Recombinant nucleocapsid-like particles from dengue-2 virus induce protective CD4\textsuperscript{+} and CD8\textsuperscript{+} cells against viral encephalitis in mice

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Abstract

Virus-like particles are a highly effective type of subunit vaccine that mimics the overall structure of virus particles without containing infectious genetic material. In this work, a particulate form of the recombinant capsid protein from dengue-2 was evaluated in mice to determine the level of protection against viral challenge and to measure the antigen-induced cell-mediated immunity (CMI). The nucleocapsid-like particles (NLPs) adjuvanted with alum did not induce antiviral antibodies. However, splenocytes from the immunized animals secreted high levels of IFN-γ upon virus stimulation, and a significant protection rate was achieved after challenge with lethal dengue-2 virus. Finally, both IFN-γ secretion and protection against viral encephalitis were demonstrated to be dependent on CD4\textsuperscript{+} and CD8\textsuperscript{+} cells. This study provides new evidences regarding the protective role of the CMI in the mouse model without the induction of neutralizing antibodies. Further studies in non-human primates or humanized mice should be carried out to elucidate the usefulness of the NLPs as a potential vaccine candidate against dengue disease.

Introduction

Dengue virus (DENV) infection is a major emerging disease of tropical and subtropical countries, transmitted by the bite of an infected mosquito, usually Aedes aegypti. Many infections are asymptomatic while the clinical manifestations can range from a self-limiting febrile illness (dengue fever) to a life-threatening disease, characterized by increased vascular permeability, thrombocytopenia, hemorrhagic manifestations and shock (dengue hemorrhagic fever) (1). It is estimated that nearly half of the world population is at risk of infection and up to 50 million people are infected each year. DENVs are positive-stranded RNA viruses belonging to the Flaviviridae family. There are four distinct serotypes (DENV-1, DENV-2, DENV-3 and DENV-4), which show substantial sequence divergence of ~30\% (2).

For several years, researchers have associated the generation of neutralizing antibodies as a premise to reach protection against DENV. However, dengue is a non-cytopathic virus that up-regulates the surface expression of MHC class I molecules in the infected cells (3); thus, the cellular immune response should constitute an important mediator of the adaptive immune system against this pathogen. Several works have arisen about the pathogenic role of the T-cell response during secondary dengue infections. It has been reported that serotype cross-reactive T cells are preferentially activated in the heterologous infection, a phenomenon termed ‘original antigenic sin’ (4). These cross-reactive T cells exhibit suboptimal degranulation (5) and altered cytokine production (6, 7), contributing to severe disease. On the contrary, little is known about the protective role of T cells against this pathogen. To our knowledge, only three reports provide evidences about this issue in the mouse model. The first report describes the contribution of...
CD8\(^{+}\) cells in protecting mice immunized with the yellow fever–dengue chimeric virus (8). In a second report, our group demonstrated the role of the cellular immune response against DENV-2 after infection with the homologous virus in mice (9). Finally, it has also been shown that the immunization of mice with four CD8\(^{+}\) T-cell epitopes from DENV-2, which are immunodominant in this animal model, enhances viral clearance (10). Further studies on cell-mediated immunity (CMI) to better understand the immunopathology of dengue and the immunogenicity of vaccine candidates are required (11).

Despite inducing adequate cellular and humoral immune responses, vaccine candidates based on live attenuated strains of dengue have several important safety concerns. The most significant ones are the reactogenicity and the theoretical risk of enhanced disease following DENV vaccination. Also, live attenuated vaccines include cell culture–derived adventitious agents, community spread of vaccine virus by resident vector mosquitoes, vaccine virus neurovirulence and the effects of vaccine administration to immunocompromised hosts (12). In addition, due to the immunodominance phenomenon and/or the viral interference in tetravalent formulations, several doses have been required to induce an equivalent immune response against the four serotypes (13, 14). As an alternative, recombinant subunit vaccines based on viral proteins, capable of mimicking the overall structure of virus particles and of inducing a proper immune response, constitute a promising approach. In this sense, virus-like particles (VLPs) have been a highly effective sort of subunit vaccine with successful results for different pathogens (15).

Considering the potential protective role of the CMI against dengue, we have focused our work on the DENV-2 capsid protein. This protein has been identified as a target of the antiviral T-cell response (16). In a previous study, a semi-purified fraction of the capsid protein, expressed in *Escherichia coli*, induced partial protection in the mouse *Escherichia coli* model without the induction of a functional humoral response (17).

In the present work, the highly purified protein in a particular form was evaluated in mice to determine the level of protection against viral challenge and to measure the antigen-induced CMI. The nucleocapsid-like particles (NLPs) adjuvanted with alum did not induce antibodies against the whole virus, whereas splenocytes of the immunized animals secreted high levels of IFN-\(\gamma\) upon virus stimulation. In addition, a significant level of protection was obtained after challenge with lethal DENV-2. Finally, the contribution of CD4\(^{+}\) and CD8\(^{+}\) cells to IFN-\(\gamma\) secretion and to protection against the disease was demonstrated.

**Methods**

**Viruses**

A preparation from suckling mice brain infected with DENV-2 [New Guinea C (NGC) strain] was used as antigen for antibody detection (18). A similar preparation obtained from brain of non-inoculated mice was used as negative control.

For animal immunization and virus challenge, a preparation of infective DENV-2 (NGC strain) \([5.6 \times 10^5\) plaque-forming units (pfu) ml\(^{-1}\)\] was used. It was obtained by homogenization of suckling mice brain infected with DENV-2 using the RPMI-1640 medium (Sigma-Aldrich, UK).

A concentrated preparation of virus (DENV-2 antigen) was used for the *in vitro* stimulation of mouse splenocytes. Supernatant from Vero cells infected (100 ml), with 10\(^6\) pfu ml\(^{-1}\) of SB8553 dengue-2 viral strain (kindly provided by Dr. M. J. Cardosa, University Sarawak, Malaysia), was concentrated by centrifugation at 80,000 \(\times g\) for 4 h at 4°C. The pellet containing the virus was resuspended in 1 ml of PBS (Gibco, Paisley, UK). A mock preparation was similarly prepared from the supernatant of uninfected Vero cells.

**Recombinant protein purification and in vitro assembly**

The capsid protein from DENV-2 was expressed in *E. coli* as previously described (17). Pellets were resuspended in 30 ml of 10 mM Tris (pH 8.0) (buffer lysis), and cells were disrupted in French Press (Ohtake, Japan) at 1500 kgf cm\(^{-2}\) with two passes on ice. After centrifugation of the lysate from the disruption at 20,000 \(\times g\) for 20 min at 4°C, the supernatant was collected. The disruption supernatant in buffer lysis was exchanged to 10 mM Tris, 7 M urea and 0.5% Tween 20 (pH 8.0) using pre-package G-25 columns (Pharmacia, Sweden). Later, the supernatant was gently shaken during 12 h at 4°C before the application onto the resin. The supernatant in this condition was applied onto the previously equilibrated SP-Sepharose FF resin (Pharmacia). The column was washed with 7 M urea, 30 mM diethanolamine (DEA), 350 mM NaCl and 0.5% Tween 20 (pH 10.3) to eliminate contaminants, and then, the recombinant protein was eluted using 7 M urea, 30 mM DEA, 750 mM NaCl and 0.5% Tween 20 (pH 10.3).

Finally, the highly purified protein was subjected to an *in vitro* assembly procedure as previously described (19). Briefly, 20 \(\mu\)g of the protein was incubated with 0.25 \(\mu\)g of single-stranded DNA oligonucleotides (random sequences) for a protein : nucleic acid molecular ratio of 100 : 1 in assembly buffer [25 mM HEPES, 100 mM KAc and 1.7 mM MgAc (pH 7.4)]. The reaction mixture was incubated for 30 min at 30°C and finally stored at 4°C. Particles with a diameter between 25–30 nm were visualized by electron microscopy.

**Mice**

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

**Mouse immunizations and protection assay**

*Immunization schedule no. 1*. Groups of 10 mice were injected by intraperitoneal (i.p.) route with different formulations of the recombinant capsid protein. Three doses of each immunogen were administered on days 0, 15 and 30. All formulations had a volume of 100 \(\mu\)l and were prepared employing aluminum hydroxide (Alhydrogel) (Brenntag...
Biosector, Denmark) as adjuvant at a final concentration of 1.44 mg ml\(^{-1}\).

- Group 1: 10 µg of capsid protein (non-particulate form).
- Group 2: 10 µg of NLPs.
- Group 3: 20 µg of NLPs.
- Group 4: Placebo (0.25 µg of oligonucleotides per mouse in assembly buffer) and
- Group 5 (positive control): Received one dose (0.5 ml) of infective DENV-2 without adjuvant.

Mice were bled and splenectomized 30 days after the last dose, and sera were collected for further immunological analysis.

**Immunization schedule no. 2.** A second immunization schedule was designed as follows:

- Group 1: 20 µg of NLPs,
- Group 2: Placebo (0.25 µg of oligonucleotides per mouse in assembly buffer) and
- Group 3 (positive control): Received one dose (0.5 ml) of infective DENV-2 without adjuvant.

The procedure, formulations and immunological evaluation were similar to those described in the previous schedule. A total of 66 animals per group were employed.

One month after the last dose, 56 animals of each group were divided into four subgroups (A, B, C and D) (Fig. 1). The subgroups A and B were inoculated by i.p. route with a single dose of 100 µg of the highly purified anti-CD8 (clone YTS 169.4) and the anti-CD4-depleting (clone YTS 191.1) mAbs, respectively (kindly provided by Dr. J. V. Gavilondo, Pharmaceutical Department, Center for Genetic Engineering and Biotechnology [CIGB]). Subgroup C received PBS. As isotype-matched control antibody administration, subgroup D was inoculated with the mAb 2C4, specific for the V3 region of the gp120 protein of the HIV-1 MN isolate (kindly provided by Dr. C. Duarte, Vaccine Division, CIGB). Three days after the administration of the depleting antibodies, animals were injected intracerebrally with 20 µl of a preparation of infective DENV-2 (NGC strain) containing 50 LD\(_{50}\) (20 pfu). Mice were observed daily during 15 days for mortality. On the same day, two animals of each subgroup were splenectomized and cell-specific depletion was confirmed by flow cytometry. More than 94% of the CD4\(^+\) or CD8\(^+\) cells were depleted in animals inoculated with the specific mAb (data not shown).

**ELISA**

An ELISA system was used to detect the anti-capsid antibodies. Polystyrene 96-well plates (Costar, USA) were coated 2 h at 37°C with 100 µl per well of dengue-2 capsid protein (5 µg ml\(^{-1}\)) in coating buffer [0.16% Na\(_2\)HPO\(_4\) and 0.29% NaHCO\(_3\) (pH 9.5)] and, next, were blocked in coating buffer containing 1% BSA for 1 h at 37°C. After three washes with PBS, 0.05% Tween 20 (PBS-T), 100 µl per well of individual sera from each group were tested by 2-fold serial dilutions in PBS-T, starting at 1 : 1000. Plates were incubated for 1 h at 37°C and washed as aforementioned. Later, 100 µl per well of 1 : 6000 diluted anti-mouse IgG-peroxidase conjugate (Amersham Pharmacia, UK) was 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\(_2\)HPO\(_4\) 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\(_2\)SO\(_4\). Absorbance was measured at 492 nm in a Sensident Scan (Merck, Finland). The positive cutoff value was considered as twice the mean absorbance’s values of the negative control sera.

In order to determine the anti-DENV antibodies, a capture ELISA system was used as previously described (9).

**In vitro neutralization measured in vivo**

Sera from the Immunization Schedule No. 1 were pooled per group. Each pool was incubated with a preparation of infective DENV-2 (NGC strain) during 1 h at 37°C. The final dilution of each pool in the mixtures was 1 : 10. The viral dose corresponded to 50 LD\(_{50}\) (20 pfu). The mAb 4G2 (30 µg per mouse) was used as positive control (20), whereas RPMI-1640 medium was added to the viral preparation as negative control.

Six groups of six mice, 14 weeks old, were inoculated by intracranial route with 20 µl of the aforementioned mixtures. Mice were observed daily during 15 days for survival assessment.

**Cell culture and viral stimulation**

Spleen cells were obtained in aseptic conditions, and erythrocytes were lysed by adding NH\(_4\)Cl 0.83% solution. Cells from each animal were washed twice with PBS-2% fetal bovine serum (FBS) (PAA Laboratories, Ontario, Canada) and resuspended at 2 × 10\(^5\) cells ml\(^{-1}\) in RPMI-1640 medium supplemented with 100 U ml\(^{-1}\) penicillin, 100 µg ml\(^{-1}\) streptomycin (Gibco), 2 mM glutamine (Gibco), 5 × 10\(^{-5}\) M 2-mercaptoethanol (Sigma, St Louis, MO, USA) and 5% FBS. Finally, 2 × 10\(^5\) cells per well were cultured in 96-well round bottom plates with the antigens (100 pfu of DENV-2 antigen or mock preparation). Con A (Sigma) was used as a positive control. In all the experiments, three wells were plated for each antigen. After 4 days of culture, culture supernatants were collected and stored at −20°C.

**In vitro cells depletion**

For CD4\(^+\) or CD8\(^+\) cells depletion, splenocytes at 5 × 10\(^6\) cells ml\(^{-1}\) in PBS were incubated for 30 min at 37°C with 100 µg of highly purified anti-CD8-depleting or anti-CD4-depleting mAbs, respectively, and rabbit complement (Cedarlane, Ontario, Canada). Later, the cells were washed twice with PBS-2% FBS and cell-specific depletion was confirmed by flow cytometry (data not shown).

**Flow cytometry analysis**

For cell-specific depletion analysis, 1 × 10\(^6\) splenocytes in PBS were incubated for 30 min at 4°C with anti-CD8-FITC antibody (Serotec Ltd, UK) and anti-CD4-allophycocyanin (APC) antibody (Serotec Ltd). Samples were analyzed in duplicate in a Partec flow cytometer (Partec GmbH, Germany).
Dead cells were excluded by propidium iodide incorporation. The gates used in the sample acquisition were saved and analyzed by the WinMDI software version 2.8 (Purdue University, WL, USA). The percentage of CD4⁺ or CD8⁺ cells was determined by gating on the positive lineages of the CD4⁺ or CD8⁺ subsets.

Cytokine detection

The culture supernatants of splenocytes previously stimulated with each antigen were analyzed in duplicate to determine the IFN-γ concentration by ELISA using mAbs pairs (INF-γ; Mabtech, Nacka, Sweden). ELISA protocol recommended by manufacturers was used with slight modifications. The lower limit of detection of cytokine was 4 pg ml⁻¹.

In silico T-cell epitope prediction

Human and murine T-cell epitopes in the capsid protein were predicted employing a computational epitope prediction system (http://tools.immune epitope.org/analyze/html/mhc_binding.html) (21), based on a beta version of the new Immune Epitope Database (IEDB) Analysis Resource. This tool provided a stabilized matrix method to predict MHC class I epitopes, combining the MHC I–peptide-binding prediction with immunoproteasome cleavage and transporter for antigen-processing predictions. Peptides (8–10 mer) predicted for humans and mice, with an IC₅₀ (half maximal inhibitory concentration) <1000 nM, were selected.

Statistical analysis

The analysis of data from ELISA was assessed using the Newman–Keuls multiple comparison test. Data from IFN-γ secretion were analyzed by the non-parametric Mann–Whitney test or two-way analysis of variance with Bonferroni post-test. Data from protection assay were analyzed by the log-rank test. In all cases, the GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA) (http://www.graphpad.com) was employed.

Results

NLPs induce a functional immune response against DENV-2 in mice

The immunogenicity of NLPs adjuvanted with alum was evaluated in mice using two different doses (10 and 20 µg). As a control, one group of animals received 10 µg of the non-particulate protein with the same adjuvant. We decided to use only the lower dose for the control group as previous studies had shown that neither 10 nor 20 µg of the non-particulate protein provided protection against challenge with infective virus (unpublished results). Thirty days after the third dose, both humoral and cellular immune responses were determined.

The humoral immune response generated against NLPs neither recognized DENV-2 nor neutralized in vivo viral infection. To measure the antibody response, two ELISA systems were used, which mainly differed on the antigen employed: the capsid protein or the DENV-2. As a result, all mice from the groups immunized with the formulations containing NLPs developed a good humoral immune response against the capsid protein, although statistical differences were observed between the two dose groups (P < 0.01) (Fig. 2A). On the other hand, the antibody titers determined for the group of animals immunized with the non-particulate protein protected mice against challenge with infective virus (unpublished results). Thirty days after the third dose, both humoral and cellular immune responses were determined.

In vivo protection

The ability of NLPs to provide protection against DENV-2 challenge was evaluated in mice. The mice were challenged with 50 LD₅₀ of infectious DENV-2 (NGC strain) 3 days after the last injection. The survival of the mice was monitored for 28 days. The Kaplan–Meier survival curves were compared using the log-rank test. The values were expressed as mean ± sem. The results showed that NLPs provided significant protection against DENV-2 challenge (P < 0.01) (Fig. 2B).

Given the lack of antiviral antibodies measured by ELISA in the groups receiving the capsid protein, two additional assays were performed to ensure the absence of humoral activity against the virus: in vitro neutralization assay measured in challenged mice and plaque reduction neutralization test (PRNT) (22). For the first one, pooled sera from animals of each group were incubated with a lethal dose of
virus and then naive mice were inoculated with the mixture
by intracranial route. The mAb 4G2, with neutralizing activity
reported against DENV (20), was used as positive control.
Consistently with the capture ELISA, none of the pools from
mice inoculated with the capsid protein neutralized the
in vivo viral infection (Fig. 2C). The same results were
obtained with the PRNT since individual sera from animals
immunized with the capsid protein did not neutralize the viral
infection (geometric mean titer <10).

The immunization with NLPs generates a cellular immune
response. Culture supernatants from mock-treated or DENV-
2-infected splenocytes from immunized animals of each
group were tested by ELISA to measure the concentration of IFN-γ. As shown in Fig. 3, high levels of the cytokine (354.8 ± 44.4 pg ml⁻¹ and 586.2 ± 16.6 pg ml⁻¹) were se-
creted by splenocytes from animals immunized with 10 and
20 μg of NLPs, respectively. Interestingly, DENV-2-immu-
nized animals developed a response similar to that of the
higher-dose group (642.2 ± 41.3 pg ml⁻¹). On the contrary,
animals immunized with 10 μg of the non-particulate capsid
protein showed a low IFN-γ secretion (124.1 ± 7.1 pg ml⁻¹),
without statistical differences with respect to the placebo
group (53.5 ± 10.7 pg ml⁻¹).

The cellular immune response generated by NLPs plays
a crucial protective role against DENV-2

Based on the previous results, the 20 μg formulation of NLPs
was selected to perform a second experiment in mice in or-
der to characterize the cellular immune response and its pos-
sible relationship with protection against dengue challenge.

Immunization of mice with NLPs induces CD4⁺ and CD8⁺ cell-
dependent IFN-γ secretion. In the first study, a high in vitro
IFN-γ secretion from splenocytes of animals immunized with
20 μg of NLPs was detected. We performed a second experi-
ment to determine the contribution of CD4⁺ and CD8⁺ cells to
this secretion. As shown in Fig. 3, and in agreement with the
first study, high levels of IFN-γ were induced in the spleno-
cytes of mice immunized with NLPs, statistically similar to
those of DENV-2-immunized animals. In turn, the concen-
tration of the cytokine was decreased in both CD4⁺ and CD8⁺
cell-depleted splenocytes, exhibiting significant differences
with respect to the non-depleted ones (P < 0.001). A similar
behavior was observed for splenocytes from DENV-2-immune
animals (Fig. 4).

NLPs-specific CD4⁺ and CD8⁺ cells protect against dengue
viral encephalitis. The effect of in vivo depleting either CD4⁺

**Fig. 2.** Humoral immune response generated after inoculation with different formulations of the recombinant DENV-2 capsid protein. The IgG antibody titers were measured by ELISA. (A) Anti-capsid antibody response. (B) Antiviral antibody response using DENV-2 as antigen. In both cases, data represent the geometric mean with 95% confidence interval of two different immunization experiments. Statistical analysis was performed by one-way analysis of variance using the Newman–Keuls multiple comparison test (***P < 0.01, ****P < 0.001). (C) Survival curves of animals inoculated by intracranial route with a lethal dose of DENV-2 mixed with pooled sera from immunized animals, with mAb 4G2 or with RPMI medium. Data are representative of two independent experiments (n = 10). Statistical analysis was performed by the log-rank test (***P < 0.01).
or CD8⁺ cells on protection against dengue challenge was also studied. One month after the last dose, a subgroup of NLPs- and DENV-2-immunized animals were inoculated with anti-CD8- or anti-CD4-depleting mAbs, and 3 days later, they were challenged with neurovirulent DENV-2.

After the observation period, 78% of animals that received NLPs (non-depleted mice) survived intracranial challenge with the homologous virus, whereas all mice of the negative control group died (Fig. 5A). In turn, consistent with previous reports, the positive control group exhibited high levels of protection (9, 17, 23). On the other hand, in the groups of mice immunized with NLPs that were additionally inoculated with either CD4⁻ or CD8⁻ depleting mAbs, the survival rate decreased significantly to 16.7% in both cases, without statistical differences with respect to the negative control group (Fig. 5B). Additionally, similar to a previous report, only CD8⁺ cell depletion affected the survival of DENV-2-immune animals (9). However, the survival rate in the groups of non-depleted mice was not affected by the inoculation of mAb 2C4, an isotype-matched control antibody (data not shown). Moreover, the mortality rate in mice from the placebo group was not affected by CD4⁺ or CD8⁺ cell depletion (data not shown).

The capsid protein contains more antigenic determinants for HLA class I molecules than their murine H-2d counterparts

To evaluate the possible functionality of the NLPs in humans, the specificity of the MHC class I-restricted CD8⁺ T-cell response to the DENV-2 capsid protein was mapped using the IEDB Analysis Resource, a web-based interactive tool for T-cell epitope prediction. For the present analysis, alleles of HLA: A0201, A0301 and B0702, corresponding to the supertypes A2, A3 and B7, were selected since they
represent ~89% of the world population (24), whereas all H-2d alleles were analyzed for BALB/c mice. Predicted peptides, with $IC_{50} \leq 1000$ nM, were separated in five ranges. As a result, for all ranges analyzed, there was a higher number of CD8$^+$ T-cell-restricted epitopes for humans than for mice (Fig. 6).

**Discussion**

VLPs are a highly effective type of subunit vaccine that mimics the overall structure of virus particles without containing infectious genetic material. In practical terms, VLPs have the ability to stimulate B-cell-mediated immune response, CD4 proliferative response and CTLs (25–27).

Previously, our group published that the recombinant capsid protein of DENV-2 produced in *E. coli* induced partial protection in mice against challenge with the homologous virus (17). Recently, we developed a purification procedure and *in vitro* assembly processes of this molecule (19), which allowed obtaining NLPs with a size similar to that of the native viral capsid (~30 nm) (28). In the present study, we characterized the immune response and the protection induced by NLPs in the mouse encephalitis model.

The main caveat conferred to the mouse encephalitis model is the inoculation of very high viral doses of mouse-adapted strains using the intracranial route. This procedure provokes disease manifestations, irrelevant to human dengue disease, given that nervous system involvement in DENV infections is rare (29). However, the infection of immunocompetent mice provides a useful immunological model to study DENV-specific T-lymphocytes response. In fact, T-cell epitopes on structural and non-structural proteins, capable of inducing antiviral activity, have been defined in BABL/c and C57BL/6 mice (10, 30, 31).

Our results showed that after immunization with NLPs, all mice generated high titers of anti-capsid antibodies. However, these antibodies neither recognized DENV-2 nor neutralized *in vivo* viral infection. Similarly, sera from DENV-2-immune mice did not recognize the capsid protein. It is well known that none of the regions of the viral nucleocapsid is exposed on the virion surface (28, 32), and therefore, they are not likely to interact with B cells during infection. Moreover, the inoculation of mice with DENV by the i.p. route induces a short-lasting viremia, which is rapidly controlled by the innate immune system (33).

While no antiviral humoral immune response was detected after NLPs inoculations, CMI was elicited since spleen cells from immunized mice produced high levels of IFN-γ upon *in vitro* restimulation with DENV-2. This cytokine has been described as a mediator of the cellular immune response and plays a role in the antiviral activity against DENV (34). Additionally, the levels of IFN-γ secretion depended on the dose of the NLPs inoculated as well as on the particulate nature of the antigen. Whether the enhanced functionality of this formulation is related to the particulate stage of the molecule or to the presence of oligonucleotides within the particles remains as an unanswered question. Particles or aggregated protein species may be cross-presented more efficiently than soluble antigens (35). Indeed, VLPs of several viruses induce dendritic cell maturation and secretion of cytokines that stimulate CD4$^+$ and CD8$^+$ T cells (36). Our results also demonstrated that IFN-γ was secreted by CD4$^+$ and CD8$^+$ cells, in accordance with previous reports (5, 37). On the other hand, there are also several studies showing that oligonucleotides have adjuvant capacity for the induction of CMI to target antigens (38). It has been reported that the dose range required for the optimal adjuvant effect of oligonucleotides in mice is 10–50 μg (39). However, in the present study, only 0.25 μg were added, 40-fold lower than the inferior limit reported as adjuvant. Based on the high sensitivity of oligonucleotides to degradation by interstitial nucleases (40), we can suggest that the NLPs are encapsulating the oligonucleotides, protecting them from nucleolytic degradation and favoring entry into the APC due to its aggregated nature. Once inside the cells, the oligonucleotides can interact with intracellular receptors and trigger the stimulation process (41).

Despite the lack of antiviral antibodies, a high survival rate in the group immunized with NLPs was attained. In parallel, when CD4$^+$ and CD8$^+$ cells were depleted before dengue challenge, the survival rate was clearly affected. This observation, consistently with the *in vitro* experiment, supports that protection was dependent on both cell populations.

![Fig. 6. CD8$^+$ T-cell epitopes predicted from DENV-2 capsid protein. MHC class I-restricted T-cell response was mapped using the IEDB Analysis Resource. The analysis was performed for HLA A0201, A0301 and B0702, corresponding to the supertypes A2, A3 and B7, and all H-2d alleles for BALB/c mice. Data represent the number of peptides for each IC$_{50}$ range.](image-url)
Accordingly, previous studies have reported the relationship between CD8 cytotoxic activity and IFN-γ secretion (5, 10, 42). On the other hand, CD4+ T cells could be directly involved in protecting against the disease since subsets of this cell population that are able to lyse infected cells have been characterized for flaviviral diseases (43), including dengue (16, 37, 44, 45).

These experimental findings suggest a potential protective role of CMI in the mouse model against DENV, in accordance with studies previously reported (8–10). In addition, the induction of a functional T-cell response after vaccination in humans with live attenuated viruses was demonstrated (42,46–49). As for the humoral immune response, a potential pathogenic role has been described for CMI in heterologous natural infections. Evidences from several studies suggest that cross-reactive memory T cells are involved in the immunopathogenesis of the disease (2, 4, 5, 50). Consequently, a future vaccine candidate should include the mixture of the recombinant proteins corresponding to the four dengue serotypes. Finally, the functionality of the NLPs as vaccine candidate against dengue disease is foreseen based on in silico predictions showing that the capsid protein contains more CD8+ T-cell predicted epitopes for the three common HLA alleles than for mouse MHC molecules. Although this result is not conclusive, since the analyses were done over predicted peptides, it suggests that it is possible to find immunodominant epitopes for humans. In fact, the protective capacity of two epitopes from the capsid protein, predicted in the present analysis, has been recently confirmed in mice experiments (10).

Our findings provide new evidences on the protective role of the CMI in mice, without the induction of a functional humoral immune response. Concerning the limitations associated to the mouse encephalitis model, further studies in non-human primates or humanized mice should be conducted to define the suitability of the NLPs as a possible vaccine candidate against dengue disease.

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Abbreviations
APC aliphophycocyanin
CIGB Center for Genetic Engineering and Biotechnology
CMI cell-mediated immunity
DEA diethanolamine
DENV dengue virus
FBS fetal bovine serum
IEDB Immune Epitope Database
i.p. intraperitoneal
NGC New Guinea C
NLPs nucleocapsid-like particles
PBS-T PBS, 0.05% Tween 20
pfu plaque-forming units
PRNT plaque reduction neutralization test
VLPs virus-like particles

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NLPs-specific CD4 and CD8 cells protect against DENV


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