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A novel dengue virus serotype-2 nanovaccine induces robust humoral and cell-mediated immunity in mice

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ABSTRACT

Dengue virus (DENV), a member of the *Flaviviridae* family, can be transmitted to humans through the bite of infected *Aedes* mosquitoes. The incidence of dengue has increased worldwide over the past few decades. Inadequate vector control, changing global ecology, increased urbanization, and faster global travel are factors enhancing the rapid spread of the virus and its vector. In the absence of specific antiviral treatments, the search for a safe and effective vaccine grows more imperative. Many strategies have been utilized to develop dengue vaccines. Here, we demonstrate the immunogenic properties of a novel dengue nanovaccine (DNV), composed of ultraviolet radiation (UV)-inactivated DENV-2, which has been loaded into the nanoparticles containing chitosan/*Mycobacterium bovis* Bacillus Calmette-Guerin cell wall components (CS/BCG-NPs). We investigated the immunogenicity of DNV in a Swiss albino mouse model. Inoculation with various concentrations of vaccine (0.3, 1, 3 and 10 μg/dose) with three doses, 15-day apart, induced strong anti-dengue IgM and IgG antibodies in the mouse serum along with neutralizing antibody against DENV-2 reference strain (16681), a clinical-isolate strain (00745/10) and the mouse-adapted New Guinea-C (NGC) strain. Cytokine and chemokine secretion in the serum of DNV-immunized mice showed elevated levels of IFN-γ, IL-2, IL-5, IL-12p40, IL-12p70, IL-17, eotaxin and RANTES, all of which have varying immune functions. Furthermore, we observed a DNV dose-dependent increase in the frequencies of IFN-γ-producing $CD4^+$ and $CD8^+$ T cells after *in vitro* stimulation of nucleated cells. Based on these findings, DNV has the potential to become a candidate dengue vaccine.

1. Introduction

Dengue virus (DENV), of the genus *Flavivirus*, family *Flaviviridae*, can be grouped into four antigenically distinct serotypes (DENV 1–4). DENV is transmitted to humans through the bite of DENV-infected *Aedes aegypti* and *Aedes albopictus*. Natural DENV infections are thought to induce lifelong protection against the infecting homologous serotype and short-term cross protection against heterologous serotypes. Recent estimates indicate that up to 390 million infections occur annually worldwide, with approximately 96 million symptomatic cases [1].

DENV infections generally present with a wide range of clinical symptoms, varying from asymptomatic infection, to undifferentiated dengue fever (DF), to dengue hemorrhagic fever (DHF), dengue shock syndrome [2], or other severe forms of dengue [2]. The mechanisms underlying severe dengue disease are believed to be related to pathogenic manifestations of the immune response, including antibody cross-reactivity to the vascular endothelium, disease-enhancing antibodies, complement proteins and byproducts, and soluble mediators such as cytokines and chemokines [3]. How each of these elements contributes to disease pathology is not clear. DHF and DSS occur in secondary heterologous DENV

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infection at a 15–80 fold higher frequency than in primary infection. These levels underscore the significance of preexisting anti-DENV antibodies, which may enhance infections by heterologous DENV serotypes. There is no DENV-specific treatment available, increasing the need for an effective vaccine that can provide simultaneous protection against all four serotypes with minimal reactogenicity. Currently, there are no licensed dengue vaccines. However, there are several vaccine candidates under development, all using different strategies to provide protection. These include live-attenuated vaccines [4–6], purified inactivated vaccines [7,8], recombinant vaccines [9], chimeric vaccines [10], virus-like particles vaccines [11,12] and subunit vaccines [13,14]. Many of these are in preclinical and clinical trials. Vaccine development is hampered by the complexity of the immune responses, the potential for interference when all four DENV serotypes are delivered simultaneously, and the theoretical safety risk of vaccine-related immune enhancement.

Subunit and recombinant protein vaccines are thought to be safer than live-attenuated vaccines since their composition is devoid of replicative forms of the virus, usually associated with reactogenicity. However, subunit and recombinant vaccines can fail to elicit robust cell-mediated immune responses and are also easily degraded by lysosomal enzymes [15,16]. The dengue/yellow fever chimeric vaccine (CYD-TDV) is the most advanced candidate dengue vaccine, currently in phase III clinical trials [17]. It is unknown how the lack of DENV non-structural (NS) proteins, a source of T cell epitopes, in the CYD-TDV will affect long-term immunogenicity and protection. Purified inactivated vaccines can be done by several methods including heat-inactivation, chemical inactivation and UV irradiation. Formalin inactivation is widely used in many vaccines such as polio virus, JEV, dengue and influenza virus. It is known that formalin treatment destroys important viral epitopes [18,19]. A more recent approach in vaccine development links DENV to the phosphoreactive psoralen, 4′-aminomethyltrioxalen hydrochloride [20], which cross-links pyrimidine residues when exposed to UV-A radiation [21]. The AMT-UV inactivated virus freely penetrates the cell phospholipid bilayer, while retaining intact structural and non-structural epitopes. This approach may increase the vaccine-induced cytotoxic T cell responses, similar to what has been reported for NS3[22].

Improving vaccine immunostimulatory properties may require the use of adjuvants and improved delivery systems. Nanotechnology is fast becoming a widely used approach for drug and vaccine delivery. Several methods are used to generate nanoparticles (NPs) including coacervation [23], emulsion [24] and polymerization [25]. Because of their small size, NPs easily cross the blood brain barrier and access the interior of cells and various intracellular compartments [26]. NPs stimulate antigen uptake by antigen-presenting cells (APCs) [27], while reducing toxicity and other side effects associated with drugs [28,29]. In animal models, NPs have been used to improve delivery and efficacy of influenza [30–32] and hepatitis B vaccines [33,34], underscoring NPs applicability to other experimental vaccines. The cell wall components of *Mycobacterium bovis* Bacillus Calmette-Gurin (BCG-CWCs) are widely used as adjuvant for several vaccine developments. BCG-CWCs serve as a ligand for TLR2/4 that pasty share their signaling pathway through MyD88, an adaptor that is essential for effective cytotoxic T lymphocytes induction, and TIRAP, lead to the activation of NF-κB and productions of cytokine such as TNFα, IL-1β, IL-6, IL-8 and chemokines like MIP [35]. It has been reported that mice treated i.p. with BCG showed robust antigen presentation and increased IL-1, IL-6 and TNFα production in peritoneal macrophages [36,37]. Since BCG-CWCs have the capability of modulating T cell responses, CS/BCG-NPs were used as an adjuvant and delivery system for DNV in this study.

Table 1
Composition of DNV. A constant amount of the BCG-CWCs adjuvant was loaded into CS-NPs by adsorption–mixing method. Varying amounts of UVI-DENV were loaded by adsorption–mixing method to form DNV.

Groups	CS-NPs (μg/ml)	BCG-CWCs (μg/ml)	UVI-DENV antigen (μg/ml)
Saline control	–	–	–
Adjuvant	45	20	–
0.3 μg of DNV	45	20	3
1 μg of DNV	45	20	10
3 μg of DNV	45	20	30
10 μg of DNV	45	20	100

Here, we report the ability of DNV, composed of AMT-UV-inactivated whole DENV2 loaded chitosan (CS)/*M. bovis* Bacillus Calmette-Guerin (BCG) cell wall components nanoparticles (CS/BCG-NPs), to stimulate humoral and cellular immunogenicity in a Swiss albino mouse model.

2. Materials and methods

2.1. Adjuvant preparation and vaccine formulation

CS/BCG-NPs were used as the adjuvant delivery carriers for dengue nanovaccine (DNV). Preparation of the adjuvant was started from chitosan core-shell nanoparticles synthesis by an emulsifier-free emulsion-polymerization method [38]. Cell wall components of *M. bovis* BCG were isolated from heat-inactivated *M. bovis* BCG Tokyo 172 by French pressure cell press [39]. No toxicity was found when the adjuvant was tested in primary human DCs, THP-1 cells and LLC-MK2 cells (data not shown). The integrity of the component was checked using an anti-lipoarabinomannan (LAM) antibody. The antigenicity of the sucrose gradient-purified AMT-UV-inactivated DENV-2 strain 16681 (UVI-DENV) [7,21,40] was tested by typing ELISA using 4G2, 3H5 and 2H2 monoclonal antibodies (data not shown). Protein concentration was determined by bicinchoninic acid (BCA) assay using bovine serum albumin as a standard (Bio-Rad Laboratories, USA). The complete inactivation of the UVI-DENV-2 antigen was verified by inoculating the antigen in LLC-MK2 cells and incubating the cells 6 days, after which we were unable to detect any plaque-forming units in the undiluted preparation (data not shown).

To generate DNV, BCG and UVI-DENV were sequentially loaded onto CS-NPs by an absorption-mixing method at 250 rpm for 16 h [41]. A constant amount of adjuvant was used in all the groups of immunized mice, except in the saline group (Table 1). DNV was cleaned by centrifugation at 10,000 rpm for 1 h to remove unbound components. Vaccine particle size and surface charge were determined by zetasizer (NanoZS 4700, Malvern Instruments, UK). Loading efficacy of UVI-DENV was determined by staining the DNV with FITC-conjugated anti-flavivirus antibodies. The frequency of UVI-DENV present on NPs was observed by BD LSRFortessa™ Cell Analyzer (BD Biosciences, data not shown).

2.2. Animals

Adult female Swiss albino mice, *Mus musculus* ICR outbred strain aged 6 to 8 weeks and of 25–30 g of body weight were maintained at the Department of Veterinary Medicine, United States Army Medical Component-Armed Forces Research Institute of Medical Sciences (USAMC-AFRIMS) (Bangkok, Thailand) under good animal welfare conditions. All procedures involving mice were performed in compliance with national laws and institutional policy and with the permission of the AFRIMS Institutional Animal Care and Use Committee.

2.3. Vaccine immunization

Seventy two mice were separated into six group ($n = 12$ per group) and i.p. vaccinated with three doses (100 μl, 15 days apart) of various vaccine formulations including saline as a negative control, adjuvant alone (CS/BCG-NPs) or DNV (0.3, 1, 3 and 10 μg/dose). Blood was collected from each group of immunized mice (3 mice per group in each time point) on days 0, 14, 16, 17, 29, 31, 32, 37, 44 and 58. Individual serum samples were used in each experiment to determine the level of anti-DENV-1, -2, -3, -4 IgM/IgG antibodies, DENV-2 neutralizing antibody and cytokine/chemokine productions. Spleens were collected on days 29, 37, 44 and 58 to investigate cell-mediated immune responses induced by DNV.

2.4. Cytokine production

Collected serum samples were tested individually for the presence of interleukin (IL)-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p40, IL-12p70, IL-17, interferon (IFN)-γ, G-CSF, GM-CSF, MCP-1, MIP-1α, MIP-1β, RANTES and TNF-α using the bead-based multiplex Bio-Plex assay system (Bio-Rad Laboratories, USA) following the manufacturer protocol. The assay was performed in triplicate wells. Briefly, dye bead-coupled capture antibodies were incubated with standards or serum samples for 30 min on an 800 rpm shaker at room temperature. Unbound material was washed prior to incubation with biotinylated detection antibodies for 30 min on an 800 rpm shaker at room temperature. After washing away unbound biotinylated antibodies, a reporter streptavidin–phycoerythrin conjugate was added to the beads and incubated for 10 min on an 800 rpm shaker at room temperature. After removing excess streptavidin–phycoerythrin, the bound beads were counted *via* a dual laser flow-based reader, which measures the fluorescence of the bound SA-PE in terms of mean fluorescence intensity (MFI).

2.5. Cell-mediated immune response

Cell-mediated immune responses in individual PBMC's were determined in three independent experiments (each in triplicates) by measuring the frequencies of IFN-γ producing cells among antigen-stimulated $CD4^+$ or $CD8^+$ T cells. Splenocytes were seeded into a 24-well plate at a concentration of 2×10^6 cells/ml. Ten μg per well of DNV was added into the splenocytes to stimulate IFN-γ secretion. Cells were incubated at 37 °C for 48 h, harvested and stained with PE-conjugated rat anti-mouse CD4 (clone RM4-5, BD Biosciences, USA), and APC-conjugated rat anti-mouse CD8 (clone 53-6.7, BD Biosciences). Cells were permeabilized with BD Cytofix/Cytoperm™ (BD Biosciences) prior to staining with FITC-conjugated anti-mouse IFN-γ (clone XMG1.2, BD Biosciences, USA). The frequency of IFN-γ secreting cells was measured by BD LSRFortessa™ Cell Analyzer (BD Biosciences). Twenty-thousand events were collected to calculate the positive frequency.

2.6. Anti-dengue IgM/IgG ELISA

Serum anti-DENV-2 IgM/IgG antibodies were measured by capture ELISA [42]. Briefly, serum was diluted as 1:10 with PBS prior to adding it into anti-mouse IgM or IgG-coated wells. One microgram per well of UVI-DENV antigen was added followed by horse radish peroxidase (HRP)-conjugated human anti-flavivirus secondary antibody (Pooled human anti-flavivirus antibody lot 21 Aug 13). Sureblue TMB solution (KPL, Inc., USA), HRP substrate, was added. The reaction was stopped at 30 min with 0.2 mM sulfuric acid.

For the detection of cross-reactive anti-DENV IgM/IgG antibodies, we used the same protocol as described above with the exception of the antigen added to the wells. In this case, we used pooled 16 HA units of DENV-1 (Hawaii, lot#08/02/48, 5120 HA titer), 16 HA units of DENV-3 (1006 lot#08/02/48, 640HA titer) and 8 HA units of DENV-4 (H241, lot# 08 Jan 04, 640HA titer) antigens, instead of the UVI-DENV antigen.

The optical density (OD) values were measured at 492 nm and used to calculate the ELISA units using the following formula: $[(OD_{Test} - OD_{Negative\ control})/(OD_{Positive\ control} - OD_{Negative\ control})] \times 100$. The results from three independent experiments testing individual samples (each in duplicates) were used to calculate the mean ELISA units in each group.

2.7. Dengue neutralizing antibody

To determine the immunogenicity of DNV, we measured vaccine-induced neutralizing (NT) antibodies in each individual serum sample against DENV-2 reference strain (16681), a clinical-isolate strain (00745/10) and the mouse-adapted strain (NGC) [43] by plaque reduction neutralization test ($PRNT_{50}$) [44]. The assay was done in three independent experiments (in duplicates). Serum was diluted in a four-fold serial dilution and mixed with the virus in equal volumes before incubation in a 35 °C water bath for 1 h. The mixture was inoculated into Rhesus monkey kidney epithelial cell (LLC-MK2 cells) in a 12-well plate and incubated at RT for 1 h on a rocker platform. The excess volume of the inoculum was removed. First medium over layer containing low melting point agarose gel (LMP, Ultra Pure™ LMP agarose, Invitrogen, USA) was added. DENV-infected cells were incubated for 6 days in a 5% CO_2 incubator at 35 °C before staining with 4% neutral red (Sigma, USA) in a second medium over layer with LMP. The number of plaques was determined by manual counting. NT antibody titer was calculated by SPSS program, using regression with probit analysis at 50% reduction which was identified as the highest dilution that can reduce the number of plaques by 50% as compared to the number of plaques in virus control well.

2.8. Statistical analysis

Mann–Whitney *U* test/Kruskal–Wallis analysis was used to compare the difference of anti-dengue IgM/IgG antibodies, neutralizing antibodies, and cytokine/chemokine production among control and other groups. A p-value of less than 0.05 was considered statistically significant. Descriptive statistical analysis was used to describe the basic features of cell-mediated immunity (CMI) [45].

3. Results

DNV was shown to be spherical in shape with a diameter of 372 ± 11 nm, and polydispersity index of 0.227–0.254, indicating narrow size distribution. Its zetapotential was +20.6 mV, referring to cationic surface.

3.1. Cytokine production

We measured 18 different cytokines in the serum of vaccinated mice at designated times. We categorized these cytokines into five groups including: proinflammatory cytokines (IL-1α, IL-1β, IL-6, IL-17 and TNF-α), Th1 cytokines (IFN-γ, IL-2, IL-12p40 and IL-12p70), Th2 cytokines (IL-4, IL-5 and IL-10), chemokines (MCP-1, MIP-1α, MIP-1β and RANTES), and growth factors (G–CSF and GM–CSF). These are shown as heat maps in Fig. 1. More detailed data are presented in graphs in Suppl. Fig. 1.

Mouse groups receiving the CS/BCG-NP adjuvant had higher levels of proinflammatory cytokines in their serum than the saline

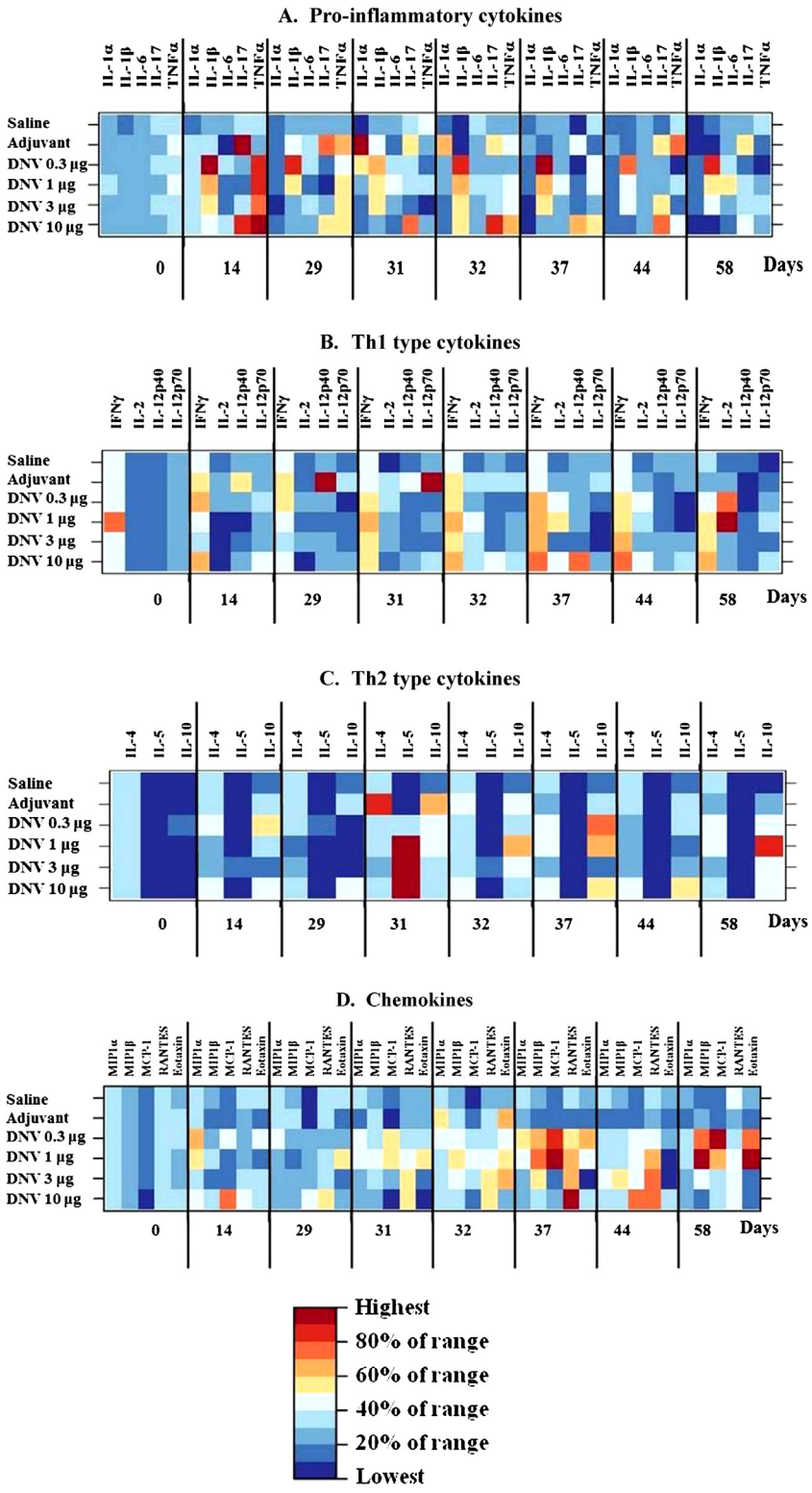

Fig. 1. Cytokines/chemokines production in the serum of saline, adjuvant and various concentrations of DNV-immunized mice (0.3, 1, 3 and 10 μg) were determined by bioplex assay. All cytokines were categorized into pro-inflammatory cytokines (A), Th1 type cytokines (B), Th2 type cytokines (C) and chemokines (D). The results presented in the mean value ($n = 3$) of each cytokines at a designated time points.

group (Fig. 1A, Suppl. Fig. 1). In particular, while there was little IL-1α generated other than in the group receiving adjuvant alone, IL-1β in the DNV-inoculated groups (all doses) was present by day 14 and stably produced until day 58 at levels that were significantly higher than in the saline or adjuvant group (p-value <0.05, suppl. Fig. 1). TNF-α production was more transient, with significantly higher levels being detected in the DNV-inoculated groups (all doses) by day 14 (p-value <0.05, Suppl. Fig. 1). IL-6 was detected only at very low concentrations in the serum (2–10 pg/ml). Nonetheless, we were able to detect a significant increase of IL-6 in

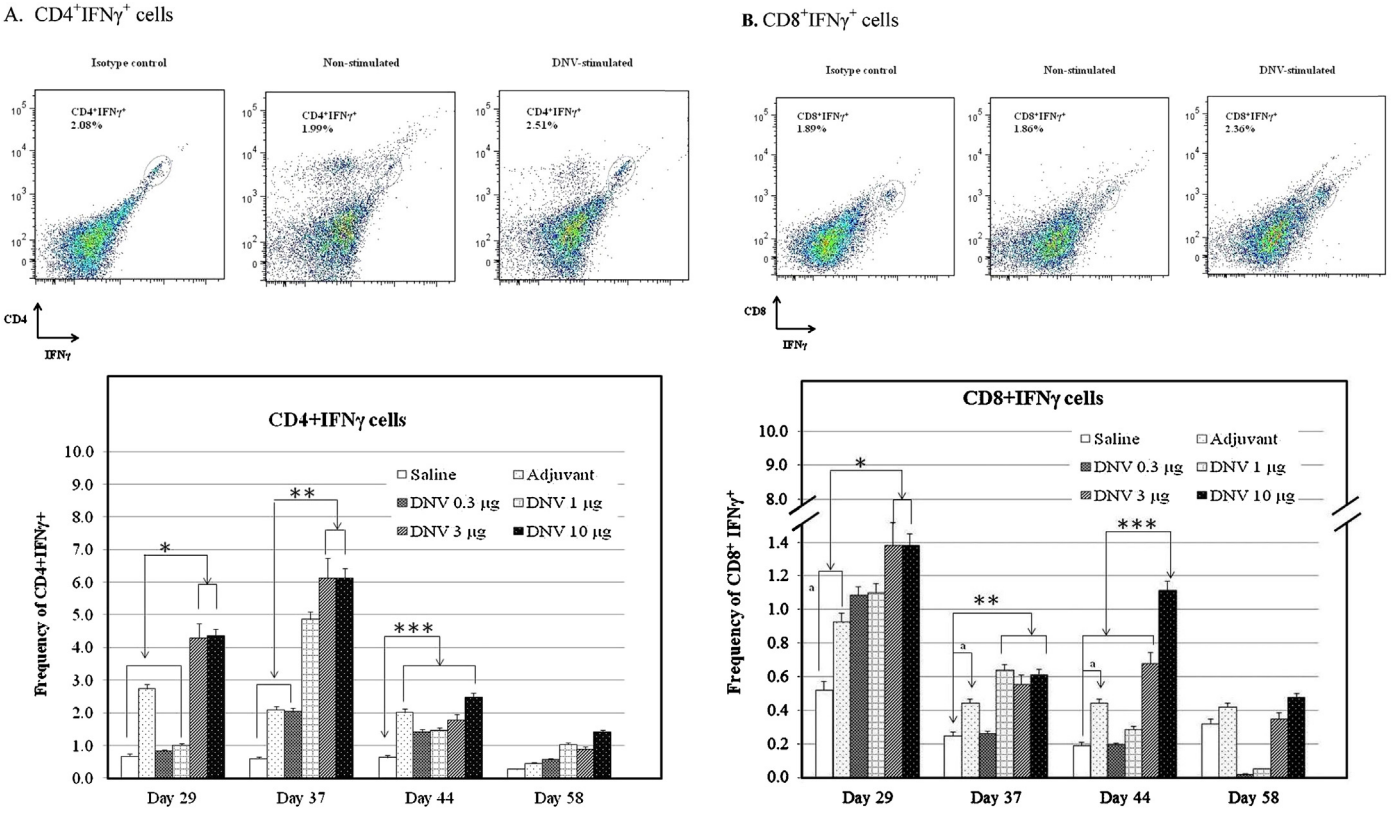

Fig. 2. Cellular immune responses induced by DNV. Splenocytes collected from saline, adjuvant and various concentration of DNV 0.3, 1. 3 and 10 μg/dose immunized mice were stimulated with 10 μg of DNV prior to staining of CD4 (A), CD8 (B) and IFNγ.(saline, adjuvant, DNV 0.3 μg, DNV 1 μg, DNV 3 μg, DNV 10 μg). [a] Indicates significant differences between adjuvant and saline, * Indicates significant differences between 10 μg of DNV and other concentrations of DNV, ** indicates significant differences between saline/adjuvant and various concentrations of DNV.

the 1 μg DNV group at days 31, 37 and 58 above all other groups. Adjuvant alone was a significant inducer of IL-17 at various time points.

IFN-γ and IL-2 appeared to be the only Th1-associated cytokines induced by DNV, albeit at very low concentrations (Fig. 1B, Suppl. Fig. 1). Levels of IFN-γ were significantly higher in all DNV-vaccinated groups by day 37 (7 days post dose three) and continued so until day 58, as compared with the adjuvant and saline groups (p-value <0.05, Suppl. Fig. 1), although some DNV-vaccinated groups generated higher levels of IFN-γ by day 14. Levels of IL-2 were only detectable at significant (p-value <0.05, suppl. Fig. 1) levels above the adjuvant and saline groups on days 31 to 44, although not consistently in all DNV-vaccinated groups. There is little evidence that DNV induced secretion of IL-12p40 and IL-12p70 in mice, with most of these cytokines generated by the adjuvant alone.

We were able to detect a sharp spike in the secretion of the Th2-associated cytokine IL-5 in the serum of DNV-inoculated mice (all doses) by day 31 (1 day post dose 3), although levels normalized soon afterward (Fig. 1C, Suppl. Fig. 1). We measured other Th2 cytokines (IL-4 and IL-10), with little evidence that DNV induced detectable levels of either of them. DNV induction of IL-10 may be attributable to the adjuvant alone.

Production of the chemokines MIP-1α, MIP-1β, MCP-1, RANTES and eotaxin varied (Fig. 1D, Suppl. Fig. 1). MCP-1 production was detected at levels significantly higher above the adjuvant and saline groups (p-value <0.05, Suppl. Fig. 1) as early as day 14 (0.3 μg DNV group) and continued until day 58 for most DNV-vaccinated groups. Interestingly, MCP-1 was lower at the highest DNV doses. DNV-induced MIP-1β secretion above the adjuvant and saline groups was most significant on days 37 (7 days post dose 3) and 58 (p-value <0.05, Suppl. Fig. 1), although one low DNV dose group (1 μg DNV) induced the highest levels of MIP-1β by days 31 and 32 (1 and 2 days post dose 3). We found a similar pattern for the induction of RANTES, with all DNV-inoculated groups secreting significant levels (p-value <0.05) above the adjuvant and saline groups by days 31 and 32 (1 and 2 days post dose 3) which then continued until day 44 and normalizing by day 58. Induction of MIP-1α in the DNV-inoculated groups was evident on day 14 (14 days post dose 1), and were higher than the adjuvant and saline groups (p-value <0.05, suppl. Fig. 1). Other cytokines investigated included the growth factors, G–CSF and GM–CSF, of which there were no evidence of induction in our experiments.

3.2. Cell-mediated immune response

We measured DNV cell-mediated immune responses by the frequency of $CD4^+$ $IFN\gamma^+$ or $CD8^+$ $IFN\gamma^+$ T cells after *in vitro* stimulation with 10 μg of DNV for 48 h (Fig. 2). Mice were inoculated (3 doses, 15 days apart) with saline or DNV at different concentrations (0.3, 1, 3 or 10 μg per dose). Spleens were collected at various times and used for splenocyte isolation. Flow cytometry revealed higher frequencies of $CD4^+$ $IFN\gamma^+$ and $CD8^+$ $IFN\gamma^+$ T cells collected from the adjuvant and vaccine immunized mice. The group vaccinated with 10 μg of DNV generated the highest frequency of $CD4^+/IFN\gamma^+$ T cells (4.35%, 6.13%, 2.48% and 1.40% for days 29, 37, 44 and 58, respectively) and $CD8^+/IFN\gamma^+$ T cells (1.38%, 0.61%, 1.11% and 0.48% for days 29, 37, 44 and 58, respectively) throughout the length of the experiment. Inoculation with DNV at 3 and 10 μg per dose generated frequencies of $CD4^+/IFN\gamma^+$ T cells significantly higher ($p < 0.05$) than any other group on days 29 and 37. Similarly,

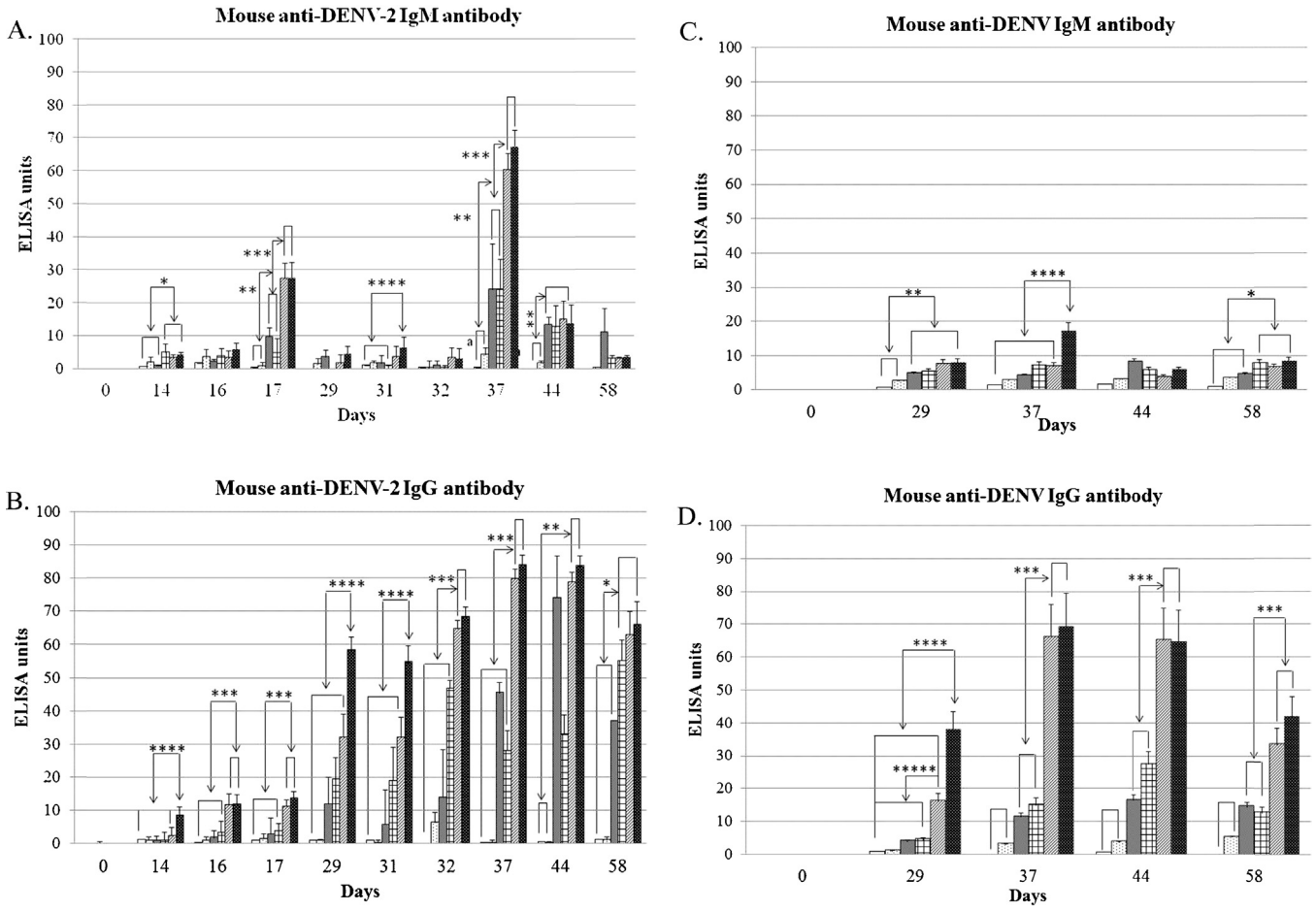

Fig. 3. Mice were immunized with saline, adjuvant or various concentrations of DNV (0.3, 1, 3 and 10 μg) for three doses (15-day apart), blood was collected at designated time points to determine the level of anti-DENV2 IgM antibodies (A), anti-DENV2 IgG antibodies (B), anti-DENV1, 3, 4 IgM antibodies (C) and anti-DENV1, 3, 4 IgG antibodies (D) by capture ELISA.(Saline, Adjuvant, DNV 0.3 μg, DNV 1 μg, DNV 3 μg, DNV 10 μg). [a] Indicates significant differences between adjuvant and placebo, * indicates significant differences between saline/adjuvant/0.3 μg of DNV and other concentrations of DNV, ** indicates significant differences between saline/adjuvant and various concentrations of DNV, *** indicates significant differences between 0.3 μg/1 μg of DNV and 3 μg/10 μg of DNV, **** indicates significant differences among 10 μg of DNV with other groups, ***** indicates significant differences among 3 μg of DNV with other groups.

inoculation with DNV at the highest doses (in particular 10 μg) generated $CD8^+/IFN\gamma^+$ T cells at frequencies that were higher than the adjuvant and saline groups on days 29, 37 and 44.

3.3. Anti-dengue IgM/IgG ELISA

We tested the ability of DNV to generate antigen-specific IgM and IgG antibodies using an ELISA method. Mice were inoculated (3 doses, 15 days apart) with saline or DNV at different concentrations (0.3, 1, 3 or 10 μg per dose). Anti-DENV-2 IgM antibodies were detected in all DNV vaccinated groups on day 17 (two days post dose 1), day 37 (7 days post dose 3) and day 44 (14 days post dose 3) at levels that were significantly higher (p-value <0.05) than the saline or adjuvant groups (Fig. 3A). As expected, IgM antibodies were induced in a dose-dependent manner. Peak IgM levels were 60.4 ± 4.9 and 67.3 ± 5.0 ELISA units for 3 μg and 10 μg of DNV, respectively, while immunization with 0.3 μg and 1 μg of DNV induced peak IgM levels of 24 ± 14.0 and 24 ± 9.2 ELISA units, respectively, on day 37. Differences among the DNV-inoculated groups were not detectable by day 44.

Serum anti-DENV2 IgG antibodies were also detected in all vaccinated groups in a dose-dependent manner (Fig. 3B), with the two highest dose-groups generating significantly higher levels of IgG as early as day 16 (p-value <0.05) and the group receiving 10 μg per dose generating higher IgG levels (p-value <0.05) as early as day 14 (two weeks post dose 1). IgG levels gradually increased until day 58 for all vaccine doses. All DNV vaccinated groups except the 0.3 μg DNV group generated levels of IgG from day 29 to day 58 which were significantly higher (p-value <0.05) than in the saline and adjuvant groups. Production of IgG peaked on days 37 and 44 which were 7 and 14 days after the third vaccine dose.

We investigated the generation of cross-reactive antibodies in the serum using pooled DENV-1, -3 and -4 antigens in an ELISA format. IgM cross-reactivity in the DNV-immunized groups was evident by day 29 and continued until day 58. These levels were nonetheless lower than those found in ELISA using DENV-2 as antigen (Fig. 3A). Cross-reactive anti-DENV IgM antibodies in the 3 and 10 μg DNV immunized mice were significant higher than other DNV dose groups on day 37 (p-value = 0.03 and 0.03 for 3 and 10 μg of DNV, respectively) and 44 (p-value = 0.002 and 0.002 for 3 and 10 μg of DNV, respectively). We detected similar trends for IgG antibody production (Fig. 3D).

3.4. Dengue neutralizing antibody

Vaccine-induced neutralizing antibody levels against DENV2 reference strain (16681), a clinical-isolate strain (00745/10) and the mouse-adapted NGC strain [43] were determined by plaque

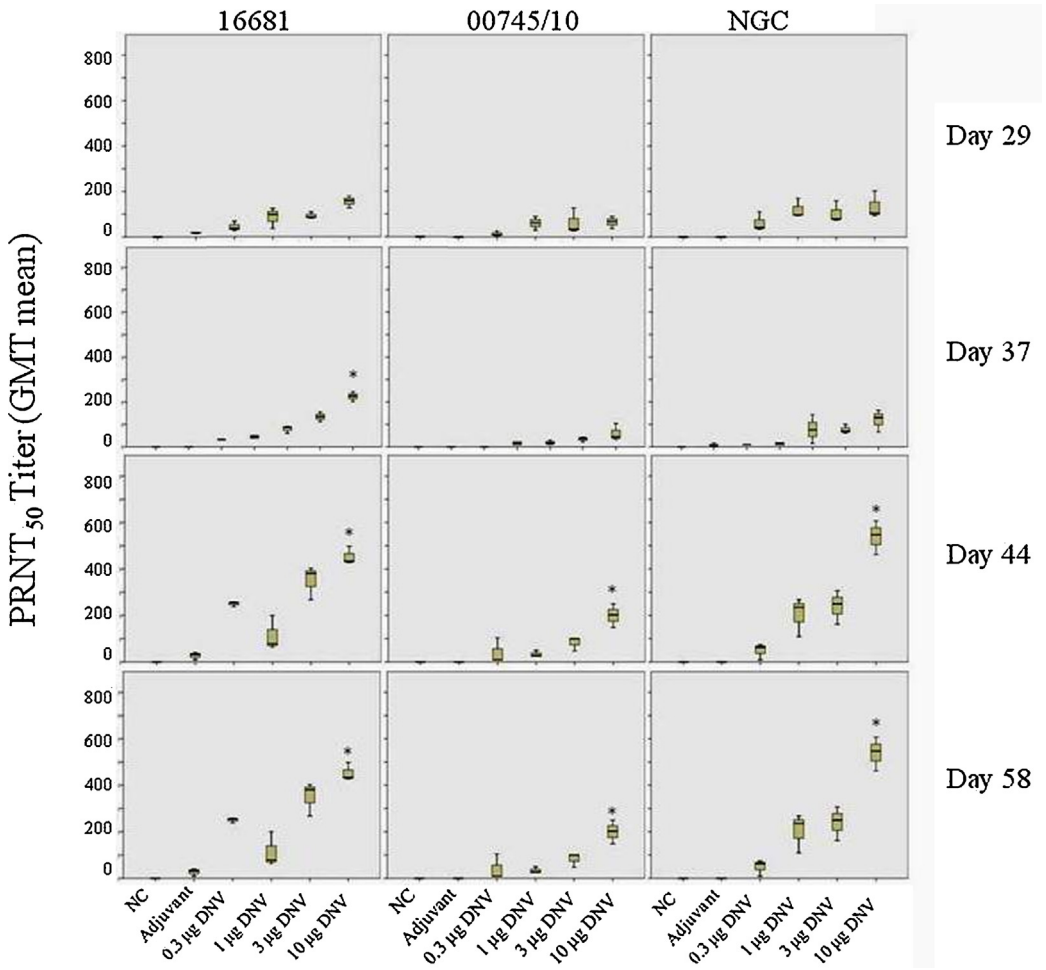

Fig. 4. Neutralizing antibody titer determined by $PRNT_{50}$ against DENV-2 reference strain (16681), clinical isolated strain (00745/10) and mouse adapted strain (NGC) in the serum of saline control (NC), adjuvant and various concentrations (0.3, 1, 3 and 10 μg) of DNV immunized mice at day 29, 37, 44 and 58.* Indicates significant differences between 10 μg of DNV and other concentrations of DNV.

reduction neutralization test ($PRNT_{50}$) on serum collected on days 0, 29, 37, 44 and 58. The results are summarized in Fig. 4. There were no NT antibodies in the saline (NC) group, and only very low levels in the adjuvant group against the DENV-2 reference strain (16681) on days 29 and 44. Overall, generation on NT antibodies against the clinical isolate 00745/10 was lowest across all days and DNV groups. In the DNV groups, NT antibodies against DENV-2 16681 and NGC were detected on day 29 and throughout the experiment. NT titers against DENV-2 00745/10 were lower in all DNV groups. DNV-immunized mice showed significant higher of NT titers than saline and adjuvant group only on days 44 and 58. We also found that DNV-induced NT antibodies against DENV-2 16681 and 00745/10 were produced in a dose-dependent manner on days 29, 37 and 58 and against the NGC strain on day 44. DNV at 10 μg was the only dose capable of stimulating significantly higher titers of NT antibodies against all three DENV-2 viruses (day 44, $p < 0.05$). We observed varying levels of NT antibodies among the three DENV2 strains used in the PRNT, but consistent within each PRNT strain. The results also indicated that mice vaccinated with 1, 3 or 10 μg/dose consistently yielded titers that were higher than the control groups throughout the length of the study.

4. Discussion

Dengue virus poses a significant public health problem affecting a large segment of the world population. An effective and safe dengue vaccine is required to control the spread of DENV. Several prototypic dengue vaccines have been tested over the years, each using different development strategies. Recently, the live-attenuated tetravalent dengue-yellow fever 17D chimeric virus vaccine (CYD-TDV) completed a phase III clinical trials, conducted in five countries in Asia. The vaccine demonstrated 56.5% overall efficacy against dengue, but only 35%, efficacy against DENV-2. Additional options for candidate dengue vaccines are clearly still needed.

We developed a novel dengue nanovaccine composed of the UVI-DENV antigen loaded on CS/BCG-NPs. UVI-DENV retained intact epitopes that are necessary to generate humoral and cellular immune responses. Psoralen and UV-inactivation have already been used to develop an inactivated DENV-1 vaccine currently in pre-clinical testing [7]. NPs were chosen as the vaccine delivery system due to their small size and fast up-take by APCs, probably the result of the positive surface charge of NPs, which increases NP adhesion to cell surfaces. NPs have been shown to activate innate immunity by inducing the productions of various cytokines, in particular IFN-γ [46] and expression of CD80, CD86 and HLA-DR in dendritic cells as well as the secretion of IL-1β, IL-6 and IL-12p70 [47].

Several cytokines induced by adjuvant and DNV play important roles in response to DENV infection, including IL-1α, IL-2 [48], IL-10 [49], IL-17 [50], IFN-γ [51] and other chemokines [52]. IL-17 has the ability to induce production of other proinflammatory cytokines

in response to *Mycobacterium tuberculosis* [53] and viral infections like DENV [54]. IL-17 is secreted from Th17 cells and other immune cells such as NK cells, NKT cells and macrophages. IL-12p40 and IL-12p70 play an important role in induction of Th1 responses in response to DENV infection. Surprisingly, in our experiments, adjuvant alone induced higher IL-17, IL-12p40 and IL-12p70 productions than DNV did, even though the amount of adjuvant used was similar. It is possible that UVI-DENV present in the DNV delayed or accelerated the production of those three cytokines. This may be explained by the differences in the size of the vaccine particle; where the DNV has a diameter of 372 nm, and the adjuvant (composed of the BCG-CWC captured in the CS-NP) with a diameter of only 299 nm (data not published). The smaller particle size of the adjuvant, combined with a higher positive surface charge (measured by zetasizer and not published here) may have increased the mobility and cell internalization of the adjuvant. It may also make the adjuvant more available to different cytokine-secreting cell types. Additionally, UVI-DENV and BCG-CWC, both competing for surface space in the NPs may hindrance each other's access to the targeted cells. DNV also induced secretion of various other immune mediators like TNF-α, an endogenous pyrogen normally found during acute DENV infection [55]; IFN-γ and IL-2, effectors of viral clearance and T cell proliferation; IL-4 and IL-5, involved in B cell differentiation into plasma cells; and chemokines like RANTES, MCP-1 and MIP-1β which are associated with cell migration, proliferation and activation of immune cells [56,57].

The role of CMI in protection against dengue disease remains unclear, but inclusion of T-cell epitopes in the vaccine has been shown to improve viral clearance in murine [58] and NHP models [59]. While T-cell responses may play a critical role in viral clearance during primary infections, low affinity interactions between epitopes and T-cells during secondary infections may contribute to the pathology of the disease [60]. Inactivated vaccines tend to induce little or no CMI [61]. However, with the help of CS/BCG-NPs, an adjuvant, our DNV was able to induce significant CMI responses.

Humoral immunity is essential for protection against DENV infection and infection with one DENV serotype normally generates long lasting antibodies against such serotype. However, heterotypic DENV protection is short-lived. In this study, DNV-immunized mice secreted cross reactivity anti-DENV-1,-3 and -4 antibodies as determined by ELISA test, demonstrating that DNV was highly immunogenic. These findings are in agreement with a prior study showing that long term humoral immunity was achieved in Swiss albino mice inoculated with three doses of Psoralen-inactivated DENV-1 [7]. DNV induced secretion of anti-DENV-2 IgG antibodies sooner than what was demonstrated using psoralen-inactivated DENV-1. This underscores the effect of CS/BCG-NPs, an adjuvant, in improving the ability of UVI-DENV antigen to stimulate host immune responses.

The potential protective properties of DNV in mice were demonstrated by the similar trends in secretion of NT antibodies against DENV2 16681 reference strain, the clinical-isolate strain (00745/10) and the mouse-adapted NGC strain. Nonetheless, the amino acid sequences on EdIII protein of the clinical-isolate strain differ from reference strain, and this may account for the lowest NT antibodies generated against this strain. As of today, PRNT assay is the most predictive test for protection against dengue.

In conclusion, our novel DNV stimulated both humoral and cell-mediated immune responses. High production of NT antibodies and stimulation of T-cells support the potential use of DNV as a dengue vaccine candidate. This study is the first step of DNV development. Several questions remain to be elucidated. The ultimate goal of this study is to develop a safe and effective tetravalent DNV with the capability to protect against all four dengue serotypes.

Conflict of interest statement

All authors declare to have no commercial or financial conflict of interest.

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The views expressed in this article are those of the authors and do not represent the official policy or position of the US Department of the Army, Department of Defense, or US Government.

Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adhered to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 2011 edition.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2015.02.016.

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