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Strategies to improve the immunogenicity of prM+E dengue virus type-2 DNA vaccine

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Abstract

Background: An important goal for dengue vaccines is to induce a high and durable level of neutralizing antibody.

Objective: Three strategies were investigated for improving the immunogenicity of a prM+E dengue serotype 2 (DENV-2) DNA vaccine: 1) expression in two different plasmids; 2) adjustment of dose; and, 3) introduction of the E sequence of *Japanese encephalitis virus* (JEV) at the carboxy-terminal portion of DENV-2 E.

Methods: Expression cassettes were designed to encode a full-length prM+E sequence of DENV-2 virus employing human-preferred codons (D2prME^{opt}), or a chimeric prM+E sequence in which the 100-residue carboxy-terminal region of E was derived from JEV (D2prMEJE20^{opt}). pHIS and pCMVkan in the presence and absence of CpG motif, respectively, were used for cassette expression. The immunogenicity was compared in mice.

Results: Three injections of full-length-D2prME^{opt} in pHIS and pCMVkan induced a comparable neutralizing antibody titer at post-week-2-injection and post-week-4-injection. The 100-μg DNA dose induced a numerically but not statistically higher neutralizing antibody titer than the 10-μg dose. The chimeric-D2prMEJE20^{opt} produced higher extracellular prM and E protein levels in transfected Vero cells, but had a tendency to induce a lower neutralizing antibody titer in mice when compared with the full-length-D2prME^{opt}. To optimize the immunogenicity of D2prME^{opt}-DNA candidate, both expression plasmids can be used to generate reproducible high neutralizing titer. A higher dose of DNA immunogen may induce a higher neutralizing antibody response.

Conclusion: The strategy of the C-terminal region chimeric counterpart with JE20 did not improve but may have reduced the induction of neutralizing antibodies.

Keywords: dengue serotype 2 virus, DNA vaccine, needle-free injection, neutralizing antibody, Japanese encephalitis, chemokine

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Abbreviations:
IFA, indirect immunofluorescence assay;
ID, intradermal;
IP, intraperitoneal;
IM, intramuscular;
PRNT, plaque reduction neutralization test;
DENV-2, dengue serotype 2 virus;
JEV, Japanese encephalitis virus;
prM, pre-membrane;
E, envelope

Introduction

Many dengue vaccine studies have utilized live attenuated and chimeric viruses. These types of vaccines are able to induce both antibody and cellular immune responses, but when used together to combat the four serotypes of dengue may result in poorer immunogenicity. It is possible that the effects of each vaccine may cancel out the immune response of the other. Therefore, a DNA vaccine may be a better alternative strategy to overcome this concern because it has been shown that tetravalent administration can induce high titers of neutralizing antibodies without causing any interference.[1,2]

The main antigens used in the dengue DNA vaccine are the envelope (E) and pre-membrane (prM) proteins of the dengue virus. Co-expression of prM and E genes in mammalian and insect cells leads to the formation and release of virus-like particles, which are immunogenic in mice.[3] Chimeric constructs consisting of prM and the amino-terminal 80% of E (residues 1-397) of DENV-2 directly fused with the carboxy-terminal 20% of E (residues 397-450) derived from Japanese encephalitis virus (JEV) can remove the endoplasmic reticulum-retention signal that is inherent in the dengue virus E protein. This technique can enhance the secretion of virus-like particles from transfected mammalian cells, and elicits a higher titer of neutralizing antibodies in mice.[4]

There are various techniques that can be used to enhance the efficacy of DNA vaccines such as modification of the vector to include agents that have immunostimulatory effects, optimization of immunogenic codon sequences as well as its route and method of administration.[5] For example, it has been shown that vector modification can affect the level of antigen expression and/or immune responses.[6] Other examples include the addition of unmethylated CpG 2006 motifs in pHIS[7,8] or utilization of the humanized cytomegalovirus immediate-early (CMV_{ie}) promoter in pCMVkan,[9] which has been shown to be able to enhance the expression of the antigen and improve the immune response. However, no one has directly compared the effects of these two expression vectors.

In addition, the vaccine administration route can significantly affect the immune response to the antigens. For instance, DNA vaccines administered intramuscularly had poor immunogenic responses compared to the intradermal route. The low immunogenic response may be due to the lack of antigen-presenting cells in the muscle tissues.[10] Besides the route of administration, the method used to deliver the vaccine can also improve the immune response. For example, needle-free injection has been shown to be more effective. Using high pressure injection, small amounts of DNA vaccine can be widely dispersed and diffused into the tissue, resulting in antigen uptake by the immune cells.[11,12] This approach has been used in several vaccines to enhance the immune responses to HIV, JE, hepatitis A and hepatitis B.[11,13-15] Another method recently used in HIV clinical trials[16] and cancer vaccines[17] is *in vivo* electroporation, which has been shown to increase the immunogenicity of DNA-based vaccines. Since there are many techniques to enhance the immunogenicity of DNA-based vaccines, for this study, we explored a number of strategies, including the use of different vectors, the injecting doses and chimeric immunogens, to improve the immunogenicity of the DENV-2 DNA vaccine.

Methods

Cells and viruses

African green monkey kidney-derived Vero cells, $LLC\text{-}MK_2$ cells, and a DENV-2 strain 16681 were provided by the Armed Forces Research Institute of Medical Sciences, Thailand. A newer DENV-2 isolate, strain 03-0420, was provided by Dr. Prida Malasit, Thailand. Vero cells and $LLC\text{-}MK_2$ were propagated in minimum essential medium (MEM with Earle salts) supplemented with 10% fetal bovine serum (FBS, Gibco BRL, Gaithersburg, MD) and in Medium 199 supplemented with 20% FBS, respectively; cells were incubated at 37°C with 5% CO_2. DENV-2 viral stock was propagated in Vero cells. Virus titers were determined by a plaque assay utilizing a monolayer of $LLC\text{-}MK_2$ cells.[18]

Expression vectors

pHIS (Pfizer Global Research and Development, Canada)[7] and pCMVkan (National Cancer Institute, Frederick, Maryland, USA)[19] were used to construct the DENV-2 DNA vaccine. Both vectors contained the cytomegalovirus immediate-early promoter, the bovine growth hormone (bGH) polyadenylation signal, and the kanamycin resistance gene. pHIS also contained the humanized CpG 2006 motifs.

Constructs and optimizations of the human codon for the DENV-2 vaccine

A DENV-2 prM+E consensus amino acid sequence was generated from 126 sequences obtained from the GENBANK database for the Thailand DENV-2 from 1974-2004. The JEV consensus E sequence was derived from 12 and 10 amino acid sequences from strains isolated in Thailand and Japan, respectively, from 1999-2007. Codon usage database (http://www.kazusa.or.jp/codon/) was used to obtain the optimized human codon. The Kozak and prM signal sequence (Met-Val-Asn-Arg-Arg-Arg-Arg-Ser-Ala-Gly-Met-Ile-Ile-Met-Leu-Ile-Pro-Thr-Val-Met-Ala) were inserted at the N-terminal. For the chimeric prM+E, the C-terminal 100-residue sequence (E401-E500) of E was derived from the homologous sequence of JEV. The sequences were synthesized by GENEART (Regensburg, Germany).

The full-length sequence was inserted into the XhoI-KpnI sites of pHIS and NotI-SalI sites of pCMVkan (designated: pHIS-D2prME^{opt} and pCMVkan-D2prME^{opt}, respectively). The chimeric constructs were derived from pHIS-D2prME^{opt} and pCMVkan-D2prME^{opt} by digesting with XmnI and NotI, followed by replacement of the JEV C-terminal sequence (designated as pHIS-D2prMEJE20^{opt} and pCMVkan-D2prME JE20^{opt}, respectively) (**Figure 1**). All plasmid constructs were transformed into *Escherichia coli* DH5αF' (Invitrogen, California, USA) and their sequences confirmed by nucleotide sequence analysis.

The native codon of prM+E was amplified from a plasmid subclone containing nt134-2504 of a DENV-2 isolate from 2003, strain 03-0420 (GenBank ID: JN556046.1), by using D2-prM-S

(5'GGGCTCGA-GACCATGGTGAATAGGAGACGCAG GTCTGC3') and D2-2401-R (5'GAGGCGGCC-GCTTAGGCC TGCACCATGACTCCCAA3') primers. The PCR product was digested with XhoI and NotI, and cloned into pHIS.

Analysis of the protein expression after the transfection of the plasmid

Vero cells were transfected with 4 μg of recombinant plasmids or empty vectors, using lipofectamine 2000 (Invitrogen). At 24-72 hours after transfection, cells were analyzed by indirect immunofluorescence and supernatants were subjected to western blot analysis.

Indirect immunofluorescence assay

Transfected cells were fixed with 3.7% formaldehyde, and permeabilized with 2% Triton X-100 and then incubated with an anti-DENV-2 E antibody (clone 3H5) (20), and an anti-NS1 antibody, clone 2E11 (or NS1-1F).21 Intracellular viral proteins were visualized with a combination of Cy3-conjugated anti-mouse IgG antibody (Jackson ImmunoResearch, PA, USA) and Alexa 488-conjugated anti-mouse IgM antibody (Invitrogen). Nuclei were counterstained with 4,6-diamino -2-phenylindole hydrochloride (DAPI) (Sigma–Aldrich, MO, USA).

Quantitative immunoblot analysis of extracellular viral proteins

The transfected (18.75 μl culture supernatants + 6.25 μl 4x non reducing sample buffer) culture supernatants were applied to polyacrylamide gels (5% stacking gel and 13% resolving gel). A standard curve was included with each experiment. The linear range for prM and E was determined by using serial dilutions of DENV-2 strain 03-0420 from 0.16 to 5 μL.

The gels were electrophoresed and the proteins were transferred onto nitrocellulose membrane. Blots were blocked with non-fat skim milk. E and prM proteins were detected using an anti-flavivirus E antibody, clone 4G2,[22] and an anti-DENV prM antibody, clone prM-6.1,[23] respectively. The membrane was incubated with horseradish peroxidase-conjugatedanti-mouse IgG antibody (Invitrogen), and an enhanced chemiluminescence substrate (Perkin Elmer, MA, USA), and then exposed to X-ray film. The protein band densities were imaged and analyzed with SynGene Gene Tools software (G:BOX chemi systems, Syngene, Cambridge, UK).

Immunization of mice

Mice

ICR mice, aged 3-6 weeks, were purchased from the National Laboratory Animal Centre, Bangkok, Thailand. The animal care was in compliance with the National Institutes of Health guidelines. Two separate experiments were performed to assess the effects when different strategies were used (**Figure 4, 5**). An additional experiment was performed in a large group of mice to confirm whether the results were reproducible or not (**Figure 6**). All studies were approved by the Committee of Animal Care and Use of the Faculty of Medicine, Chulalongkorn University.

Needle-free immunization

Mice were immunized with 10 or 100 μg of plasmid DNA which was resuspended in PBS. The total final volume for the vaccine was 100 μL which was administered at weeks 0, 2, and 4 by a needle-free jet injector (ShimaJET, Shimadzu, Kyoto, Japan) in both thighs. DENV-2 strain-16681 (105 PFU/mouse) was used as a positive control. The empty pHIS (63 μg) and the empty pCMVkan (66 μg) were used as the negative controls. Bleedings were performed at week 2 and 4 post-last injection for neutralizing antibody tests.

Evaluation of the serotype-specific response to DENV-2 DNA vaccine

Five mice were needle-free immunized with three doses of 25 μg of pCMVkan-D2prME^{opt} (as described above). Two weeks post-last injection, mice were bled and sera were pooled for neutralizing antibody assays against DENV-1 strain 16007, DENV-2 strain 16681, DENV-3 strain 16562 and DENV-4 strain 1036.

Plaque reduction neutralization test (PRNT)

A PRNT[18,24] was employed with slight modifications to assess the neutralizing antibody activity against the DENV-2 strain 16681. Briefly, the sera collected from the mice were inactivated at 56°C for 30 min, two-fold serially diluted in 10% MEM (1:10-1:1280), and then incubated at 37°C for 1 hour with an equal volume of diluted DENV-2 strain-16681 so the total final concentration of the virus was 500 PFU/mL. The virus-antibody mixes were incubated on LLC-MK$_2$ for 1 hour. Following virus absorption, cells were overlaid with the first overlay medium containing 1.8% low-melting agarose (Invitrogen). After 4 days of incubation at 37°C with 5% CO_2, the cells were stained with the second overlay medium containing 4% [w/v] neutral red (Sigma-Aldrich). Plaques were counted after 24 hours of incubation at 37°C.

Statistical analysis

The comparisons of prM and E protein expressions from the plasmid transfected-cells and supernatants were determined using a one-way ANOVA with Tukey's multiple comparison test. The differences of $PRNT_{50}$ titers between groups of immunized mice at each time point were performed by Mann-Whitney test. The GraphPad Prism software (La Jolla, USA), version 3.0, was used for the analyses. The values were considered significant at $p < 0.05$.

Results

Design of DENV-2 DNA vaccine

The inserted codon-optimized full-length DENV-2 prM+E expression cassette (D2prMEopt) and its chimeric derivative (D2prMEJE20^{opt}) in both pHIS and pCMVkan are shown in **Figure 1**. The native prM+E expression cassette was generated by amplifying the whole prM+E coding region from a DENV-2 isolated, strain 03-0420, and cloned into pHIS.

In vitro protein expression

At 24 hours after transfection, cytoplasmic expression of the E protein was observed with all recombinant plasmids

(**Figure 2**). Transfected pHIS-D2prMEJE20^{opt} cells had very high expressions of the E protein but this was not statistically significant (mean±SD, 44.1±9.5%, n=6) compared to in pHIS-D2prME^{native} (37.7±5.7%, n=3) or pHIS-D2prME^{opt}-transfected cells (38.4±12.0%, n=7). Similar results were observed following transfections with pCMVkan-D2prMEJE20^{opt} (42.9±8.3, n=5) and pCMVkan-D2prME^{opt} (34.3±10.1%, n=5). None of the plasmid-transfected cultures reacted with anti-NS1 antibody. Cells infected with strain-16681, which served as the positive control, were positive for both anti-E and anti-NS1 antibodies. Cells with empty pHIS, which served as the negative control, were negative for both anti-E and anti-NS1 antibodies.

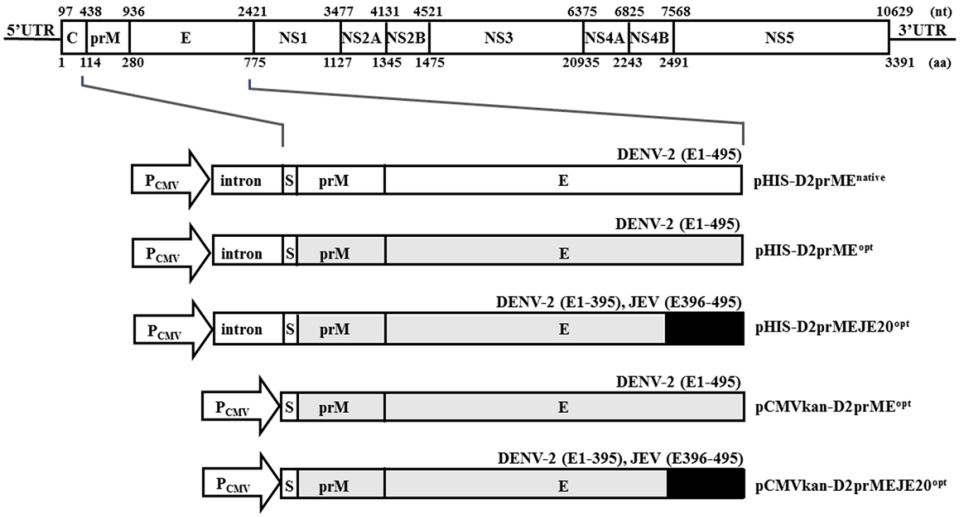

Figure 1. Schematic representations of the dengue virus genome and various prME expression cassettes. Upper panel, organization of the RNA genome of the dengue serotype 2 virus strain-16681. Nucleotide (upper line) and amino acid (lower line) positions are indicated separately. Lower panel, organization of the prME expression cassettes inserted into the pHIS or pCMVkan vectors. Clear and shaded boxes represent regions encoded with the native codons and human-optimized codons, respectively. The black box indicates the 100-residue C-terminal region derived from the human-optimized codons from the JEV. S, signal sequence derived from the C-terminal region of the capsid gene.

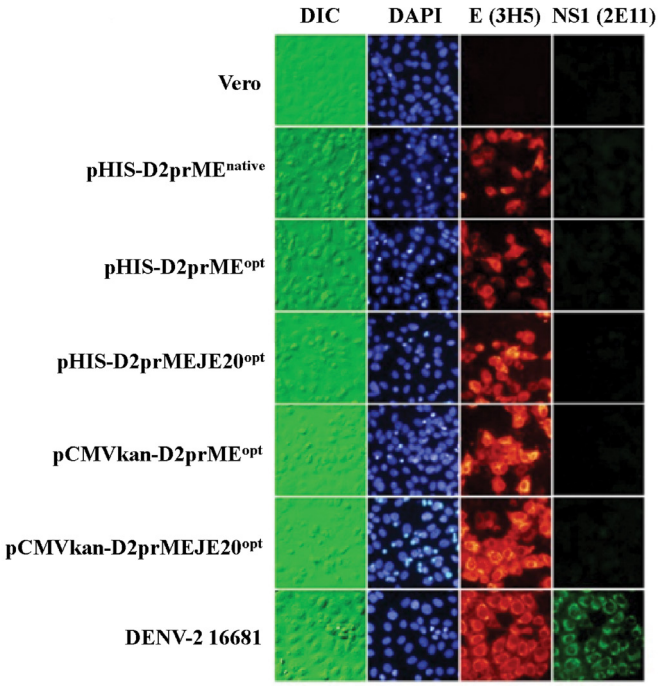

Figure 2. Expression of DENV-2 prME plasmid constructs in Vero cells. Vero cells were transfected with indicated recombinant plasmids by employing lipofectamine 2000, incubated at 37°C for 24 hours, and then fixed, permeabilized and reacted with monoclonal antibodies specific for DENV-2 E protein (3H5, IgG) and NS1 protein (2E11, IgM). The expressions of E and NS1 were visualized with Cy3-conjugated anti-mouse IgG antibody and Alexa 488-conjugated anti-mouse IgM antibody, respectively. DAPI was used to stain the nuclei. Vero cells infected with dengue serotype 2 virus strain-16681 served as the positive control for the staining and detection processes. DIC, image of cells captured using differential interference contrast.

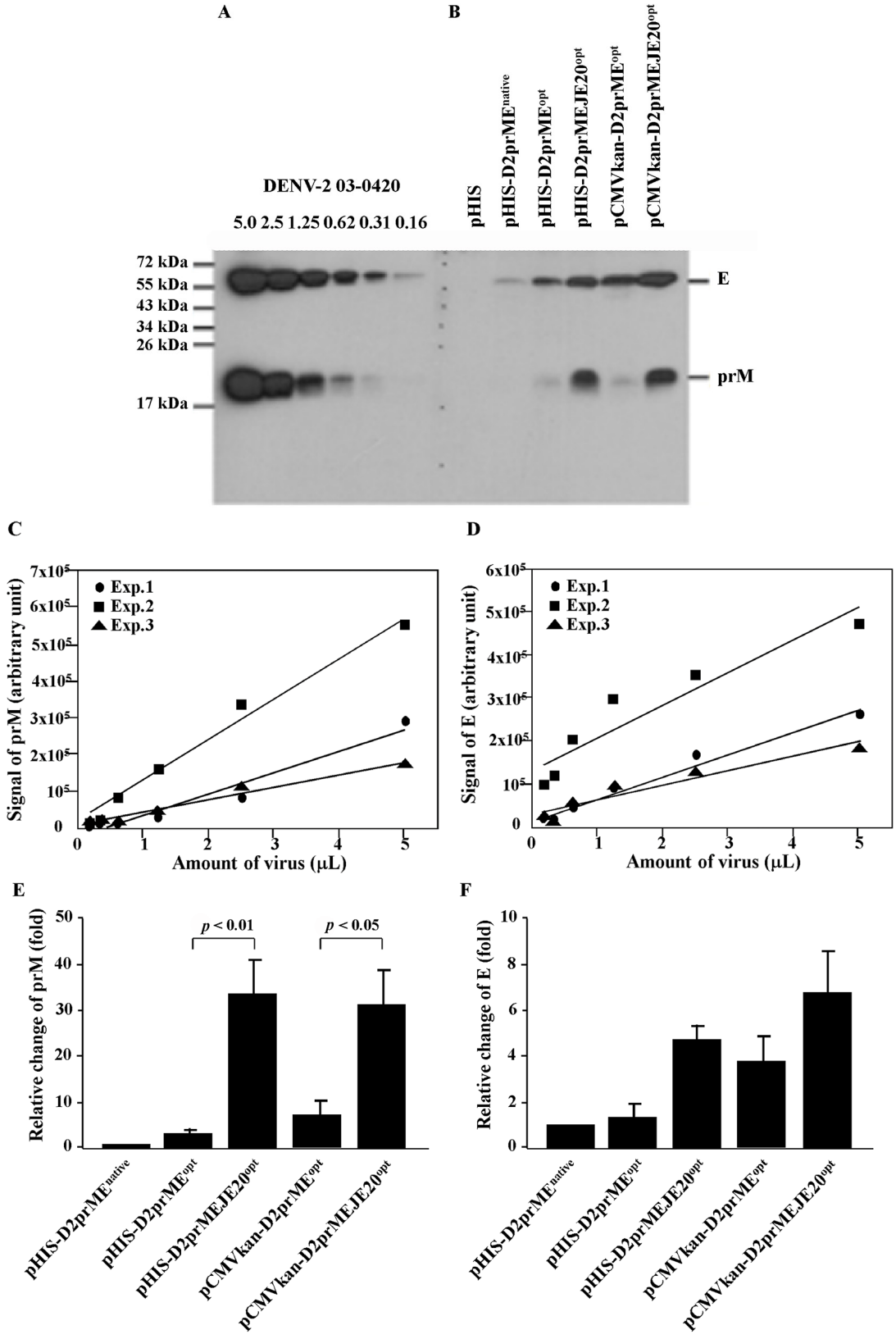

Figure 3. Quantitation of extracellular prM and E proteins by immunoblot analysis. Known amount of the dengue serotype 2 virus strain-03-0420 (5-0.16 μL) (A)were blotted to generate standard curves for: (C)prM and (D)E. Equal volumes of plasmid transfected supernatants (B) were blotted for protein quantitation. All samples were probed with monoclonal antibodies specific for flavivirus E protein(4G2), and dengue virus prM protein (prM-6.1) and then detected by the alkaline phosphatase-conjugated anti-mouse IgG antibody and a chemiluminescent substrate. Blots were exposed to X-ray film and the band intensities were analyzed by densitometry. The amount of protein expression from the indicated plasmid constructs were determined by quantitative immunoblot analysis and showed as the relative fold change for (E) prM and (F) E. The results shown are representatives of three separate experiments.

Immunoblot analyses showed that all plasmid constructs had prM and E bands of approximately 18 and 55 kDa in size, respectively, in the supernatants (**Figure 3B**). The DENV-2 strain 03-0420 was used to generate the standard curves for prM and E (**Figure 3A**). The intensities of the bands from all plasmid constructs were compared to the standard curve to assess the production of prM and E proteins (**Figure 3E, 3F**). Densitometric analysis indicated that the chimeric-D2prMEJE20^{opt} pHIS and pCMVkan generated significantly higher levels of prM (**Figure 3D**, p<0.01 and 0.05, respectively). The production of the E protein was observed to increase in the chimeric plasmids, but this trend did not reach statistical significance (**Figure 3F**). The optimized human codon prM+E construct had a modest increase in signal intensity for both prM and E bands over the native codon construct. No viral protein band was observed in the empty plasmids (**Figure 3B**).

Comparison of the immunogenicity of the pHIS expression plasmid with full-length prM+E and chimeric prM+EJE20

At weeks 0, 2, and 4, a dosage of 10 or 100 μg of the pHIS-D2prME^{opt} and pHIS-D2prMEJE20^{opt} was administered by a needle-free injector (**Figure 4A**). The full-length-D2prME^{opt} induced significantly higher levels of neutralizing antibody than the chimeric-D2prMEJE20^{opt} at the 100-μg dosage (median PRNT50 titers=1:640 *vs.* 1:40; p=0.008), but not at the 10-μg dosage (**Figure 4B**). The immune response appears to be dose-dependent. In contrast, the chimeric-D2prMEJE20^{opt} was less immunogenic when injected at a higher dosage compared to the lower dosage (1:40 *vs.* 1:160, p=0.032).

As for the pCMVkan full-length-D2prME^{opt} and chimeric-D2prMEJE20^{opt}, three dosages of 100-μg of the full-length generated a stronger immune response but this was not statistically significant. The median PRNT50 titer at both 2 and 4 weeks post-last injection for pCMVkan-D2prMEJE20^{opt} was higher than the chimeric-D2prMEJE20^{opt} (1:640 *vs.* 1:160 and 1:320 *vs.* 1:160, respectively) (**Figure 5B and 5C**).

Comparison of the immunogenicity between pHIS and pCMVkan-based vectors

Here, 10 or 100 μg per dosage of pHIS and pCMVkan-based recombinant DNA immunogens were administered at weeks 0, 2 and 4 by a needle-free injector (**Figure 5A**). There were no significant differences of the median PRNT50 titers between both groups 2 weeks post-last injection (**Figure 5B**). However, it should be noted that the pHIS-D2prMEopt at 100 μg induced the highest neutralizing antibody response (1:960) when compared to the 10 μg dosage of pHIS-D2prMEopt (1:80, p=0.016). The pHIS-D2prMEopt at 100 μg induced a higher immune response compared to the pCMVkan-D2prMEopt at 100 μg (1:320, p=0.032) (**Figure 5C**).

Specificity of the antibody response

In order to assess the specificity of the antibody response to the vaccine, a different dosage was used. The mice were immunized with 25 μg dosages of pCMVkan-D2prME^{opt} at weeks 0, 2, and 4 (**Figure 5A**). Blood was collected from the mice at two weeks post-week-4-injection. Pooled sera at two weeks after the last injection revealed the highest PRNT50

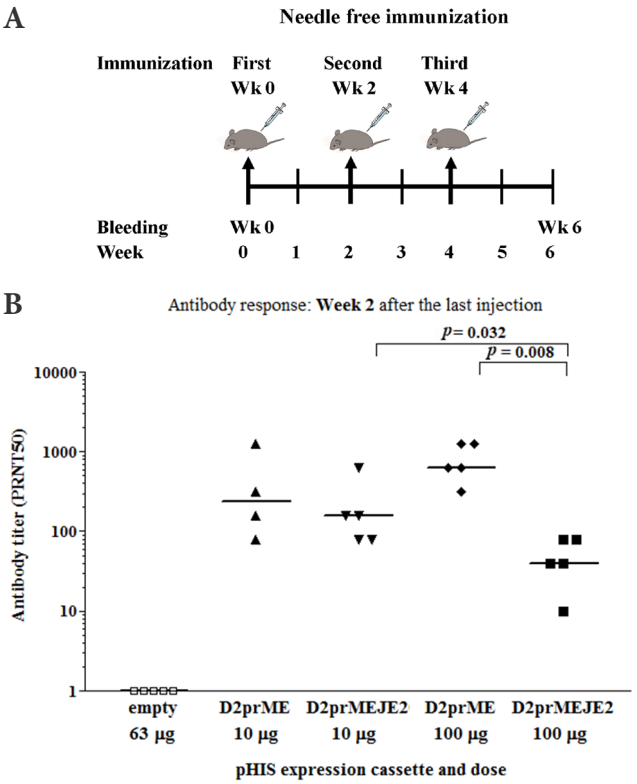

Figure 4. The induction of the neutralizing antibodies to the pHIS-based recombinant plasmids. (A) Vaccine immunization schedule. ICR mice were immunized by needle-free injectors with 10 or 100 μg of pHIS-D2prME^{opt} and pHIS-D2prMEJE 20^{opt} at weeks 0, 2 and 4. At week 6, the mice were bled. A 63 μg of pHIS empty plasmid was used as the negative control. (B) Individual serum samples were serially diluted from 1:10 to 1:2560 and tested via the PRNT. DENV-2 strain-16681 was used as the target virus. Horizontal line represents the median PRNT50 titer for each group of mice.

(1:320) against the DENV-2 targeted virus. There were some cross-reactive neutralizing activities detected against DENV-1, -3 and -4 viruses. The PRNT50 titers against DENV-1, -3 and -4 viruses were 1:40, 1:20 and 1:10, respectively (**Figure 5D**).

Reproducibility study of DENV-2 DNA vaccine immunization

To assess the DENV-2 DNA vaccine reproducibility, an additional experiment was performed in which the number of mice was increased to 10 per group. The mice received three doses of 100-μg DENV-2 DNA vaccines per time at weeks 0, 2, and 4 (**Figure 5A**). Both pHIS and pCMVkan-based full-length-D2prME^{opt} induced a comparable level of neutralizing antibody titer post-week-2-injection and post-week-4-injection (**Figure 6A and 6B**). For both vectors encoding the full-length-D2prME^{opt}, the levels of the neutralizing antibody were higher than the chimeric -D2prMEJE20^{opt} at post-week-2-injection. However, when the full-length construct was compared to the chimeric construct for the pCMVkan-based vector, we noticed a significant difference in its ability to elicit neutralizing antibodies. The full-length construct was able to induce a

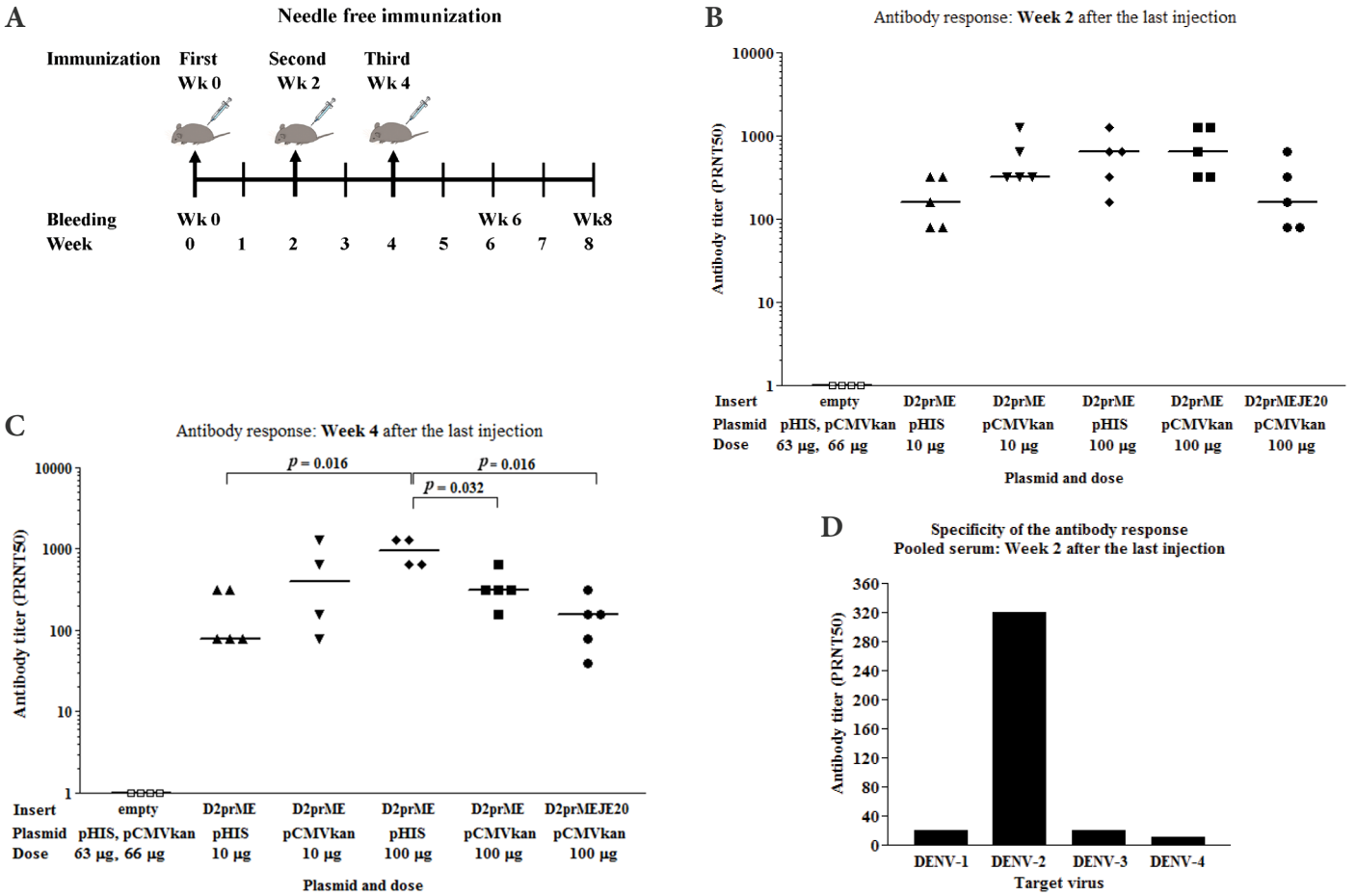

Figure 5. The induction of the neutralizing antibodies to the recombinant pHIS- and pCMVkan-based plasmids. (A) Vaccine immunization schedule. ICR mice were immunized by needle-free injectors with three doses of 10 or 100 µg of pHIS-based or pCMVkan-based plasmids encoded with the prME protein of DENV-2 at weeks 0, 2 and 4. An additional plasmid, pCMVkan-D2 prMEJE20^{opt}, was assessed at 100-µg dose in parallel. Individual serum samples at post-week-2-injection (B) and post-week-4 -injection (C) were tested for anti-DENV-2 neutralizing antibodies. The median PRNT50 titer is shown for each group of immunized mice as horizontal line. (D) Serotype specificity of the immune response. Five mice were immunized with three doses of 25 µg of pCMVkan-D2prME at weeks 0, 2 and 4. At week 6, the sera were pooled and tested via the PRNT by using the following target viruses: DENV-1 strain-16007, DENV-2 strain-16681, DENV-3 strain-16562, and DENV-4 strain-1036.

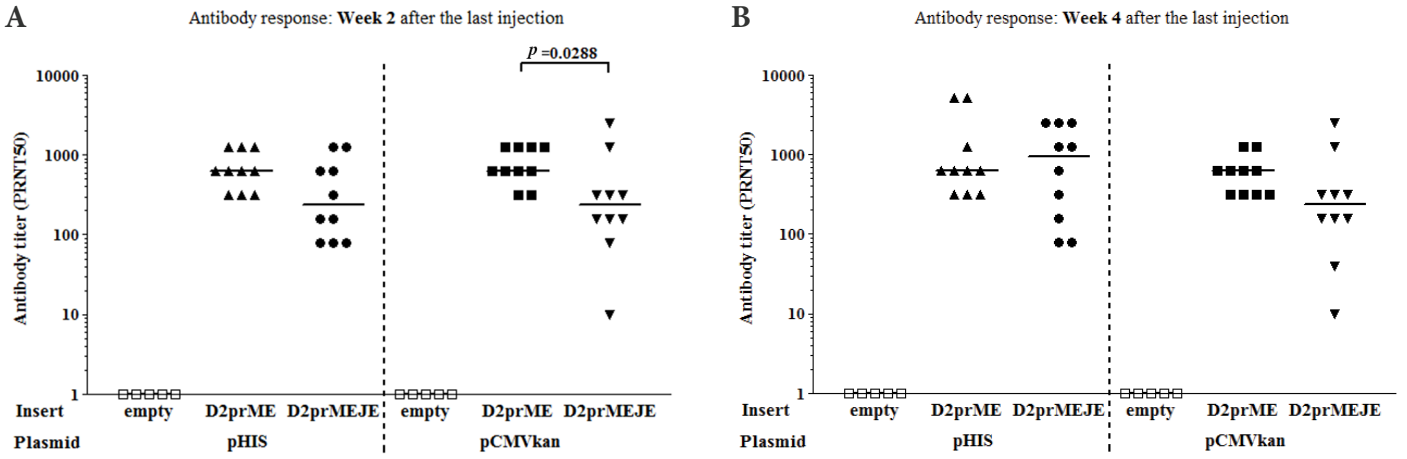

Figure 6. The induction of the neutralizing antibodies to the DENV-2 DNA vaccines from the reproducibility experiment. Based on the same immunizing schedule as **Figure 5A**, three doses of 100 µg of pHIS-based or pCMVkan-based plasmids encoded with the prME^{opt} or prMEJE20^{opt} were administered to ICR mice (10 mice/group) by needle-free injectors at weeks 0, 2 and 4. At week 6, the mice were bled. Individual serum samples at post-week-2-injection (A) and post-week-4-injection (B) were tested for anti-DENV-2 neutralizing antibodies. The median PRNT50 titer is shown for each group of immunized mice as horizontal line.

very high level of neutralizing antibodies compared to the chimeric pCMVkan-based construct (median PRNT50 titers (IQR) = 1:640 (1:640-1280) *vs.* 1:240 (160-320)). This was also seen at post-week-2-injection (1:640 *vs.* 1:240). However, when the full-length construct was compared to the chimeric construct for the pHIS vector, there was no significant difference detected in its ability to elicit neutralizing antibodies.

Discussion

Three strategies were investigated to see whether the immunogenicity of our DNA vaccine can be improved, such as the utilization of certain plasmid vectors, modification of the dosages, and the presence of the endoplasmic reticulum retention signal. Two plasmid vectors, pHIS and pCMVkan, were chosen based on previous pre-clinical and clinical HIV vaccine trials.[7-9,25] Both plasmids showed impressive immunogenicity in the preclinical studies, but were less immunogenic in humans. These two vectors have not been investigated for development of the dengue DNA vaccine. When our DENV-2 DNA vaccine was used at the highest dose (100-μg), the production of neutralizing antibody was high and comparable between both vectors. However, when the DENV-2 DNA vaccine was administered at a lower dose (10-μg), the pCMVkan-based construct showed a tendency to generate more antibodies than pHIS. These findings indicated that pCMVkan is not only cost-effective but immunogenic, so it can be used in future studies for the development of tetravalent vaccine.

Other efforts to develop a DENV-2 DNA vaccine have resulted in various degrees of success.[26-30] Our DENV-2 DNA vaccine constructs appear to elicit the neutralizing antibodies that are of higher magnitude than those obtained by other DENV-2 DNA vaccine candidates. There are two studies of DENV-2 DNA vaccine candidates whose constructs are similar to the one used in this study but resulted in low neutralizing antibody titers. In one of these studies, the vaccine had to be administered three times intramuscularly with 100 μg of plasmid at two-week intervals and had only 10 PRNT90 titer.[27] This result was in agreement with the other study in which immunized mice did not develop any neutralizing antibodies when they were injected intramuscularly with 100 μg of DNA every four weeks for a total of four times. When the interval was reduced to three weeks, the PRNT50 titer was ≥100.[30] There may be several reasons why our DNA vaccine was able to elicit strong neutralizing antibody responses. It is possible that the plasmid vectors used were highly immunogenic and the needle-free administration technique may have contributed to the higher production of the neutralizing antibody. We are currently evaluating whether *in vivo* electroporation technique will further improve the antibody response to our DNA vaccine candidates.

Another strategy investigated is modification of the codons. Our finding corroborates previous HIV-1 DNA vaccine results,[31] showing that the codon optimization moderately enhanced the *in vitro* expression of the desired protein. For this study, the dengue E protein expression was modest compared to the native codons. This may be due to the strong transcriptional promoter of the selected plasmids which allowed high level protein expression without the need for optimizing the codon.[9] From our reproducible experiment, the chimeric construct was associated with the higher production of extracellular prM level but induced lower anti-DENV-2 neutralizing antibody titers compared to the full-length construct. These unexpected results indicated that the strong transcriptional promoters became dominant. An enhanced expression of E protein may override the influence of the endoplasmic reticulum retention signal of the dengue virus E protein,[32,33] resulting in a comparable amount of E protein being transported out of the endoplasmic reticulum for both constructs. On the other hand, the absence of the endoplasmic reticulum retention signal in the chimeric E construct may affect the rate of export of the virus-like particles after their assembly from prM and E in the lumen of the endoplasmic reticulum to the Golgi apparatus. A reduced cleavage of prM may occur when the trans-Golgi-resident furin is overwhelmed by high prM load, resulting in an increased production of extracellular immature and partially mature particles.[23] Despite these processes, the immunogenicity of the various types of dengue virus particles is not well understood. It is likely that prM-containing particles do not induce a strong neutralizing antibody response because the receptor-binding EDIII domain is inaccessible at the immature status.[34] The high level of protein expression and absence of the endoplasmic reticulum retention signal for the export of virus-like particles may lower the efficacy of the chimeric construct in inducing the neutralizing antibody response in mice.

Because of this, it is very important that the DNA vaccine is able to elicit a high level of neutralizing antibody. Recently, the results from the phase IIb clinical trial of a live-attenuated chimeric tetravalent dengue vaccine (CYD-1-4) in Thailand showed poor response to DENV-2 (9.2% protection) which is the most pandemic serotype but had a robust production of DENV-2 neutralizing antibodies.[35] This unexpected finding may be due to the antigen mismatch between the vaccine and the circulating DENV-2 strain. As a result, we also compared the prME homology of an isolated virus from 2011 to our DENV-2 DNA or CYD-2 vaccine. Our DENV-2 DNA has high homology (99.8%) compared to the recent circulating virus. There were no mismatch sequences at E83, E226 and E228 which can contribute to a CYD-2 failure.

In conclusion, the immune responses to our monovalent DENV-2 DNA vaccine candidates, pHIS and pCMVkan, were comparable and dose-dependent. The chimeric prM+E did not improve the immunogenicity. These findings can be used to design vaccines for DENV-1, DENV-3 and DENV-4 as well as a tetravalent DNA vaccine for dengue.

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Conflict of interest

KR was supported by the Senior Researcher Scholar, Thailand Research Fund (TRF). All other authors declare no conflict of interest.

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