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ORIGINAL ARTICLE

Nucleocapsid-like particles of dengue-2 virus enhance the immune response against a recombinant protein of dengue-4 virus

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Abstract In this study, we evaluate in mice a novel formulation containing nucleocapsid-like particles of dengue-2 virus (recNLP) co-immunized with a chimeric protein composed of the dengue-4 envelope domain III fused twice within the meningococcal P64k protein of *Neisseria meningitidis* (PD24). The animals receiving the PD24–recNLP mixture showed the highest levels of antiviral antibodies. Similar results were obtained for IFNγ secretion levels, indicating a functional Th1 cellular response. Consistently, the percentage of mice surviving after viral challenge was significantly higher for those immunized with the mixture than for those inoculated with PD24 protein alone. In addition, in vivo depletion experiments demonstrated the decisive role of $CD4^+$ and $CD8^+$ cells in the protection conferred by immunization with PD24–recNLP. In conclusion, this report demonstrates for the first time the adjuvant capacity of dengue-2 virus recNLP. Additionally, the evidence presented highlights the potential of these particles for enhancing the immune response against heterologous recombinant proteins.

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Introduction

Infection with dengue viruses (DEN) remains a health problem worldwide, with no vaccines yet available for its prevention. Although the development of recombinant subunit vaccines has been proposed as an alternative to overcome the drawbacks associated with live-attenuated vaccines, the current candidates have been shown to be poorly immunogenic [1].

Virus-like particles (VLP) are an effective type of subunit vaccine that mimics the overall structure of the virus. The relatively large size of the VLP often leads to efficient antigen processing and presentation by dendritic cells, thus promoting maturation and migration [2]. Different VLP have been successfully evaluated as vaccines [3–6] or as adjuvants for different purposes, e.g. as carriers for presenting foreign epitopes [7, 8], as partners for the chemical conjugation of peptides [9] and for the delivery of independent antigenic molecules [10, 11].

The variety of molecular assemblies typical of viral structures offers several alternatives for obtaining virus-like particles. The capsid proteins of enveloped viruses, in particular, have been widely used for this purpose, and the VLPs obtained in these cases are often named nucleocapsid-like particles (NLP) [2]. In 2007, we developed, for the first time, a recombinant construct of the capsid protein of dengue-2 virus (DEN2) in *Escherichia coli*. After a process of partial purification, the highly aggregated recombinant protein was shown to induce partial protection in the murine viral encephalitis model [12]. Subsequently, the protein was subjected to purification and in vitro assembly processes. As a result, morphologically similar NLPs were obtained with a size of 30 nm, independently of the sequence and length of the oligonucleotides employed in the assembly process [13]. These particles were able to

induce functional cell-mediated immunity (CMI) in mice and protection upon challenge with the homologous serotype [14].

Our group has previously reported the genetic construction, antigenic characterization and immunological evaluation of chimeric proteins composed of domain III from the envelope protein corresponding to the four serotypes of dengue virus fused to the C-terminal region of the P64k protein from *Neisseria meningitidis* [15–17]. The P64k protein has been extensively evaluated as carrier for diverse antigens [18], and its safety and immunogenicity in humans have been also demonstrated [19]. Domain III from the envelope protein of DEN virus is believed to function as the putative cellular-receptor-binding domain and contains multiple neutralizing epitopes [20]. Additionally, antibodies that bind to structural domain III are very efficient at neutralizing viral infectivity and also tend to be virus type or subtype specific [21]. The fusion proteins of serotypes 1, 2 and 3, were found to be immunogenic and protective [17, 20, 22]; however, the protein corresponding to serotype 4 was the least immunogenic (unpublished data). We recently reported the cloning, expression and immunological evaluation in mice of a chimeric antigen composed of the DEN4 envelope domain III inserted at two different sites within the P64k protein, code-named PD24 [23]. Although this strategy was followed in order to increase the immunogenicity of the recombinant protein corresponding to serotype 4, this protein elicited a low humoral response and induced only partial protection after viral challenge in mice [23].

In this study, we evaluate the enhancing capacity of in vitro-assembled capsid protein of DEN2 (recNLP) on the immunogenicity of the PD24 protein. Additionally, we characterize the immune response that is induced and determine the role of cellular immunity on protection after challenge with a lethal dose of infectious virus.

Materials and methods

Viruses and antibodies

For the immunization of positive control groups and viral challenges, a preparation of 10^5 PFU/mL of infectious DEN4 virus (strain H241) [24] was employed. The immunogen was obtained by homogenizing the brains of suckling mice infected with DEN4 virus in RPMI-1640 medium. Antigens for antibody detection assays were prepared from brain homogenates from suckling mice infected with DEN4 virus (strain H241) according to the method of Clarke and Casals [25]. A similar preparation obtained from the brains of non-inoculated mice was used as a negative control. The viral stock for the neutralization assay was obtained from clarified cell culture supernatant fluid harvested from Vero cells infected with DEN4 virus (strain H241). A concentrated preparation of DEN4 virus (strain H241) was used for in vitro stimulation in the IFNγ detection assay. This preparation was obtained by centrifugation of 100 mL of supernatant from infected Vero cells at $80{,}000 \times g$ for 4 h at 4°C, followed by resuspension in 1 mL of phosphate-buffered saline (PBS). A mock preparation was prepared similarly from the supernatant of uninfected Vero cells. The depleting antibodies employed in the in vivo depletion experiment were kindly provided by Dr. J. V. Gavilondo, Pharmaceutical Department, CIGB.

Recombinant proteins

The recombinant protein PD24 (the dengue-4 envelope domain III inserted at two sites within P64k) was obtained as inclusion bodies in *E. coli* and was subjected to a denaturation–renaturation procedure. After a process of partial purification, the protein reached a purity of 60% [23].

The capsid protein from DEN2 was expressed in *E. coli* as described previously [12]. The recombinant protein was purified by ion-exchange chromatography under denaturing conditions, and the highly pure protein was subjected to an in vitro assembly procedure. Briefly, the protein was incubated with single-stranded oligodeoxynucleotides (ODNs) at a molecular ratio of protein to nucleic acid of 100:1 in assembly buffer (25 mM HEPES, 100 mM KAc, 1.7 mM MgAc, pH 7.4). Finally, homogeneous particles with a diameter of 30 nm, quite close to the diameter of the native capsid particles, were obtained [13].

Immunization and protection assays

Immunization schedule no. 1

Groups of 20–22 6-week-old female BALB/c mice, from CENPALAB (Centro Nacional para la Producción de Animales de Laboratorio, Havana City) were injected with the appropriate formulation at days 0, 15, 30 and 45. All formulations had a volume of 100 µL and were prepared using aluminum hydroxide as an adjuvant at a final concentration of 1.44 mg/mL.

Group 1 (negative control)	Each mouse was inoculated with 20 µg of P64 k
Group 2	Each mouse was inoculated with 10 µg of recNLP
Group 3	Each mouse was inoculated with 20 µg of PD24
Group 4	Each mouse was inoculated with 20 µg of PD24 mixed

	with 10 μg of recNLP before combining with aluminum hydroxide (PD24–recNLP)
Group 5 (positive control)	Each mouse was inoculated with only one dose (0.5 mL) of the preparation of DEN4 virus described above (subsection "Viruses and antibodies") without adjuvant

For further immunological analysis, 10 mice from each group were bled 15 days after the last dose, and sera were collected. One month after the last inoculation, the rest of the mice in each group were challenged by intracranial inoculation with 20 μL of a lethal preparation of infective DEN4 virus containing 50 median lethal doses (LD_{50}). Then, the mice were observed daily for 21 days to monitor their survival.

Immunization schedule no. 2

In this scheme, we used Groups 1, 3, 4 and 5, as described above, each with 46 6-week-old female BALB/c mice, (CENPALAB, Cuba). The procedure, formulations and immunological evaluation were the same as in the previous schedule.

Three days before viral challenge, the groups (36 non-bled animals each) immunized with PD24–recNLP and PD24 were divided into three subgroups of 12 mice each. One of them was inoculated intraperitoneally with 100 μg of an anti-CD4 depleting monoclonal antibody (clone YTS 191.1), another one was inoculated intraperitoneally with 100 μg of anti-CD8 depleting monoclonal antibodies (clone YTS 169.4), and the last one was inoculated with PBS only. Depletion was measured by flow cytometry, using two mice per subgroup. More than 94% of the $CD4^+$ or $CD8^+$ cells were depleted in the animals inoculated with the specific monoclonal antibody (data not shown). The remaining mice were challenged intracranially with 20 μL of a preparation of lethal infectious DEN4 virus containing 50 median lethal doses (LD_{50}). Mice were observed daily for 21 days to monitor their survival.

In both immunization schedules, vaccine preparations were administered by the intraperitoneal route. This route was found to offer the highest functional response and protection in the viral encephalitis murine model in a preliminary comparison with the subcutaneous and intramuscular routes.

The maintenance and care of experimental animals used in this research complied with the CIGB's Laboratory Animal Care and Use Guidelines.

Enzyme-linked immunosorbent assay

Anti-DEN4 antibody levels were measured using an amplified sandwich ELISA system. Polystyrene plates with 96 wells (Costar, USA) were coated for 2 h at 37°C with 100 μL per well of a mixture of anti-dengue human immunoglobulins (IgG) (10 μg/mL) in coating buffer (0.16% Na_2CO_3, 0.29% $NaHCO_3$, pH 9.5); plates were blocked with coating buffer containing 1% bovine serum albumin (BSA) for 1 h at 37°C and washed three times in PBS, 0.05% Tween (PBS-T). Viral antigens from suckling mouse brain infected with DEN4 virus (100 μL per well) and uninfected suckling mouse brain as a negative control were incubated overnight at 4°C. After three washes with PBS-T, 100 μL per well of sera from each group was tested by serial dilution in PBS-T, starting at 1:100. Plates were incubated for 1 h at 37°C and washed as described above. Later, 100 μL per well of anti-mouse IgG-peroxidase conjugate (Amersham-Pharmacia, UK) diluted 1:5,000 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 2% Na_2HPO_4 1% citric acid buffer, pH 5.0) was added. The plates were kept at 25°C for 30 min, and the reaction was stopped by adding 50 μL 12.5% H_2SO_4 to each well. An automated ELISA reader recorded the absorbance at 492 nm. A value was considered positive if it had an absorbance twofold higher than that of the control preparation.

Anti-PD24 antibody levels were measured in a similar way. Polystyrene plates (96 wells, Costar, USA) were coated for 2 h at 37°C with 100 μL of PD24 per well at 5 μg/mL in coating buffer. After blocking and washing, 100 μL of serum per well from each group was tested by serial dilution in PBS-T, starting at 1:5,000. The remaining steps were similar to those of the antiviral ELISA described above.

Plaque-reduction neutralization test

Neutralizing antibody titers were measured by the plaque-reduction neutralization test (PRNT) using DEN4 virus (strain H241) with BHK-21 cells as described previously [26]. The neutralization titer was defined as the dilution yielding a 50% reduction in the maximum number of plaque-forming units.

Cell culture

One month after the last dose, three mice per group were splenectomized under aseptic conditions.

The cells were washed twice with 2% fetal bovine serum (FBS) in PBS and resuspended at 2×10^6 cells/mL in

RPMI-1640 medium (Sigma–Aldrich, Ayrshire KA, UK) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco), 2 mM glutamine (Glutamax, Gibco), 5×10^{-5} M 2-mercaptoethanol (Sigma, St. Louis, MO, USA) and 5% FBS. The cells (2×10^5 cells/well) were cultured in 96-well round-bottom plates with the relevant antigens (dengue virus and mock preparation). Concanavalin A (ConA; Sigma, St. Louis, MO, USA) was used as positive control.

Cytokine detection

Culture supernatants from splenocytes that had been stimulated with DEN4 virus were analyzed in duplicate to measure the IFNγ concentration by ELISA using monoclonal antibody pairs (Mabtech (IFNγ; Nacía, Sweden). The ELISA protocol recommended by the manufacturers was used with slight modifications. The lowest limit of detection of the cytokine in this assay was 10 pg/mL.

Flow cytometry analysis

To confirm the cell-specific depletion, 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-APC antibody (Serotec Ltd., UK). Optimal compensation and gain settings were determined for each experiment based on unstained samples. Samples were analyzed in duplicate in a PasIII flow cytometer (Partec GmbH, Germany). Dead cells were excluded by propidium iodide incorporation. The gates used in the sample acquisition were saved and analyzed using 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.

Statistical analysis

The ELISA data were analyzed using the Newman-Keuls Multiple Comparison Test and the Mann–Whitney test. The data from the protection assay were analyzed using the Logrank test. In all cases, the software application GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA, http://www.graphpad.com) was employed.

Results

Immunization schedule no. 1

To investigate the adjuvant capacity of in vitro-assembled capsid protein of DEN2 (recNLP) on the immunogenicity of the recombinant protein PD24, five groups of mice were immunized by the intraperitoneal route as described in "Materials and methods".

Humoral response

Fifteen days after the last dose, the sera were tested for antiviral antibodies by a capture ELISA assay. No anti-DEN4 antibodies were detected among the mice immunized with either P64k protein or recNLP alone, and the animals immunized with PD24–recNLP exhibited antibody titers that were higher than those in the group receiving PD24 ($p < 0.05$) and similar to those of the positive control

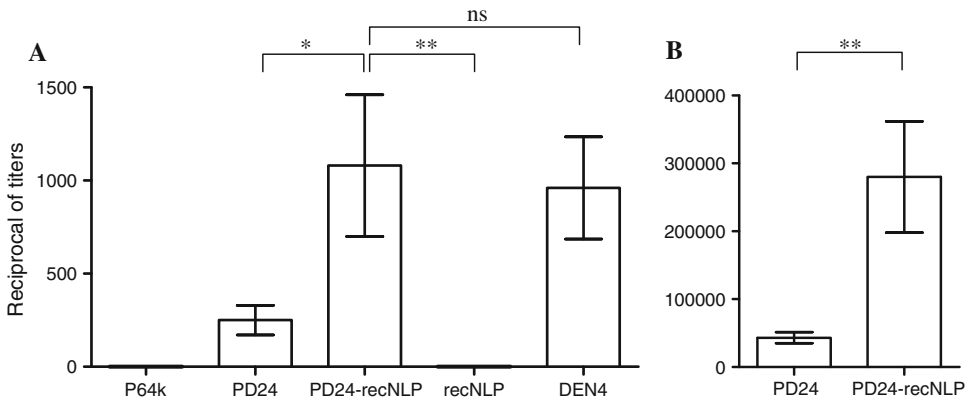

Fig. 1 Evaluation of the humoral response. **a** Anti-DEN4 antibody response. **b** Anti-PD24 antibody response. Fifteen days after the last dose, 10 animals per group were bled. Sera were collected and analyzed by ELISA for the presence of anti-DEN4 or anti-PD24 antibodies. Statistical significance was evaluated using the Newman–Keuls Multiple Comparison Test for antiviral antibodies and the Mann–Whitney test for anti-PD24 antibodies (**$p < 0.01$; *$p < 0.05$; ns $p > 0.05$). The data represent the mean ± SD ($n = 10$ for each group)

group (Fig. 1a). Consistently, the antibody response against the recombinant protein was higher in mice immunized with PD24–recNLP than in those receiving only PD24 ($p < 0.01$) (Fig. 1b).

The functionality of these antiviral antibodies was assessed by a plaque-reduction neutralization test. The antibodies did not neutralize viral infectivity in our assay in any of the experimental groups, even at dilutions of 1:20.

Animal protection assay

One month after the last dose, the animals that were not bled were challenged with neurovirulent infectious DEN4 virus. After 21 days of observation, the survival rates were 50 and 92% for the groups immunized with PD24 and PD24–recNLP, respectively, with statistically significant differences between them ($p < 0.05$). There was no statistically significant difference between the survival rates of the negative-control group (10%) and the group immunized with recNLP (15%). In the positive-control group, 90% of the animals survived the challenge (Fig. 2).

Immunization schedule no. 2

Because of the poor humoral response induced by the PD24–recNLP formulation and the significant protection it afforded upon viral challenge, we implemented a second immunization schedule to characterize the immune response elicited and to determine its relationship to protection.

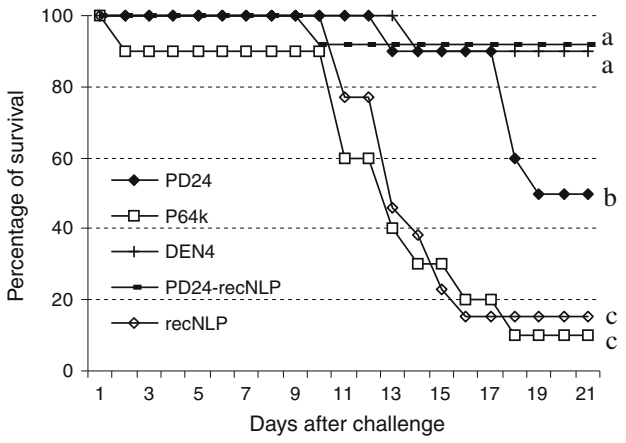

Fig. 2 Protection assay (Immunization schedule no. 1). One month after the last inoculation, mice were injected intracranially with a lethal dose of infectious DEN4 virus, and they were observed daily for 21 days. Survival rates were of 1/10 (P64k), 2/12 (recNLP), 5/10 (PD24), 9/10 (DEN4 and 11/12 (PD24–recNLP). *Different letters* mean statistical differences by the Logrank test ($p < 0.05$)

Humoral response

In agreement with the results obtained with "Immunization schedule no. 1", none of the mice immunized with the P64k protein showed anti-DEN4 antibodies, and mice immunized with PD24–recNLP exhibited antibody titers that were higher than in those immunized with the PD24 protein alone (data not shown).

Similarly, the functionality of these antiviral antibodies was assessed using a plaque-reduction neutralization test. Only two mice immunized with the formulation PD24–recNLP showed neutralizing titers at a dilution of 1:10. The rest of the animals did not exhibit antibodies that were able to neutralize viral infection in BHK-21 cells (data not shown).

Cellular immune response

One month after the last dose, the splenocytes of three mice per group were stimulated in vitro with infectious DEN4 virus. After 3 days of incubation, culture supernatants were harvested and analyzed by ELISA for mouse IFNγ. Levels of IFNγ secretion in the splenocytes of the group of mice immunized with PD24–recNLP were similar to those of the positive-control group, and higher than those of mice immunized with the recombinant protein alone (Fig. 3).

Animal protection assay

One month after the last dose, the animals that were not bled were challenged with neurovirulent infectious DEN4 virus. After 21 days of observation, the survival rate for the animals immunized with the PD24 protein was 50%, compared with a 100% survival rate among the animals immunized with the PD24–recNLP formulation. This difference was statistically significant ($p < 0.05$) (Fig. 4a). In the negative- and positive-control groups, the survival rates were 0 and 100%, respectively.

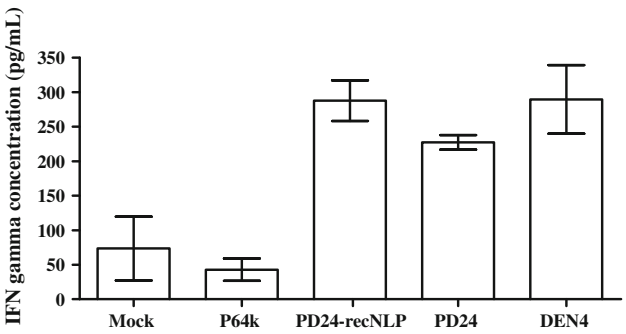

Fig. 3 IFNγ secretion measured by ELISA in splenocytes stimulated with DEN4. One month after the last dose, three animals per group were splenectomized, and splenocytes were cultured and stimulated with 10^3 pfu of DEN4 virus. Data represent the mean ± SD ($n = 3$ for each group)

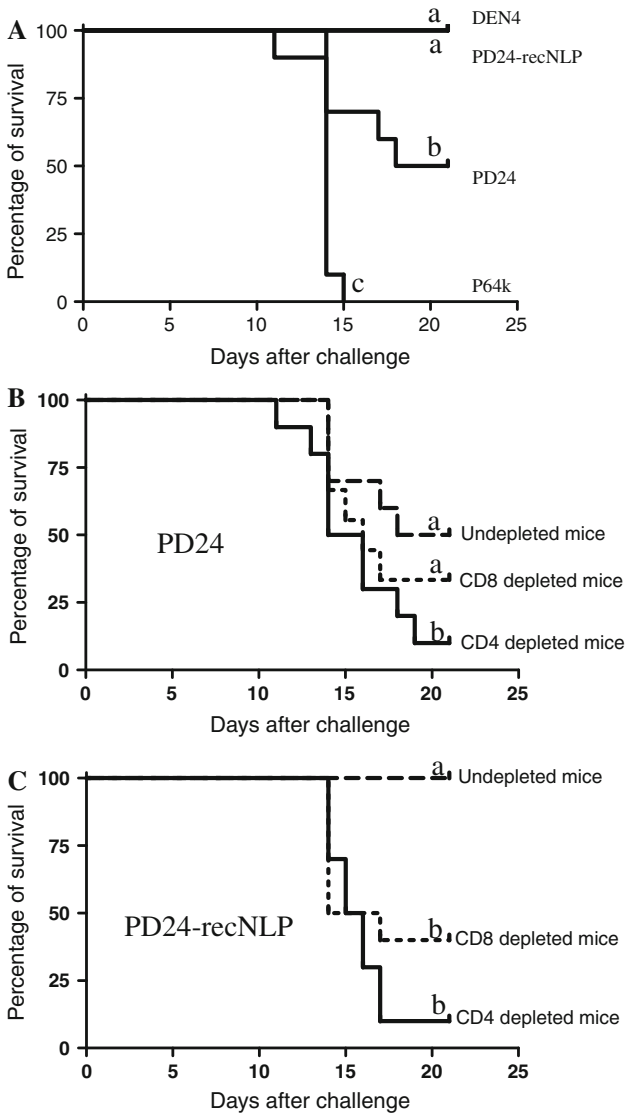

Fig. 4 Protection assay against lethal DEN4 virus after in vivo depletion. Three days before the viral challenge, the groups of immunized mice (Immunization schedule no. 2) were divided in three groups of 10 mice each. One was inoculated with 100 μg of an anti-CD4 depleting monoclonal antibody, another one was inoculated with 100 μg of anti-CD8 depleting monoclonal antibody, and the last one was inoculated with PBS only. Three days after, depleted and non-depleted mice were challenged with a preparation of infectious DEN4 virus containing 50 LD_{50}. Mice were observed daily for 21 days. **a** Survival curves of the non-depleted groups. **b** Survival curves of mice immunized with the PD24 protein. **c** Survival curves of mice immunized with the formulation PD24–recNLP. *Different letters* mean statistical differences by the Logrank test ($p < 0.05$)

In vivo depletion assay

The groups of mice immunized with either PD24 or PD24–recNLP were further subdivided into three subgroups. Three days before viral challenge, one subgroup was inoculated with a CD4-depleting monoclonal antibody, another was inoculated with a CD8-depleting monoclonal antibody, and a third was inoculated with PBS only. After challenging the mice with a preparation of infectious DEN4 virus, their survival was monitored by daily observation for 21 days. The rate of survival of mice immunized with PD24 alone dropped from 50 to 10% ($p < 0.05$) after the depletion of $CD4^+$ cells; no effect was observed for the depletion of $CD8^+$ cells. Among the animals immunized with the PD24–recNLP formulation, the survival rates decreased significantly ($p < 0.05$), from 100 to 10%, for the subgroup treated with the anti-CD4 antibody, and from 100 to 40% for the subgroup depleted of $CD8^+$ cells (Fig. 4b, c).

Discussion

In a previous work, we reported that immunization of mice with a semipurified recombinant capsid protein from DEN2 virus produced in *E. coli* conferred protection against challenge with the homologous virus [12]. We also published a purification and in vitro-assembly procedure for this molecule that allowed recombinant nucleocapsid-like particles (recNLP) [13] with a similar size to the native viral capsid to be obtained [27]. In another report, it was shown that these recNLPs are not able to induce a humoral immune response against DEN2 virus, consistent with the fact that none of the regions of the viral nucleocapsid are exposed on the virion surface [14]. However, these authors also found that spleen cells from recNLP-immunized mice produced high levels of IFN gamma upon in vitro stimulation with DEN2 virus, and recNLP-immunized mice were protected after homologous viral challenge [14].

In this work, the low immunogenicity of the serotype 4 recombinant protein compared to the remaining serotypes prompted us to select the PD24 protein (DEN4 envelope domain III fused twice with the meningococcal P64k protein) as a model to examine the enhancing capacity of the recNLP on the immunogenicity of this antigen.

As a first finding, the humoral response induced by the PD24–recNLP and PD24 formulations were similarly low, in agreement with previous results with recombinant PD24 [23]. However, since the envelope domain III of dengue virus has been described as a target for neutralizing antibodies [28], this result might have arisen from the aggregated nature of PD24 [23] or due to a possible incorrect conformation of domain III after the denaturation/renaturation procedures. We previously reported the recognition of the PD24 protein by five human sera and four different lots of hyperimmune murine ascitic fluids (both with neutralizing titers against DEN4 virus higher than 1:250) by ELISA, suggesting a correct folding of the viral fragment and its exposure on the aggregates [23]. On the other hand, mice immunized with this viral dose also did

not develop neutralizing antibodies. The induction of a low neutralizing response against DEN4 virus has been reported after the evaluation of different vaccine candidates based on live attenuated virus, fusion proteins including domain III or the whole envelope protein [29–31]. Moreover, based on epidemiological data, DEN4 has been described as a naturally attenuated virus, since there have been few reports of primary DEN4 outbreaks [32]. Another important issue in this sense is that the PRNT has been described as having low sensitivity for measuring neutralizing antibodies against DEN4 virus [33]. Taking all of this into account, we suppose that the low humoral response found in this study could be a consequence of the low sensitivity of the technique employed, together with the low immunogenicity of this serotype *per se*.

Despite this low level of neutralizing antibodies, the anti-DEN4 and anti-PD24 titers obtained for the PD24–recNLP group were higher than those of the PD24 group, indicating a positive influence of the recNLP on the immunogenicity of the final formulation.

The in vivo protection assay provided the main results of the present work. In agreement with the findings discussed above, the group co-immunized with PD24 and recNLP (PD24–recNLP) reached higher levels of protection than the mice immunized with the recombinant protein alone. These results confirm the enhancement provided by recNLP on the protection induced by PD24.

These significant levels of protection in the absence of a high humoral response led us to address the functionality of the induced cellular immune response. First, we determined IFNγ secretion levels after stimulation with DEN4 virus. In accordance with the results from the protection experiments, the formulation containing recombinant PD24 and recNLP induced the highest levels of IFNγ secretion, similar to those induced by viral infection. Second, an in vivo depletion experiment was performed in order to define the role of $CD8^+$ and $CD4^+$ cells in protection. This assay demonstrated that protection of mice immunized with the PD24–recNLP formulation depends on both cell populations, which is in agreement with earlier reports in which VLPs of several viruses induced dendritic cell maturation and secretion of cytokines able to stimulate $CD4^+$ and $CD8^+$ T cells [34]. In turn, the protection reached in the group of mice immunized with the PD24 protein alone was affected only by $CD4^+$ depletion, consistent with the finding that $CD4^+$ T cell subsets are able to lyse infected cells in diverse flaviviral diseases including dengue [35, 36]. Indeed, a protective effect mediated by IFNγ-secreting $CD4^+$ T cells [37] and a direct relationship between $CD8^+$ cytotoxic activity and IFNγ secretion [38, 39] have been observed previously. In addition, the "helper" role of $CD4^+$ T cell subsets should be considered. These results allow us to speculate that recNLP possibly creates an environment that favors a Th1 immune response against PD24, in agreement with previous reports in which it was described that a cytotoxic T-cell epitope within domain III from DEN2 virus conferred protection in the absence of a neutralizing humoral response [40].

Moreover, the enhancement provided by the recNLP of the cell-mediated immunity induced by the PD24 protein in the viral encephalitis murine model suggests a possible noncovalent interaction between recNLP and PD24 that guarantees the uptake of the whole aggregate by dendritic cells and, consequently, their maturation and selective polarization toward a Th1 response. Similar results have been reported previously from experiments employing a multiepitopic recombinant protein of HIV and the hepatitis B core antigen [41].

Another essential point regarding the adjuvanticity of these recNLP is the presence of oligodeoxynucleotides (ODNs) within their structure. There are several studies describing ODNs as adjuvants that are capable of enhancing cell-mediated immunity [10, 42, 43]. Moreover, it has been reported that the dose range required for the optimal adjuvant effect of ODNs in mice is 10–50 μg [44]. In the present study, only 0.125 μg was employed, almost ten times less than the lowest limit reported. This fact, together with the known high sensitivity of ODNs to degradation by interstitial nucleases [45], suggest that the recNLP protects the ODNs against nucleolytic degradation and favors their entry into 'antigen-presenting cells' due to its aggregated nature. Once inside the cells, ODNs can interact with intracellular receptors and trigger the stimulation process [46].

Dengue virus is a non-cytopathic virus that up-regulates the surface expression of MHC class I molecules in infected cells [47]. Therefore, the cellular immune response is a critical defense mechanism in dengue virus infections. The present study constitutes another example of the important role of the cellular immune response in protecting against dengue virus in the mouse model, confirming the evidence provided by Van der Most et al. [39] and Gil et al. [48]. Nonetheless, a contribution of the antiviral antibodies in protection should not be totally dismissed. Despite the lack of neutralizing antibodies, the antiviral response might be involved in protection through other mechanisms such as antibody-dependent cell-mediated cytotoxicity or the complement activation.

In conclusion, this report demonstrates for the first time the adjuvant capacity of dengue-2 virus recNLP. Further studies of these recNLP as enhancers of the immune response against recombinant proteins of other dengue serotypes are in progress. This issue is critical for obtaining a tetravalent formulation capable of protecting against the four dengue serotypes, in which the recNLP could be used as adjuvant for the P64k-domIII proteins of serotypes 1, 3

and 4 and, at the same time, as an antigen inducing a functional immune response against DEN2.

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