in Whey buffer. Then, 100 µl of horseradish peroxidase (HRP)-conjugated streptavidin (Vector Laboratories) diluted at 1:5000 in Whey buffer was added to each well and incubated for 1 hour. To detect the mouse antibody, a similar protocol was used except that the plates were incubated for 1 hour at 37°C with 100 µl of HRP-conjugated goat-anti-mouse IgG diluted at 1:10,000 in Whey buffer. After the final wash, the plates were developed with 3,3',5,5' Tetramethylbenzidine (TMB) solution at 100 µl per well (Sigma, St. Louis, MO) for 3.5 minutes. The reactions were stopped by adding 25 µl of 2 M H₂SO₄, and the plates were read at an OD of 450 nm. The end titration titer was determined as the highest serum dilution that has an OD reading above twice of that from the negative control serum.

IgG1 or IgG2 isotype-specific ELISA was conducted, as previously described [47]. This assay is similar to the above total IgG ELISA, with the exception that horseradish peroxidase (HRP)-conjugated goat-anti-mouse IgG1 or IgG2 (Southern Technology Associates, AL) at 1:2000 dilution was used. The concentrations for HA-specific mouse IgG1 or IgG2a were calculated from the standard curve using a known amount of purified mouse IgG.

Gamma interferon enzyme-linked immunospot (IFN- γ ELISPOT) assay

IFN- γ ELISPOT assays were performed on fresh mouse splenocytes, as previously described [48,49]. The IFN- γ ELISPOT assay for HA-specific T cells used the mouse IFN- γ ELISPOT kit from U-CyTech Biosciences, Netherland and Multiscreen Immobilon P membrane White Sterile 96-well plates (IP plates) from Millipore, MA. The assay was performed according to manufacturers' directions. Briefly, the plates were coated with 5 μ g/ml of purified rat anti-mouse IFN- γ IgG1 in PBS at 4°C overnight. After the plates were washed three times with PBS, each plate was blocked by the addition of 200 μ l of the blocking buffer in each well for 1 h at 37°C. The known H2^d mouse restricted HA epitope (IYSTVASSL) [50] was employed for measuring HA-specific T cells. This HA epitope is well-conserved in the H5 HA sequence for H5N1 A/VietNam1203/04. The non-HA peptide was from HIV Env V3 region (IGPGRAFYT) as a negative control. Peptides were purchased from AnaSpec Corp. (San Jose, CA). The peptides (final concentration 4 μ g/ml) were added to the wells with 100 μ l of freshly isolated splenocytes (500,000 cells/well in R10 medium) in duplicate. The plates were incubated for 24 h overnight at 37°C in 5% CO₂. The plates were then washed, incubated with 100 μ l of biotinylated rat anti-mouse IFN- γ Rat IgG1 (1 μ g/ml in dilution buffer in the kit), and incubated at 4°C overnight. After additional washes, 100 μ l of HRP-conjugated streptavidin complex was added to each well in above dilution buffer for 2 h at room temperature. The plates were washed, and spots representing individual IFN- γ -producing cells were detected after a 25-min color reaction using AEC coloring system. IFN- γ spot-forming cells (SFC) were counted. The results were expressed as the number of SFC per 10⁶ input cells. The number of peptide-specific IFN- γ -secreting T cells was calculated by subtracting the background (no-peptide) control value from the established SFC count.

Assays for antibody secreting cells (ASC)

IP-plates were coated with H5 HA antigen produced from the transient transfection of 293T cells at a concentration of ~2 μ g/ml in PBS at 4°C overnight, then blocked as described above. Freshly isolated splenocytes (100 μ l/well, 500,000 cells/well) in R10 medium with 0.1% β -ME were incubated in duplicate wells for 4 hours at 37°C. The plates were then washed, incubated with 100 μ l of biotinylated goat-anti-mouse IgG diluted at 1:1000 in dilution buffer from the ELISPOT kit above for 1 hour. After additional washes, 100 μ l of HRP-conjugated streptavidin complex diluted at 1:2000 in the dilution buffer was added to each well for 1 h at 37°C, then spots were developed using AEC coloring system as described above.

Statistical analysis

Student's t test was used to analyze the differences in antibody responses between animal immunization groups, as measured by antibody titers and spot forming cells in ASC and IFN- γ ELISPOT assays. A *p* value of less than 0.05 was considered significant.

Discussion

Since the discovery of DNA vaccination as a novel technology to induce antigen-specific immune responses, different DNA plasmid delivery approaches can be grouped into two major types. The first type includes "chemical approaches", relying on the chemical and biochemical interactions between DNA molecules and the target cells. With this type of delivery, DNA plasmids are dissolved in various solutions, with or without carrier polymers (in lipid-form or

other chemical natures), and delivered by conventional intramuscular or intradermal needle injections, transdermally or through mucosal administration [4,51–57]. The second type of DNA delivery includes “physical approaches”. With this type of approach, the delivery of DNA plasmids is based on various forms of physical forces, such as shock wave, high pressure gas and electrical pulse. In general, the physical approaches require special devices that can produce external forces, such as a gene gun or an electroporation device [3,12,13,58,59]. There have been limited studies to directly compare these two types of DNA vaccine delivery approaches, but available data indicates that the physical method is more effective in eliciting higher immune responses in animal studies and that the type of immune responses elicited by these two types of delivery methods may also be different [15,60,61].

The difference between the chemical and physical approaches is further highlighted by the results from early phase DNA vaccine studies in humans. DNA vaccines delivered by chemical methods, including those with facilitating chemical agents, have not been shown to be particularly immunogenic. In the first series of DNA vaccine studies in humans, a group of asymptomatic HIV-infected individuals who received a DNA vaccine encoding an *env* gene and a *rev* gene from the HIV-1_{MN} isolate via conventional intramuscular needle injection. While this study confirmed the safety of DNA immunization, no consistent change in CD4 or CD8 T lymphocyte counts or in plasma HIV concentrations was observed [62,63]. In another study, it was found that although a DNA vaccine encoding the *P. falciparum* circumsporozoite protein for malaria, could elicit low level antigen-specific CTL responses through conventional intramuscular injection [64], measurable antibody responses were not detected in any of the 20 volunteers [65]. On the other hand, gene gun delivery of a plasmid expressing hepatitis B surface antigen (HBsAg) in naive volunteers showed promising immune responses in humans [1]. Three administrations of plasmid DNA by this approach resulted in the generation of seroprotective antibody levels in all 12 volunteers. Furthermore, all 12 volunteers showed cell-mediated immune responses to HBsAg post-administration, highlighting the ability of the physical delivery approach to generate broad-based immune responses. More recently, a phase I human study using gene gun technology examined the safety and immunogenicity of a DNA vaccine, pPJV1671, which expresses the HA from the H3 Panama strain of the human influenza virus in healthy adults [2]. Volunteers in the 4µg dose group (highest dose) achieved the criteria on all three parameters required for licensure by the Committee for Proprietary Medical Products (CPMP) in the European Union (seroconversion, seroprotection and GMT) 21 days after a single vaccination. These data highlight the potential of DNA vaccines in humans, when delivered by a physical method.

The above results suggest that the ability of DNA vaccines to elicit an immune response in a host appear to greatly depend upon the route of administration (i.e., chemical vs. physical delivery). A high level immune response appears to rely on the ability of the DNA plasmids to efficiently enter targeted cells and not so much on the amount of DNA that is administered. DNA immunization via gene gun administration allows the DNA plasmids to penetrate directly into the cytoplasm [3,66,67], presumably resulting in the DNA being processed by antigen presenting cells (APCs) and subsequently presented to T cells [68]. The same holds true for any method that increases the ability of the DNA to enter directly into the intracellular environment rather than into the extracellular space, as is true for conventional needle delivery.

Recently, additional methods based on physical principles, such as electroporation (EP), have demonstrated increased immunogenicity when compared to the chemical delivery method via needle injection alone approach, as EP presumably creates transient pores in the cell membranes, and increases movement of the DNA into the cells due to an electrophoretic effect [69]. The effectiveness of EP has been shown in a number of studies [34,39–43,61,70–72]. The application of electroporation, regardless of the site of injection, should favor the transfection of a greater variety of cells, including APCs. Furthermore, as an additional

mechanism, mild tissue damage, which may be induced by electroporation, could provoke an influx of APCs, induce danger signals, and enhance the release of antigen from injured cells, thereby increasing antigen presentation, and also possibly provide adjuvant effect [73,74]. The chemical approach of delivering DNA with conventional needle delivery has a particular disadvantage in that the DNA is deposited into the extracellular space and subsequently needs to be taken up by the cells –a critical step, which so far has not been able overcome by any particular chemical carriers.

In the current study, we compared the relative immunogenicity of the two leading physical delivery methods: gene gun and electroporation, in both rabbit and mouse models. Our data show that both of these methods are highly effective in inducing antigen-specific immune responses, and both are more immunogenic than the conventional needle injection method. The difference in immunogenicity between these two physical approaches is somewhat limited. While the gene gun approach is significantly more effective in eliciting antibody responses in rabbits and only slightly more effective in mice, when compared to the EP approach, this advantage appears to disappear when multiple immunizations are given. On the other hand, EP may be more effective in inducing higher Th1-type antibody immune responses as shown in our study while GG predominantly induced Th2-type antibody immune responses as well documented in literature [75,76]. This is interesting because both are “physical delivery methods” and raise the question of whether IM injection, even after electroporation, is the key factor in controlling these differences. In the current study, we did not directly measure secreted cytokines because it is not clear whether changes in the Th subtypes that would have been examined in mice would be valid in non-human primates or humans, the “real” targets for improved DNA vaccine delivery. With the increasing use of EP in large animal studies and the pending phase I clinical study in healthy human volunteers, a more complete understanding of antigen-specific T cell responses, including the unique profiles of cell-mediated immune responses related to the use of different DNA delivery methods, will be gained by using polyfunctional cytokine analyses.

EP delivery is clearly more effective than IM delivery in our study but the magnitude of increase was less than previously reported [34], reasons for which there may be a number of factors. First, the immunogenicity of DNA vaccines is highly dependent on individual antigens. The HA antigen is known to be highly immunogenic and since the HA gene insert used in the current study is codon optimized, the immunogenicity of the HA DNA vaccine is further improved [45]. Other factors which influence the immunogenicity of DNA vaccines irrespective of whether or not GG or EP is used include the choice of DNA vaccine vector and the design of the testing antigen as part of the antigen engineering process [77,78]. In combination, these factors may improve the immunogenicity of the DNA vaccine included in the current study which may result in a smaller difference observed between IM and EP deliveries.

In the current study, a caliper electrode model electroporator was used. Currently, there is no standardized technology in EP. There are at least 3–4 different major models in the field. The results learned from the current report may not completely represent the results from other models of electroporators. Future studies may need to overcome the commercial barrier and conduct parallel studies to examine the safety and efficacy of different models of electroporators, especially in humans using the same antigens.

While, in the current study, we utilized an ELISA to measure antibody responses to vaccination, other assays such as hemagglutination inhibition (HI) would have been a more useful measure of protective antibodies against influenza. However, the main goal of the current study was to examine the immunogenicity, rather than the protective qualities of the different vaccination

approaches and given that the same antigen is used, the levels of protective antibodies are usually proportional to the level of overall antibody response.

Previously, the relative immunogenicity of DNA vaccine delivery methods by EP or GG was studied in an anti-tumor model in transgenic mice [61] where the overall antigen-specific immune responses between these two methods were very similar. The only difference was that GG is less effective than EP in controlling the incidence and the growth of spontaneous tumors, however, there was no data to rule out a non-antigen-specific effect in this finding. Future studies should include the development of standardized protocols for EP and to compare the different EP models that are available in the field, especially for human clinical studies for vaccines against infectious diseases. Furthermore, since immune responses may differ dramatically with the use of this immunization method based on the innate properties of the antigen used, future studies should also examine the factors that determine the immunogenicity of various individual antigens when EP is used as the immunization method. The availability of more than one physical delivery approach in the field of DNA vaccine is a positive sign that DNA vaccine technology is maturing and proliferating before being accepted as a vaccination method for general use.

Acknowledgments

This work was supported in part by the grants U01 AI056536 and R01 AI065250 from the National Institute of Allergy and Infectious Diseases, USA and Jiangsu Province Natural Science Foundation grant BK2006728 and grant support for the Jiangsu Province Key Lab in Infectious Diseases, China. The authors thank Dr. Jill M. Grimes-Serrano for critical reading of the manuscript.

References

1. Roy MJ, Wu MS, Barr LJ, et al. Induction of antigen-specific CD8+ T cells, T helper cells, and protective levels of antibody in humans by particle-mediated administration of a hepatitis B virus DNA vaccine. *Vaccine* 2000;19(7–8):764–78. [PubMed: 11115698]
2. Drape RJ, Macklin MD, Barr LJ, Jones S, Haynes JR, Dean HJ. Epidermal DNA vaccine for influenza is immunogenic in humans. *Vaccine* 2006 May 22;24(21):4475–81. [PubMed: 16150518]
3. Tang DC, Devit M, Johnston SA. Genetic immunization is a simple method for eliciting an immune response. *Nature* 1992;356(6365):152–4. [PubMed: 1545867]
4. Ulmer JB, Donnelly JJ, Parker SE, et al. Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science* 1993;259(5102):1745–9. [PubMed: 8456302]
5. Robinson HL, Hunt LA, Webster RG. Protection against a lethal influenza virus challenge by immunization with a haemagglutinin-expressing plasmid DNA. *Vaccine* 1993;11(9):957–60. [PubMed: 8212843]
6. Lu S, Santoro JC, Fuller DH, Haynes JR, Robinson HL. Use of DNAs expressing HIV-1 Env and noninfectious HIV-1 particles to raise antibody responses in mice. *Virology* 1995;209(1):147–54. [PubMed: 7747464]
7. Wang B, Ugen KE, Srikantan V, et al. Gene inoculation generates immune responses against human immunodeficiency virus type 1. *Proc Natl Acad Sci U S A* 1993 May 1;90(9):4156–60. [PubMed: 8483929]
8. Davis HL, Demeneix BA, Quantin B, Coulombe J, Whalen RG. Plasmid DNA is superior to viral vectors for direct gene transfer into adult mouse skeletal muscle. *Hum Gene Ther* 1993 Dec;4(6):733–40. [PubMed: 8186288]
9. Wang S, Kennedy J, West K, et al. Balanced cellular and antibody responses induced by the polyvalent DNA prime-protein boost HIV-1 vaccine formulation DP6-001 in healthy human volunteers. *Vaccine*. 200810.1016/j.vaccine.2007.12.024
10. Pertmer TM, Eisenbraun MD, McCabe D, Prayaga SK, Fuller DH, Haynes JR. Gene gun-based nucleic acid immunization: elicitation of humoral and cytotoxic T lymphocyte responses following epidermal delivery of nanogram quantities of DNA. *Vaccine* 1995;13(15):1427–30. [PubMed: 8578820]

11. Dobano C, Widera G, Rabussay D, Doolan DL. Enhancement of antibody and cellular immune responses to malaria DNA vaccines by in vivo electroporation. *Vaccine* 2007;25(36):6635–45. [PubMed: 17669562]
12. Fuller DH, Loudon P, Schmaljohn C. Preclinical and clinical progress of particle-mediated DNA vaccines for infectious diseases. *Methods* 2006 Sep;40(1):86–97. [PubMed: 16997717]
13. Dean HJ, Haynes J, Schmaljohn C. The role of particle-mediated DNA vaccines in biodefense preparedness. *Adv Drug Deliv Rev* 2005 Jun 17;57(9):1315–42. [PubMed: 15935876]
14. Talaat AM, Lyons R, Johnston SA. A combination vaccine confers full protection against co-infections with influenza, herpes simplex and respiratory syncytial viruses. *Vaccine* 2001 Nov 12;20(3–4):538–44. [PubMed: 11672920]
15. Fynan EF, Webster RG, Fuller DH, Haynes JR, Santoro JC, Robinson HL. DNA vaccines: protective immunizations by parenteral, mucosal, and gene-gun inoculations. *Proc Natl Acad Sci U S A* 1993;90(24):11478–82. [PubMed: 8265577]
16. Macklin MD, McCabe D, McGregor MW, et al. Immunization of pigs with a particle-mediated DNA vaccine to influenza A virus protects against challenge with homologous virus. *J Virol* 1998 Feb;72(2):1491–6. [PubMed: 9445052]
17. Lodmell DL, Ray NB, Parnell MJ, et al. DNA immunization protects nonhuman primates against rabies virus. *Nat Med* 1998;4(8):949–52. [PubMed: 9701249]
18. Fuller DH, Rajakumar PA, Wilson LA, et al. Induction of Mucosal Protection against Primary, Heterologous Simian Immunodeficiency Virus by a DNA Vaccine. *J Virol* 2002 August;76(7):3309–17. [PubMed: 11884556]
19. Roy MJ, Wu MS, Barr LJ, et al. Induction of antigen-specific CD8+ T cells, T helper cells, and protective levels of antibody in humans by particle-mediated administration of a hepatitis B virus DNA vaccine. *Vaccine* 2000 Nov 22;19(7–8):764–78. [PubMed: 11115698]
20. Tacket CO, Roy MJ, Widera G, Swain WF, Broome S, Edelman R. Phase 1 safety and immune response studies of a DNA vaccine encoding hepatitis B surface antigen delivered by a gene delivery device. *Vaccine* 1999 Jul 16;17(22):2826–9. [PubMed: 10438052]
21. Rottinghaus ST, Poland GA, Jacobson RM, Barr LJ, Roy MJ. Hepatitis B DNA vaccine induces protective antibody responses in human non-responders to conventional vaccination. *Vaccine* 2003 Nov 7;21(31):4604–8. [PubMed: 14575774]
22. Roberts LK, Barr LJ, Fuller DH, McMahon CW, Leese PT, Jones S. Clinical safety and efficacy of a powdered Hepatitis B nucleic acid vaccine delivered to the epidermis by a commercial prototype device. *Vaccine* 2005 Sep 23;23(40):4867–78. [PubMed: 15985318]
23. McConkey SJ, Reece WH, Moorthy VS, et al. Enhanced T-cell immunogenicity of plasmid DNA vaccines boosted by recombinant modified vaccinia virus Ankara in humans. *Nat Med* 2003 Jun;9(6):729–35. [PubMed: 12766765]
24. Neumann E, Schaefer-Ridder M, Wang Y, Hofschneider PH. Gene transfer into mouse lymphoma cells by electroporation in high electric fields. *Embo J* 1982;1(7):841–5. [PubMed: 6329708]
25. Potter H. Electroporation in biology: methods, applications, and instrumentation. *Anal Biochem* 1988 Nov 1;174(2):361–73. [PubMed: 3071177]
26. Tsong TY. Electroporation of cell membranes. *Biophys J* 1991 Aug;60(2):297–306. [PubMed: 1912274]
27. Sukharev SI, Klenchin VA, Serov SM, Chernomordik LV, Chizmadzhev Yu A. Electroporation and electrophoretic DNA transfer into cells. The effect of DNA interaction with electropores. *Biophys J* 1992 Nov;63(5):1320–7. [PubMed: 1282374]
28. Wolf H, Rols MP, Boldt E, Neumann E, Teissie J. Control by pulse parameters of electric field-mediated gene transfer in mammalian cells. *Biophys J* 1994 Feb;66(2 Pt 1):524–31. [PubMed: 8161705]
29. Harrison RL, Byrne BJ, Tung L. Electroporation-mediated gene transfer in cardiac tissue. *FEBS Lett* 1998 Sep 11;435(1):1–5. [PubMed: 9755847]
30. Rols MP, Delteil C, Golzio M, Dumond P, Cros S, Teissie J. In vivo electrically mediated protein and gene transfer in murine melanoma. *Nat Biotechnol* 1998 Feb;16(2):168–71. [PubMed: 9487524]
31. Aihara H, Miyazaki J. Gene transfer into muscle by electroporation in vivo. *Nat Biotechnol* 1998 Sep;16(9):867–70. [PubMed: 9743122]

32. Mir LM, Bureau MF, Gehl J, et al. High-efficiency gene transfer into skeletal muscle mediated by electric pulses. *Proc Natl Acad Sci U S A* 1999 Apr 13;96(8):4262–7. [PubMed: 10200250]
33. Gehl J, Mir LM. Determination of optimal parameters for in vivo gene transfer by electroporation, using a rapid in vivo test for cell permeabilization. *Biochem Biophys Res Commun* 1999 Aug 2;261(2):377–80. [PubMed: 10425193]
34. Widera G, Austin M, Rabussay D, et al. Increased DNA vaccine delivery and immunogenicity by electroporation in vivo. *J Immunol* 2000 May 1;164(9):4635–40. [PubMed: 10779767]
35. Bloquel C, Fabre E, Bureau MF, Scherman D. Plasmid DNA electrotransfer for intracellular and secreted proteins expression: new methodological developments and applications. *J Gene Med* 2004 Feb;6(Suppl 1):S11–23. [PubMed: 14978747]
36. Mathiesen I. Electroporation of skeletal muscle enhances gene transfer in vivo. *Gene Ther* 1999 Apr;6(4):508–14. [PubMed: 10476210]
37. Rizzuto G, Cappelletti M, Maione D, et al. Efficient and regulated erythropoietin production by naked DNA injection and muscle electroporation. *Proc Natl Acad Sci U S A* 1999 May 25;96(11):6417–22. [PubMed: 10339602]
38. Khan AS, Pope MA, Draghia-Akli R. Highly efficient constant-current electroporation increases in vivo plasmid expression. *DNA Cell Biol* 2005 Dec;24(12):810–8. [PubMed: 16332178]
39. Dupuis M, Denis-Mize K, Woo C, et al. Distribution of DNA vaccines determines their immunogenicity after intramuscular injection in mice. *J Immunol* 2000 Sep 1;165(5):2850–8. [PubMed: 10946318]
40. Hooper J, Golden J, Ferro A, King A. Smallpox DNA vaccine delivered by novel skin electroporation device protects mice against intranasal poxvirus challenge. *Vaccine* 2007;25(10):1814–23. [PubMed: 17240007]
41. Tollefsen S, Vordermeier M, Olsen I, et al. DNA injection in combination with electroporation: a novel method for vaccination of farmed ruminants. *Scand J Immunol* 2003 Mar;57(3):229–38. [PubMed: 12641651]
42. Luckay A, Sidhu MK, Kjekken R, et al. Effect of plasmid DNA vaccine design and in vivo electroporation on the resulting vaccine-specific immune responses in rhesus macaques. *J Virol* 2007 May;81(10):5257–69. [PubMed: 17329330]
43. Tsang C, Babiuk S, van Drunen Littel-van den Hurk S, Babiuk LA, Griebel P. A single DNA immunization in combination with electroporation prolongs the primary immune response and maintains immune memory for six months. *Vaccine* 2007 Jul 26;25(30):5485–94. [PubMed: 17408815]
44. Babiuk S, Tsang C, van Drunen Littel-van den Hurk S, Babiuk LA, Griebel PJ. A single HBsAg DNA vaccination in combination with electroporation elicits long-term antibody responses in sheep. *Bioelectrochemistry* 2007;70(2):269–74. [PubMed: 17118714]
45. Wang S, Taaffe J, Parker C, et al. Hemagglutinin (HA) proteins from H1 and H3 serotypes of influenza A viruses require different antigen designs for the induction of optimal protective antibody responses as studied by codon-optimized HA DNA vaccines. *J Virol* 2006;80(23):11628–37. [PubMed: 16987975]
46. Wang, S.; Joshi, S.; Lu, S. Delivery of DNA to Skin by Particle Bombardment. In: Heiser, WC., editor. *Gene Delivery to Mammalian Cells*. Humana Press; 2004.
47. Wang S, Heilman D, Liu F, et al. A DNA vaccine producing LcrV antigen in oligomers is effective in protecting mice from lethal mucosal challenge of plague. *Vaccine* 2004 Sep 3;22(25–26):3348–57. [PubMed: 15308359]
48. Chen X, Rock MT, Hammonds J, et al. Pseudovirion particle production by live poxvirus human immunodeficiency virus vaccine vector enhances humoral and cellular immune responses. *J Virol* 2005 May;79(9):5537–47. [PubMed: 15827168]
49. Rock MT, Crowe JE Jr. Identification of a novel human leucocyte antigen-A*01-restricted cytotoxic T-lymphocyte epitope in the respiratory syncytial virus fusion protein. *Immunology* 2003 Apr;108(4):474–80. [PubMed: 12667209]
50. Tamura M, Kuwano K, Kurane I, Ennis FA. Definition of amino acid residues on the epitope responsible for recognition by influenza A virus H1-specific, H2-specific, and H1- and H2-cross-

- reactive murine cytotoxic T-lymphocyte clones. *J Virol* 1998 Nov;72(11):9404–6. [PubMed: 9765498]
51. Felgner PL, Tsai YJ, Sukhu L, et al. Improved cationic lipid formulations for in vivo gene therapy. *Ann N Y Acad Sci* 1995 Nov 27;772:126–39. [PubMed: 8546385]
 52. Wolff JA, Malone RW, Williams P, et al. Direct gene transfer into mouse muscle in vivo. *Science* 1990 Mar 23;247(4949 Pt 1):1465–8. [PubMed: 1690918]
 53. Nabel GJ, Nabel EG, Yang ZY, et al. Direct gene transfer with DNA-liposome complexes in melanoma: expression, biologic activity, and lack of toxicity in humans. *Proc Natl Acad Sci U S A* 1993 Dec 1;90(23):11307–11. [PubMed: 8248244]
 54. Raz E, Carson DA, Parker SE, et al. Intradermal gene immunization: the possible role of DNA uptake in the induction of cellular immunity to viruses. *Proc Natl Acad Sci U S A* 1994 Sep 27;91(20):9519–23. [PubMed: 7937799]
 55. Herrmann JE, Chen SC, Jones DH, et al. Immune responses and protection obtained by oral immunization with rotavirus VP4 and VP7 DNA vaccines encapsulated in microparticles. *Virology* 1999;259(1):148–53. [PubMed: 10364499]
 56. Chen SC, Jones DH, Fynan EF, et al. Protective immunity induced by oral immunization with a rotavirus DNA vaccine encapsulated in microparticles. *J Virol* 1998;72(7):5757–61. [PubMed: 9621034]
 57. Selinsky C, Luke C, Wloch M, et al. A DNA-based vaccine for the prevention of human cytomegalovirus-associated diseases. *Hum Vaccin* 2005 Jan-Feb;1(1):16–23. [PubMed: 17038834]
 58. Haynes JR. Particle-mediated DNA vaccine delivery to the skin. *Expert Opin Biol Ther* 2004 Jun;4(6):889–900. [PubMed: 15174971]
 59. Haynes JR, McCabe DE, Swain WF, Widera G, Fuller JT. Particle-mediated nucleic acid immunization. *J Biotechnol* 1996 Jan 26;44(1–3):37–42. [PubMed: 8717384]
 60. Pertmer TM, Roberts TR, Haynes JR. Influenza virus nucleoprotein-specific immunoglobulin G subclass and cytokine responses elicited by DNA vaccination are dependent on the route of vector DNA delivery. *J Virol* 1996;70(9):6119–25. [PubMed: 8709236]
 61. Smorlesi A, Papalini F, Amici A, et al. Evaluation of different plasmid DNA delivery systems for immunization against HER2/neu in a transgenic murine model of mammary carcinoma. *Vaccine* 2006 Mar 10;24(11):1766–75. [PubMed: 16288939]
 62. MacGregor RR, Boyer JD, Ugen KE, et al. First human trial of a DNA-based vaccine for treatment of human immunodeficiency virus type 1 infection: safety and host response. *J Infect Dis* 1998 Jul; 178(1):92–100. [PubMed: 9652427]
 63. Boyer JD, Chattergoon MA, Ugen KE, et al. Enhancement of cellular immune response in HIV-1 seropositive individuals: A DNA-based trial. *Clinical immunology (Orlando, Fla)* 1999;90(1):100–7.
 64. Wang R, Doolan DL, Le TP, et al. Induction of antigen-specific cytotoxic T lymphocytes in humans by a malaria DNA vaccine. *Science* 1998;282(5388):476–80. [PubMed: 9774275]
 65. Le TP, Coonan KM, Hedstrom RC, et al. Safety, tolerability and humoral immune responses after intramuscular administration of a malaria DNA vaccine to healthy adult volunteers. *Vaccine* 2000 Mar 17;18(18):1893–901. [PubMed: 10699338]
 66. Eisenbraun MD, Fuller DH, Haynes JR. Examination of parameters affecting the elicitation of humoral immune responses by particle bombardment-mediated genetic immunization. *DNA Cell Biol* 1993 Nov;12(9):791–7. [PubMed: 8216850]
 67. Yang NS, Burkholder J, Roberts B, Martinell B, McCabe D. In vivo and in vitro gene transfer to mammalian somatic cells by particle bombardment. *Proc Natl Acad Sci U S A* 1990 Dec;87(24): 9568–72. [PubMed: 2175906]
 68. Condon C, Watkins SC, Celluzzi CM, Thompson K, Falo LD Jr. DNA-based immunization by in vivo transfection of dendritic cells. *Nat Med* 1996 Oct;2(10):1122–8. [PubMed: 8837611]
 69. Zaharoff DA, Barr RC, Li CY, Yuan F. Electromobility of plasmid DNA in tumor tissues during electric field-mediated gene delivery. *Gene Ther* 2002 Oct;9(19):1286–90. [PubMed: 12224011]
 70. Babiuk LA, Pontarollo R, Babiuk S, Loehr B, van Drunen Littel-van den Hurk S. Induction of immune responses by DNA vaccines in large animals. *Vaccine* 2003 Jan 30;21(7–8):649–58. [PubMed: 12531334]

71. Wang Z, Troilo PJ, Wang X, et al. Detection of integration of plasmid DNA into host genomic DNA following intramuscular injection and electroporation. *Gene Ther* 2004 Apr;11(8):711–21. [PubMed: 14724672]
72. Heller LC, Ugen K, Heller R. Electroporation for targeted gene transfer. *Expert Opin Drug Deliv* 2005 Mar;2(2):255–68. [PubMed: 16296752]
73. Prud'homme GJ. DNA vaccination against tumors. *J Gene Med* 2005 Jan;7(1):3–17. [PubMed: 15538731]
74. Prud'homme GJ, Glinka Y, Khan AS, Draghia-Akli R. Electroporation-enhanced nonviral gene transfer for the prevention or treatment of immunological, endocrine and neoplastic diseases. *Curr Gene Ther* 2006 Apr;6(2):243–73. [PubMed: 16611045]
75. Feltquate DM, Heaney S, Webster RG, Robinson HL. Different T helper cell types and antibody isotypes generated by saline and gene gun DNA immunization. *J Immunol* 1997;158(5):2278–84. [PubMed: 9036975]
76. Weiss R, Scheiblhofer S, Freund J, Ferreira F, Livey I, Thalhamer J. Gene gun bombardment with gold particles displays a particular Th2-promoting signal that overrules the Th1-inducing effect of immunostimulatory CpG motifs in DNA vaccines. *Vaccine* 2002 Aug 19;20(25–26):3148–54. [PubMed: 12163266]
77. Wang S, Farfan-Arribas DJ, Shen S, et al. Relative contributions of codon usage, promoter efficiency and leader sequence to the antigen expression and immunogenicity of HIV-1 Env DNA vaccine. *Vaccine* 2006 May 22;24(21):4531–40. [PubMed: 16140431]
78. Lu, S.; Manning, S.; Arthos, J. Antigen engineering in DNA immunization. In: Lowrie Da, WR., editor. *Methods in Molecular Medicine DNA Vaccines*. Humana Press; 1999. p. 355-74.

Fig. 1. Temporal anti-H5 HA IgG responses in rabbits measured by ELISA at 1:500 serum dilution. (A) Rabbits received empty DNA vector via intramuscular injection (IM, Vc-1), electroporation (EP, Vc-2) or gene gun (GG, Vc-3 ~ Vc-5). Rabbits received H5 HA DNA immunizations via different vaccination approaches: (B) IM, (C) EP, or (D) GG. The arrows indicate the time of DNA immunizations. Each curve represents one rabbit.

Fig. 2. Peak level serum anti-H5 HA IgG antibody responses in rabbits induced by different vaccination approaches: intramuscular injection (IM), electroporation (EP) or gene gun (GG). All rabbits received H5 HA DNA immunizations and sera were collected at two weeks after the 4th DNA immunization. Each bar represents an individual rabbit serum. The broken horizontal lines indicate the mean antibody titer of the relevant group. The statistical difference between each group was determined and the differences with $p < 0.05$ are indicated.

Fig. 3.

H5 HA-specific antibody responses in mice. (A) Temporal anti-H5 HA IgG responses in mice measured at 1:500 serum dilution. Each curve represents the average antibody responses for each group of mice from three independent assays. “H5 HA-IM”, “H5 HA-EP” and “H5 HA-GG” indicate the groups receiving H5 HA DNA immunizations via intramuscular injection (IM), electroporation (EP) or gene gun (GG), respectively. “Vector-GG” indicates the mice receiving empty DNA vector by gene gun. Group pooled mouse sera from 5 mice in each group were used in the ELISA. The arrows indicate the time of DNA immunizations. (B) Peak level serum anti-H5 HA IgG responses in mice immunized with empty DNA vector or with H5 HA DNA vaccine via IM, EP or GG immunization. Each bar represents the average titer of each

group of 5 mice with standard error. Mouse sera were collected at two weeks after the 4th DNA immunization. The statistical difference between each group was determined and the differences with $p < 0.05$ are indicated.

Fig. 4.

H5 HA-specific antibody secreting cells (ASC) in splenocytes of immune mice as measured by ELISPOT. Mice were immunized with empty DNA vector or with H5 HA DNA vaccine via IM, EP or GG immunization, as indicated. (A) Actual sample wells of H5 HA-specific ASC spots with either mock antigen coated or H5 HA antigen coated plates. (B) Frequency of H5 HA-specific ASC per million splenocytes in each group. Data represent the average of spot forming cells (SFCs) per million of splenocytes from 5 mice/group plus standard deviation. The splenocytes were collected 2 weeks after the 4th DNA immunization. The statistical difference between each group was determined and groups with $p < 0.05$ are indicated.

Fig. 5. ELISPOT analysis for IFN- γ secretion in mouse splenocytes immunized with empty DNA vector or with H5 HA DNA vaccine via IM, EP or GG immunization, as indicated. (A) Actual sample wells with mock or HA peptide stimulations. (B) Frequency of HA peptide-specific spots per million splenocytes in each group. Data represent the average of spot forming cells (SFCs) per million of splenocytes from each group plus standard deviation. The splenocytes were collected 2 weeks after the 4th DNA immunization.

Fig. 6.

Subtype analysis of H5 HA-specific IgG in mouse sera raised by the H5 HA DNA vaccine through different vaccination approaches: IM, EP or GG. The H5 HA-specific IgG1 and IgG2a were measured by IgG isotype ELISA. The IgG1/IgG2a ratios were based on the concentration of IgG1 or IgG2a isotypes calculated using the standard curves established with known concentration of mouse IgG1 or IgG2a. The mouse sera were collected 2 weeks after the 3rd DNA immunization. The bars show the average IgG1/IgG2a ratios in each group plus standard deviation.

Table 1Rabbit immunization groups[^]

Groups	Rabbit ID	DNA vaccines	Route [*]	Dose (µg)
A	IM-1, IM-2, IM-3	H5 HA DNA vaccine	IM	200
B	EP-1, EP-2, EP-3	H5 HA DNA vaccine	EP	200
C	GG-1, GG-2, GG-3	H5 HA DNA vaccine	GG	30
D	Vc-1, Vc-2, Vc-3	Empty vector	GG	30
E	Vc-4	Empty vector	EP	200
F	Vc-5	Empty vector	IM	200

[^] All rabbits received DNA immunizations at weeks 0, 2, 4 and 6.

^{*} GG: gene gun immunization; IM: intramuscular injection; EP: electroporation

Table 2Mouse immunization groups[^]

Groups	No. of mice	DNA vaccines	Route	Dose (μg)
A	5	H5 HA DNA vaccine	IM	100
B	5	H5 HA DNA vaccine	EP	100
C	5	H5 HA DNA vaccine	GG	6
D	5	Empty vector	GG	6

[^] All mice received DNA immunizations at weeks 0, 2, 4 and 6.

^{*} GG: gene gun immunization; IM: intramuscular injection; EP: electroporation