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# Enhanced immunogenicity and protective efficacy of a tetravalent dengue DNA vaccine using electroporation and intradermal delivery

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ABSTRACT

Phase 1 clinical trials with a DNA vaccine for dengue demonstrated that the vaccine is safe and well tolerated, however it produced less than optimal humoral immune responses. To determine if the immunogenicity of the tetravalent dengue DNA vaccine could be enhanced, we explored alternate, yet to be tested, methods of vaccine administration in non-human primates. Animals were vaccinated on days 0, 28 and 91 with either a low (1 mg) or high (5 mg) dose of vaccine by the intradermal or intramuscular route, using either needle-free injection or electroporation devices. Neutralizing antibody, IFN-γ T cell and memory B cell responses were compared to a high dose group vaccinated with a needle-free intramuscular injection delivery device similar to what had been used in previous preclinical and clinical studies. All previously untested vaccination methodologies elicited improved immune responses compared to the high dose needle-free intramuscular injection delivery group. The highest neutralizing antibody responses were observed in the group that was vaccinated with the high dose formulation via intradermal electroporation. The highest IFN-γ T cell responses were also observed in the high dose intradermal electroporation group and the $CD8^+$ T cells were the dominant contributors for the IFNγ response. Memory B cells were detected for all four serotypes. More than a year after vaccination, groups were challenged with dengue-1 virus. Both the low and high dose intradermal electroporation groups had significantly fewer days of dengue-1 virus RNAemia compared to the control group. The results from this study demonstrate that using either an electroporation device and/or the intradermal route of delivery increases the immune response generated by this vaccine in non-human primates and should be explored in humans.



## 1. Introduction

Dengue fever is the most common viral disease transmitted by a mosquito. It is caused by infection with one of the four serotypes of dengue virus, a flavivirus in same family as yellow fever and West Nile virus. The geographic distribution of the *Aedes* mosquitoes capable of transmitting dengue viruses spans tropical and subtropical regions, which represent over 100 countries. It is estimated that 390 million dengue infections occur globally each year [1], resulting in approximately 500,000 hospitalizations and 20,000 deaths annually [2]. There is an urgent need for an effective tetravalent dengue vaccine that can induce protective immunity against all four dengue serotypes.

Currently, there is only one licensed vaccine for dengue, Dengvaxia® from Sanofi Pasteur. However, this vaccine is only suitable for individuals who have previously been infected with dengue virus [3] and thus is not suitable for populations that do not live in endemic areas. Other live attenuated vaccines (LAV) are the most advanced alternative vaccine products; however, these vaccines have their own challenges in terms of production and tolerability. Viral interference, balancing attenuation to produce acceptable tetravalent immunogenicity with minimal reactogenicity, and achieving uniformity of immune responses to four serotypes in a mixed tetravalent formulation may be recurrent problems for multivalent live attenuated dengue vaccines [4,5]. A tetravalent dengue DNA vaccine remains a viable prophylactic vaccine candidate

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because it offers numerous potential advantages compared with other conventional vaccines such as simplicity, scalability, excellent stability, rapid construction, noninfectious state, and the ability to induce humoral and cellular immune responses [5].

DNA vaccines for dengue virus infections have been extensively tested in pre-clinical models [6–11] and two phase 1 clinical trials have been completed [12,13]. The two clinical trials demonstrated the safety and favorable tolerability of the dengue DNA vaccine; however, consistent with other clinical trials for DNA vaccines, while moderate cell-mediated immune responses were observed, neutralizing antibody responses were suboptimal among the subjects. The low antibody responses generated by DNA vaccines has in part been attributed to a relatively low transfection efficiency of the DNA vaccine [14], and a growing body of literature suggests that the success of a DNA vaccine relies on its efficiency in transfecting antigen presenting cells (APCs) [15,16].

The delivery of DNA vaccines by electroporation (EP) has improved the performance of DNA vaccines. EP is believed to enhance the immunogenicity of DNA vaccines by increasing transfection efficiency of target cells [17,18] and by recruiting immune cells to the vaccination site [19,20]. The advantages of this vaccine delivery method were demonstrated in numerous preclinical and clinical trials of DNA vaccines for the prevention of several diseases including HIV [21], malaria [22,23] Chagas disease [24], Ebola, Marburg [25], and hemorrhagic fever with renal syndrome [26].

Intradermal (ID) vaccination is another feasible method to improve transfection efficiency by introducing the plasmid DNA vaccine to the dermal region, a skin layer abundant in professional APCs in the form of dendritic dermal cells (DDCs) and Langerhan cells (LCs) [15]. A number of clinical trials have demonstrated that ID immunization with a reduced dose (typically one-fifth of the IM dose) of rabies, hepatitis B, influenza and inactivated poliovirus vaccines have resulted in at least equivalent immune responses compared with the standard dose delivered by the IM route [27–30].

To determine if the immunogenicity of a tetravalent dengue DNA vaccine could be increased, we explored these alternate methods of vaccine administration in non-human primates. Animals were vaccinated by the intradermal or intramuscular route, using either needle-free jet injection or electroporation devices. The immunogenicity results as well as the results from a live virus challenge are presented here.

## 2. Materials and methods

### 2.1. Vaccine constructs

TVDV is a mixture of equal amounts of four monovalent plasmid DNA vaccines produced under current Good Manufacturing Practices conditions in the United States. Each monovalent plasmid contains the pre-membrane (prM) and envelope (E) genes of dengue 1, 2, 3, or 4 viruses cloned into the backbone plasmid VR1012 (Vical Incorporated, San Diego, CA) [10]. The dengue-1 DNA sequences are derived from West Pac 74, while the dengue-2 dengue-3, and dengue-4 DNA sequences are derived from low passage Philippine strains. For dengue-2, the C-terminal transmembrane and cytoplasmic domains of E were replaced with those of the lysosome-associated membrane protein 1 (LAMP-1). The substitution of the LAMP-1 sequences significantly enhances dengue 2 neutralizing antibody responses [9,31].

### 2.2. Animals and vaccine administration

The study protocol was reviewed and approved by the Wake Forest University Institutional Animal Care and Use Committee and the US Army Medical Research and Materiel Command Animal Care and Use Review Office in compliance with all applicable Federal regulations governing the protection of animals in research. Twenty-eight dengue virus antibody negative *Macaca fascicularis* monkeys were divided into seven groups of four animals each (Table 1). The animals were vaccinated with either a 5 mg or 1 mg total DNA dose of vaccine on days 0, 28 and 91 via the intradermal or intramuscular route, using either a needle-free jet injection (Pharmajet Stratis or Tropis) or an electroporation (TriGrid, Ichor) device.

**Table 1**
Vaccination groups.

| Group | N | Dose | Vaccination method |
|---|---|---|---|
| 1 | 4 | 5 mg | IM EP |
| 2 | 4 | 1 mg | IM EP |
| 3 | 4 | 5 mg | ID EP |
| 4 | 4 | 1 mg | ID EP |
| 5 | 4 | 5 mg | ID NFI |
| 6 | 4 | 1 mg | ID NFI |
| 7 | 4 | 5 mg | IM NFI |

IM = intramuscular, EP = electroporation, ID = intradermal, NFI = needle-free injection.

### 2.3. Anti-dengue neutralizing antibody responses

Anti-dengue neutralizing antibody in serum was assayed using a high throughput dengue microneutralization (MN) test. Two hundred $TCID_{50}$ of each dengue virus serotype (1–4) was incubated with two-fold serial dilutions of serum samples for 60 min in a 96 well flat bottom plate. Vero81 cells ($2 \times 10^4$) were then added to each well of the microtiter plate and incubated at 37 °C for 4–5 days depending on serotype. After incubation, cells were fixed and dengue specific antigens were measured using rabbit anti-dengue polyclonal antibody and a peroxidase labeled anti-rabbit IgG secondary using a standard ELISA format. The highest serum dilution that resulted in ≥50% reduction in absorbance compared to control was the determined 50% neutralization titer. The results are expressed as $MN_{50}$ titers, which represent the reciprocal serum dilution giving a 50% reduction in absorbance readout when compared with a virus dose control lacking serum.

### 2.4. ELISPOT assay for IFNγ response

PBMCs were sampled prior to vaccination and at days 35 and 240. T-cell IFNγ responses were measured using an ELISpot assay described previously [12]. Briefly, frozen peripheral blood mononuclear cells (PBMCs) were thawed, washed and placed in a 37 °C 5% $CO_2$ incubator in complete cell culture medium, RPMI1640 supplemented with 10% fetal bovine serum (Hyclone, Logan, Utah) and 1% penicillin-streptomycin (Corning) overnight for viability recovery. Cells were then plated in ELISPOT plates (MAIPSU10, Millipore) pre-coated with anti-IFNγ monoclonal antibody (kit # 3421M-2A, Mabtech AB, Sweden) at $1 \times 10^5$ cells per well for mock- and antigen-stimulated cultures and $3.3 \times 10^4$ cells per well for PHA control wells. Antigens used to stimulate PBMCs were four prME peptide pools comprised of 15–20 mer peptides overlapping by 5–11 amino acids from prM (synthesized by GenScript USA Inc, NJ) and E proteins (BEI Resources) corresponding to each of the four dengue serotypes at a final concentration of 1 μg/ml per peptide. Negative controls (mock control) were cultures treated with diluted solvent (dimethyl sulfoxide) only, while positive controls were cultures treated with the mitogen phytohemagglutinin (PHA) (Sigma Aldrich) at 2 μg/ml final concentration. Samples were run in duplicate or triplicate depending on

the amount of cells recovered after thawing. After a 24-hour incubation, the plates were washed and ELISPOT was developed using a kit (#3421M-2A, Mabtech AB, Sweden) according to the manufacturer's instruction. The spots were counted on an automated spot counter (AID ELISPOT Reader, Autoimmun Diagnostika GmbH, Germany), normalized based on input cells per well and presented as spot forming units (SFUs) per $10^6$ PBMCs. A positive response to DENV antigen is scored when the mean SFUs in peptide-stimulated cultures is at least 4-fold higher than that of the corresponding mock control and greater than 50 SFU/$10^6$ cells. If a mock control has a count of zero, it is set to 1 to enable the fold increase calculation. If a sample has a response to PHA with SFUs < 695/$10^6$ PBMCs, the sample is considered to have poor viability and is not included for further data analysis. Based on available cell numbers, for day 35 samples, 15 samples were run in triplicate and 13 in duplicate. For Day 240 samples, all samples were run in duplicate.

### 2.5. Intracellular staining

PBMCs from day 240 were thawed and stimulated with antigens, solvent only or PHA in the same manner as described above for the ELISPOT assay but in normal U-bottom 96-well cell culture plates. Briefly, after an overnight antigen stimulation, PBMCs were treated with Golgi-plug (BD Biosciences) at a final concentration of 1:1000 for four hours. The cells were washed once in 96-well plates with PBS and stained with CD4-PerCp (clone L200) and CD8-FITC (clone PRA-T8) for 30 min on ice. After two washes with PBS, the cells were fixed and permeablized using PermFix and PermWash buffers (BD Biosciences) as recommended by the manufacturer. The cells were stained with anti-IFN$\gamma$-PE (clone 4S.B3) in PermWash buffer for 30 min at room temperature. After two washes, the cells were run on a FACS CANTO II using a pre-optimized template with lymphocytes, CD4+ and CD8+ cell subsets properly gated and displayed. Three thousand events within the lymphocyte gate were collected for each sample. The percentage of IFN$\gamma^+$ cells for each subset of cells was calculated. The dengue antigen-specific response was calculated by subtracting the response in the mock-stimulated cultures from the response in the antigen stimulated cultures.

### 2.6. Memory B cell responses

The B cell ELISPOT assay for NHPs was adopted from a method described by Crotty et al. [20]. Briefly, PBMCs were thawed, counted, adjusted to $10^6$/ml, and seeded into 24-well plates at 2 ml/well. The cells were polyclonally stimulated with a mitogenic stimulus from a Monkey IgG B cell ELISPOT Kit (U-Cytech Biosciences) for six days. The mitogen-stimulated PBMC were harvested from the 24-well plates, washed and adjusted to $4 \times 10^6$/ml according to the original seeding cell counts. The mitogen stimulated cells were then added to DENV antigen-coated wells (MAIPSWU10 ELISPOT plates, Millipore) for assessment of antigen-specific memory B cells, KLH-coated wells for negative controls, and anti-IgG Ab (provided by the same kit, U-Cytech) coated wells for enumeration of total IgG secreting cells. DENV antigens used for the assay were E polypeptides (Hawaii Biotech) from DENV1 strain West Pac 74, DENV-2 strain 16803, DENV-3 strain CH53489, and DENV4 341750. The E polypeptides were synthesized in drosophila cells and consisted of 80% of the whole protein length (provided by the same kit, U-Cytech). For assessing total IgG secreting cells, the mitogen-stimulated PBMCs were diluted 10 to 100 times before being added to the ELISPOT plates. The plates were developed using the reagents provided by the same kit (U-Cytech) according to the manufacturer's instructions. The assay was stopped and spots were counted using an automated ELISPOT counter (AID Autoimmun Diagnostika GmbH). Samples with <1000 total IgG SFUs/$10^6$ cells were removed from further analysis [22]. Samples were scored as positive if SFUs/well was greater than its corresponding KLH control and greater than five SFUs/well. Five SFUs/well was used as a cutoff based on previous results from 52 KLH tests on 52 NHP samples that demonstrated that ninety-three percent of the responses to KLH were between 0 and 5 SFU/well. Due to limited cell numbers, four samples were tested in a single well, the rest were tested in duplicate. Data presented are percentage of antigen-specific SFUs per total IgG SFUs.

### 2.7. Live virus challenge

At day 392, all vaccinated and a control group of NHPs were challenged by injecting $2 \times 10^5$ plaque forming units of dengue-1 (WP-74) virus via the subcutaneous route. Blood was collected immediately before and for 10 days after challenge. The presence of dengue-1 virus RNA was quantitated using digital droplet PCR. RNA was isolated from 40 μL of sera using MagMAX Viral/pathogen total nucleic acid kit (Applied Biosystems) in a KingFisher Flex automated magnetic particle processor (ThermoFisher Scientific). Dengue-1 virus RNA quantitation in the extracted nucleic acid samples was performed using One-step Reverse-Transcriptase digital droplet PCR advanced kit for probes (BIO-RAD) and DENV-1 specific primers and probes [32]. Briefly, RT-ddPCR droplets were made by mixing 20 μL of RT-PCR reactions with 70 μL of droplet generation oil in the QX200 Droplet Generator (BIO-RAD). After thermocycling, the fluorescence intensity in each droplet was read on a QX200 Droplet Reader (BIO-RAD) and the data was analyzed by QuantaSoft software.

### 2.8. Data analysis

In order to test for differences in mean peak neutralizing antibody titers, peak IFN-$\gamma$ responses, days of DENV-1 RNAemia, and peak DENV-1 copy numbers a one-way ANOVA with Tukey's honestly significant difference (HSD) post hoc test was performed using GraphPad Prism 7. Pearson correlation coefficients were calculated using GraphPad Prism 7.

## 3. Results

### 3.1. Neutralizing antibody responses

Dengue virus antibody negative non-human primates were divided into seven groups of four animals each. For each group, vaccinations were conducted as indicated in Table 1 on days 0, 28 and 91. Serum was processed for antibody analysis on days 56, 119, 140, 197, 240 and 392. In the high dose intradermal (ID) electroporation (EP) group, all animals developed tetravalent neutralizing antibody responses by day 56 (Fig. 1). By day 119, 100% of the animals in all groups had developed tetravalent neutralizing antibodies to all four dengue serotypes. More than a year after initial vaccination (day 392), all animals in the high dose ID EP ($ID_{5mg}EP$) and high dose ID needle-free injection ($ID_{5mg}NFI$) groups and 50% of the animals in the high dose intramuscular EP ($IM_{5mg}EP$) and low dose ID EP ($ID_{1mg}EP$) groups still had tetravalent neutralizing antibody titers $\geq$ 40 (Fig. 1).

Peak neutralizing antibody titers for almost all groups for all serotypes were observed at day 119. The only exception was the $ID_{5mg}NFI$ group where peak neutralizing antibody titers for DENV-1 and DENV-3 were observed at day 140. For DENV-1, the highest mean microneutralization $(MN)_{50}$ titers were seen in the $ID_{5mg}EP$ and $ID_{1mg}EP$ groups and were significantly higher than the $IM_{5mg}NFI$ group (p 0.044) (Fig. 2). For DENV-2, mean $MN_{50}$

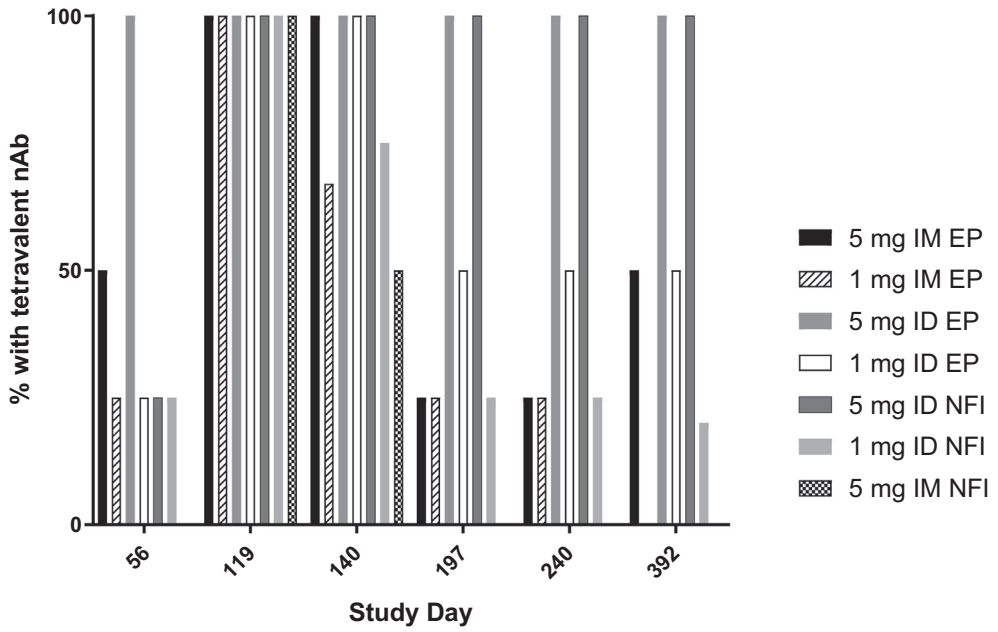


**Fig. 1.** Tetravalent neutralizing antibodies. Percentage of NHP with neutralizing antibodies to all four serotypes of dengue virus by group and study day. Titer $\geq$ 40 is considered positive.

titers were highest for the $ID_{5mg}EP$ group and were significantly greater than the $IM_{1mg}EP$ (p 0.025) and $IM_{5mg}NFI$ groups (p 0.016). For DENV-3, the $ID_{5mg}EP$ and $ID_{5mg}NFI$ groups had the highest mean titers, but they were not significantly different than the other groups. For DENV-4, the $ID_{5mg}EP$ group had the highest mean neutralizing antibody titers and they were significantly higher than the $IM_{5mg}EP$ (p 0.018), $IM_{1mg}EP$ (p 0.007), $ID_{1mg}EP$ (p 0.007), $ID_{1mg}NFI$ (p 0.018) and $IM_{5mg}NFI$ groups (p = 0.005) (Fig. 2).

### 3.2. T cell responses

PBMCs were sampled prior to vaccination and at days 35 and 240. Eight NHPs (two each from the $IM_{5mg}EP$, $IM_{1mg}EP$, and the $ID_{5mg}NFI$ groups, and one each from the $ID_{5mg}EP$ and $ID_{1mg}EP$ groups) were assessed for pre-vaccination IFNγ T cell responses. Pre-vaccination samples from the remaining NHPs were not tested due to low cell recovery. Seven of the eight NHPs tested were negative for dengue specific pre-vaccination IFNγ response. One NHP in the high dose ID group had a positive pre-vaccination response to DENV2 (107 SFU/$10^6$ PBMCs), but was negative for the other three serotypes (data not shown). This NHP was excluded from further DENV2 analysis. At both days 35 and 240, one NHP in the $ID_{5mg}NFI$ group consistently had a high background response and was excluded from further analysis.

At day 35, the percentage of NHPs with a positive IFN-γ response was highest in the $ID_{5mg}EP$ and $IM_{5mg}EP$ groups and lowest in the $ID_{1mg}NFI$ and $IM_{5mg}NFI$ groups (Table 2). At day 240, the percentage of NHPs with a positive IFN-γ response was highest in the EP groups, moderate in the $IM_{5mg}NFI$ group and lowest in the ID NFI groups (Table 2).

For all serotypes and all groups, peak IFN-γ responses were observed at day 240. For DENV-1, the highest mean IFN-γ response occurred in the high dose $IM_{5mg}EP$ group, but was not significantly different than any of the other groups (Fig. 3). For DENV-2, the mean IFN-γ response was highest in the $ID_{5mg}EP$ group and was significantly higher than the mean responses in the $ID_{1mg}NFI$ group (p 0.023) and the $IM_{5mg}NFI$ group (p 0.044). For DENV-3, the $IM_{5mg}EP$, $ID_{5mg}EP$ and $ID_{1mg}$ EP groups had the highest mean IFN-γ responses, but were not significantly different than any of the other groups. For DENV-4, the $IM_{5mg}EP$ group had the highest mean IFN-γ response but was not significantly different than any of the other groups (Fig. 3).

### 3.3. T cell subsets contributing to the IFNγ response

Due to limited cell numbers, 11 NHPs for DENV-1 and 10 NHPs for DENV-2,-3-, and 4 were analyzed by intracellular cytokine staining (ICS) to determine the cell types contributing to the IFNγ response (Fig. 4). Lymphocytes from the day 240 samples were gated on based on forward scatter and side scatter and then separated into CD4 and CD8 subpopulations. The expression of CD8 within the $CD8^+$ subpopulation showed a range of intensity and thus was further gated into $CD8^{dim}$ and $CD8^{bright}$. While CD4, $CD8^{dim}$ and $CD8^{bright}$ T cells all produced some amount of IFNγ, the dominant population contributing to IFNγ production was the CD8 $^{dim}$ T cells (Fig. 4). Good correlation between $CD8^{dim}$ IFNγ ICS response and IFNγ ELISPOT response was observed for all four serotypes with correlation efficiencies (r) of 0.7015, 0.8142, 0.7215 and 0.7696 for DENV-1, -2, -3, and -4 respectively. The correlation efficiencies between $CD4^+$ IFNγ ICS and IFNγ ELISPOT were 0.0803, 0.3883, 0.6287, and 0.2451 for DENV-1, -2, -3, and -4 respectively and the correlation efficiencies between $CD8^{bright}$ IFNγ ICS with IFNγ ELISPOT were 0.4554, 0.8449, 0.1961, and 0.6263 for DENV-1, -2, -3 and -4 respectively. Therefore, the correlation between $CD8^+$ IFNγ ICS with IFNγ ELISPOT is better than that of $CD4^+$ T cells, and among the $CD8^+$ T cells, the $CD8^{dim}$ cells are the major contributors.

### 3.4. Memory B cell responses

Due to limited availability of PBMCs, samples from only 17 NHPs were assessed for memory B cell responses at day 240. The $ID_{5mg}EP$ group had a 100% response rate across all four serotypes

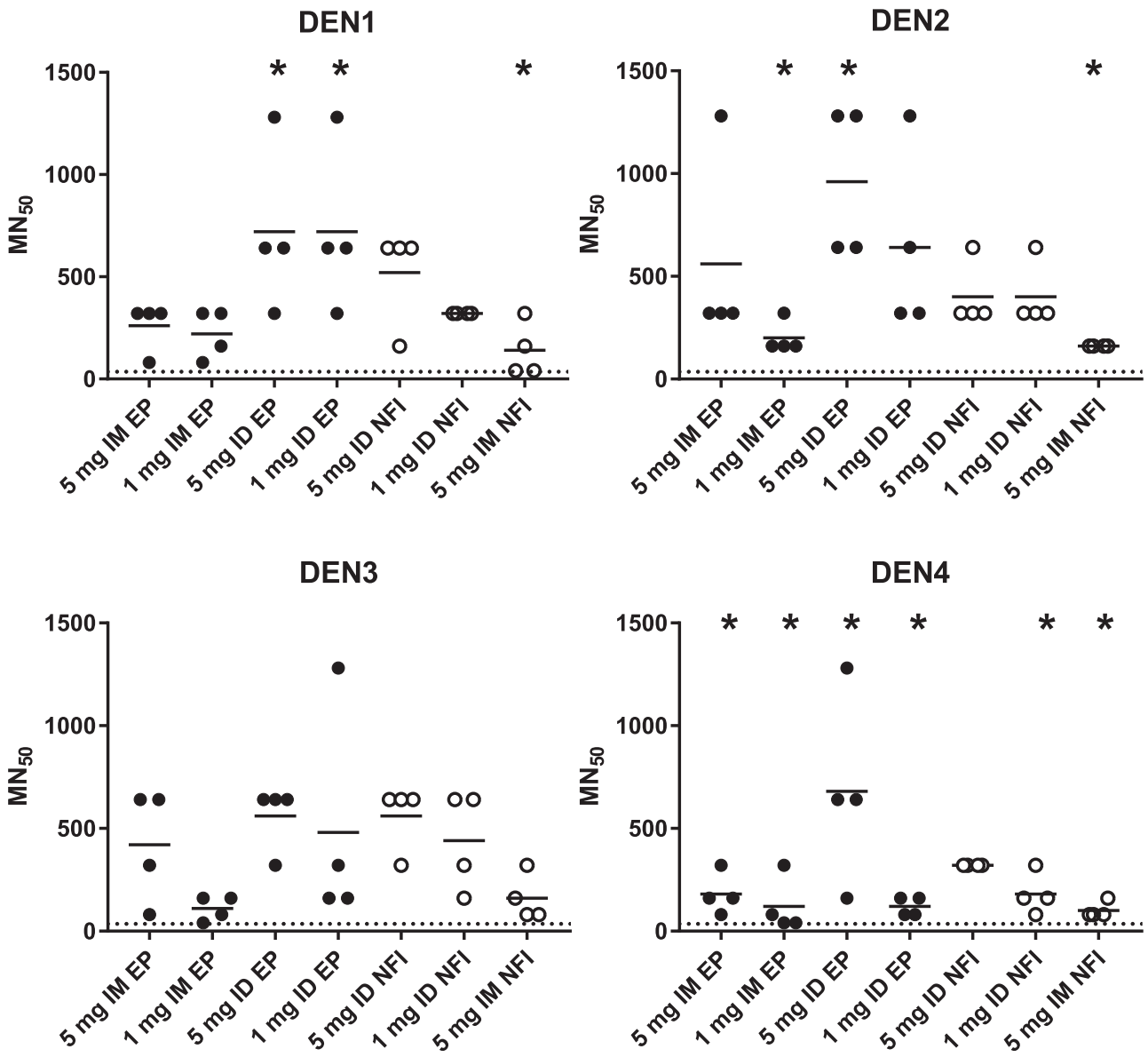


**Fig. 2.** Peak neutralizing antibody titers. Peak microneutralization$_{50}$ ($MN_{50}$) titers for each animal by group and serotype. Peak $MN_{50}$ titer for each animal for each serotype is represented by a dot. The geometric mean titers for each group for each serotype is represented by a horizontal line. Significantly different groups are marked by asterisks. The dotted horizontal line represents the limit of detection for the assay.

**Table 2**
Percentage of NHPs by group and serotype with dengue specific IFN-γ responses.

| Group | n | Day 35 | | | | Day 240 | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | DENV-1 | DENV-2 | DENV-3 | DENV-4 | DENV-1 | DENV-2 | DENV-3 | DENV-4 |
| IM EP 5 mg | 4 | 75 | 75 | 100 | 100 | 100 | 100 | 100 | 100 |
| IM EP 1 mg | 4 | 75 | 75 | 75 | 25 | 75 | 100 | 100 | 50 |
| ID EP 5 mg | 4 | 100 | 100 | 100 | 75 | 75 | 100 | 100 | 75 |
| ID EP 1 mg | 4* | 50 | 100 | 75 | 0 | 100 | 100 | 100 | 100 |
| ID NFI 5 mg | 3** | 67 | 50 | 67 | 33 | 33 | 50 | 67 | 33 |
| ID NFI 1 mg | 4 | 25 | 0 | 0 | 0 | 75 | 50 | 75 | 25 |
| IM NFI 5 mg | 4 | 0 | 0 | 0 | 0 | 75 | 75 | 75 | 75 |

IM = intramuscular, EP = electroporation, ID = intradermal, NFI = needle-free injection, n = number of NHPs.
* n = 3 for all serotypes at Day 240.
** n = 2 for DENV-2 at Day 35 and Day 240.

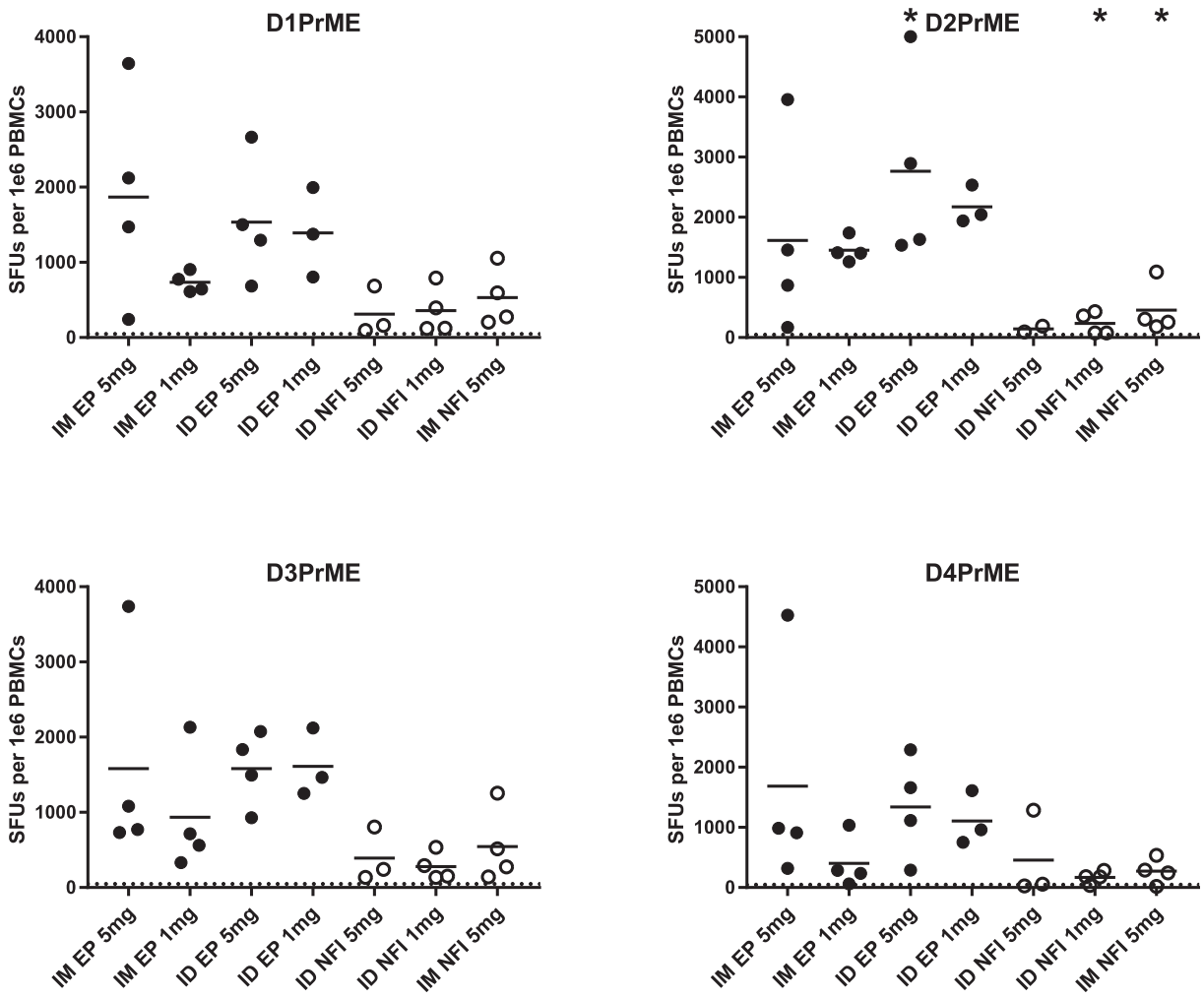


**Fig. 3.** Peak IFN-γ T cell responses. Peak spot forming units of IFN-γ producing cells per $10^6$ PBMCs for each animal by group and serotype. SFU per $10^6$ PBMCs for each animal is represented by a dot. The mean for each group by serotype is represented by a horizontal line. Significantly different groups are marked by asterisks. The dotted horizontal line represents the limit of detection for the assay.

while no memory B cell responses were detected in the $ID_{1mg}$NFI group (Table 3). Total IgG responses ranged from 10.5 to 157 SFUs/well/$10^3$ input cells and individual responses to E proteins reached up to 0.610% (Fig. 5). The $IM_{1mg}$EP, $ID_{1mg}$NFI, and $IM_{5mg}$NFI groups had the lowest responses (Fig. 5). Modest correlation was observed between day 240 $MN_{50}$ titers and the percentage of memory B cells (r = 0.3709, 0.4385, 0.4292 and 0.6910 for DENV-1, -2, -3, and -4 respectively).

### 3.5. Live virus challenge

On day 392 all vaccinated and four control animals were challenged with dengue-1 virus. Blood was collected daily for 10 days after challenge and the presence of viral RNA was quantitated by digital droplet PCR. The control group had an average of 6.75 days of RNAemia and both the low (3 days, p = 0.0027) and high dose (1.5 days, p = <0.0001) intradermal electroporation groups had significantly fewer days of dengue-1 virus RNAemia (Table 4). Peak DENV-1 copy numbers were significantly lower compared to the control group in the low (p = 0.0176) and high (p = 0.0200) dose ID EP, $IM_{5mg}$EP (p = 0.0255), and $IM_{5mg}$NFI groups. (p = 0.0330). At the time of challenge, neutralizing antibody titers $\geq$ 40 against dengue-1 were detected in all of the animals in the ID EP groups (both high and low dose), the $IM_{5mg}$EP group, and the $ID_{5mg}$NFI group. Neutralizing antibodies against dengue-1 were detected in 50% of the animals in the $IM_{1mg}$EP and $ID_{1mg}$NFI groups. Only 25% of the animals in the $IM_{5mg}$NFI group had detectable neutralizing antibodies to dengue-1 at the time of challenge (Table 4).

## 4. Discussion

DNA vaccines remain an attractive option due to their simplicity and versatility. However, while results have been promising in preclinical models, results from clinical trials have not been as promising. Consistent with this pattern, preclinical studies with a DNA vaccine for dengue demonstrated the generation of protective immunogenicity in non-human primates, while clinical trials demonstrated minimal neutralizing antibodies and moderate T cell responses. Two strategies to increase the immune responses elicited by DNA vaccines include electroporation (EP) and intradermal (ID) delivery. In this study, we examined the effect of electroporation, intradermal delivery or both on the immunogenicity of a dengue DNA vaccine in non-human primates.

While a definitive correlate of protection has not been identified for dengue, neutralizing antibodies clearly play an important role. Overall, in terms of magnitude, frequency and durability of

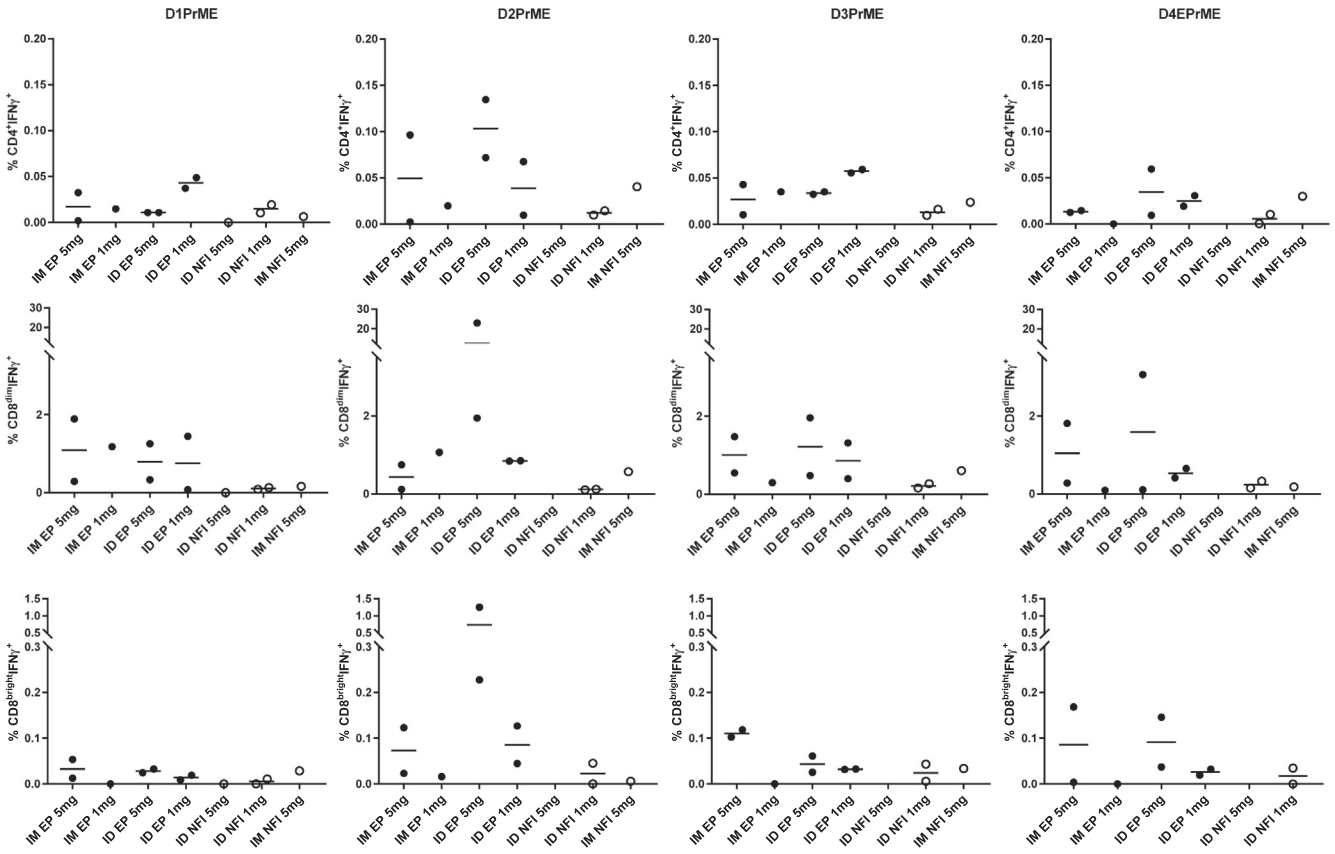


**Fig. 4.** T cell subsets contributing to the IFN-γ response. Percentage of IFN-γ producing cells by T cell subset (CD4, $CD8^{dim}$, $CD8^{bright}$) for each animal by group and serotype. Individual sample results are represented by a dot and the mean for each group is represented by a horizontal line.

**Table 3**
Percentage of NHPs by group and serotype with dengue specific memory B cell responses at day 240.

| Group | n | D1E | D2E | D3E | D4E |
|---|---|---|---|---|---|
| IM EP 5 mg | 3 | 67 | 67 | 67 | 67 |
| IM EP 1 mg | 3 | 67 | 0 | 33 | 33 |
| ID EP 5 mg | 3 | 100 | 100 | 100 | 100 |
| ID EP 1 mg | 2 | 50 | 50 | 100 | 50 |
| ID NFI 5 mg | 2 | 50 | 50 | 100 | 50 |
| ID NFI 1 mg | 2 | 0 | 0 | 0 | 0 |
| IM NFI 5 mg | 2 | 50 | 50 | 50 | 50 |

IM = intramuscular, EP = electroporation, ID = intradermal, NFI = needle-free injection, n = number of NHPs.

neutralizing antibody responses, delivery of vaccine via electroporation and/or via the intradermal route resulted in better responses than by intramuscular needle-free injection. Consistent with previous studies, a dose sparing effect was observed for intradermal delivery in both the EP and NFI groups as the lower dose ID groups had higher mean neutralizing antibody titers than the high dose IM groups. Comparing the same route of administration and dose, electroporation groups overall had higher mean neutralizing antibody titers than the corresponding NFI groups.

T cell responses also play a role in protection from dengue. For T cell responses, a dose sparing effect of intradermal delivery was not apparent. At day 35, for the electroporation groups, the $IM_{5mg}EP$ group had higher IFN-γ responses than the $ID_{1mg}EP$ group, but at day 240 the low dose ID group had higher IFN-γ responses than the high dose IM group. For the NFI groups, at both days 35 and 240 the responses between the high dose IM and low dose ID groups were similar. Comparing the same route of administration and dose, electroporation groups overall had higher IFN-γ responses at both days 35 and 240.

The generation of memory B cells by a vaccine is important in generating a rapid and robust response to infection. In evaluating the generation of memory B cells, there was no clear dose sparing effect of intradermal delivery as the high dose IM and low dose ID groups for both EP and NFI delivery were similar. Comparing the same route of administration and dose, electroporation groups had higher memory B cell responses for the high dose IM and low dose ID groups while the responses were comparable for the high dose ID groups.

In order to determine which vaccination route and delivery method for the dengue DNA vaccine best induced protective immunity, we challenged the vaccinated and a control group of NHPs with dengue-1 virus. Based upon nucleic acid amplification assays, only

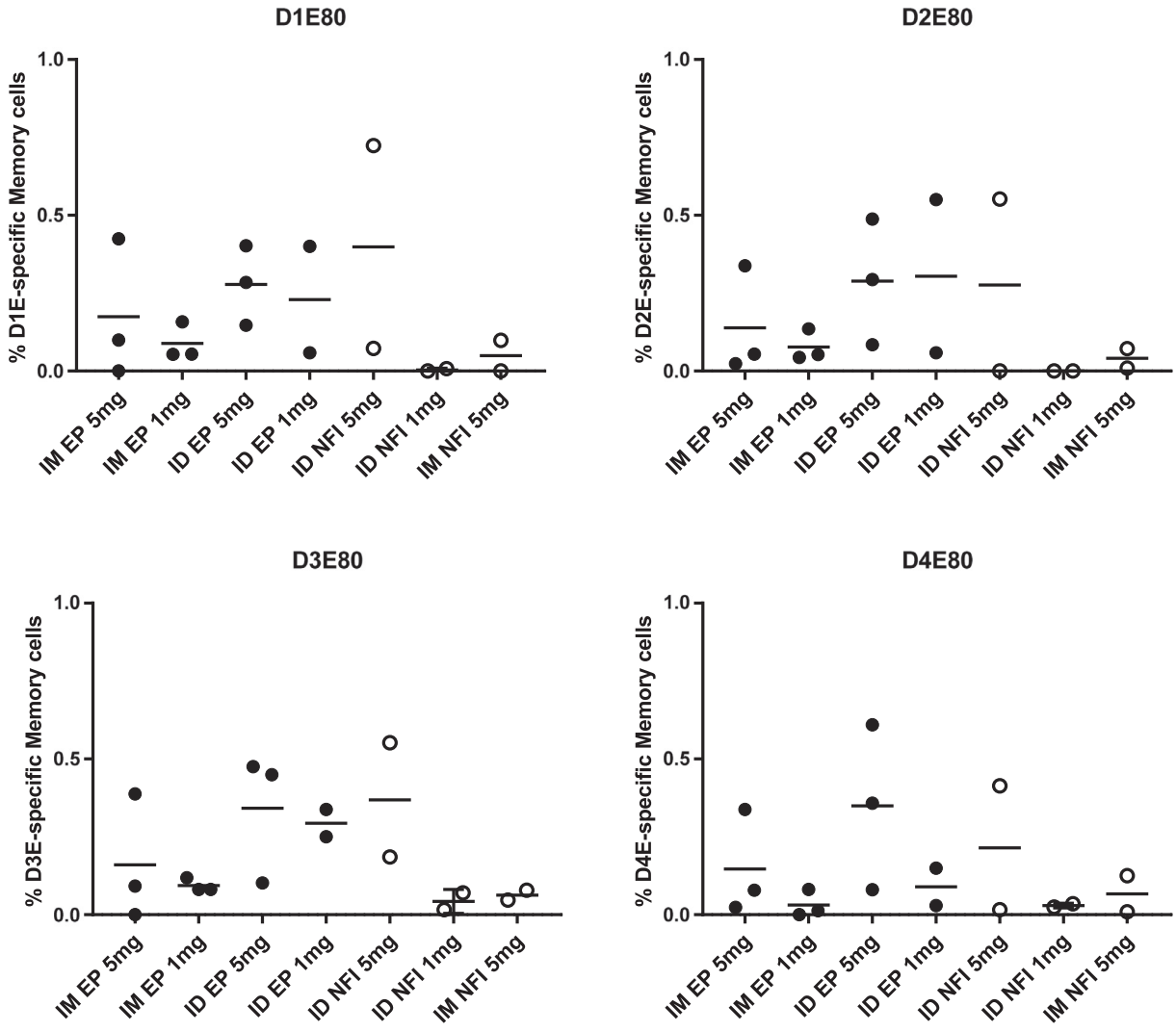


**Fig. 5.** Memory B cells. Percentage of dengue-specific memory B cells for each animal by group and serotype. Percentage of serotype specific memory B cells for each animal is represented by a dot. The mean for each group by serotype is represented by a horizontal line.

the low and high dose intradermal electroporation groups had significantly fewer days of dengue-1 virus RNAemia compared to the control group.

This study had several limitations. In examining the T cell responses, we were not able to assess pre-vaccination dengue specific T cell responses for all of the NHPs. While all of the NHPs were negative for dengue antibodies before vaccination, it is possible that they still could have been positive for dengue specific T cell responses. In our experience, while this is rare, it is possible and in fact, one of the NHPs that was tested for pre-vaccination dengue-specific IFN-$\gamma$ responses was positive for dengue-2. In addition, due to limited cell numbers some of the group sizes for the cellular immune response analyses were reduced from their original intended sample size. Thus, one must be cautious in interpreting the statistical analysis results given that power was reduced for some groups.

In this study, we wanted to directly compare equivalent vaccine doses delivered via alternate routes and devices. While dose, route of delivery and device used for delivery all had an effect on the immune response generated from the dengue DNA vaccine, overall, the device used for delivery had the largest effect. Groups receiving vaccine via EP had both higher antibody and T-cell responses. While increased antibody responses were also observed when the vaccine was delivered via the intradermal route, this effect is limited by the fact that only a small volume of vaccine can be delivered in one administration via the intradermal route. Previous clinical trials with this dengue DNA vaccine administered the vaccine IM using either needle and syringe or a needle-free injection device [12,13]. Delivery of DNA vaccines using electroporation is advantageous in that the use of electroporation has been shown to increase the amount of plasmid taken up by cells 100–1000 fold compared to the delivery of plasmid DNA without electroporation. The increased cellular uptake of plasmid DNA using electroporation leads to increased antigen expression and has been shown to result in a 10–100 fold enhancement of the immune response for several different DNA vaccines [33]. Vaccination using an ID route is advantageous in that the dermis and epidermis of the skin contains a high concentration of antigen presenting cells. As these cells are critical for processing and presenting incoming antigens, vaccine delivery to the dermal region allows for more efficient antigen presentation and a corresponding increase in the induced immune responses. The results from this NHP study indicate that using either an EP device and/or the ID route of delivery for this dengue DNA vaccine may increase the immune response generated by this vaccine in humans and should be explored.

**Table 4**
Dengue-1 RNAemia in vaccinated and control animals.

| Group | ID | $MN_{50}$ | DENV-1 copy number/μl sera, days post-challenge | | | | | | | | | | RNAemia | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | Total days | Mean days | P value |
| 5 mg IM EP | 48 | 80 | – | 21 | 5 | 2 | 5 | 3 | – | – | – | – | – | 5 | 4.25 | 0.0631 |
| | 57 | 80 | – | 1 | – | – | – | 2 | – | – | – | – | – | 2 | | |
| | 59 | 40 | – | – | – | 3 | 2 | 112 | 1 | – | – | – | – | 5 | | |
| | 60 | 80 | – | 7 | 1 | – | 2 | 1 | – | – | – | – | – | 5 | | |
| 1 mg IM EP | 44 | 80 | – | 1 | 1 | 6 | 108 | 763 | 5 | – | – | – | – | 6 | 5.75 | 0.8056 |
| | 50 | 20 | – | 10 | 1 | 40 | 61 | 340 | 30 | 2 | 1 | – | – | 8 | | |
| | 51 | 80 | – | 7 | 2 | 1 | 5 | 6 | – | – | – | – | – | 5 | | |
| | 64 | <20 | – | – | – | – | 1 | 17 | 2 | 1 | – | – | – | 4 | | |
| 5 mg ID EP | 42 | 320 | – | – | – | 1 | – | 8 | – | – | – | – | – | 2 | 1.5 | <0.0001 |
| | 43 | 160 | – | – | – | – | – | – | – | – | – | – | – | 0 | | |
| | 46 | 80 | – | – | – | 3 | 6 | 40 | – | – | – | – | – | 3 | | |
| | 55 | 160 | – | – | – | – | – | 39 | – | – | – | – | – | 1 | | |
| 1 mg ID EP | 47 | 160 | – | 1 | – | 1 | – | 2 | – | – | – | – | – | 3 | 3 | 0.0027 |
| | 49 | 40 | – | 52 | 4 | 21 | 16 | 1 | – | – | – | – | – | 5 | | |
| | 53 | 80 | – | 2 | – | – | 1 | 3 | – | – | – | – | – | 3 | | |
| | 58 | 160 | – | – | – | – | – | – | 1 | – | – | – | – | 1 | | |
| 5 mg ID NFI | 45 | 80 | – | 8 | 3 | 4 | 4 | 6 | – | – | – | – | – | 5 | 4.75 | 0.1837 |
| | 52 | 320 | – | – | – | 1 | 13 | 109 | 1 | – | – | – | – | 4 | | |
| | 62 | 160 | – | 1 | – | 3 | 20 | 23 | – | – | – | – | – | 4 | | |
| | 63 | 320 | – | 2 | – | 3 | 8 | 175 | 14 | 5 | – | – | – | 6 | | |
| 1 mg ID NFI | 40 | <20 | – | 1 | 2 | 9 | 29 | 525 | 431 | 37 | – | – | – | 7 | 5.5 | 0.6238 |
| | 61 | 80 | – | 14 | 1 | 1 | 2 | 15 | – | – | – | – | – | 5 | | |
| | 65 | <20 | – | 41 | 77 | 43 | 8 | 4 | – | – | – | – | – | 5 | | |
| | 66 | 80 | – | 28 | 6 | 248 | 80 | 18 | – | – | – | – | | 5 | | |
| 5 mg IM NFI | 39 | 20 | – | 10 | 17 | 6 | 8 | 7 | 2 | – | – | – | – | 6 | 5 | 0.2933 |
| | 41 | 80 | – | 1 | 2 | 1 | 5 | – | 13 | – | – | – | – | 5 | | |
| | 54 | 20 | – | 4 | – | 1 | 2 | 3 | – | – | – | – | – | 4 | | |
| | 56 | <20 | – | 168 | 111 | 80 | 11 | 3 | – | – | – | – | – | 5 | | |
| Control | 77 | <20 | – | 313 | 213 | 62 | 7 | 6 | 2 | – | 1 | – | – | 7 | 6.75 | |
| | 78 | <20 | – | 225 | 397 | 254 | 63 | 23 | 4 | 2 | 1 | – | – | 8 | | |
| | 79 | <20 | – | 820 | 915 | 318 | 35 | 10 | 2 | 1 | – | – | – | 7 | | |
| | 80 | <20 | – | 399 | 104 | 64 | 2 | 1 | – | – | – | – | – | 5 | | |

ID = animal identification number, $MN_{50}$ = 50% microneutralization titers for DENV-1 at day 392.

## Disclaimers

The views expressed in this article reflect the results of research conducted by the author and do not necessarily reflect the official policy or position of the Department of the Navy, Department of Defense, nor the United States Government. I am a military Service member [or employee of the U.S. Government]. This work was prepared as part of my official duties. Title 17, U.S.C., §105 provides that copyright protection under this title is not available for any work of the U.S. Government. Title 17, U.S.C., §101 defines a U.S. Government work as a work prepared by a military Service member or employee of the U.S. Government as part of that person's official duties.

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## Declaration of Competing Interest

K.R. and K.R.P. are patent holders on US Patent No. US 6455509 title "Dengue nucleic acid vaccines that induce neutralizing antibodies."

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