A dose-response study in mice of a tetravalent recombinant dengue envelope domain III protein secreted from insect cells

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Abstract

Background: DENV is the most globally prevalent mosquito-transmitted virus. Induction of a broadly and potently immune response is desirable for dengue vaccine development.

Methods: We constructed a secreted tetravalent ED protein from eukaryotic cells and established a MAC-ELISA method for DENV diagnosis. This work aimed to evaluate the immune responses in mice of several formulations of rEDIII containing different amounts of the tetravalent protein.

Results: We demonstrated that the tetravalent protein induced humoral immunity against all four serotypes of DENV, even at the lowest dose assayed. Besides, cellular immunities against DENV-1 and DENV-2 were elicited by medium dose group. Importantly, the immune responses induced by the tetravalent formulation were functional in clearing DENV-2 in circulation of mice.

Conclusions: We believe that the tetravalent formulation of secreted ED protein is a potential vaccine candidate against DENV and suggest further detailed studies of this formulation in nonhuman primates.

Keywords: Dengue virus; Tetravalent; Humoral immunity; Cellular immunity; Vaccine

Background

Dengue virus infection is an arthropod-borne viral disease caused by dengue viruses which are transmitted by the bites of infected mosquitoes Aedes aegypti and Aedes albopictus [1, 2]. These viruses belong to the Flaviviridae family and contain a positive-sense single-strand RNA genome encoding three structural (C, prM and E) and seven nonstructural proteins [3]. Unlike other flaviviruses, there are four serotypes, referred to as DENV1, 2, 3, 4, that are genetically similar but antigenically distinct [4]. Owing to the wide distribution of mosquito vectors, more than 2.5 billion people, representing 40% of the world's population, are at risk of dengue infection. In recent years, the geographical extension, number of cases, and disease severity of dengue have greatly increased, and it is estimated about 390 million dengue infections, of which 96 million manifest clinically and 22,000 deaths per year [5, 6].

Infection with any of the four DENV serotypes can result in a broad spectrum of clinical manifestations from a mild fever to classical dengue fever with hemorrhage (DHF) or dengue shock syndrome (DSS) [7, 8]. Primary infection with any one DENV serotype confers life-long immunity to that serotype, whereas secondary infection with a different serotype can cause the severe forms of the disease because of antibody-dependent enhancement (ADE) [9–11]. Hence, it is necessary for an effective DENV vaccine to induce a balanced and durable immune response specific for all four DENV serotypes simultaneously, avoiding the risk of severe disease through ADE. Several approaches for the research of tetravalent dengue vaccines are in different stages of development [12–19]. To date, the only DENV vaccine, Dengvaxia, from Sanofi-Pasteur, has been licensed in several dengue-endemic countries [20]. Nevertheless, this vaccine shows an overall efficacy of 60.3% and declined protection against DENV in seronegative vaccinees [21, 22], and is suspected to sensitise naive recipients to increased risk of

hospitalisation at later times [17]. Therefore, development of a tetravalent dengue vaccine inducing balanced and long-life immunity is still a huge challenge.

It is well established that the envelope protein is the major structural protein on the surface of the mature dengue virions, and the domain III region (DIII) of E protein is targeted by neutralizing antibodies in humans upon virus infection [23, 24]. Although there are some evidences showing domain III as an irrelevant target for neutralizing antibodies in humans [25, 26], vaccination with the domain III of the envelope protein indeed induces a neutralizing antibody response, conferring protection against re-infection [27–29]. In addition, DIII is involved in the recognition of the DENV receptor on target cells, and the cellular immune response should play an important role in the control of viral infection [30]. Taken together, the immunogenicity, protective capacity, and serotype specificity of DENV envelope protein DIII merits this antigen an ideal candidate for inducing a functional humoral and cell-mediated immune response.

In our previous study, we developed a tetravalent protein by connecting the receptor-binding envelope domain III (EDIII) of the four dengue virus serotypes using flexible peptide linkers, and found that the recombinant rEDIII protein was expressed successfully in Spodoptera frugiperda (sf9) lepidopteron cells and secreted into the supernatant of cell culture in a stable form with preserved native conformation. Based on these findings, we established and evaluated a MAC-ELISA method, which has potential utility in the diagnosis of dengue virus infections [31]. However, the immunogenicity of the tetravalent rEDIII protein was not fully studied. In the present study, we further analyzed the profile of humoral and cellular immune responses against DENV in mice after immunization with chimeric protein. Our results indicated that effective immune responses were generated in rEDIII-immunized mice, highlighting its functionality as a promising tetravalent dengue vaccine candidate, which could be potentially applied in DENV hyper-endemic areas.

Methods

Cells and viruses

African monkey kidney (Vero), K562 and C6/36 cells were kindly provided by Xiao-Hui Zou of the National Institute for Viral Disease Control and Prevention, China CDC, Beijing. Cells were grown at 37 °C in Eagle's minimal essential medium (MEM) or RPMI medium supplemented with 10% heat-inactivated fetal bovine serum (FBS). The viral strains GZ (DENV-1), ZS (DENV-2), H-87 (DENV-3) and H241 (DENV-4) were used for the plaque-reduction neutralization test (PRNT), animal inoculation and animal challenge experiment. Virus propagation was implemented in C6/36 cell and viral titers were determined by plaque assay on Vero cells.

Animals and ethics approval

BALB/c mice (female, 6–8 weeks old) were purchased from Pengyue Laboratory Animal Co., Ltd. (Jinan), and housed in appropriate animal care facilities during the experimental period. The animals were kept at

the Laboratory Animal Center of Weifang Medical University. All of the mouse experiments were carried out in strict accordance with the guidelines of the Animal Committee of Weifang Medical University. The study was reviewed and approved by the Ethic Committee of Weifang Medical University. All mice were euthanized by cervical dislocation after the experiment. All sections of this report adhere to the ARRIVE Guidelines for reporting animal research. A completed ARRIVE guidelines checklist is included in Checklist S1.

Recombinant protein

The design, cloning, expression and purification of the tetravalent dengue protein were described previously [31]. In brief, the DIII regions were fused with linker to generate a rEDIII chimeric protein of each DENV serotype with a 6-His-tag at their C-terminal ends. The recombinant plasmid was transfected sf9 cells and the target protein was secreted into the supernatant culture. The supernatant fluid was clarified and subjected to chromatography on a Ni-NTA column attached to an AKTA purifier system (GE Healthcare Life Sciences, Sweden). At last, protein were collected and quantified. All recombinant protein were lyophilized and stored at -20 °C until use. The tetravalent formulations were prepared with different quantities of the rEDIII protein and incubated with 500 μg aluminum for 36 hours at 4 °C (on a rocking platform), clarifed by centrifugation (5,000 rpm, 5 min) and re-suspended in 100 μl sterile 1 × PBS.

Animal experiments

The immunogen was injected by the intraperitoneal route into five groups of 16 female BALB/c mice on days 0, 15 and 45. Groups 1 to 4 received different tetravalent formulations containing rEDIII protein and aluminum adjuvant. Mice that were injected with aluminum alone (without vaccine candidates) served as negative controls. Blood and spleens of ten mice from each group were obtained for further immunological analysis 15 days after the last dose injection. Meanwhile, eight mice from each group were prepared for the animal challenge experiment. All sera were prepared and stored at -20 °C until use.

Measurement of humoral immune response

The presence of rEDIII-specifc IgG in the sera was determined by enzymelinked immunosorbent assay (ELISA). A 2-fold serial dilution (starting at 1:10 for negative control or 1:100 for other groups) of each sample was prepared. Polystyrene 96-well plates (Costar, USA) were each coated with 100 μl of EDIII(D1-D4) protein (3 μg/mL) overnight at 4 °C in coating buffer (0.16% Na_2CO_3, 0.29% $NaHCO_3$, pH 9.5), respectively. Then they were blocked in coating buffer containing 5% skimmed milk for 1 h at 37 °C. After five washes with PBS containing 0.05% Tween 20 (PBS-T), 100 μl per well of sera from each group were tested by serial dilutions in PBS-T, starting at 1:5000. The sera of healthy mice were used as negative control. Plates were incubated for 1 h at 37 °C and washed as described above. Later, 100 μl per well of 1:30 000 diluted Goat anti mouse IgG peroxidase conjugate (Amersham Pharmacia, Beckinghamshire, UK) were added and the plates were incubated for 1 h at 37 °C. After washing, 100 μl per well of substrate, 3, 3', 5, 5'-tetramethylbenzidine (TMB), was added for color development. The plates were kept for 15 min at room temperature and the reaction was stopped with 50 μl per well of 2.5 M H_2SO_4.

Absorbance was read at 450 nm in a SensIdent Scan device (Merck, Helsinki, Finland). The positive cutoff value was set as twice the mean absorbance value of negative control sera.

Plaque reduction neutralization test

Neutralizing antibody titers were measured by plaque reduction neutralization test (PRNT) in Vero cells as previously described [32]. In brief, Vero cells (2×10^3 cells/well) were dispensed into 96-well plates and incubated for 16 h at 37 °C in a humidified 5% CO_2 environment. Equal amounts of diluted serum sample and diluted DENV were mixed and incubated at 37 °C for 1 h. Thereafter, 100 μl of the neutralized liquid was inoculated into three wells per dilution and allowed to adsorb for 36 h at 37 °C and 5% CO_2. Neutral red staining was then performed before final plaque counts were made. The neutralizing antibody titers were identified as the highest serum dilution that reduced the number of virus plaques by 50% (PRNT50) compared with control samples containing the virus alone. The sera were diluted by the continuous double dilution method starting at 1:2.

Measurement of cellular immune response

Mice were sacrificed and splenocytes were prepared 30 days after the third dose injection. The frequency and intensity of IFN-γ-producing cells were determined by mouse IFN-γ ELISPOT kits (Dakewe). All operations were conducted according to the manufacturer's procedures. In brief, 100 μl of IFN-γ-specific mAb was coated onto 96-well plates with PVDF membranes and incubated overnight at 4 °C. The plates were washed three times with PBS and blocked with RPMI medium supplemented with feta bovine serum (10%) at 37 °C for 1 h to prevent nonspecific binding in later steps. The splenocytes were seeded at a concentration of 1×10^6 cells/well with each EDIII protein of DENV 1–4. Quadruplicate wells were set up for each stimulation and PBS or concanavalin A (5 μg/mL) were included as controls in parallel. After stimulation for 2 days at 37 °C in a 5% CO_2 humidified incubator, the cells were removed from the plates by washing five times with 0.05% (w/v) Tween 20 in PBS. Secondary biotin-conjugated antibody was added to each well and incubated at 37 °C for 2 h. The wells were washed five times with 0.05% Tween 20 in PBS, and peroxidase-labeled streptavidin was added at a 1:1000 dilution for 1 h. Then the plates were washed five times with 0.05% (w/v) Tween 20 in PBS and three times with PBS alone. Finally, a 100 μl aliquot of 3-amine-9-ethylcarbazole staining solution was added to each well to develop the spots. The reaction was stopped after 30 min by placing the plates under tap water. Plates were dried, and spots were counted under an ELISPOT reader. The results were expressed as the number of spot-forming units (SFU) per 10^6 splenocytes.

Animal challenge with DENV-2 infected K562 cells

Eight immunized mice from each group received a intraperitoneal injection of 5×10^7 DENV-2 infected K562 cells that were suspended in 0.5 mL of serum-free RPMI medium one month after the last dose injection, of which the mice immunized with aluminum (without vaccine candidates) were taken as negative control. One day after challenge, the mice were euthanized to measure viral loads in plasma by

quantification on Vero cells. The blood (0.2 mL) was immediately mixed with 0.02 mL of 3.8% sodium citrate pre-chilled on ice. The plasma was isolated, and the viremia level was evaluated using PRNT with Vero cells.

Statistical analysis

Prism software version 5.00 (GraphPad Software, San Diego, CA, USA) was used for calculating the means, standard deviations, standard errors, and statistics analyses. Direct or transformed (Log2, Log10) data that passed the normality test (Kolmogorov–Smirnov or D'Agostino and Pearson omnibus normality test) and showed variance homogeneity (Bartlett's test) were analyzed by ANOVA parametric tests. Data that did not fulfill normality and/or variance homogeneity tests, even after transformations, were analyzed by the nonparametric test (Kruskal-Wallis test with LSD's multiple comparison tests). Differences with $p < 0.05$ were considered to be statistically significant.

Results

The tetravalent rEDIII protein elicits a broad profile of humoral immune responses in mice.

BALB/c mice were immunized with the rEDIII protein three times and antibody responses were monitored later. Serum samples were collected 15 days after the last immunization and tested for antibody responses against EDIII of all 4 serotypes by ELISA. All mice immunized with the tetravalent rEDIII protein developed an anti-EDIII IgG response against all four DENV serotypes (Fig. 1A-D). The anti-EDIII antibody titers of all cases were higher than 10^3, and no anti-EDIII antibody response were detected in animals from the aluminum group. As was expected, the strongest anti-EDIII antibody response with titers of all immunized animal groups exceeding 10^4 was detected against DENV-2. Statistical analysis showed a dose-response behavior. The immune response against D1EIII, D2EII, D4EIII measured in animals immunized with 50 μg of rEDIII had significant differences compared with the lowest dose group (2 μg of rEDIII) (Fig. 1ABD). However, the response against D2EIII, D3EII, D4EIII detected in animals immunized with 50 μg of rEDIII was not statistical different from the response in the highest dose group (250 μg of rEDIII) (Fig. 1BCD). In general, animals receiving 50 μg of rEDIII showed a stronger response against all four DENV serotypes than those immunized with the lowest dose ($p < 0.05$).

The capacity of the antibodies to neutralize dengue virus was evaluated using an in vitro neutralization test (PRNT). The results revealed that sera from rEDIII protein immunized animal neutralized in vitro viral infection with all four DENV serotypes (Fig. 2). The neutralizing antibody titer against dengue-3 or 4 was lower than that against dengue-1 or 2. Meanwhile, no neutralizing activity against DENV was detected in the sera of animals immunized with aluminum adjuvant. In accordance with the result of ELISA, the highest neutralizing activity was also detected against DENV-2. In addition, no statistical differences were observed between 10 μg and 50 μg groups in the neutralizing antibody titer measured against all four DENV serotypes.

The tetravalent rEDIII protein induces a cellular immune response in mice.

In order to evaluate the cellular immune response, spleen cells were collected 30 days after the last injection, and the frequency of IFN-γ producing in vitro stimulated with each EDIII protein was measured. Regardless of the dose of rEDIII protein, a positive IFN-γ secreting cell response was induced in immunized animals after in vitro stimulation with all four DENV EDIII antigens except D3EIII (Fig. 3). In general, the intensity of cell responses to D1EIII and D2EIII were much stronger than D3EIII and D4EIII. Be consistent with the humoral immune response, the highest frequency of IFN-γ-producing cells were observed in the group that medium dose of rEDIII assayed (10 μg or 50 μg), with values of 328.5 ± 103.8 or 314.8 ± 96.17 SFU/million cells for D2EIII. A lower frequency was detected in mice immunized with 2 or 250 μg of rEDIII, which showed a frequency of 100.1 ± 66.57 or 91.38 ± 77.94 SFU/million cells for D1EIII, 141.5 ± 74.75 or 174.4 ± 107.9 SFU/million cells for D2EIII and 33.25 ± 35.49 or 79 ± 103.5 SFU/ million cells for D4EIII. No antiviral IFN-γ secreting was observed in spleen cells from animals inoculated with aluminum adjuvant.

The tetravalent rEDIII protein significantly reduces viremia level in mice challenged with DENV-2.

Mice are usually not the permissive hosts for DENV and artificial transient viremia can be developed by intraperitoneal injection of DENV infected K562 cells, which became a reliable indicator to evaluate the protective capacities of the candidate during dengue vaccine development [33]. In our study, this method was adopted to evaluate the efficiency of virus clearance in rEDIII protein immunized mice. 30 days after the last immunization, BALB/c mice of each group were challenged with DENV-2 infected K562 cells. As was expected, high viral loads were found in blood of mice immunized with the adjuvant, with a mean of 7.3×10^5 FFU/mL of DENV-2 (Fig. 4). However, the virus titers in the blood of rEDIII protein immunized mice were significantly lower than that from the adjuvant group. And there was a significant difference between each rEDIII protein immunized group and aluminum immunized group ($p < 0.05$). These results suggested that rEDIII protein immunized mice developed functional immune responses to clear DENV-2 from the circulation.

Discussion

The envelope protein (E protein) of DENV, especially it's domain III (EDIII), is responsible for a wide range of biological activities, including binding to host cell receptors as well as fusion to and entry into host cells [34]. This domain, which is exposed and accessible on the virion surface and is involved in host cell surface receptor recognition, contains multiple type- and subtype-specific conformation-dependent neutralizing epitopes [35]. Therefore, the dengue E protein is an important antigen for diagnosis and vaccine development [36, 37]. Accumulating studies have proved that EDIII-based vaccine candidates can elicit specific neutralizing antibodies [38–40] Choosing only domain III rather than whole envelope protein could reduce the risk of ADE due to the lack of other non-neutralizing or cross-reactive epitopes. Hence, most of recent efforts have focused on utilization of EDIII to produce a subunit dengue vaccine [41–43]. We have constructed a tetravalent domain III in tandem using the Gly4SerGly4 linker, and testified its potential utility in the diagnosis of DENV infections with a high degree of sensitivity and specificity [41].

In this study, we attempted to develop this tetravalent protein (rEIII) as vaccine candidates against DENV and evaluated the immune responses in mice.

Over recent years, recombinant envelope proteins of DENV have been successfully expressed in different systems including prokaryotic expression systems and eukaryotic expression systems, its defects are obvious. The major limitation lies in protein secretion, proteolytic processing and glycosylation. In the present report, the secreted rEDIII protein produced by baculovirus expression system was adopted as vaccine candidate in anti-dengue virus. This system had several advantages over most others, including (i) a secreted protein with greater flexibility and integrity of the tertiary structure, (ii) easier methods to purify, and thus (iii) higher yields. Our study was performed to assess the immunogenicity and protective capacity in mice with different doses, containing 2, 10, 50 and 250 μg of rEDIII protein mixed equivalents aluminum adjuvant.

Our results suggested that the tetravalent formulation could induce high antibody response against all four DENV EDIII antigens. Even the lowest dose (2 μg rEDIII) induced a positive antibody response showing good immunogenicity. Importantly, the antiviral antibodies elicited by all groups, 2, 10, 50 and 250 μg of rEDIII, showed neutralizing activity against all four serotypes of DENV. Dengue virus infection is a complex viral disease that caused by four serotypes. Viral interference has been reported when a dengue vaccine candidate composed of four live-attenuated DENVs was used [44, 45]. The occurrence of this interference may cause failure in providing adequate protection against all 4 serotypes [46]. In this study, 10 μg and 50 μg groups could elicit high and comparable affinity antibodies against all 4 serotypes of DENV, indicating that both groups were suitable for vaccine candidate. It should be pointed out that the neutralizing activities against dengue-3 and 4 did not quite match up to the high level against dengue-1 and 2, which probably due to the position located in the sequence (1-3-4-2) that may lead burying some crucial antigenic determinants inside. A longer linker was more appropriate to expose the most antigenic determinants of the four domain IIIs. Besides, the strongest activity was found in neutralizing antibodies against DENV-2. This is likely due to amino acid sequence differences of EDIIIs and the position of D2EIII in tetravalent protein. Similar results have been found in other studies [34, 47, 48].

Additionally, the secreting levels of IFN-γ in mouse spleen cells after stimulation with each DEIII were measured as a surrogate of the cellular immune response. The role of IFN-γ in anti-dengue infection has been widely reported [49, 50]. Sustained levels of IFN-γ in the serum of DENV-infected patients are correlated with anti-viral protection and induction of cytotoxic T-cell response [51, 52, 53]. Our results showed that no matter of the dose used, the rEDIII formulation induced obvious cellular immune responses against the D1EIII and D2EIII, suggesting the T-cell epitopes of D1EIII and D2EIII were fully recognized by mouse spleen cells. However, nearly no response against D3EIII and D4EIII was found, which might be attribute to that the T-cell epitopes were lost or buried inside of the rEDIII protein. Nevertheless, it cannot be ruled out that the activity of cellular response will depend on the cell line, the concentration of stimulant, and even the assay performed to assess this activity. Accumulated evidence indicated that the antigen dose plays a significant role in determining the quality of antigen-specific T cells. Recently research proved that a low dose of DENV antigen generates T cells with a high-avidity TCR,

whereas a high dose only induces T cells with a low-avidity TCR [54]. Similar but not identical results were observed in our study. Compared with the medium dose (10 μg or 50 μg), the high dose (250 μg) did inhibit the cellular immune response against D2EIII. However, no stronger response against D2EIII was induced in the low dose (2 μg) than in the medium dose.

Finally, the protective capacity of the rEDIII formulations was evaluated using a mouse model for DENV infection. Although mice are not the natural host of DENV, viremia levels could be detected within 10 min after injection by the intravenous route in mice [55]. However, it can be prolonged up to 1 day by challenging with DENV -infected K562 cells [33]. In this study, mice were challenged with K562 cells infected by DENV-2 because of its high immunogenicity and virulence. Measurement of virus titers in plasma 1 day after the challenge revealed a significant reduction of virus levels in rEDIII-immune mice compared to the control group. In accordance with the results of neutralization test and ELISPOT, the 10 μg and 50 μg groups manifested better virus clearance capacity than other groups. Considering more time was needed in virus-induced cellular immune response, neutralizing antibodies may play an important role in this process. These results suggested that rEDIII formulation induced functional immune responses in eliminating DENV-2 in vivo.

Conclusions

We evaluated the immune responses in mice to the rEDIII formulation and demonstrate that the tetravalent protein induced humoral immunity against all four serotypes of DENV and elicited cellular immunity against DENV-1 and DENV-2. Importantly, the immune responses induced by the tetravalent formulation were functional in clearing DENV-2 in circulation of mice. These represent important features on dengue vaccine development. Our findings showed the promising potential of this secreted protein in developing a tetravalent dengue vaccine candidate. Further studies should be conducted to evaluate the immunogenicity and protective capacity of rEDIII formulation in non-human primates.

Abbreviations

DENV
dengue virus
EDIII
envelope domain III
DHF
dengue fever with hemorrhage
DSS
dengue shock syndrome
ADE
antibody-dependent enhancement
PRNT
plaque-reduction neutralization test

ELISA
enzyme-linked immunosorbent assay

Declarations

Ethical approval and consent to participate

The protocols were approved by the Committee on the Ethics of Animal Experiments of Weifang Medical University.

Consent for publication

Not applicable.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors have declared that no competing interests exist.

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Authors' contributions

L.S.and G.N. conceived and designed the experiments. Z.P.,Y.B. and Z.L. performed the experiments. W.L.analysed the data. G.L.and M.W. contributed reagents, materials and analysis tools. Y.G.,Z.P.,L.S.and G.N. took part in the preparation and revision of the manuscript. All authors have given approval to the final version of the manuscript.

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Figures

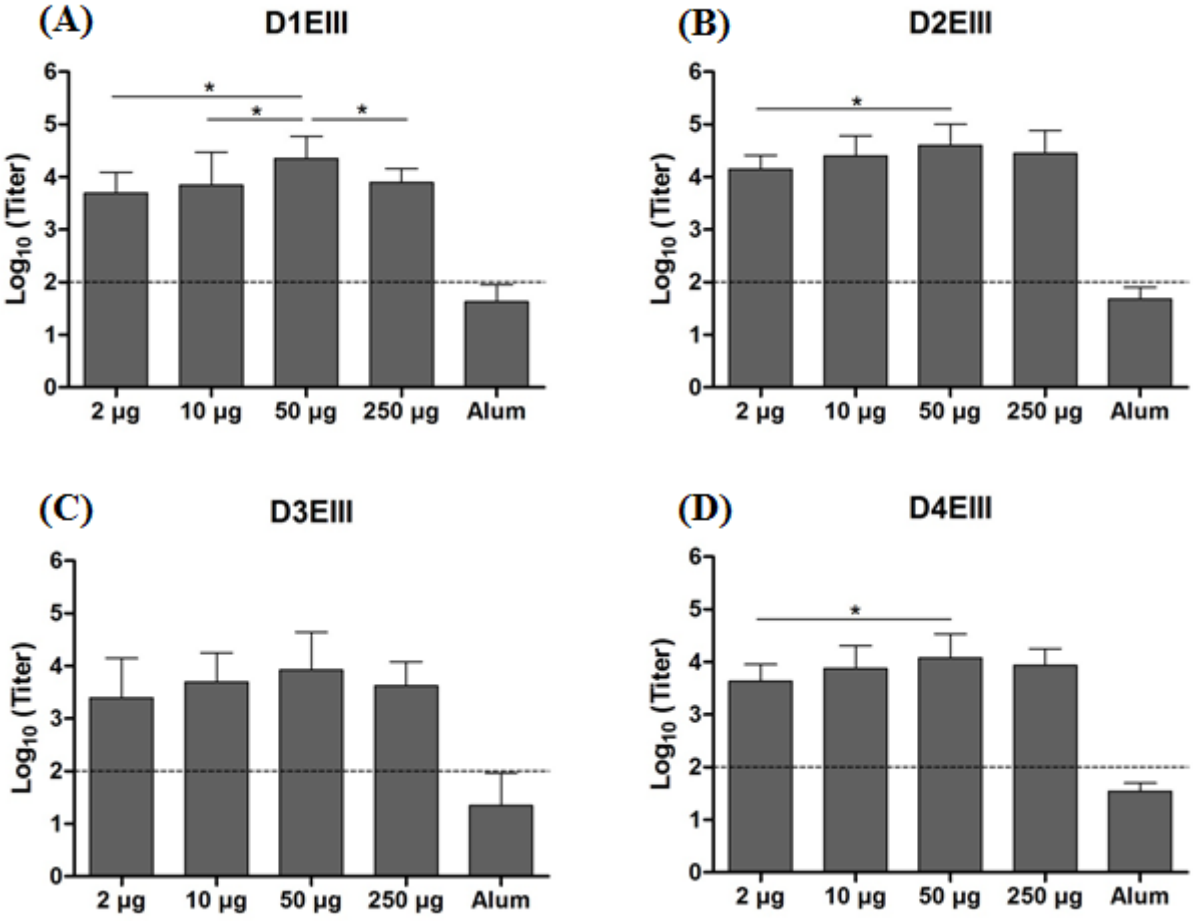

Figure 1

Anti-EIIIs antibody responses induced by tetravalent recombinant dengue EDIII in mice. Four groups of BALB/c mice were intraperitoneally immunized with different formulations of the tetravalent recombinant dengue envelope protein domain III mixed with aluminum adjuvant. As a negative control, one group received equivalent amount of aluminum adjuvant. Fifteen days after the last dose, ten animals were bled and the IgG antibodies against D1EIII (A), D2EIII (B), D3EIII(C) and D4EIII (D) in sera were measured by ELISA. The charts plot mean with standard error media (n = 10). The dashed line represents the cutoff value. The statistical analysis was performed using Kruskal-Wallis One Way ANOVA and LSD's Multiple Comparisons tests (*p < 0.05). The results are representative of two independent experiments

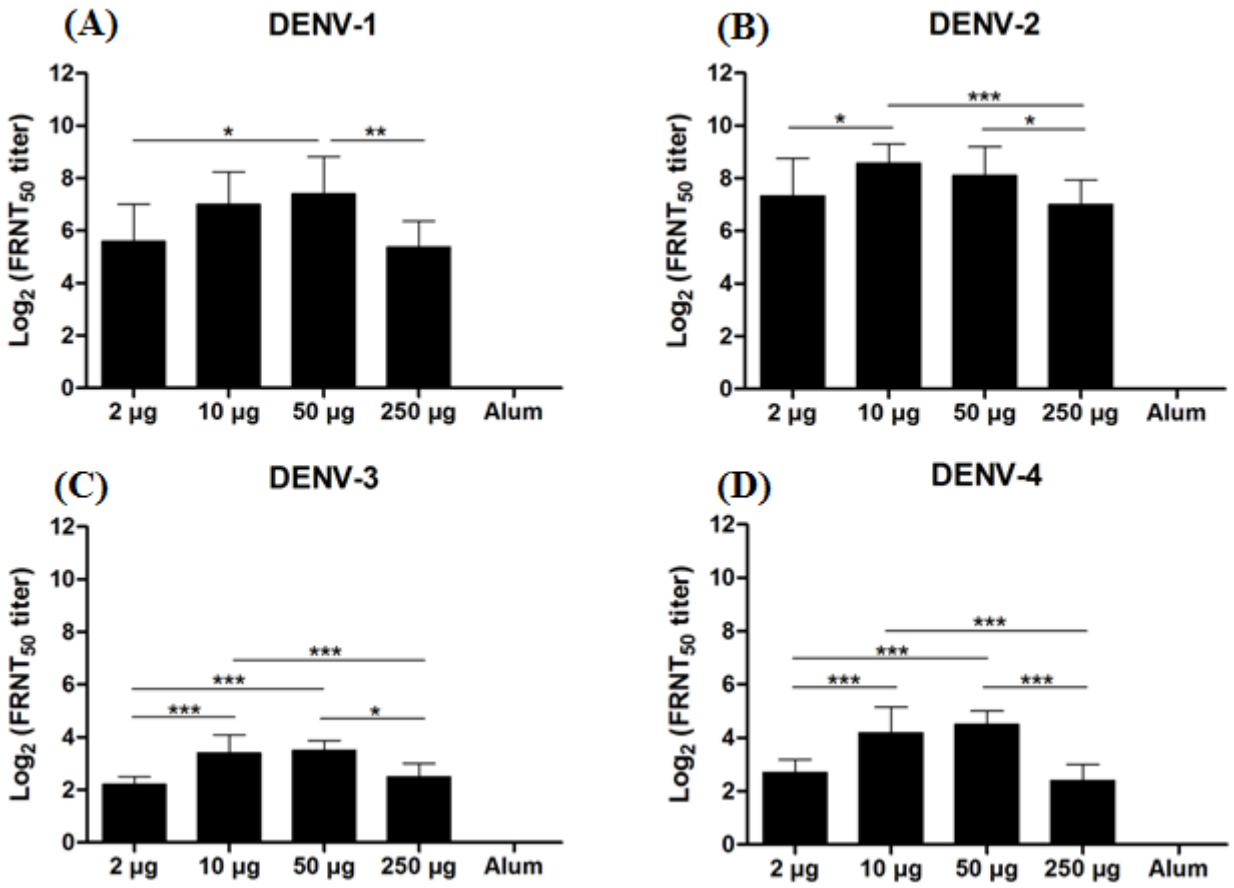

Figure 2

Neutralizing antibody responses induced by tetravalent recombinant dengue EDIII in mice. Fifteen days after the last dose, ten animals were bled and the neutralizing antibodies against DENV-1(A), DENV-2(B), DENV-3(C) and DENV-4 (D) in sera were measured by PRNT. The charts plot mean with standard error media (n = 10). The dashed line represents the cutoff value. The statistical analysis was performed using Kruskal-Wallis One Way ANOVA and LSD's Multiple Comparisons tests (*p < 0.05; **p < 0.01; ***p < 0.001). The results are representative of two independent experiments.

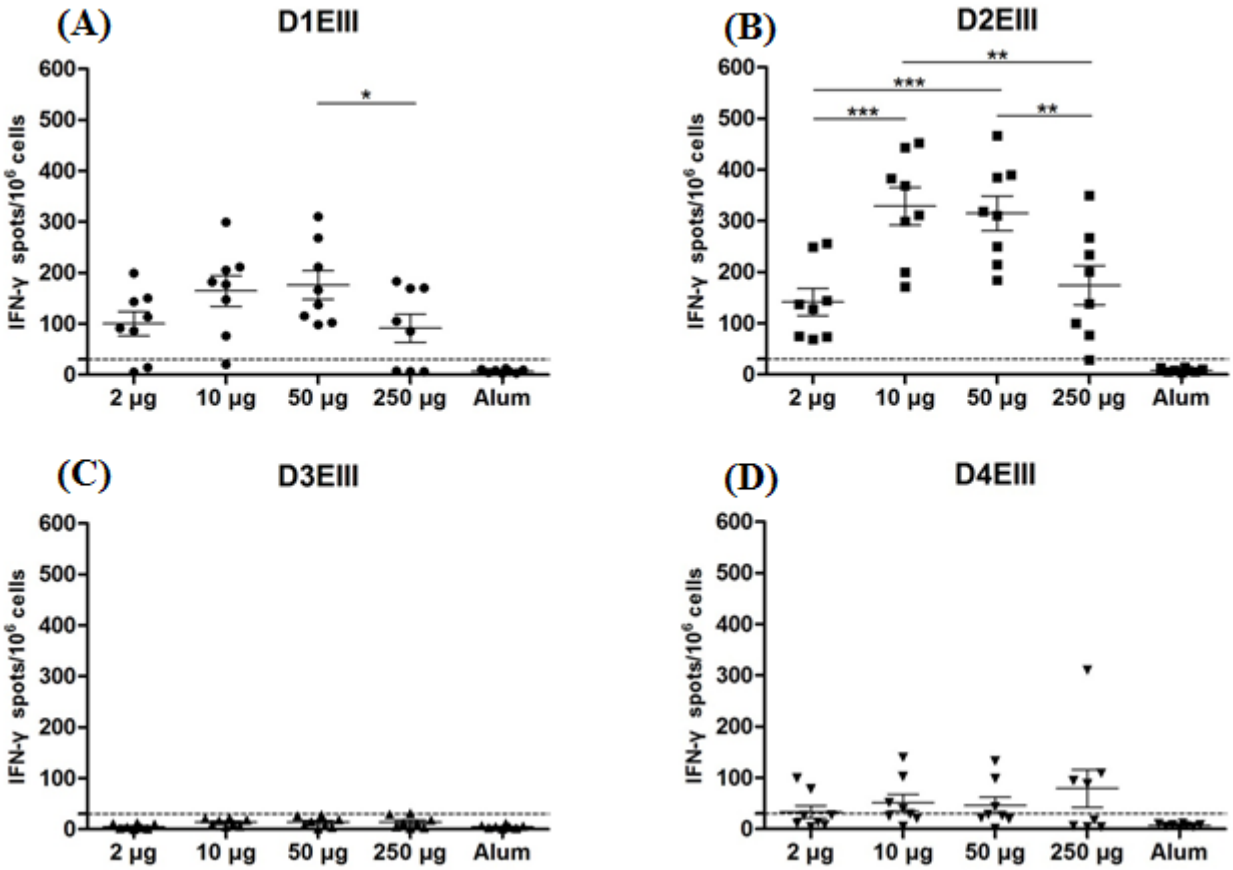

Figure 3

Cell-mediated immune responses induced by tetravalent recombinant dengue EDIII in mice. One month after the last dose, spleen cells from the eight animals were stimulated in vitro with each EDIII. The frequency of IFN-γ-secreting cells in spleens was measured by ELISPOT after in vitro stimulation with D1EIII (A), D2EIII (B), D3EIII (C) and D4EIII (D). The dashed line indicates the cutoff. The statistical analysis was performed using Kruskal-Wallis One Way ANOVA and LSD's Multiple Comparisons tests (*p < 0.05; **p < 0.01; ***p < 0.001). Data represent the mean± standard error from total 8 mice of two independent experiments. The results are expressed as the number of spot-forming units (SFU) per 106 splenocytes.

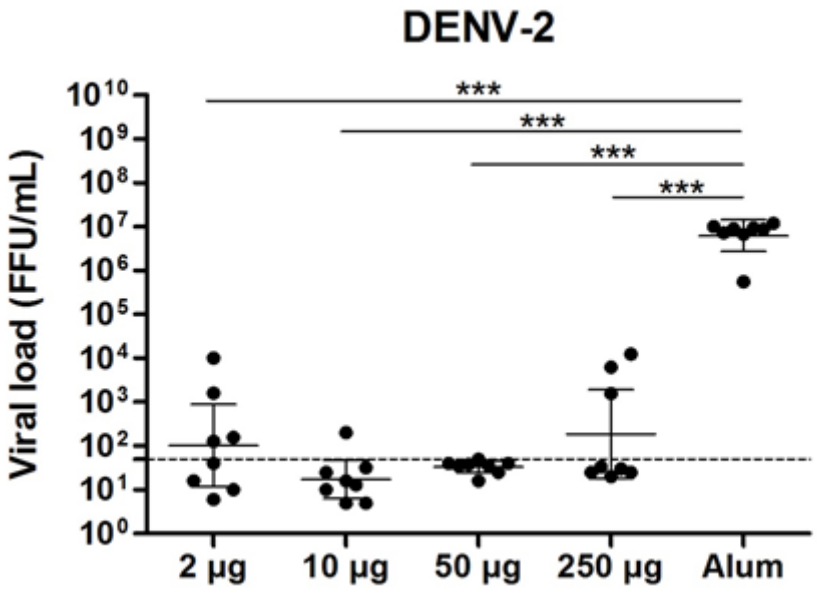

Figure 4

Reduction of viremia levels in vaccinated mice after challenged with DENV-2. Mice were intraperitoneally challenged with 5 × 107 DENV-2 infected K562 cells at one month after the last vaccination. One day after challenge, the mice were euthanized to measure viral loads in plasma by quantification on Vero cells. The charts plot mean with standard error media (n = 8). The dashed line represents the cutoff value. The statistical analysis was performed using Kruskal-Wallis One Way ANOVA and LSD's Multiple Comparisons tests (***p < 0.001). The results are representative of two independent experiments.

Supplementary Files

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