

# A dose-response study in mice of a tetravalent vaccine candidate composed of domain III-capsid proteins from dengue viruses

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**Abstract** Tetra DIIIIC is a subunit vaccine candidate based on domain III of the envelope protein and the capsid protein of the four serotypes of dengue virus. This vaccine preparation contains the DIIIIC proteins aggregated with a specific immunostimulatory oligodeoxynucleotide (ODN 39M). Tetra DIIIIC has already been shown to be immunogenic and protective in mice and monkeys. In this study, we evaluated the immunogenicity in mice of several formulations of Tetra DIIIIC containing different amounts of the recombinant proteins. The Tetra DIIIIC formulation induced a humoral immune response against the four DENV serotypes, even at the lowest dose assayed. In contrast, the highest level of cell-mediated immunity, measured as frequency of IFN $\gamma$ -producing cells, was detected in animals immunized with the lowest dose. The protective capacity of the tetravalent formulations was assessed using the mouse model of dengue virus encephalitis. Upon challenge, vaccinated mice showed significantly reduced virus replication in all tested groups. This study provides new information about the functionality of Tetra DIIIIC as a vaccine candidate and also supports the crucial role of cell-mediated immunity in protection against dengue virus.

## Introduction

Dengue is one of the most important arthropod-borne diseases. Dengue virus (DENV) causes up to 390 million infections and 20,000 deaths annually [1]. Infection may be asymptomatic or can result in a spectrum of clinical disease including self-limiting fever with manifestations of varying severity (dengue fever; DF), which can then progress to life-threatening dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [2].

DENV belongs to the family *Flaviviridae* and has four different serotypes (DENV-1 to 4), which are clinically indistinguishable. These viruses are distributed worldwide in the tropics and in subtropical regions [3]. Infection with one serotype confers life-long immunity against the infecting virus, whereas secondary infection with a different serotype can cause the severe forms of the disease [4]. A tetravalent vaccine formulation is therefore necessary to achieve effective immunity against this pathogen.

Phase I–III clinical trials have been conducted to examine the immunogenicity and safety profiles of different vaccine candidates against DENV [5–11]. To date, only Dengvaxia from Sanofi-Pasteur has been licensed in 13 countries during the last two years [12]. Nevertheless, this vaccine has an overall efficacy of 60.3% [13] and shows waning protection, especially in seronegative vaccinees [9].

Our group has developed a subunit vaccine candidate based on domain III (DIII) of the envelope protein and the capsid (C) protein of DENV. These viral fragments are potential inducers of neutralizing antibodies and cell-mediated immunity (CMI). The resulting chimeras, named DIIIIC proteins (DIIIIC-1–4), are efficiently expressed in *Escherichia coli* and are easily purified using a combination of ion exchange and ion metal affinity chromatography. In our first studies, a monovalent formulation of

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DIIC-2 aggregated with oligodeoxynucleotides proved to be immunogenic and protective in mice and monkeys [14–16]. Later, we demonstrated the immunogenicity of DIIC proteins aggregated with an immunostimulatory oligodeoxynucleotide (ODN 39M) and in a tetravalent formulation (Tetra DIIC) in both animal models [17]. In parallel, a study evaluating the immunogenicity in mice of different amounts of the aggregated protein DIIC-2 revealed that the best immunogenicity profile was obtained with the lowest quantity assayed [18]. Based on those results, in the present study, we evaluate in mice the immunogenicity of five formulations of Tetra DIIC, containing different amounts of the recombinant proteins.

Our results show that, regardless of the dose tested, Tetra DIIC induces a humoral and cellular immune response against DENV. In addition, we observed for the first time that even a low dose of this vaccine candidate (4 µg of Tetra DIIC) induces an immune response that controls DENV infection.

## Material and methods

### Virus

Animal immunizations were carried out using DENV-1 (Jamaica strain), DENV-2 (SB8553 strain), DENV-3 (Nicaragua strain) and DENV-4 (Dominica strain). DENV-1 (Hawaii strain), DENV-2 (New Guinea C strain), DENV-3 (H-87 strain) and DENV-4 (H241 strain) were used for detection of IgG antibodies [19]. DENV-1 (West Pacific 74 strain), DENV-2 (S16803 strain), DENV-3 (CH53489 strain), and DENV-4 (TVP-360 strain) were used for the plaque reduction neutralization test (PRNT) as recommended by WHO [20]. A neuroadapted DENV-1 (Hawaii strain) from virus-infected suckling mouse brain was employed in the challenge experiment.

### Mice

Female BALB/c (Bc, H-2d) mice (aged 6–8 weeks) were purchased from CENPALAB (Havana, Cuba) and housed in appropriate animal-care facilities during the whole experimental period. The maintenance and care of animals used in this research complied with the Cuban Institute of Health guidelines for the human use of laboratory animals. The Ethics Committee of Animal Experiments of CIGB approved the experimental protocol used.

### Recombinant proteins

The design, cloning, expression and purification of the recombinant proteins DIIC-1, DIIC-2, DIIC-3 and

DIIC-4 were described previously [21]. The sequence coding for the domain III fragment (amino acids 286–426) or C protein were amplified from the viral genome of the following dengue strains: DENV-1 Jamaica (AF425621); DENV-2 Jamaica (M20558.1), DENV-3 Nicaragua (FJ882576), and DENV-4 Dominica 814669 (AF326573). The DIII and C regions were fused to generate DIIC chimeric proteins of each DENV serotype with a 6-His-tag at their N-terminal ends. Recombinant proteins were expressed in *Escherichia coli*, purified and aggregated *in vitro* [21]: 400 µg of the recombinant proteins were incubated with 40 µg of ODN 39M (5'-ATCGACTCTCGA GCGTTCTCGGGGGACGATCGTCGGGGG-3') in TE buffer (10 mM Tris, 6 mM EDTA, pH 7.4). Finally, different quantities of the DIIC proteins were evaluated in mice using a ratio of 1:1:1:1 from each serotype. All formulations were prepared in 200 µL with aluminum hydroxide (alum; Alhydrogel, Brenntag Biosector) at a final concentration of 1.44 mg/mL.

### Mouse experiments

Three doses of each immunogen were injected by the intraperitoneal route into five groups of 15 female BALB/c mice on days 0, 15 and 45. Groups 1 to 5 received different tetravalent formulations containing aggregated DIIC proteins (Table 1). Five additional groups were used as negative or positive controls.

Ten mice from each group were partially bled 15 and 30 days after the third dose injection, and sera were collected for further immunological analysis. In addition, one month after the last dose, 6–8 animals per group were splenectomized to study the cellular immune response. For the challenge experiment, seven animals from each group received a 20 µL intracranial injection of a suspension of neuroadapted DENV-1 containing 50 times the median lethal doses (LD<sub>50</sub>) one month after the last dose injection. Seven days after this challenge, mice were euthanized to measure the brain viral load. This preclinical study was repeated twice.

### Anti-DENV IgG antibody response

The antibody IgG response was measured by enzyme-linked immunosorbent assay (ELISA). Flat-bottomed 96-well plates (Costar, USA) were coated with 5 µg/mL of a mixture of anti-DENV human immunoglobulins for 2 h at 37 °C in coating buffer (10 mM Na<sub>2</sub>CO<sub>3</sub>, 35 mM NaHCO<sub>3</sub>, pH 9.5). The wells were washed three times with PBS containing 0.05% Tween 20 (PBS-T) after each step of the ELISA. The plates were blocked with 5% (w/v) skim milk in coating buffer for 1 h at 37 °C. Serum samples were serially diluted in PBS-T and incubated for 1 h at

**Table 1** Study design

Group	Total protein	Composition	Volume of inoculum	Adjuvant
1	4 µg of Tetra DIIC	1 µg of each DIIC	200 µL	Alum
2	10 µg of Tetra DIIC	2.5 µg of each DIIC	200 µL	Alum
3	20 µg of Tetra DIIC	5 µg of each DIIC	200 µL	Alum
4	40 µg of Tetra DIIC	10 µg of each DIIC	200 µL	Alum
5	80 µg of Tetra DIIC	20 µg of each DIIC	200 µL	Alum
6	Placebo	8 µg of ODN 39M	200 µL	Alum
7	DENV-1	Infectious virus (10 <sup>5</sup> PFU/mL)	500 µL	Without adjuvant
8	DENV-2	Infectious virus (10 <sup>5</sup> PFU/mL)	500 µL	Without adjuvant
9	DENV-3	Infectious virus (10 <sup>5</sup> PFU/mL)	500 µL	Without adjuvant
10	DENV-4	Infectious virus (10 <sup>5</sup> PFU/mL)	500 µL	Without adjuvant

37 °C. Anti-mouse IgG-peroxidase conjugate (Sigma, USA) was added, and the plates were incubated for 1 h at 37 °C. After washing, substrate solution (500 µg/mL *o*-phenylenediamine and H<sub>2</sub>O<sub>2</sub> in buffer containing 53 mM Na<sub>2</sub>HPO<sub>4</sub> and 24 mM citric acid, pH 5.5) was added. Plates were kept at 25 °C for 30 min, and the reaction was stopped with 2.5 M H<sub>2</sub>SO<sub>4</sub>. Absorbance at 492 nm was read in a microplate reader (SensIdent Scan; Merck, Germany). Titers were defined as the dilution of serum giving twice the absorbance value of the negative control serum. The positive cutoff value was taken as twice the antibody titer of the negative control serum, defined arbitrary as 50.

Neutralizing antibody titers were measured by PRNT in LLC-MK2 cells as described previously [22]. The neutralizing antibody titer was defined as the highest serum dilution that reduced the number of virus plaques by 50% (PRNT<sub>50</sub>). As positive control, hyperimmune murine ascitic fluids from each dengue serotype were used. Titers higher than 10 were considered positive.

### Cell culture and *in vitro* stimulation

Spleen cells were obtained under aseptic conditions, and erythrocytes were eliminated by lysis in 0.155 mM NH<sub>4</sub>Cl. Cells were washed twice with PBS-2% fetal bovine serum (FBS) (PAA Laboratories, Ontario, Canada) and resuspended at 2 × 10<sup>6</sup> cells/mL in RPMI-1640 medium (Sigma Aldrich) supplemented with 100 U of penicillin per mL, 100 µg of streptomycin (Gibco, UK) per mL, 2 mM glutamine (Gibco, UK), 5 × 10<sup>-5</sup> M 2-mercaptoethanol (Sigma St. Louis, MO) and 5% FBS. Finally, 2.5 × 10<sup>5</sup> cells/well were cultured in 96-well round-bottom plates with each recombinant DIIC protein (10 µg/mL) or a mock preparation. Concanavalin A (ConA); (Sigma, St. Louis, MO) was used as a positive control. In all of the experiments, three wells were plated for each antigen. After four days of *in vitro* stimulation, culture supernatants were collected and stored at -20 °C.

### Detection of IFNγ-secreting cells

Nitrocellulose-backed 96-well plates (MultiScreen-IP filter plates, Millipore, Bedford, MA, USA) were coated with 100 µL of IFN-γ-specific mAb (5 µg/mL; Mabtech Inc., Cincinnati, OH, USA) overnight at 4 °C, washed three times with PBS, and blocked with supplemented RPMI medium and 10% FBS at 37 °C for 1 h. Splenocytes that had been stimulated for two days were transferred to nitrocellulose plates (2 × 10<sup>5</sup> cells per well) and incubated for 48 h at 37 °C in 5% CO<sub>2</sub> in RPMI medium and 5% FBS. The same positive and negative stimulation controls described above were included. Later, the plates were washed three times with PBS and five times with 0.05% Tween 20 in PBS. Secondary biotin-conjugated antibody (1 µg/mL; Mabtech) was then added and incubated for 2 h at room temperature. The wells were washed five times with 0.05% Tween 20 in PBS, and peroxidase-labeled streptavidin (Sigma) was added at a 1:1000 dilution for 1 h. The plates were then washed again with 0.05% Tween 20 in PBS and afterwards with PBS. Finally, the spots were developed by adding 100 µL of 3-amino-9-ethylcarbazole (Sigma) solution. After 15 min, the reaction was stopped with tap water. Plates were dried, and spots were counted under a dissection microscope. The results were expressed as the number of spot-forming units (SFU) per 10<sup>6</sup> splenocytes.

### Detection of viremia

Brains were aseptically homogenized in 1 mL of PBS containing 2% FBS and supplemented with 100 U of penicillin and 100 µg of streptomycin per mL. The suspensions were centrifuged for 10 min at 8000 × *g*, and supernatants were collected and stored at -80 °C. 5 × 10<sup>5</sup> Vero cells per well were cultured for 24 h in 6-well plates with MEM (Sigma Aldrich) supplemented with 100 U of penicillin per mL, 100 µg of streptomycin per mL, 2 mM

glutamine, 1 mM sodium pyruvate (Gibco), and 2% FBS. Cells were infected with different dilutions (1:10, 1:100 or 1:1000) of the infected brain supernatants in supplemented MEM medium with 1% FBS. After incubation for 4 h at 37 °C and 5% of CO<sub>2</sub> overlay medium was added (carboxymethylcellulose, 3% [w/v]) and the plates were incubated at 37 °C and 5% CO<sub>2</sub>. Infected cells were visualized by immunofocus assay. Briefly, the cells were fixed with 3.7% formaldehyde for 30 min, washed twice with PBS and then incubated in PBS-1% Triton X-100 for 15 min. After permeabilization, blocking buffer (PBS supplemented with 10% FBS) was added to each well for 30 min at room temperature. After blocking, the cells were incubated with mAb 4G2 at 1 µg/mL (in PBS and 1% FBS) for 1 h at room temperature. After two washes with PBS, polyclonal anti-mouse IgG-HRP (Sigma Aldrich) was added at a dilution of 1:2000 (in PBS and 1% FBS) and incubated for 1 h at room temperature. The cells were then washed twice with 50 mM Tris (pH 7.4), and a substrate solution of 3,3'-diaminobenzidine, 1.5 mM CoCl<sub>2</sub> and 155 mM NaCl in 50 mM Tris (pH 7.4) was added to each well. The plates were left on a shaker for 30 min until clear foci were visible for subsequent counting. The detection limit was 10<sup>2</sup> pfu/mL.

### Statistical analysis

Direct or transformed (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. All data were analyzed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, <http://www.graphpad.com>.

## Results

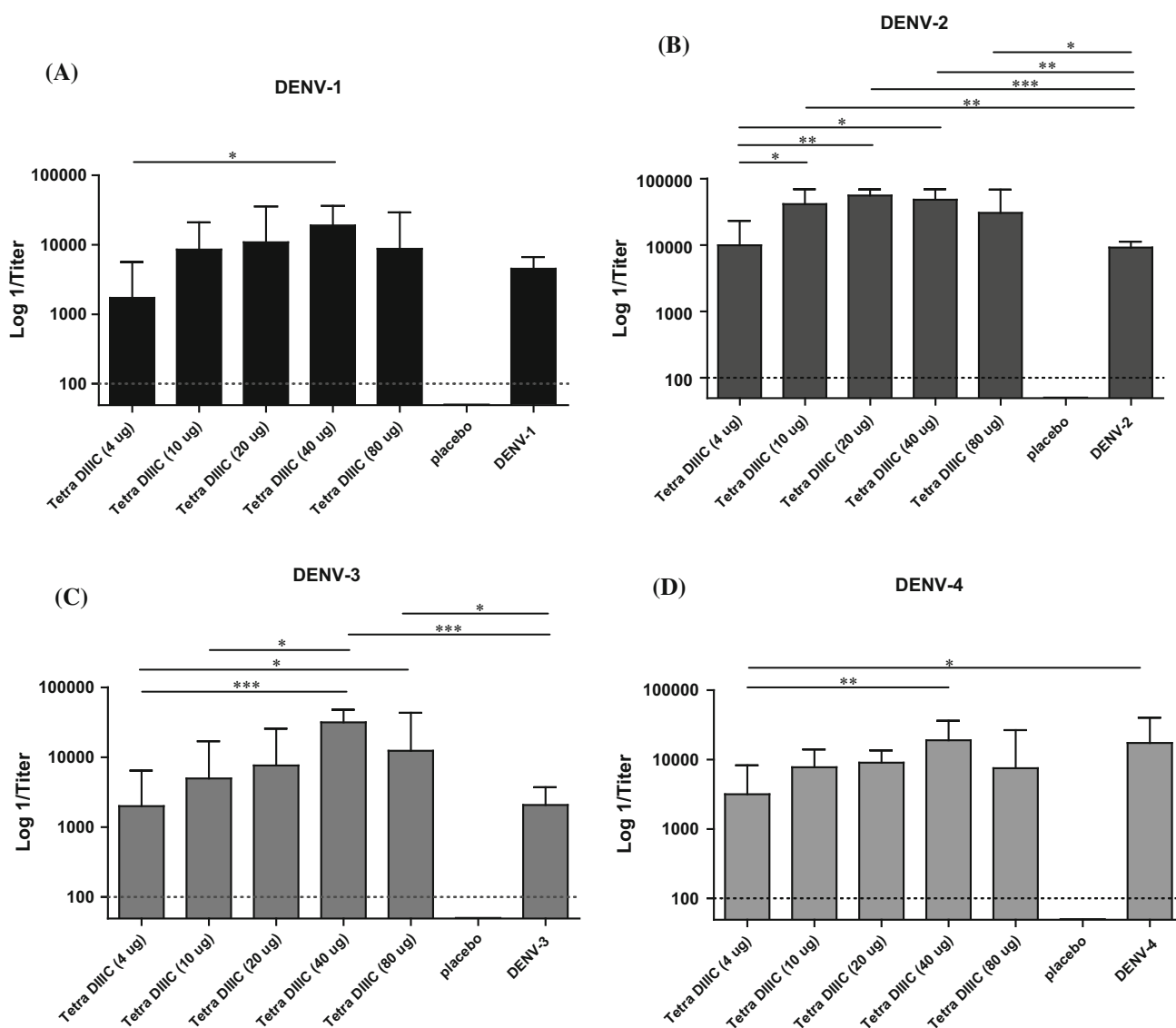
A previous study conducted by our group demonstrated the immunogenicity and protective capacity in mice of aggregated DIIC proteins as a tetravalent formulation [17]. However, in that study we assessed only one formulation of Tetra DIIC, which contained 20 µg of each recombinant protein (80 µg of Tetra DIIC). In this study, we evaluated the immunogenicity of different doses of the tetravalent formulation in mice. We immunized four groups of BALB/c mice with 4, 10, 20 or 40 µg of Tetra DIIC. An additional group received the same amount of DIIC proteins that had been evaluated previously, 80 µg of Tetra DIIC. As a negative control, one group of animals was

immunized with a placebo formulation containing the highest quantity of ODN 39M employed in the aggregation of the recombinant proteins, while four more groups were immunized with infectious DENV of each serotype, acting as positive controls. We evaluated the humoral and cellular immune responses after three immunizations. In addition, based on the low immunogenicity detected against DENV-1 in the previous evaluation of Tetra DIIC [17], in this study, we measured the protective capacity of each tetravalent formulation against this serotype.

### Tetravalent formulations of aggregated DIIC proteins elicit a strong humoral immune response

The humoral immune response was detected by measuring antiviral IgG antibodies by ELISA. All animals immunized with the tetravalent formulations of DIIC proteins developed an antiviral IgG response against all four DENV serotypes, regardless of the total amount of protein inoculated (Fig. 1A–D). In all cases, antiviral antibody titers were higher than 10<sup>3</sup>, and animals from the placebo group did not develop an antiviral antibody response. As was observed previously [17], the strongest antiviral antibody response was detected against DENV-2. Statistical comparing among the groups inoculated with the different doses of Tetra DIIC suggests a dose-response behavior. However, an analysis per viral serotype revealed that only the immune response against DENV-2 measured in animals immunized with 10, 20 or 40 µg of Tetra DIIC showed statistical differences when compared with the response detected in the group immunized with the lowest dose assayed (4 µg of Tetra DIIC) (Fig. 1B). In general, animals receiving 40 µg of Tetra DIIC showed a stronger response against the four DENV serotypes than animals inoculated with the lowest dose evaluated ( $P < 0.05$ ). However, the response detected in this specific group (40 µg of Tetra DIIC) was not always different from the response detected in animals receiving 10, 20 or 80 µg of Tetra DIIC (Fig. 1).

The functionality of the antiviral antibody response was also measured using an *in vitro* neutralization test (PRNT). The results showed that sera from animals immunized with the tetravalent formulations neutralized *in vitro* viral infection with serotypes 1, 2 and 3 (Fig. 2A–C). We did not detect neutralizing activity against DENV-4 in the sera of animals immunized with the tetravalent formulations of DIIC proteins, or even in those receiving infectious DENV-4 (Fig. 2D). In accordance with the antiviral antibody titers, the highest neutralizing activity was found against DENV-2. In addition, the neutralizing antibody response detected against DENV-1 was weaker than the one detected against DENV-2 or DENV-3. Statistical differences were only observed in the neutralizing immune



**Fig. 1** Antiviral antibody response in mice immunized with different formulations of Tetra DIIC (1-4). As a control, one group received a placebo formulation, and four groups were inoculated with infectious DENV. Fifteen days after the last dose, ten animals were bled and the antibodies in sera were measured using a capture ELISA. (A) IgG antibodies against DENV-1. (B) IgG antibodies against DENV-2. (C) IgG antibodies against DENV-3. (D) IgG antibodies against

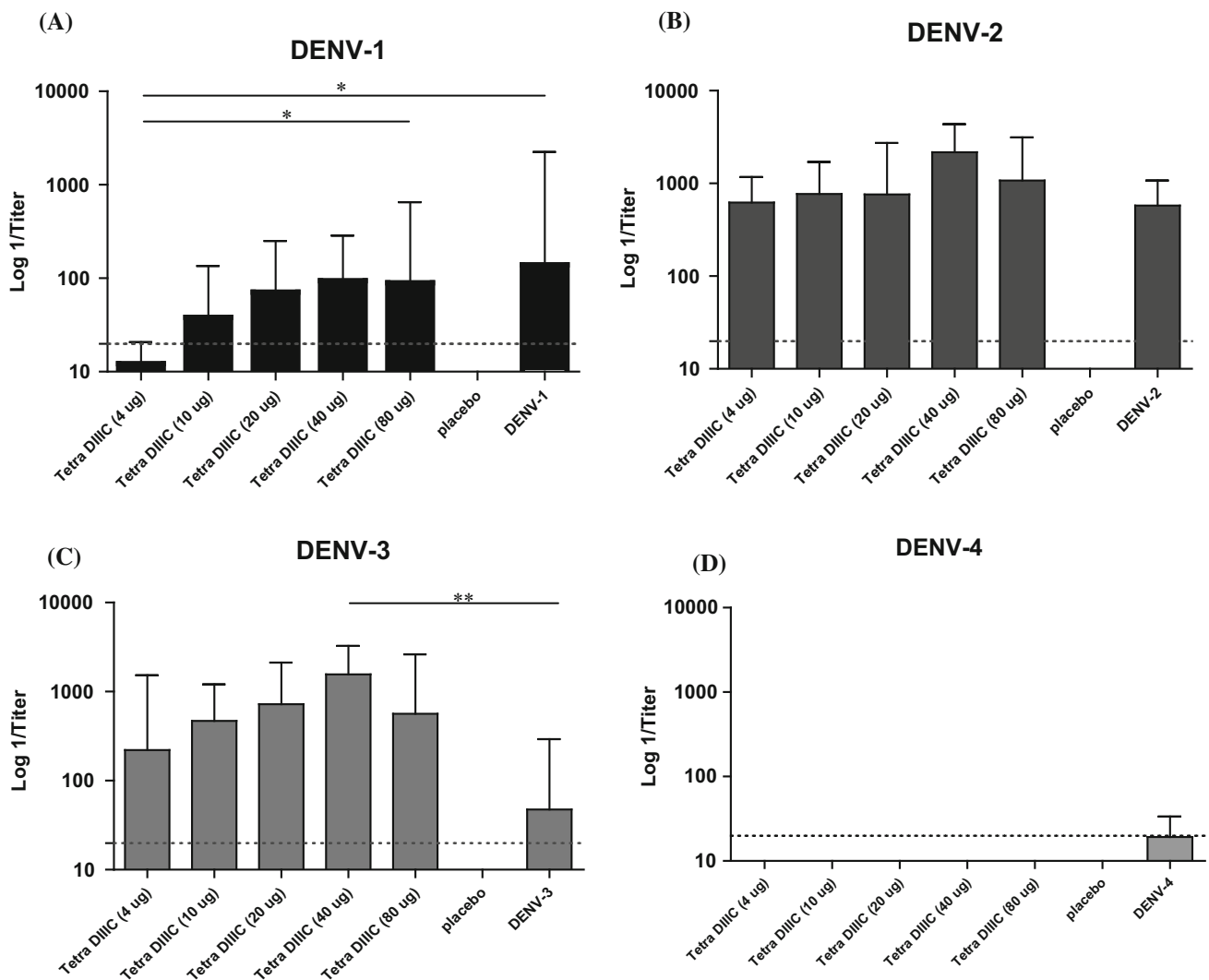
response measured against DENV-1, specifically between the groups receiving 4 µg and 80 µg of Tetra DIIC ( $P < 0.05$ , Fig. 2A).

### Tetravalent formulations of aggregated DIIC proteins induce a cellular immune response in mice

In order to evaluate the cellular immune response, the frequency of IFN $\gamma$ -producing spleen cells was measured 60 days after the last dose injection, after *in vitro* stimulation with each recombinant DIIC protein (Fig. 3A-D). Regardless of the dose of Tetra DIIC evaluated, the

DENV-4. The chart shows the geometric mean with 95% CI. The dashed line represents the cutoff (twice the mean titer of the placebo, arbitrarily defined as 50). Statistical analysis of data from ELISA was performed using Kruskal-Wallis and Dunn's multiple comparison tests (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ), ( $n = 10$  per group). The results are representative of two independent experiments

immunization induced a positive IFN $\gamma$ -secreting cell response in more than 50% of the animals after *in vitro* stimulation of mouse splenocytes with DIIC-1 (Fig. 3A), DIIC-2 (Fig. 3B) or DIIC-3 (Fig. 3C). By contrast, stimulation with the protein DIIC-4 resulted in small number of cells secreting the antiviral cytokine, with a low percentage of responder mice, mainly in the groups receiving 10, 20, 40 or 80 µg of Tetra DIIC (Fig. 3D). In contrast to the humoral immune response, we observed the highest frequency of IFN $\gamma$ -producing cells in animals receiving the lowest dose of Tetra DIIC assayed (4 µg of Tetra DIIC), with values of  $380.0 \pm 90.4$ ,  $467.0 \pm 83.4$ ,



**Fig. 2** Neutralizing antibody response in mice immunized with different formulations of Tetra DIIC (1-4). As a control, one group received a placebo formulation, and four groups were inoculated with infectious DENV. Thirty days after the last dose, mice were bled, and the antibodies in sera were measured by PRNT. (A) Neutralizing antibodies against DENV-1. (B) Neutralizing antibodies against DENV-2. (C) Neutralizing antibodies against DENV-3.

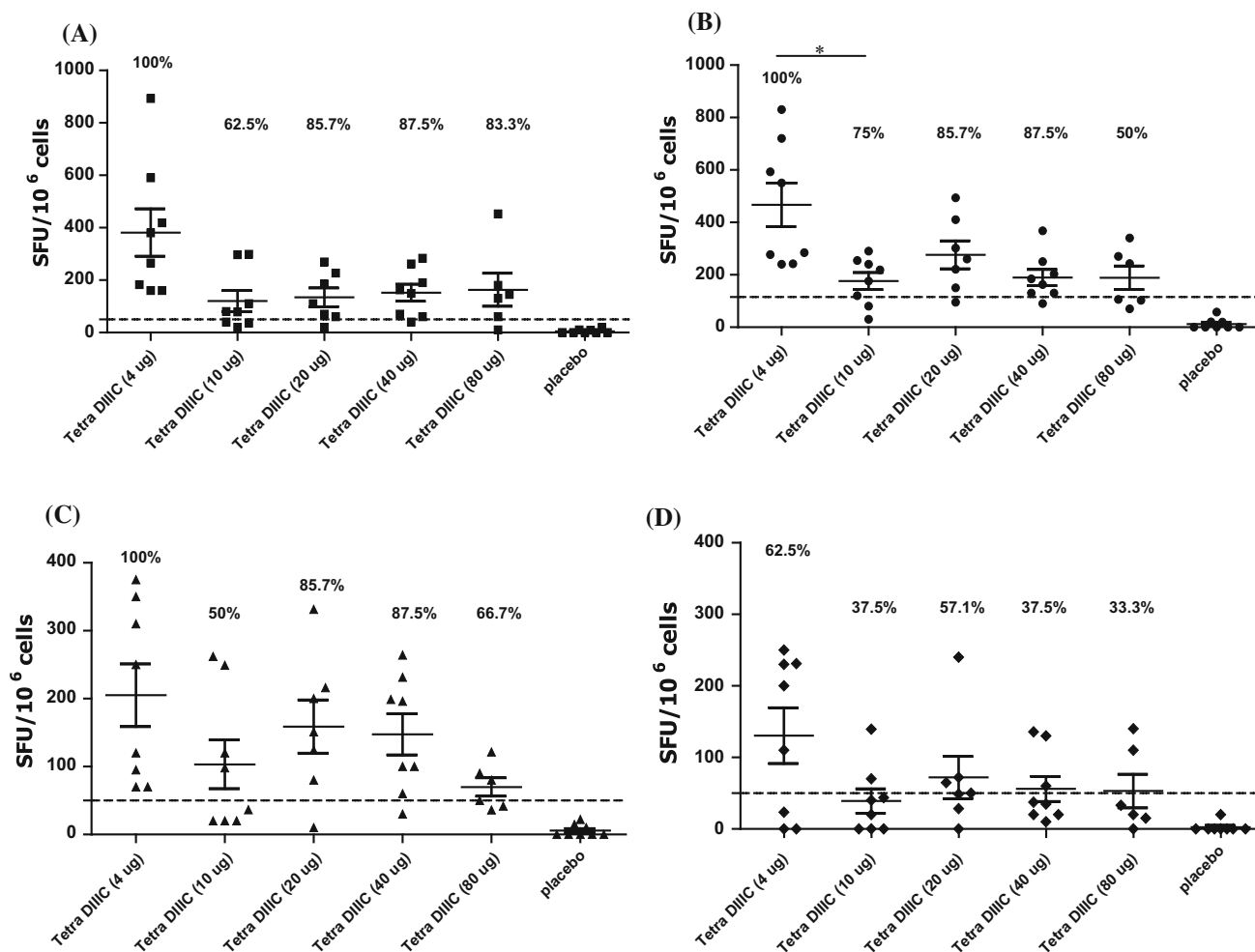
(D) Neutralizing antibodies against DENV-4. The chart shows the geometric mean with 95% CI. The dashed line represents the cutoff (twice the mean titer of the placebo, arbitrarily defined as 10). Statistical analysis of data was performed using Kruskal-Wallis and Dunn's multiple comparison tests (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ), ( $n = 8$  per group). The results are representative of two independent experiments

205.0  $\pm$  46.0 and 130.6  $\pm$  38.9 SFU/million cells for DIIC-1, DIIC-2, DIIC-3, DIIC-4, respectively, (Fig. 3). A weaker response was detected in animals immunized with the other doses evaluated. For example, mice immunized with 20 or 40  $\mu$ g of Tetra DIIC showed a frequency of IFN $\gamma$ -producing cells of 134.6  $\pm$  35.5 or 152.3  $\pm$  63.2 SFU/million cells after the stimulation with DIIC-1, 276.2  $\pm$  52.9 or 189.7  $\pm$  30.85 SFU/million cells after stimulation with DIIC-2, 159  $\pm$  39.1 or 174  $\pm$  30.4 SFU/million cells after stimulation with DIIC-3, and 72  $\pm$  29.4 or 56  $\pm$  17.6 SFU/million cells after stimulation with DIIC-4 (Fig. 3). A similar response was detected in animals immunized with 10 or 80  $\mu$ g of the tetravalent

formulation. Spleen cells from animals inoculated with the placebo formulation did not secrete the antiviral cytokine.

#### **Tetravalent formulations of aggregated DIIC proteins significantly reduce the viral load in mice intracranially challenged with DENV-1**

One month after the third immunization with the tetravalent formulations of DIIC proteins, animals of each group were challenged by the intracranial route with DENV-1. The protective capacity of Tetra DIIC formulations was assessed by measuring viral titers in brain homogenates from immunized and subsequently challenged mice. As



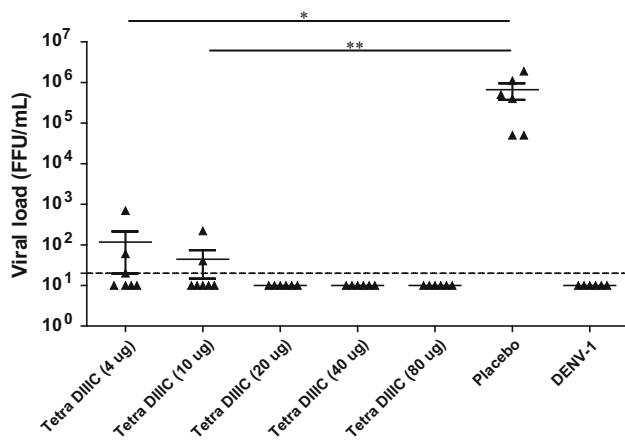
**Fig. 3** Cell-mediated immunity induced in mice by the formulations of Tetra DIIIC (1-4). Animals were immunized with the tetraivalent formulations by the intraperitoneal route on days 0, 15 and 45. Spleen cells from each mouse were cultured with each DIIIC protein at day 60 after the third dose. The frequency of IFN $\gamma$ -secreting cells was measured by ELISPOT after *in vitro* stimulation. (A) *In vitro* stimulation with DIIIC-1. (B) *In vitro* stimulation with DIIIC-2. (C) *In vitro* stimulation with DIIIC-3. (D) *In vitro* stimulation with DIIIC-4.

was expected, high virus titers were detected in brains of animals immunized with the placebo formulation, with a mean of  $6.7 \times 10^5$  FFU/mL of DENV-1 (Fig. 4). However, a significant reduction ( $P < 0.05$ ) in the viral load was found in the brains of animals immunized with any of the tetraivalent formulations. A similar result was observed in mice immunized with DENV-1 (Fig. 4).

## Discussion

The development of an effective dengue vaccine has faced several challenges: a) the vaccine must be able to protect against the four serotypes of the virus; therefore, a tetraivalent vaccine is needed, b) long term protection is

required, otherwise an individual can become susceptible to the infection due to the presence of non-functional waning immunity, and c) there are no suitable animal models for dengue in its severe form. Dengvaxia, the tetraivalent vaccine from Sanofi-Pasteur group, has recently been introduced in several countries [12], but its protective efficacy and its potential benefits for children younger than nine years old have been widely questioned [9, 23]. Our group has developed a subunit vaccine candidate against this human pathogen, combining the DIII of the viral envelope protein and the capsid protein (C) of each virus. The resulting DIIIC proteins are purified after their expression in *E. coli* bacteria, and we have observed that they form aggregates when incubated with an immunostimulatory oligodeoxynucleotide, codenamed ODN 39M



**Fig. 4** Protection against DENV-1 in mice immunized with formulations of Tetra DIIC (1-4). One month after the last immunization, animals were challenged intracranially with 50 LD<sub>50</sub> of a neuroadapted DENV-1. Seven days after challenge, the mice were euthanized to measure brain viral loads by quantification on Vero cells. The chart shows the mean  $\pm$  SEM. The dashed line represents the cutoff. Statistical analysis was conducted using Kruskal-Wallis and Dunn's multiple comparison tests, (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ), ( $n = 7$  per group). The results are representative of two independent experiments

[21]. An immunological evaluation in mice and monkeys of the aggregated proteins, injected as a tetravalent formulation adjuvanted with alum, demonstrated the generation of a functional humoral and cellular immune response in both animal models, with protective capacity against the four DENV in the mouse model of dengue virus encephalitis [17]. The present study was conducted to evaluate the immunogenicity and protective capacity in mice of different doses of the vaccine candidate, containing 4, 10, 20 and 40  $\mu\text{g}$  of Tetra DIIC. As a control, we included one group of animals immunized with the same Tetra DIIC formulation that was evaluated previously [17], containing 80  $\mu\text{g}$  of Tetra DIIC.

Our results show that regardless the dose used, the tetravalent formulation induced an antiviral antibody response against the four DENV serotypes. Even the lowest dose, containing 4  $\mu\text{g}$  of Tetra DIIC, induced a positive antiviral antibody response, although the antiviral antibodies only neutralized *in vitro* viral infection with DENV-2 and DENV-3. The antiviral antibodies elicited by the other doses assayed, 10, 20, 40 and 80  $\mu\text{g}$  of Tetra DIIC, also showed neutralizing activity against DENV-1. As was described previously [17], none of the tetravalent formulations of DIIC proteins evaluated in this study induced neutralizing antibodies against DENV-4. However, in that study, solid protection was observed in animals immunized with Tetra DIIC and even with the monovalent formulation of DIIC-4 (the chimeric protein obtained from DENV-4). The low immunogenicity of similar vaccine candidates against this serotype has been observed in earlier mouse studies [17, 24–26] and even in studies

employing live-attenuated virus [27]. On the other hand, some authors have suggested that DENV-4 should be considered a natural attenuated virus [28]. However, Tetra DIIC induced neutralizing antibodies against DENV-4 in monkeys when it was administered by the subcutaneous, intramuscular or intradermal route. This finding suggests that the domain III of this virus might not be an immunodominant region in mice [17]. Nevertheless, it cannot be ruled out that the neutralizing activity of any serum against DENV will depend on the cell line, the viral serotype, and even the assay employed to measure this activity.

In the present study, cell-mediated immunity was also evaluated by measuring the frequency of IFN $\gamma$ -producing cells after the stimulation of mouse splenocytes with each DIIC protein. Other studies have demonstrated that the capsid protein is an important target for T cells during natural infection [29–31] and that it can induce a cellular immune response with protective capacity against DENV in mice and monkeys [16, 32]. Here, we show that spleen cells from more than 50% of the animals immunized with the tetravalent formulations produced antiviral cytokines upon stimulation with DIIC-1, DIIC-2 and DIIC-3 proteins. As expected, a weaker response was detected after stimulation with DIIC-4. The antiviral and protective role of IFN $\gamma$  against dengue virus has been demonstrated previously [33, 34]. Sustained levels of this cytokine in the sera of DENV-infected individuals have been correlated with protection and subclinical disease [35, 36].

In contrast to the humoral immune response, the highest frequency of IFN $\gamma$ -producing cells was found in animals immunized with the lowest dose of Tetra DIIC assayed. Several lines of evidence indicate that the antigen dose can play an important role in determining the quality of antigen-specific T cells. The functional avidity and/or T-cell receptor (TCR) affinity of responding T cells is strongly dependent on the concentration of antigen used for the *in vitro* or *in vivo* stimulation of CD4<sup>+</sup> T cells [37, 38] and CD8<sup>+</sup> T cells [39–41]. Therefore, a low antigen dose can induce T cells with a high-avidity TCR, whereas a high antigen dose generates T cells with a low-avidity TCR [42]. Similar results were observed during the evaluation of different doses of a monovalent formulation of DIIC-2 [18]. These findings confirm the critical role of the vaccine dose in inducing an adequate immune response. Accordingly, the lowest dose of a tetravalent formulation based on 80% of the envelope protein and adjuvanted with ISCOMATRIX showed the best immunogenicity and protective profile in non-human primates [25].

Finally, we evaluated the protective capacity of the tetravalent formulations using a mouse neurotropic model for dengue virus infection. Animals were challenged with a neuroadapted viral strain of DENV-1 because of the low immunogenicity elicited by the tetravalent formulations



against this serotype. The main limitation of this model is the need for inoculation of very high doses of mouse-adapted viral strains by the intracranial route. This provokes disease manifestations that are irrelevant to the human dengue disease, since nervous system involvement in DENV infections is unusual [36]. However, infection of immunocompetent mice provides a useful immunological model to study DENV-specific responses. Also, the protective capacity of vaccine candidates against dengue measured in this animal model has been demonstrated *a posteriori* in non-human primates [25, 43, 44]. Measurement of virus titers in brains showed a significant reduction of viral loads in Tetra DIIC-immune mice when compared to animals from the placebo group. Interestingly, despite the lack of neutralizing antibodies against DENV-1 in mice inoculated with the lowest dose of Tetra DIIC, viral replication was significantly controlled. This result demonstrates once again the protective role of cell-mediated immunity against this human pathogen, taking into account the high frequency of IFN $\gamma$ -secreting cells detected in these animals. Tetra DIIC induces both arms of the adaptive immune response, and we suggest that the induced T-cell response can control any antibody-dependent enhancement of the infection potentially mediated by anti-domain-III antibodies.

In conclusion, our findings constitute an additional proof of concept of the immunogenicity and protective capacity of Tetra DIIC, even when using low doses of the vaccine candidate. The results presented here also provide evidence of the protective role of cell-mediated immunity against DENV. Further studies should be conducted to evaluate the immunogenicity and protective capacity of different doses of Tetra DIIC in non-human primates.

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#### Compliance with ethical standards

**Conflict of interest** The authors declare no conflict of interest.

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