The tetravalent formulation of domain III-capsid proteins recalls memory B- and T-cell responses induced in monkeys by an experimental dengue virus infection

Lázaro Gil1, Laura Lazo1, Iris Valdés1, Edith Suzarte1, Phuong Yen2, Rosa Ramírez3, Mayling Álvarez3, Le T Dung2, Karem Cobas1, Ernesto Marcos1, Yusleidi Pérez1, María G Guzmán2, Nguyen D Hien2, Gerardo Guillén1 and Lisset Hermida1

Tetra DIIIC is a vaccine candidate against dengue virus (DENV) composed by four chimeric proteins that fuse the domain III of the envelope protein of each virus to the corresponding capsid protein. Containing B- and T-cell epitopes, these proteins form aggregates after the incubation with an immunostimulatory oligodeoxynucleotide, and their tetravalent formulation induces neutralizing antibodies and cellular immune response in mice and monkeys. Also, Tetra DIIIC protects mice after challenge with each DENV, and the monovalent formulation obtained from DENV-2 protects monkeys upon homologous viral challenge. However, in the last years, new evidences have arisen regarding domain III of DENV envelope protein as irrelevant target for neutralizing antibodies in humans. Nevertheless, vaccination with domain III induces a neutralizing antibody response that confers protection against re-infection. In addition, it has been demonstrated that the induction of a cellular immune response is essential to protect during the infection. This response can also avoid severe manifestations of dengue disease, associated to the antibody-dependent enhancement of the infection. In this study, we observed that Tetra DIIIC was able to boost the antiviral and neutralizing antibody responses previously generated in monkeys during an experimental DENV infection, demonstrating that domain III is targeted by B cells during the viral infection. Additionally, Tetra DIIIC successfully boosted the cellular immune response generated by the viruses, probably against T-cells epitopes in the capsid proteins. These results highlight the functionality of Tetra DIIIC as a vaccine candidate against DENV.

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Dengue, caused by four dengue virus serotypes (DENV-1–DENV-4) is the most prevalent emerging disease in tropical and subtropical countries, transmitted by mosquitoes. Clinical disease range from asymptomatic infection and dengue fever to a life-threatening disease, characterized by increased vascular permeability, thrombocytopenia, hemorrhagic manifestations and shock (dengue severe). It is estimated that 390 million cases of DENV infection occur annually, 96 million of which are apparent, 500 000 severe and 20 000 fatal. DENV are positive-stranded RNA viruses belonging to the Flaviviridae family, genus flavivirus.

Unfortunately, Dengvaxia, the vaccine registered by Sanofi-Pasteur in some countries of the world has shown low and declined protection against DENV, especially in seronegative individuals. That is why, the efficacy of new and ongoing vaccine candidates should be evaluated in the upcoming future. Although live-attenuated viral strains are considered as promising vaccines due to their immunogenicity, recombinant proteins have been developed as alternative approaches, with a high safety profile. In the last years, several groups have expressed the domain III of the viral envelope protein, as a subunit vaccine to induce neutralizing antibodies against DENV. However, there are some evidences showing domain III as an irrelevant target for neutralizing antibodies in humans.

Nevertheless, these findings are not conclusive to discourage the inclusion of the domain III in vaccine candidates against this human pathogen. In fact, vaccination with the domain III of the envelope proteins induces a neutralizing antibody response, conferring protection against re-infection.

Tetra DIIIC is one of the subunit vaccine candidates expressing the domain III of the envelope protein to induce a neutralizing antibody response. In this candidate, the domain III is fused to the viral capsid...
protein to also generate a T-cell immune response. DENV are non-cytolytic viruses that upregulate the surface expression of MHC-I molecules in the infected cells, thus the cellular immune response should play an important role in the control of viral infection. In fact, the protective role of CD4+ and CD8+ T cells against DENV have been widely demonstrated in the last years, even in presence of sub-protective antibodies with enhancing capacity of the infection. The four recombinant domain III-capsid proteins (DIIIC-1–DIIIC-4) are produced in Escherichia coli and easily purified by combining the ion exchange and ion-metal affinity chromatographies. Also, these proteins form aggregates, after their incubation with the oligodeoxynucleotide (ODN) 39M, which contains immunostimulatory CpG motifs. These aggregates induce a functional humoral and cellular immune response in mice and monkeys, protecting mice against the four DENV after intracranial challenge. Also, the monovalent formulation of DIIIC-2 is able to boost neutralizing antibodies generated in monkeys previously infected with DENV-2 and protects monkeys upon homologous viral challenge. In this study, we evaluate the capacity of Tetra DIIIC to boost a memory immune response generated in DENV-immune monkeys. Our results demonstrate that Tetra DIIIC was able to recall DENV-specific memory B- and T-cell response after its administration in monkeys experimentally infected with DENV, highlighting its functionality as a promising vaccine candidate, which could be potentially administered in DENV hyper-endemic areas.

RESULTS
Experimental DENV infection produces a detectable viremia in rhesus monkeys
To simulate a DENV hyper-immune non-human primate population, we infected nine rhesus monkeys from the Reu Island in Vietnam with DENV-1, DENV-3 or DENV-4. Previous studies conducted in 55 monkeys from this island demonstrated the circulation of DENV in the island, mainly DENV-3. The first group of three animals was inoculated with 10^5 plaque-forming units (pfu) of DENV-1 Jamaica. Groups second and third (two animals in both groups) were inoculated with 10^6 and 10^4 pfu of DENV-3 Nicaragua, respectively, and the last group of two animals received 10^5 pfu of DENV-4 strain Dominica. An additional group of three naïve animals were included as control. Table 1 summarizes the features of animals and also the immune status at the beginning of the study. Blood was collected for 10 days and the virus was detected in serum samples by plaque assay on VERO cells (Figure 1). As results, animals inoculated with DENV-1 developed viremia with a mean duration of 4.7 days and maximum viral loads of 10^12 pfu ml^-1 in two monkeys and 10^13 pfu ml^-1 in the third one. Similar viral loads were detected in animals receiving DENV-3, regardless the viral dose, with ~3 days of viremia. Animals inoculated with DENV-4 had 4 days of viremia, but we measured lower viral loads (10^1.6 pfu ml^-1 and 10^1.7 pfu ml^-1). These results are in accordance with previous studies reporting DENV infection in this and other species of non-human primates.

Tetra DIIIC boosts the humoral and cellular immune response generated in DENV-immune monkeys
Eight months after DENV infection, monkeys were immunized with the tetravalent formulation of DIIIC proteins (Tetra DIIIC). Animals were bleeding the day of the dose (day 0) and 30 days after the dose, to measure the humoral (antiviral and neutralizing antibodies) and the cellular immune responses. Responses in both the time points were compared in each group of the study, and regardless the virus inoculated at the beginning of the study (day = 240).

Humoral immune response
Antibodies against the four DENV were measured using an indirect ELISA system. Viral inoculation produced an antiviral antibody response that was even detected 8 months after the infection (Figure 2). All animals had antiviral antibodies against the four viruses, regardless the virus type inoculated on day 0, with a geometric mean titer around 3000. As it was expected, this antibody response was significantly boosted (P < 0.01), after the administration of the recombinant proteins with geometric mean titer of 9332.2, 10 886.3, 14 814 and 7406.9 for DENV-1, DENV-2, DENV-3 and DENV-4, respectively (Figure 2d). No statistical differences were detected when this response was analyzed in each independent group due to the low number of animals per group (Figure 2a-c).

Additionally, the functionality of these antiviral antibodies was evaluated through a PRNT, using VERO cells and the following viruses: DENV-1 Jamaica, DENV-2 SB8553, DENV-3 Nicaragua and DENV-4 Dominica. Sera from animals immunized with DENV-1 or DENV-3 neutralized the in vitro viral infection produced for each DENV, 240 days after the experimental viral infection (Figure 3a and b). However, sera from DENV-4-immune monkeys only showed neutralizing capacity against DENV-2 and the homologous virus (Figure 3c). DENV-4 has been considered an attenuated virus and the low immunogenicity of this serotype has been also reported for vaccine candidates based on live virus. We observed an increase in neutralizing antibody titers 30 days after the administration of Tetra DIIIC (Figure 3a-c), but no statistical difference was detected with respect to the day of the vaccine dose (day 0) due to the number of animals included in each independent group. However, when this response was compared in an overall analysis, including all animals, a significant boost of neutralizing antibody titers was detected after the administration of Tetra DIIIC (Figure 3d). Besides, exclusion of the data from animals infected with DENV-1, DENV-3 or DENV-4 in the corresponding analysis per serotype, to avoid any bias in the overall analysis, did not affect the statistical results obtained (Supplementary Figure 1A and B).

Table 1 Summary of animal features and virus inoculated at the beginning of the study

| Group | Monkey | Sex | Age (months) | Weight (kg) | Immune status, day − 240 | Viral inoculum |
|-------|--------|-----|-------------|-------------|-------------------------|---------------|
| I     | 16     | Male| 16          | 1.6         | + DENV-3                | day − 240a    |
| 29    | Male   | 24  | 3.8         | + DENV-3    | DENV-1                  |               |
| 50    | Female | 16  | 3.8         | + DENV-3, DENV-4 |                        |               |
| II    | 39     | Female | 18      | 2.0         | + DENV-1                | day − 240b    |
| 45    | Female | 20  | 2.5         | Naïve       | DENV-3                  |               |
| III   | 9      | Male | 20          | 2.2         | Naïve                   | DENV-3        |
| 40    | Female | 24  | 2.7         | + DENV-1    | DENV-4                  |               |
| IV    | 52     | Male | 16          | 2.0         | + DENV-1                | day − 240b    |
| 61    | Female | 20  | 2.2         | Naïve       | DENV-4                  |               |
| V     | 54     | Male | 16          | 1.8         | Naïve                   |               |
| 57    | Male   | 16  | 1.8         | Naïve       | DENV-4                  |               |
| 59    | Female | 18  | 2.1         | Naïve       | DENV-4                  |               |

*aThe immune status at the beginning of the study (day − 240) was determined by PRNT (plaque-reduction neutralization test) and cell-mediated immune response, stimulating PBMC of animals with each DENV. Positive response was considered when the IFNγ concentration value in the stimulated PBMC was twice or higher than the IFNγ concentration in cells without viral stimulus. However, none animal had neutralizing antibodies against DENV.

*bVirus serotype inoculated at the beginning of the study (day − 240).
Figure 1  Viremia produced in monkeys after DENV inoculation. Nine *Macaca mulatta* monkeys were divided in four groups and inoculated with DENV-1 Jamaica, DENV-3 Nicaragua or DENV-4 Dominica. Animals were bleeding during 10 days after the experimental infection and the viremia was measured by direct plaque assay on VERO cells.

Figure 2  Antiviral antibody response. Nine rhesus monkeys were inoculated with DENV-1, DENV-3 or DENV-4 and eight months later they were immunized with a tetravalent formulation of DIIC proteins (Tetra DIIC). Anti-DENV IgG antibodies were measured by ELISA the day of vaccine dose (day 0) and 30 days after the dose. (a) Antiviral antibody titers in DENV-1-immune monkeys (*n*=3). (b) Antiviral antibody titers in DENV-3-immune monkeys (*n*=4). (c) Antiviral antibody titers in DENV-4-immune monkeys (*n*=2). (d) Antiviral antibody titers, including all DENV-immune monkeys (*n*=9). In all cases, the data represent the geometric mean titers of two independent experiments. The statistical analysis was performed using the Wilcoxon signed rank test (**: *P*<0.001, two-side *P*-value). The dashed line indicates the cutoff value (two times the lowest dilution of sera).
Animals from the control group receiving the dose of Tetra DIIIC did not develop antiviral and neutralizing antibodies against the viruses (Supplementary Figure 2).

Cellular immune response

The capacity of Tetra DIIIC to boost a memory cell-mediated immunity generated in DENV-immune monkeys was evaluated measuring the levels of IFNγ in the supernatants of PBMC stimulated with each DENV on day 0 and 30 days after the vaccine dose. Low IFNγ levels were detected 8 months after viral inoculation (194.21 ± 90.44 pg ml⁻¹ for DENV-1, 30.2 ± 73.45 pg ml⁻¹ for DENV-2, 92.38 ± 57.36 pg ml⁻¹ for DENV-3 and 92.38 ± 57.36 pg ml⁻¹ for DENV-4) (Figure 4), probably due to the low number of effector memory T cells that are circulating in the periphery. In vivo, these cells detect the cognate antigen and rapidly produce effector cytokines or became cytotoxic but they do not proliferate much; therefore, the immune response against the antigen may also require large numbers of effectors generated from the pool of central memory T cells, which remain in lymph nodes.28 As it was expected, after the administration of Tetra DIIIC, a significant production of IFNγ was detected in the supernatant of PBMC stimulated with each DENV (690.8 ± 382.4 pg ml⁻¹ for DENV-1, 641.4 ± 498.3 pg ml⁻¹ for DENV-2, 248.0 ± 107.1 pg ml⁻¹ for DENV-3 and 669.6 ± 525.7 pg ml⁻¹ for DENV-4) (Figure 4). No response was detected in the supernatant of PBMC from animals of the control group (Supplementary Figure 2C).

DISCUSSION

We have previously demonstrated the immunogenicity of Tetra DIIIC in mice and monkeys.14 Also, in 2011, Valdés and colleagues demonstrated that the recombinant protein DIIIC-2 (from DENV-2) was able to boost a neutralizing antibody response generated in monkeys 3 months before by an infection with DENV-2.21 However, as vaccine candidate, Tetra DIIIC will be administered in individuals from dengue hyper-endemic areas with a complex immunological background generated by one or more viral infections. With this in mind, in the present study, we infected monkeys from the Reu Island in Vietnam with or without pre-existing immunity to DENV. After the experimental virus infection, animals were immunized with Tetra DIIIC and the humoral and cellular immune responses were evaluated. Results from the analysis of antiviral and neutralizing antibody responses suggest that domain III expressed in the recombinant proteins was able to recall memory B cells specific to this viral region and generated in animals during the infection. None of the regions of the viral nucleocapsid are exposed on the surface of the virion22 and therefore they are not likely to interact with B cells...
during viral infection. In fact, studies with human sera have revealed that upon natural infection, antibodies do not recognize the capsid protein. Moreover, sera from mice and monkeys immunized with recombinant capsid proteins do not recognize the viruses neither neutralize the in vitro viral infections. On the other hand, control monkeys (that is, naive) that were immunized with only one dose of Tetra DIIIC did not develop antiviral and neutralizing antibodies, as it has been previously observed.

The experimental virus infection generated in the majority of animals a cross-neutralizing antibody response that was boosted after the administration of Tetra DIIIC. This result is in accordance with previous observations, demonstrating that each DIIIC protein is mainly recognized by humans and murine polyclonal antibodies generated against the homologous virus, but they are also recognized by antibodies generated against heterologous viruses. Taken together all results, we can assert that domain III is targeted by B cells during DENV infections, and these memory B cells are recalled after the immunization with Tetra DIIIC, supporting the use of this viral region in vaccine candidates.

Additionally, we measured the levels of IFNγ in the supernatant of PBMC stimulated with DENV, as a surrogate of the cellular immune response. The antiviral and protective role of this cytokine against dengue has been widely documented. Sustained levels of this cytokine in the sera of DENV-infected individuals have been correlated with protection and sub-clinical disease, and its secretion correlates with the induction of a cytotoxic T-cell response. Our results show that Tetra DIIIC successfully boosted the cell-mediated immunity previously generated by the experimental viral infection. Some studies suggest that the stimulation of PBMC with infectious viruses can lead to IFNγ response. In accordance, DENV-related flavivirus such as yellow fever virus can trigger TLR-2, 7, 8, and 9 on dendritic cells and it is plausible that this could result in innate cytokine production that in turn could stimulate IFNγ responses by T cells in a non-virus-specific manner. However, there are several evidences demonstrating that mouse-splenocytes and monkey-PBMC from non-immunized animals did not secrete IFNγ after the in vitro stimulation with infectious DENV, suggesting specific antiviral responses.

Capsid protein is the main target of cytotoxic and IFNγ-secreting CD4+ T cells generated during natural infection; therefore, we could speculate that DIIIC proteins containing epitopes for this T-cell subset, recalled capsid-specific effector and central memory CD4+ T cells generated during experimental DENV infections. These cells should contribute to protection against the disease, as it has been demonstrated in different mouse models of DENV infection. Also, recombinant capsid proteins from DENV form nucleocapsid-like particles after incubation with the ODN 39M, and these particles induce a cellular immune response with demonstrated protective capacity in non-human primates.

Figure 4 Cellular immune response. Nine rhesus monkeys were inoculated with DENV-1, DENV-3 or DENV-4 and eight months later they were immunized with a tetravalent formulation of DIIIC proteins (Tetra DIIIC). The day of vaccine dose (Day 0) and 30 days after the dose, PBMC from the animals were stimulated in vitro with each DENV and IFN-γ concentrations in culture supernatants were determined by ELISA. (a) Cytokine concentration in DENV-1-immune monkeys (n=3). (b) Cytokine concentration in DENV-3-immune monkeys (n=4). (c) Cytokine concentration in DENV-4-immune monkeys (n=2). (d) Cytokine concentration in all DENV-immune monkeys (n=9). Data represent the median of two independent experiments (n=9). The statistical analysis was performed using the Wilcoxon signed rank test (*: P<0.05; **: P<0.01, two-side P-value).
Further studies should be conducted to increase the number of animals and also for evaluating the viral control after the administration of Tetra DIIIC (that is, protection after viral challenge). Additionally, studies in humans will be conducted to evaluate the immunogenicity of Tetra DIIIC in DENV seronegative and seropositive individuals. Taking into account the results described in this study, we could expect a good efficacy rate for this vaccine candidate in DENV-immune recipients. In fact, Dengvaxia, that does not induce a cell-mediate immune response against DENV, has shown to be effective in seropositive vaccines. Also, contrary to Dengvaxia, Tetra DIIIC will generate humoral and cellular immune responses in DENV-seronegative individuals, which should control the virus infection even in the context of an antibody-dependent enhancement of infection. On the other hand, results described here highlight the possibility to combine our vaccine candidate with live-attenuated tetravalent vaccine (LATV) in a prime/boost strategy for immunizations. These combinations using proteins that are usually safe and without the viral interference phenomenon could induce a balanced immune response against the four DENV. Priming with recombinant proteins has the potential advantage to decrease the reactogenicity associated to LATV, since the immune response induced by the proteins can reduce the vaccine viremia without affecting the boosting capacity of the virus. On the other hand, boosting with recombinant proteins after the vaccination with the LATV, could improve the maturation and duration of the immune response against DENV.

**METHODS**

**Cells and viruses**

African monkey kidney (vero) cells were received from the National Institute for Biological Standards and Control (NIBSC). Cells were grown at 37 °C in Eagle’s minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS). The following virus strains were used for antibody detection: Hawaii (DENV-1), New Guinea C (DENV-2), H-87 (DENV-3) and H241 (DENV-4). The viral strains DENV-1 Jamaica (AF42562), DENV-2 SB8553, isolated in 1997 in Malaysia (FM986658), DENV-3 primary isolate from Nicaragua 1994 epidemic (FJ882576), DENV-4 isolate of Dominica 814669 (AF326573) were used for the plaque-reduction neutralization test (PRNT), the isolate from Nicaragua 1994 epidemic (FJ882576), DENV-4 isolate of Dominica 814669 (AF326573) were used for the plaque-reduction neutralization test (PRNT) and the in vitro stimulation of peripheral blood mononuclear cells (PBMC) and animal inoculation. Viral titers were determined by plaque assay on VERO cells.

**Animals and ethics statement**

Twelve healthy adult monkeys (Macaca mulatta) were obtained from the Reu Island located in the northern province of Quang Ninh, Vietnam. The island is administered by the Centre for Research and Production of Vaccines and Biologicals (Polvac, and the study was carried out in Polvac facilities in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals. Animals were maintained throughout the study in individual cages that permitted the evaluation and patterns conducted according to their species, size, age and sex. They were fed with fruits and vegetables, and water was available ad libitum. All immunizations and blood extractions were performed using ketamine hydrochloride, 10 mg kg⁻¹ body weight, and all efforts were made to minimize suffering. None of the monkeys was sacrificed.

All animals were screened for previous exposure to DENV by enzyme-linked immunosorbent assay (ELISA) and PRNT.

**Viral infection and measurement of the viremia**

Monkeys were subcutaneously inoculated in the upper arms with 10⁵ pfu of DENV-1 Nicaragua, 10⁵ or 10⁴ pfu of DENV-3 Nicaragua or 10⁵ pfu of DENV-4 Dominica. Blood was collected daily during 10 days to detect viremia. The presence of virus in sera was determined by inoculating 100 μl of serum diluted 1:10 onto VERO cell monolayers grown in 6-well plates. The cultures were incubated at 37 °C for 6 days and virus production was detected by plaque assays on VERO cells.

**Tetravalent formulation of DIIIC proteins (Tetra DIIIC)**

The design, cloning, expression and purification of the recombinant proteins DIIIC-1, DIIIC-2, DIIIC-3 and DIIIC-4, were previously described. All the recombinant proteins were purified in the presence of 7 M urea, basically by a procedure consisting of ammonium sulfate precipitation, followed by a cation exchange chromatography and an immobilized metal ion affinity chromatography. Purified proteins were subjected to an in vitro aggregation procedure as previously described with few modifications (10). Briefly, 400 μg of the recombinant proteins were incubated with 40 μg of ODN 39M (5′-ATC GACTCTCGAGCGTTCTCGGGGGACGATCGTCGGGGG-3′) in TE buffer (Tris 10 mM, EDTA 6 mM, pH 7.4). The reaction mixture was incubated for 30 min at 30 °C and finally stored at 4 °C.

The tetravalent formulation (Tetra DIIIC) was prepared with 100 μg of each aggregated protein and using aluminum hydroxide (Alhydrogel) (Brenntag Biosector, Frederikssund, Denmark) as adjuvant at a final concentration of 1.44 mg ml⁻¹.

**Measurement of humoral immune response**

An amplified sandwich ELISA system was used to detect the anti-DENV IgG antibodies. Polystyrene plates with 96 wells (Costar, Cambridge, MA, USA) were coated for 2 h at 37 °C with 100 μl well of monocalonal antibody 4G2 (5 μg ml⁻¹) in coating buffer (0.16% Na₂CO₃ and 0.29% NaHCO₃; pH 9.5); then they were blocked in coating buffer containing 5% skimmed milk for 1 h at 37 °C and washed three times in PBS containing 0.05% Tween 20 (PBS-T).

The viral antigen (100 μl per well) and the negative control antigen were incubated overnight at 4 °C. After three washes with PBS-T, 100 μl per well of sera from each group were tested by serial dilutions in PBS-T, starting at 1:1000. Plates were incubated for 1 h at 37 °C and washed as described above. Later, 100 μl per well of 1:35 000 diluted 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 0.04% substrate 3,3',5,5'-tetramethylbenzidine in buffer (2% Na₂HPO₄ and 1% citric acid; pH 5.0) was added. The plates were kept for 30 min at room temperature and the reaction was stopped with 50 μl per well of 2.5 M H₂SO₄. Absorbance was read at 492 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 PRNT in VERO cells as previously described. The neutralizing antibody titer was identified as the highest serum dilution that reduced the number of virus plaques by 50% (PRNT50). As a positive control, murine hyper-immune ascitic fluids from each dengue serotype were used.

**Cell culture and viral stimulation**

PBMCs were isolated as previously described. Cells were washed twice with PBS 2% FBS (PA Laboratories, Ontario, Canada) and resuspended at 2 × 10⁶ cells per ml in RPMI 1640 medium (Sigma-Aldrich, Irvine, North Ayrshire, UK) supplemented with 100 U ml⁻¹ penicillin, 100 μg ml⁻¹ streptomycin (Gibco, Paisley, UK), 2 mM glutamine (Gibco), 5 × 10⁻⁵ M 2-mercaptoethanol (Sigma St. Louis, MO, USA) and 5% FBS. Finally, 2.5 × 10⁵ cells per well were cultured in 96-well round bottom plates and stimulated with DENV at multiplicity of infection (MOI) of 0.5. Concanaavalin A (Sigma) was used as a positive control. In all experiments, three wells were plated for each virus. After 4 days of incubation period, culture supernatants were collected and stored at −20 °C.

**Cytokine detection**

The culture supernatants of PBMC previously stimulated with each DENV were analyzed in duplicate to determine the INF-γ concentration by ELISA using monoclonal antibodies (MAB) pairs (INF-γ; Mabtech, Nacia, Sweden).
ELISA protocol recommended by manufacturers was used with slight modifications. The lowest limit of detection of cytokine was 10 pg/mL.

Statistical analysis
Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA) was used for statistical analysis. The data normality was verified using D’Agostino-Pearson’s test, and homogeneity of variance was checked using Bartlett’s test. The global analysis of homoural (antiviral and neutralizing antibodies) and cellular immune response was performed using the Wilcoxon signed rank test. In all cases, *: P<0.05; **: P<0.01; ***: P<0.001.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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1 Kyle JL, Harris E. Global spread and persistence of dengue. Annu Rev Microbiol 2008; 62: 71–92.
2 Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL et al. The global distribution and burden of dengue. Nature 2013; 496: 504–507.
3 Fu J, Tan BH, Yap EH, Chan YC, Tan YH. Full-length cDNA sequence of dengue type 1 virus (Singapore strain S275/90). Virology 1992; 188: 953–958.
4 Halstead SB, Russell PK. Protective and immunological behavior of chimeric yellow fever dengue vaccine. J Virol 2006; 80: 153–153.
5 Simmons M, Porter KR, Escamilla J, Graham R, Watts DM, Eckels KH et al. Evaluation of recombinant dengue viral envelope B domain protein antigens for the detection of dengue complex-specific antibodies. Am J Trop Med Hyg 1998; 58: 144–151.
6 Hermoda L, Bernardo L, Martin J, Alvarez M, Prado I, Lopez C et al. A recombinant protein containing the domain III of the dengue-2 envelope protein is immunogenic and protective in nonhuman primates. Vaccine 2006; 24: 3165–3171.
7 Chen HT, Liu Y, Tsai JP, Chiang CY et al. A consensus envelope protein domain III can induce neutralizing antibody responses against serotype 2 of dengue virus in non-human primates. Arch Virol 2013; 158: 1523–1531.
8 Etemad B, Batra G, Raut D, Dahiya S, Khanam S, Swaminathan S et al. An envelope domain III-based chimeric antigen produced in Pichia pastoris elicits neutralizing antibodies against all four dengue virus serotypes. Am J Trop Med Hyg 2008; 79: 353–363.
9 Wahala WM, Kraus AA, Haymore LB, ccaviti-Loper MA, de Silva AM, Dengue virus neutralization by human immune serum: role of envelope protein domain III-reactive antibody. Virology 2009; 392: 103–113.
10 Williams KL, Wahala WM, Orozco S, de Silva AM, Harris E. Antibodies targeting dengue virus envelope domain III are not required for serotype-specific protection or prevention of enhancement in vivo. Virology 2012; 429: 12–20.
11 Gil L, Marcos E, Izquierdo A, Lazo L, Valdes I, Ambala PC et al. The protein DIIIC-2, aggregated with a specific oligodeoxynucleotide and adjuvated in alum, protects mice and monkeys against DENV-2. Immuno Cell Biol 2015; 43: 57–66.
12 White LJ, Sarola CA, Markova MD, Wahala MP, Yingwisesaphat V, Collier ML et al. An alphavirus vector-based tetravalent dengue vaccine induces a rapid and protective immune response in macaques that differs qualitatively from immunity induced by live virus infection. J Virol 2013; 87: 3409–3424.
13 Suzarte E, Gil L, Valdes I, Marcos E, Lazo L, Izquierdo A et al. A novel tetravalent formulation combining the four aggregated domain III-capsid proteins from dengue viruses induces a functional immune response in mice and monkeys. Int Immunol 2015; 27: 367–379.
14 Zuest R, Valdes I, Skibinski D, Lin Y, Tsoh YK, Chan K et al. Tetravalent dengue DIIIC protein together with alum and ODN elicits a Th1 response and neutralizing antibodies in mice. Vaccine 2015; 33: 1474–1482.
15 Lobigs M, Mullbacher A, Lee E. Evidence that a mechanism for efficient flavivirus budding upregulates MHC class I. Immuno Cell Biol 2004; 82: 184–188.
16 Yauch LE, Zellweger RM, Romero Y, Castro J, Puente P, Lazo L et al. The chimeric protein domain III-capsid of dengue virus serotype 2 (DEN-2) successfully boosts neutralizing antibodies generated in monkeys upon infection with DEN-2. Clin Vaccine Immunol 2011; 18: 455–459.
17 Lazo L, Suzarte E, Castro J, Yen P, Dung LT, Gil L et al. Screening for immune response against Dengue virus in Vietnamese non-human primates: implications for vaccine developers. Clin Transl Immunol 2016; 6: e1335.
18 Althouse BM, Durbin AP, Hanley KA, Halstead SB, Weaver SC, Cummings DA. Viral kinetics of primary dengue virus infection in non-human primates: a systematic review and individual pooled analysis. Virology 2014; 452-453: 237–246.
19 Weiskopf D, Angelo MA, de Azerezo EL, Sidney J, Greenbaum JA, Fernando AN et al. Comprehensive analysis of dengue virus-specific responses supports an HLA-linked protective role for CD8+ T cells. Proc Natl Acad Sci USA 2013; 110: E2046–E2053.
20 Zellweger RM, Eddy WE, Tang WW, Miller R, Shresta S. CD8+ T cells prevent antigen-induced antibody-dependent enhancement of dengue disease in mice. J Immunol 2014; 193: 4117–4124.
21 Gil L, Romero Y, Castro J, Puente P, Lazo L et al. The chimeric protein domain III-capsid of dengue virus serotype 2 (DEN-2) successfully boosts neutralizing antibodies generated in monkeys upon infection with DEN-2. Clin Vaccine Immunol 2011; 18: 455–459.
22 Lazo L, Suzarte E, Castro J, Yen P, Dung LT, Gil L et al. Screening for immune response against Dengue virus in Vietnamese non-human primates: implications for vaccine developers. Clin Transl Immunol 2016; 6: e1335.
23 Althouse BM, Durbin AP, Hanley KA, Halstead SB, Weaver SC, Cummings DA. Viral kinetics of primary dengue virus infection in non-human primates: a systematic review and individual pooled analysis. Virology 2014; 452-453: 237–246.
24 Vaughn DW. Invited commentary: Dengue lessons from Cuba. Am J Epidemiol 2000; 152: 800–803.
25 Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL et al. The global distribution and burden of dengue. Nature 2013; 496: 504–507.
26 Halstead SB, Russell PK. Protective and immunological behavior of chimeric yellow fever dengue vaccine. Vaccine 2016; 34: 1643–1647.
27 Simmons M, Porter KR, Escamilla J, Graham R, Watts DM, Eckels KH et al. Evaluation of recombinant dengue viral envelope B domain protein antigens for the detection of dengue complex-specific antibodies. Am J Trop Med Hyg 1998; 58: 144–151.
28 Kyle JL, Harris E. Global spread and persistence of dengue. Annu Rev Microbiol 2008; 62: 71–92.
hospitalized or who had mild or subclinical dengue infection. PLoS Negl Trop Dis 2015; 9: e0003673.

43 Slifka MK, Leung DY, Hammarlund E, Raue HP, Simpson EL, Tofte S et al. Transcutaneous yellow fever vaccination of subjects with or without atopic dermatitis. J Allergy Clin Immunol 2016; 133: 439–447.

44 Queiroz T, Bennouna S, Alkan S, Laouar Y, Gorden K, Flavell R et al. Yellow fever vaccine YF-17D activates multiple dendritic cell subsets via TLR2, 7, 8, and 9 to stimulate polyvalent immunity. J Exp Med 2006; 203: 413–424.

45 Gil L, Izquierdo A, Lazo L, Valdes I, Ambala P, Ochola L et al. Capsid protein: evidences about the partial protective role of neutralizing antibody-independent immunity against dengue in monkeys. Virology 2014; 456-457: 70–76.

46 Weiskopf D, Angela MA, Zapardiel J, Seumois G, de Silva A, de Silva A et al. DENV-specific CD4 T-cells dominantly recognize capsid-derived epitopes and display a cytotoxic phenotype. J Immunol 2016; 196(Suppl): 147.

47 Weiskopf D, Angela MA, Grifoni A, O'Rourke PH, Sidney J, Paul S et al. HLA DRB1 alleles are associated with different response magnitudes of dengue virus specific CD4+ T cell responses. J Infect Dis 2016; 214: 1117–1124.

48 Gil L, Bernardo L, Pavon A, Izquierdo A, Valdes I, Lazo L et al. Recombinant nucleocapsid-like particles from dengue-2 induce a functional serotype-specific cell-mediated immunity in mice. J Gen Virol 2012; 93: 1204–1214.

49 Morens DM, Halstead SB, Repik PM, Puthan R, Raybourne N. Simplified plaque reduction neutralization assay for dengue viruses by semimicro methods in BHK-21 cells. comparison of the BHK suspension test with standard plaque reduction neutralization. J Clin Microbiol 1985; 22: 250–254.

50 Kaufman BM, Summers PL, Dubois DR, Eckels KH. Monoclonal antibodies against dengue 2 virus E-glycoprotein protect mice against lethal dengue infection. Am J Trop Med Hyg 1987; 36: 427–434.

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