

## ORIGINAL ARTICLE

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

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Tetra DIIC 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 DIIC 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 DIIC 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 DIIC 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 DIIC as a vaccine candidate against DENV.

*Clinical & Translational Immunology* (2017) 6, e148; doi:10.1038/cti.2017.24; published online 23 June 2017

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).<sup>1</sup> 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.<sup>2</sup> DENV are positive-stranded RNA viruses belonging to the *Flaviviridae* family, genus *flavivirus*.<sup>3</sup>

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.<sup>4,5</sup> 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.<sup>6–9</sup> However, there are some evidences showing domain III as an irrelevant target for neutralizing antibodies in humans.<sup>10,11</sup> 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.<sup>7,12,13</sup>

Tetra DIIC 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

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Received 1 April 2017; accepted 12 May 2017

protein to also generate a T-cell immune response.<sup>14,15</sup> DENV are non-cytolytic viruses that upregulate the surface expression of MHC-I molecules in the infected cells,<sup>16</sup> thus the cellular immune response should play an important role in the control of viral infection. In fact, the protective role of CD4<sup>+</sup> and CD8<sup>+</sup> T cells against DENV have been widely demonstrated in the last years,<sup>17–19</sup> even in presence of sub-protective antibodies with enhancing capacity of the infection.<sup>20</sup>

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,<sup>12</sup> 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.<sup>14</sup> Also, the monovalent formulation of DIIIC-2 is able to boost neutralizing antibodies generated in monkeys previously infected with DENV-2<sup>21</sup> and protects monkeys upon homologous viral challenge.<sup>12</sup>

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.<sup>22</sup> The first group of three animals was inoculated with 10<sup>3</sup> plaque-forming units (pfu) of DENV-1 Jamaica. Groups second and third (two animals in both groups) were inoculated with 10<sup>3</sup> and 10<sup>4</sup> pfu of DENV-3 Nicaragua, respectively, and the last group of two animals received 10<sup>3</sup> pfu of DENV-4 strain

**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 <sup>a</sup>	Viral inoculum day – 240 <sup>b</sup>
I	16	Male	16	1.6	+ DENV-3	DENV-1
	29	Male	24	3.8	+ DENV-3	
	50	Female	15	1.6	+ DENV-3, DENV-4	
II	39	Female	18	2.0	+ DENV-1	DENV-3
	45	Female	20	2.5	Naive	
III	9	Male	20	2.2	Naive	DENV-3
	40	Female	24	2.7	+ DENV-1	
IV	52	Male	16	2.0	+ DENV-1	DENV-4
	61	Female	20	2.2	Naive	
V	54	Male	16	1.8	Naive	—
	57	Male	16	1.8	Naive	
	59	Female	18	2.1	Naive	

<sup>a</sup>The 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 IFN $\gamma$  concentration value in the stimulated PBMC was twice or higher than the IFN $\gamma$  concentration in cells without viral stimulus. However, none animal had neutralizing antibodies against DENV.

<sup>b</sup>Virus serotype inoculated at the beginning of the study (day – 240).

Dominica. An additional group of three naive 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<sup>2.1</sup> pfu ml<sup>-1</sup> in two monkeys and 10<sup>1.9</sup> pfu ml<sup>-1</sup> 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<sup>1.6</sup> pfu ml<sup>-1</sup> and 10<sup>1.7</sup> pfu ml<sup>-1</sup>). These results are in accordance with previous studies reporting DENV infection in this and other species of non-human primates.<sup>23</sup>

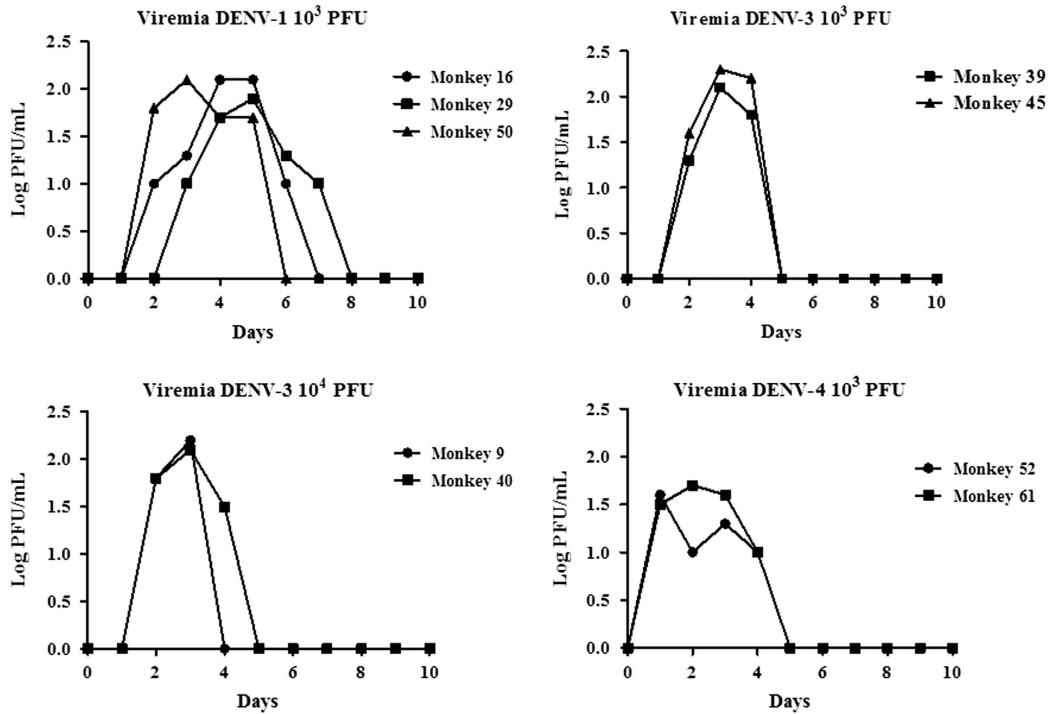
### Tetra DIIIC boosts the humoral and cellular immune response generated in DENV-immune monkeys

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).

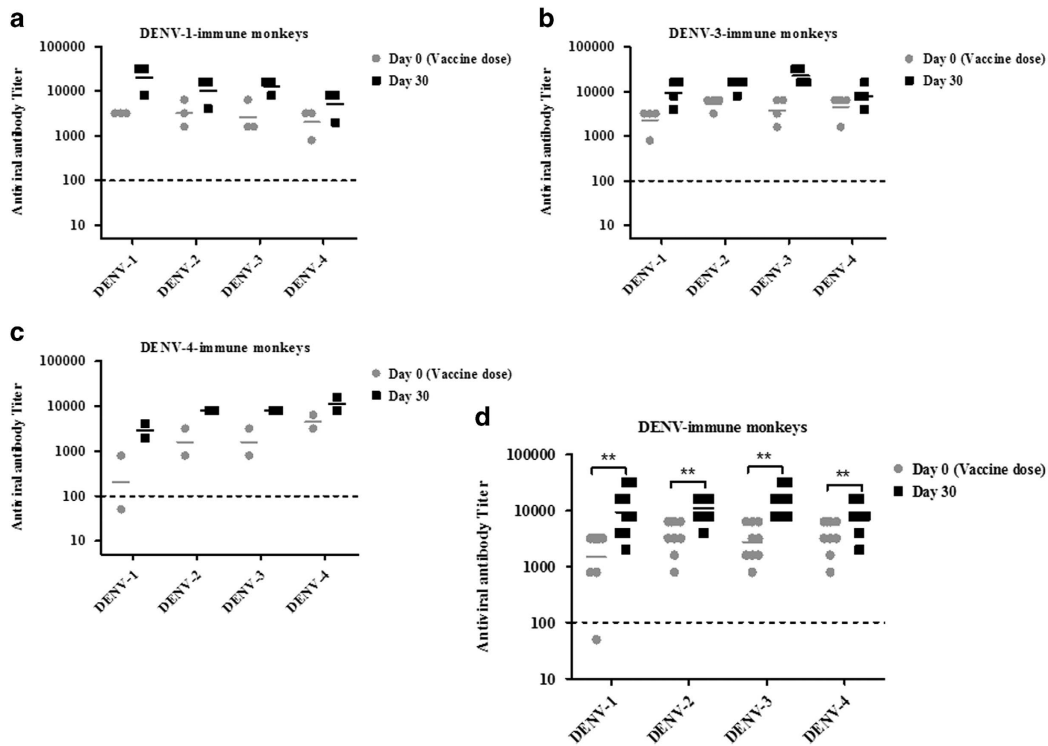
### Humoral immune response

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 – 240, 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 titers 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).

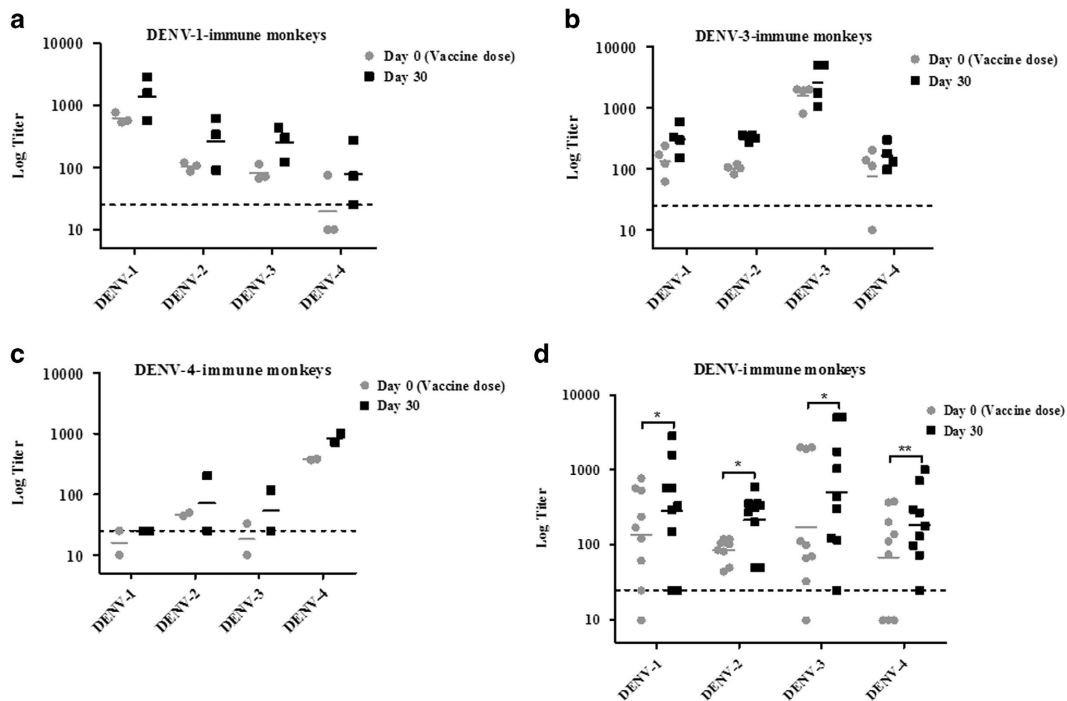
Additionally, the functionality of these antiviral antibodies was evaluated through a PRNT, using VERO cells and the following viruses: DENV-1 Jamaica, DENV-2 SB8553, DENV-3 Nicaragua and DENV-4 Dominica. Sera from animals immunized with DENV-1 or DENV-3 neutralized the *in vitro* viral infection produced 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<sup>24</sup> and the low immunogenicity of this serotype has been also reported for vaccine candidates based on live virus.<sup>25–27</sup> 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).



**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 DIIIC proteins (Tetra DIIIC). 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 (\*\*:  $P<0.01$ , two-side  $P$ -value). The dashed line indicates the cutoff value (two times the lowest dilution of sera).



**Figure 3** Neutralizing 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). Neutralizing antibodies were measured by a plaque-reduction neutralization test using VERO cells and the viral strains: DENV-1 Jamaica, DENV-2 SB8553, DENV-3 Nicaragua and DENV-4 Dominica, the day of vaccine dose (day 0) and 30 days after the dose. (a) Neutralizing antibody response in DENV-1-immune monkeys ( $n=4$ ). (b) Neutralizing antibody response in DENV-3-immune monkeys ( $n=4$ ). (c) Neutralizing antibody response in DENV-4-immune monkeys ( $n=2$ ). (d) Neutralizing antibody response, 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 (\*:  $P<0.05$ ; \*\*:  $P<0.01$ , 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 DIIC did not develop antiviral and neutralizing antibodies against the viruses (Supplementary Figure 2).

### Cellular immune response

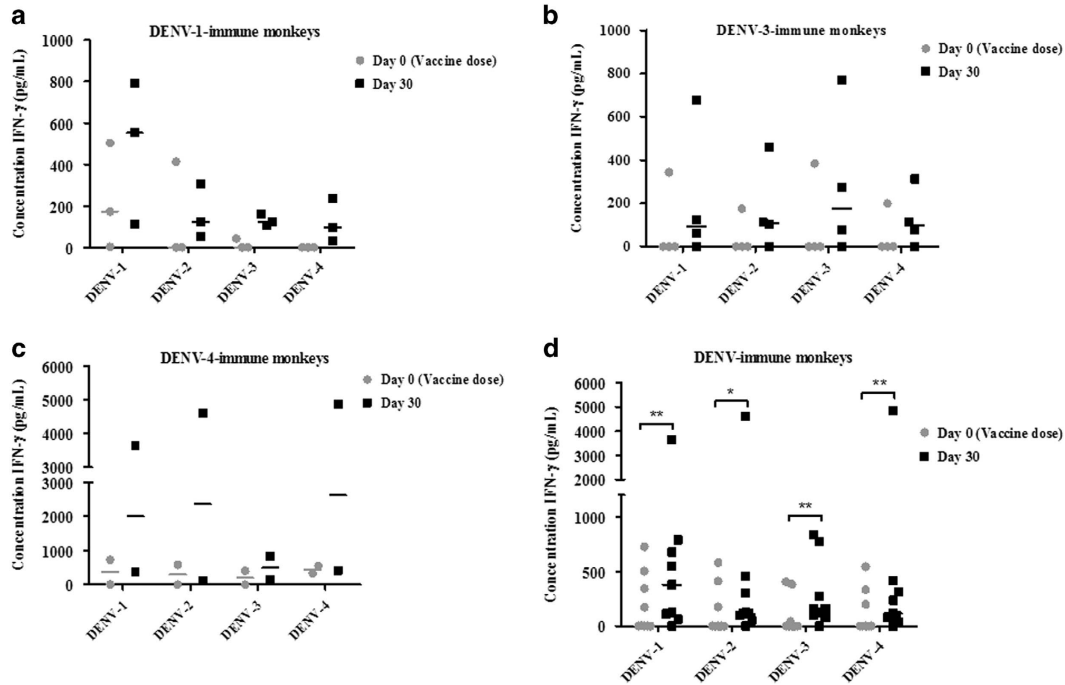
The capacity of Tetra DIIC to boost a memory cell-mediated immunity generated in DENV-immune monkeys was evaluated measuring the levels of IFN $\gamma$  in the supernatants of PBMC stimulated with each DENV on day 0 and 30 days after the vaccine dose. Low IFN $\gamma$  levels were detected 8 months after viral inoculation ( $194.21 \pm 90.44$  pg ml $^{-1}$  for DENV-1,  $30.2 \pm 73.45$  pg ml $^{-1}$  for DENV-2,  $92.38 \pm 57.36$  pg ml $^{-1}$  for DENV-3 and  $119.6 \pm 65.45$  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.<sup>28</sup> As it was expected, after the administration of Tetra DIIC, a significant production of IFN $\gamma$  was detected in the supernatant of PBMC stimulated with each DENV ( $690.8 \pm 382.4$  pg ml $^{-1}$  for DENV-1,  $641.4 \pm 498.3$  pg ml $^{-1}$  for DENV-2,  $248.0 \pm 107.1$  pg ml $^{-1}$  for DENV-3 and  $669.6 \pm 525.7$  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 DIIC in mice and monkeys.<sup>14</sup> Also, in 2011, Valdés and colleagues

demonstrated that the recombinant protein DIIC-2 (from DENV-2) was able to boost a neutralizing antibody response generated in monkeys 3 months before by an infection with DENV-2.<sup>21</sup> However, as vaccine candidate, Tetra DIIC 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, experimental infections, animals developed a detectable viremia, regardless of the virus inoculated, showing additional evidences that support a no cross-protective response among serotypes or even suggesting that homotypic protection could depend on the time between the infections. DENV infection confers lifelong protection against the homologous serotype, but this immunity might not be sterilizing depending on the interval between the primary infection and re-infection with the homologous virus. Recent epidemiological studies conducted in Iquitos, where the authors followed the serological status of many individuals, suggest that the protection afforded by natural infection against homotypic DENV re-infection is incomplete,<sup>29</sup> a result that is coherent with other studies performed on monkeys.<sup>30,31</sup>

After the experimental virus infection, animals were immunized with Tetra DIIC 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 virion<sup>32</sup> and therefore they are not likely to interact with B cells



**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 tetra valent 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- $\gamma$  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).

during viral infection. In fact, studies with human sera have revealed that upon natural infection, antibodies do not recognize the capsid protein.<sup>33</sup> Moreover, sera from mice and monkeys immunized with recombinant capsid proteins do not recognize the viruses neither neutralize the *in vitro* viral infections.<sup>34</sup> 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.<sup>14</sup>

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.<sup>35,36</sup> 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.

It could be thought that Tetra DIIIC only slightly boosted the neutralizing antibody response previously generated by the virus infection. However, similar results have been observed in DENV seropositive individuals after one, two and even three doses of Dengvaxia administered 6 months apart.<sup>37,38</sup> Contrary to the registered vaccine, Tetra DIIIC is a non-replicating antigen; therefore, our result highlights its potential capacity to boost a neutralizing antibody response in humans previously infected with DENV. Also, shorter immunization schedules can be used with our vaccine candidate in order to administrate more than one dose.

Additionally, we measured the levels of IFN $\gamma$  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<sup>39,40</sup> 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,<sup>41,42</sup> and its secretion correlates with the induction of a cytotoxic T-cell response.<sup>19</sup> 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 IFN $\gamma$  response.<sup>43</sup> In accordance, DENV-related flavivirus such as yellow fever virus can trigger TLR-2, 7, 8, and 9 on dendritic cells<sup>44</sup> and it is plausible that this could result in innate cytokine production that in turn could stimulate IFN $\gamma$  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 IFN $\gamma$  after the *in vitro* stimulation with infectious DENV,<sup>12,14,45</sup> suggesting specific antiviral responses.

Capsid protein is the main target of cytotoxic and IFN $\gamma$ -secreting CD4<sup>+</sup> T cells generated during natural infection;<sup>46,47</sup> therefore, we could speculate that DIIIC proteins containing epitopes for this T-cell subset, recalled capsid-specific effector and central memory CD4<sup>+</sup> 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.<sup>18,48</sup> 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.<sup>34,45</sup>

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-mediated immune response against DENV, has shown to be effective in seropositive vaccinees.<sup>4</sup> 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 *in vitro* stimulation of peripheral blood mononuclear cells (PBMC) and animal inoculation. Viral titers were determined by plaque assay on VERO cells.<sup>49</sup>

### 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 (Polyvac, 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<sup>-1</sup> 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 10<sup>3</sup> pfu of DENV-1 Nicaragua, 10<sup>3</sup> or 10<sup>4</sup> pfu of DENV-3 Nicaragua or 10<sup>3</sup> 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.<sup>12</sup>

### 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.<sup>36</sup> 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 GACTCTCGAGCGTTCTCGGGGACGATCGTGGGGG-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<sup>-1</sup>.

### 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 per well of monoclonal antibody 4G2<sup>50</sup> (5 µg ml<sup>-1</sup>) in coating buffer (0.16% Na<sub>2</sub>CO<sub>3</sub> and 0.29% NaHCO<sub>3</sub>; 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 O-phenylenediamine in buffer (2% Na<sub>2</sub>HPO<sub>4</sub> 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 H<sub>2</sub>SO<sub>4</sub>. 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.<sup>49</sup> 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<sup>20</sup>. Cells were washed twice with PBS 2% FBS (PAA Laboratories, Ontario, Canada) and resuspended at 2 × 10<sup>6</sup> cells per ml in RPMI 1640 medium (Sigma-Aldrich, Irvine, North Ayrshire, UK) supplemented with 100 U ml<sup>-1</sup> penicillin, 100 µg ml<sup>-1</sup> streptomycin (Gibco, Paisley, UK), 2 mM glutamine (Gibco), 5 × 10<sup>-5</sup> M 2-mercaptoethanol (Sigma St. Louis, MO, USA) and 5% FBS. Finally, 2.5 × 10<sup>5</sup> cells per well were cultured in 96-well round bottom plates and stimulated with DENV at multiplicity of infection (MOI) of 0.5. Concanavalin 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 IFN-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<sup>-1</sup>.

### 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 humoral (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.

### ACKNOWLEDGEMENTS

We thank the staff from the Animal Facility for the Reu Island located in the Northern province of Quang Ninh, Vietnam, for their technical support in the handling and maintenance of the animals. The island is administered by the Centre for Research and Production of Vaccines and Biologicals. This work was supported by CIGB.

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