

Production of a Recombinant Dengue Virus 2 NS5 Protein and Potential Use as a Vaccine Antigen

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Dengue fever is caused by any of the four known dengue virus serotypes (DENV1 to DENV4) that affect millions of people worldwide, causing a significant number of deaths. There are vaccines based on chimeric viruses, but they still are not in clinical use. Anti-DENV vaccine strategies based on nonstructural proteins are promising alternatives to those based on whole virus or structural proteins. The DENV nonstructural protein 5 (NS5) is the main target of anti-DENV T cell-based immune responses in humans. In this study, we purified a soluble recombinant form of DENV2 NS5 expressed in *Escherichia coli* at large amounts and high purity after optimization of expression conditions and purification steps. The purified DENV2 NS5 was recognized by serum from DENV1-, DENV2-, DENV3-, or DENV4-infected patients in an epitope-conformation-dependent manner. In addition, immunization of BALB/c mice with NS5 induced high levels of NS5-specific antibodies and expansion of gamma interferon- and tumor necrosis factor alpha-producing T cells. Moreover, mice immunized with purified NS5 were partially protected from lethal challenges with the DENV2 NGC strain and with a clinical isolate (JHA1). These results indicate that the recombinant NS5 protein preserves immunological determinants of the native protein and is a promising vaccine antigen capable of inducing protective immune responses.

Dengue fever is an acute disease caused by dengue virus (DENV), an arbovirus belonging to the *Flaviviridae* family, transmitted by *Aedes* mosquitoes (1, 2). In urban settings, DENV is found as four immunologically distinct types (serotypes), DENV1 to DENV4, which have approximately 30% divergence in their genomic nucleotide sequences (3–7). The disease may present with different degrees of manifestations that are classified by the WHO as dengue fever, dengue with warning signs, and severe dengue (8). A recent study showed that, annually, an average of 96 million DENV-infected people develop symptoms with severity sufficient to change their routines (1); according to previous studies, about 500,000 cases develop into severe forms of disease, which may include hemorrhage and shock syndrome (9). The mortality rate in this group reaches 10% in hospitalized patients, increasing to 30% in nontreated DENV-infected patients (9–11). Despite the high epidemiological importance of dengue fever, no effective anti-DENV drug or vaccine formulation is in clinical use for treatment or prevention of the disease.

Several strategies have been explored, aiming toward the development of an effective dengue vaccine (12–21). Nonetheless, a tetravalent vaccine formulation requires a neutralizing response to all four serotypes (22–25). A poor vaccine-induced response to structural proteins of any of the four serotypes may pose a risk of the onset of antibody-dependent enhancement (ADE) of virus replication upon infection by a subneutralized serotype (22–25). Moreover, protection based on the induction of neutralizing antibodies targeting structural proteins may be negatively impacted by the genetic diversity found among DENV serotypes. These potential complications have delayed significantly the development and the release of an efficient anti-DENV vaccine. The use of DENV structural proteins as vaccine targets may be attributed to the difficulties faced by the most studied anti-DENV vac-

cine candidate to achieve higher protective levels in clinical trials carried out in several countries (26–28).

DENV nonstructural proteins (NSs) have been shown to be promising vaccine antigen candidates in monovalent formulations by inducing T cell-based immune responses (17, 18, 29–32). In addition, NSs are highly conserved among serotypes and are not known to be involved in the ADE phenomenon (33). Also, NSs are relevant targets for T cell-based immune responses capable of controlling infection caused by DENV in humans (34–37). This evidence indicates that NSs are also an alternative to the development of anti-DENV vaccines.

The DENV NS5 is a protein of approximately 103 kDa, containing two major functional/structural domains, (i) N-terminal residues 1 to 368, which comprise the 2'-O-methyltransferase region, and (ii) residues 405 to 900, which comprise an RNA-dependent RNA polymerase (38), as well as an interdomain region with two nuclear localization sequences (NLSs) that direct the protein

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to the nucleus (39, 40). During natural infection, the NS5 is processed by the proteasome pathway, and specific epitopes are presented to T lymphocytes (41, 42). In fact, CD8⁺ T cells are known to target NS5 epitopes after experimental DENV infection in rhesus monkeys (31). In addition, human CD8⁺ T cells preferentially target NS3 and NS5 after DENV natural infection (37). Recently, subjects receiving a live attenuated tetravalent dengue vaccine developed T cell responses to highly conserved epitopes in the NS5 (34). Moreover, it has recently been demonstrated that protective immunity against DENV experimentally induced using live viruses in mice is achieved mainly by CD8⁺ T cells targeting NSs, particularly NS5 (43). These studies indicate that the NS5 protein is a promising candidate antigen for a dengue vaccine formulation based on nonstructural proteins. Thus, the generation of recombinant NS5 with preserved immunological features represents a priority for different studies, such as those focused on the development of anti-dengue vaccines and validation of the T cell-based response to protective immunity to dengue.

In this study, we report conditions leading to the expression and purification of a recombinant DENV2 NS5 expressed in *Escherichia coli* at high yields and purity levels. Sera from DENV-infected patients recognized the purified NS5 in an epitope-conformation-dependent manner. Immunization of mice with the purified NS5 alone induced large amounts of NS5-specific antibodies and expansion of NS5-specific T cells secreting tumor necrosis factor alpha (TNF- α) and gamma interferon (IFN- γ). In addition, mice immunized with nonadjuvanted purified NS5 expressed partial protection from lethal challenges with two different DENV2 isolates. Collectively, our results demonstrate that the recombinant NS5 retains immunological determinants of the native virus protein and, more relevantly, is a promising vaccine antigen.

MATERIALS AND METHODS

Animals and ethics statement. Six- to 8-week-old male BALB/c mice were maintained under specific-pathogen-free conditions at the isogenic mouse facility of the Parasitology Department, University of São Paulo, Brazil. All mice were handled according to procedures approved by the Committee for the Ethical Use of Laboratory Animals from the Institute of Biomedical Sciences of the University of São Paulo. All procedures involving human serum handling followed the recommendations of the Institutional Review Board of the Hospital das Clínicas, University of São Paulo (protocol number 0652/09), and all subjects provided written informed consent.

Expression of the recombinant NS5 protein. The customized pUC-NS5 plasmid, encoding the DENV2 JHA1 strain NS5 sequence, was purchased from GenScript (USA). The NS5 sequence was obtained by digestion of the pUC-NS5 with BamHI and XhoI and then ligation into the corresponding restriction sites of the digested expression vector pET28a(+) (Novagen, USA), generating the pETNS5 plasmid. This plasmid was used to transform the chemically competent *Escherichia coli* BL21-CodonPlus (DE3)-RIL strain (Stratagene, USA), resulting in a BLNS5 lineage capable of expressing N-terminally His-tagged constructs for full-length NS5. For the expression test, this bacterial strain was cultivated in LB medium containing 50 μ g/ml of kanamycin and 30 μ g/ml of chloramphenicol at 37°C. Isopropyl beta-D-thiogalactoside (IPTG) was added at a concentration of 0.5 mM when the culture reached an absorbance at 600 nm (A_{600}) of 0.5 (induction for 4 h at 37°C) or an A_{600} of 2.0 (induction for 16 h at 18°C). Culture samples were collected for analysis before and after IPTG induction. After induction, bacterial cells were suspended in lysis buffer (20 mM Na₂PO₄, 0.5 M NaCl, 50 mM L-arginine,

50 mM L-glutamine acid, 10 mM MgSO₄, 5 mM imidazole, 5 mM β -mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol; pH 7.0) and lysed by sonication. After centrifugation (5,000 \times g for 20 min), the soluble and insoluble fractions were recovered. The same amounts of noninduced and induced culture whole extracts, as well as the soluble and insoluble fractions, were dissolved in loading buffer and subjected to SDS-PAGE and Western blot analysis (44).

Western blot analysis. After transfer by blotting, membranes were blocked in blocking buffer (5% of skimmed milk in phosphate-buffered saline [PBS]-Tween 0.05%) overnight at 4°C. Western blot analysis was carried out with 1 h of incubation with a specific anti-NS5 monoclonal antibody (MAb) (GT361, Genetex) (1:5,000 dilution in blocking buffer) at room temperature. After washing, the membrane was incubated with a goat anti-mouse horseradish peroxidase-IgG2a conjugate (1:3,000; Southern Biotechnology) for 1 h at room temperature and then developed with chemiluminescence (SuperSignal kit; Thermo Fisher).

Purification of the recombinant NS5 protein in two steps. Purification of the recombinant NS5 protein was carried out as previously described (38), with modifications. Bacterial cultures were grown in 4 liters of LB medium containing 50 μ g/ml of kanamycin and 30 μ g/ml of chloramphenicol until reaching an A_{600} of 2.0. Protein expression was induced with IPTG at a final concentration of 0.5 mM. Induced cultures were grown for 16 h at 18°C. Cells were harvested by centrifugation at 5,000 \times g for 10 min at 4°C, suspended in lysis buffer, and then lysed by mechanical shearing using an Aplab-10 model homogenizer (Artepeças, Brazil). The lysates were centrifuged at 20,000 \times g for 60 min at 4°C. The supernatants were filtered through a 0.22- μ m filter and subjected to affinity chromatography using HisTrap HP columns (GE Healthcare Life Sciences, United Kingdom) previously equilibrated with buffer A (20 mM Na₂PO₄, 0.5 M NaCl, 10% glycerol; pH 7.0) on an Akta fast-performance liquid chromatograph (Amersham Pharmacia Biotech, United Kingdom). Columns were washed with buffer A, and protein elution was carried out with increasing defined concentrations of imidazole (150, 300, 500, and 1,000 mM imidazole in 20 mM Na₂PO₄, 0.5 M NaCl, and 10% glycerol; pH 7.0). Eluted fractions containing protein were then pooled, dialyzed overnight against dialysis buffer (50 mM Tris buffer [pH 8.0] with 500 mM NaCl and 10% glycerol), and then applied onto a HiPrep 16/60 Sephacryl S-100 HR gel filtration column previously equilibrated with the dialysis buffer. Fractions containing the full-length NS5 were pooled and stocked in endotoxin-free glass vials until use. All glassware was autoclaved for 45 min and then heated at 190°C in a dry oven for 4 h, and water used was pyrogen free. Purified protein samples were quantified using the bicinchoninic acid protein assay kit (ThermoScientific, USA) during all steps of purification. Endotoxin levels in recombinant protein were determined by the *Limulus* amoebocyte lysis method, using the kit LAL QCL-1000 (Lonza, Switzerland) according to the manufacturer's instructions.

Enzyme-linked immunosorbent assay. NS5-specific antibodies (IgG) present in serum samples from both NS5-immunized mice and DENV-infected patients were titrated by enzyme-linked immunosorbent assay (ELISA), as previously described (17). Briefly, polystyrene Maxisorp Nunc microplates (Sigma-Aldrich, USA) were coated with intact or heat-treated (100°C for 20 min) purified recombinant NS5 expressed in *E. coli* (400 ng/well). The plates were washed 3 times with PBS containing 0.05% Tween 20 (PBST) and blocked with 1 \times PBST containing 3% skim milk for 1 h at 37°C. After a new wash cycle, serum samples were serially diluted (log₂) starting at 1:100 and incubated at room temperature for 2 h. After a new wash cycle, the anti-human or anti-mouse IgG antibody, conjugated to peroxidase (Sigma-Aldrich, USA), was added to wells and incubated again for 2 h. After a final washing, plates were developed with sodium citrate buffer (pH 5.8) containing *ortho*-phenylenediamine dihydrochloride (Sigma-Aldrich) and H₂O₂, and the reaction was stopped after 20 min with the addition of 50 μ l of H₂SO₄ at 2 M. The optical density reading was performed at 492 nm with a plate reader (Labsystems Multiscan, ThermoScientific, USA).

Conservancy analysis of *in silico*-predicted NS5 human B cell epitopes. Human B cell epitopes in the DENV2 JHA1 NS5 protein amino acid sequence were predicted *in silico* using the Bepipred linear epitope prediction method (45). Predicted epitopes with higher scores (cutoff of 1.0 for each amino acid) were selected for conservancy analysis. Conservancy analysis was carried out using the epitope conservancy analysis method of the Immune Epitope Database and Analysis Resource (46). The conservancy of human B cell epitopes predicted for the DENV2 NS5 was estimated among the four serotypes of DENV, using four samples of NS5 amino acid sequences representative of DENV1 and DENV3 and five representatives of DENV2 and DENV4. The minimum identity for selection was set to $\geq 60\%$. The number of protein sequences matching a minimal identity of 60% with DENV2 JHA1 strain NS5 protein was determined.

Immunization and challenge. Male BALB/c mice, 6 to 8 weeks old, were subjected to an immunization regimen of three doses of vaccine formulation administered subcutaneously (s.c.) on days 0, 14, and 28. Mice ($n = 10$) were inoculated with 10 μg of NS5 only in PBS or admixed with 22 μg of alum (Rehydragel; Reheis, USA). A control group ($n = 10$) was injected with endotoxin-free PBS (pH 7.0). Mice were bled from the retro-orbital plexus 2 weeks after each vaccine dose. Serum samples were collected after blood coagulation at 37°C for 30 min and at 4°C for 30 min, followed by centrifugation at $3,000 \times g$ for 15 min at 4°C. Serum samples were stored at -20°C until testing. For the challenge assay, mice ($n = 10$) were intracranially (i.c.) challenged with the DENV2 NGC strain or with the neurovirulent DENV2 JHA1 clinical isolate, as previously described (47). Mice were immunized with 10 μg of NS5 (s.c.) in three doses, with the same interval between doses as described above, and the control group was injected with PBS. On day 14 after the last immunization dose, mice were anesthetized with a mixture of ketamine and xylazine and then inoculated by the intracerebral route with 20 μl of viral suspension corresponding to approximately 5×10^5 PFU of NGC DENV2 strain and 1.5×10^2 PFU of JHA1 DENV2, which, in our hands, caused 70% and 100% mortality, respectively, in nonimmunized BALB/c mice. After the challenge, mice were monitored daily for 25 days.

***In silico* prediction of major histocompatibility complex class I- and major histocompatibility complex class II-restricted NS5 epitopes.** DENV2 JHA1 NS5 peptides were predicted *in silico* using a previously described algorithm (48–50) hosted at the Immune Epitope Database and Analysis Resource. Two high-score-predicted peptides were selected for H2-Kd and H2-Dd (major histocompatibility complex class I [MHC-I] allotypes): $^{479}\text{LGARFLEF}^{486}$ and $^{765}\text{MYFHRRDL}^{772}$, and $^{709}\text{CSHHFHEL}^{716}$ and $^{758}\text{YAQMWSLM}^{765}$, respectively. One high-score-predicted peptide was selected for H2-Iad and H2-Ied (major histocompatibility complex class II [MHC-II] allotypes): $^{759}\text{AQMWSLMYFHRRDLR}^{773}$ and $^{401}\text{KKVRSNAALGAIFTD}^{415}$, respectively.

Spleen cell isolation. Mice were euthanized with CO_2 2 weeks after the third immunization, and the spleens were removed aseptically, as previously described (18). Splenocytes were suspended and washed once with RPMI 1640 medium (Sigma) with 2% fetal bovine serum (FBS; Gibco). Red blood cells were lysed with 3 ml of ACK solution (150 mM NH_4Cl , 10 mM KHCO_3 , 0.1 mM EDTA) per spleen for 3 min at room temperature. After two additional washes with RPMI and 2% FBS, the spleen cells were resuspended in R10 medium (RPMI supplemented with 10% fetal bovine serum). Cell viability was assessed using 0.1% trypan blue dye exclusion, and cells were counted using a Neubauer chamber.

Evaluation of IFN- γ and TNF- α by ELISPOT assays. Mice immunized with three doses of 10 μg of NS5 or with PBS (control) were euthanized 2 weeks after the last vaccine dose; their spleens were removed and processed, and their cells were used in the enzyme-linked immunosorbent spot (ELISPOT) assays. Splenocytes (2×10^5 cells/well) were cultured in 96-well plates (Millipore, USA) previously coated with capture antibody to IFN- γ or TNF- α (BD Biosciences, USA) at a concentration of 10 $\mu\text{g}/\text{ml}$. Cells were incubated for 48 h in the presence or absence of the pool of peptides restricted by MHC-I or -II (100 ng of each peptide/well). After

stimulation, cells were discarded, and plates were washed 3 times with PBS and 5 times with PBST, followed by incubation with biotinylated mouse monoclonal antibody against IFN- γ or TNF- α (BD Biosciences, USA) to a final concentration of 2 $\mu\text{g}/\text{ml}$. After 16 h, the plate was again washed and then incubated with a solution containing peroxidase-labeled streptavidin (BD Biosciences, USA) (200 ng/well) for 2 h at 25°C. After washing, plates were developed with diaminobenzidine solution for 30 min. The spots were counted with the aid of a magnifying glass. The number of IFN- γ - or TNF- α -producing cells/ 10^6 splenocytes was calculated after subtracting the negative-control values (number of spots in the wells containing medium only).

Data analysis. Statistical significance values (P values) were calculated using two-way analysis of variance and Bonferroni's test. Student's t test and the Mann-Whitney posttest were used to compare means when necessary. The survival curves were compared using the log-rank test. All tests were calculated using Prism 5 software (GraphPad Software). Differences with P values of < 0.05 were considered statistically significant.

RESULTS

Expression and purification of the recombinant NS5. The DENV2 NS5 coding sequence was inserted in the expression vector pET28a(+), and the plasmid construct was used to transform the *E. coli* BL21-CodonPlus (DE3)-RIL strain. Protein expression was carried out under two initial conditions after the addition of IPTG: for 16 h at 18°C and for 4 h at 37°C. The protein expression was monitored by SDS-PAGE and Western blotting. The recombinant NS5 (107 kDa) was expressed under the tested conditions and in both soluble and insoluble fractions, as shown in Figure 1. Protein expression carried out at 18°C for 16 h (Fig. 1a and c) reduced protein degradation with regard to cells incubated at 37°C for 4 h (Fig. 1b and d). The amount of soluble NS5 expressed in cells kept at 18°C for 16 h was also larger than that detected under the other expression condition.

The soluble NS5 protein was purified by affinity chromatography and eluted with 300 to 500 mM imidazole (Fig. 2a). The eluted fractions contained mainly the full-length NS5 and a 35-kDa degradation product (Fig. 2a). The pooled fractions were subjected to size-exclusion chromatography, and three elution peaks were observed. The first corresponded to NS5 coeluted with bacterium-derived proteins of various molecular masses (retention time, 97 min). The second peak contained only NS5 (107 kDa), with a retention time of 113 min, and the third peak, with a retention time of 143.75 min, corresponded to the 35-kDa degradation product. Under ordinary conditions, we obtained 7.64 mg of purified NS5 protein per liter of culture, with a recovery yield of 37.8% over the total protein obtained after the affinity chromatography step (Table 1). Endotoxin levels per 10 μg of NS5 were between 0.5 and 2.0 endotoxin units (EU)/ml, which is below the maximum value allowed in vaccine formulations for humans (53). These results indicate that recombinant DENV2 NS5 was expressed in high yields and high purity after two purification steps and had small amounts of endotoxin contamination.

The recombinant NS5 preserves immunological determinants of the native viral protein. The recombinant NS5 was tested as solid-phase bound antigen in reactions with serum samples collected from humans infected with different DENV serotypes (Fig. 3). All tested serum samples reacted with the recombinant protein in a conformation-dependent manner, since the detected reading values were significantly reduced after heat denaturation of the protein. The recombinant NS5 was also recognized by an

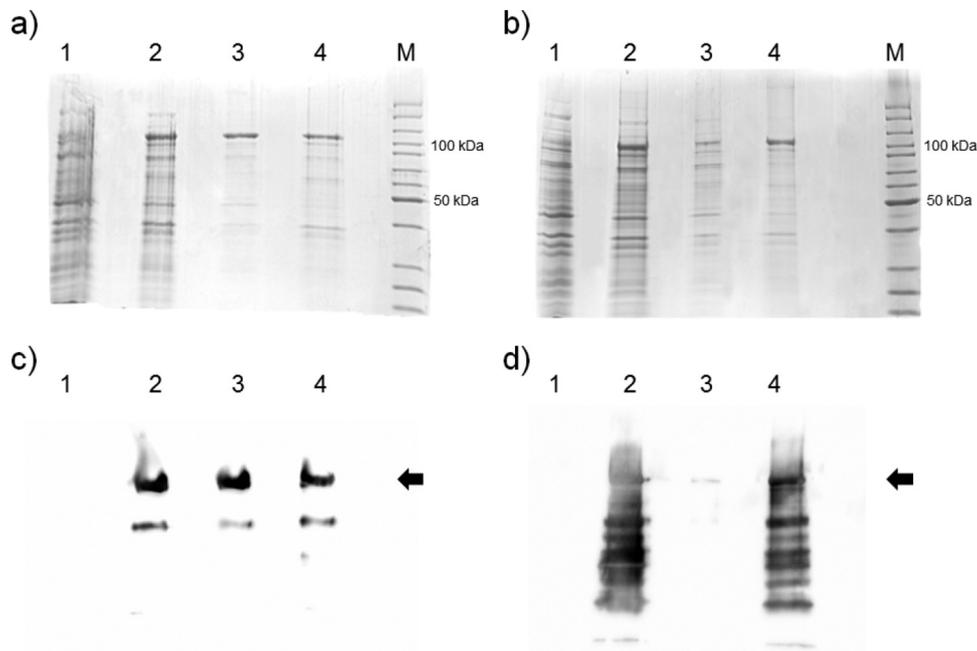


FIG 1 Expression of the recombinant DENV2 NS5 protein produced by *E. coli*. Coomassie blue-stained polyacrylamide gels of whole bacterial cell extracts induced after cultivation with IPTG for 16 h at 18°C (a) or 4 h at 37°C (b). Western blot analysis of the whole-cell extracts for the first (c) and second (d) induction conditions probed with a mouse anti-NS5 MAb; the black arrows indicate the full NS5 expressed (107 kDa). Lane 1, whole-cell extract of noninduced BLNS5 culture; lane 2, whole-cell extract of induced BLNS5 culture; lane 3, soluble protein fraction of BLNS5 culture after IPTG induction; lane 4, insoluble protein fraction of BLNS5 culture after IPTG induction; lane M, molecular size marker.

NS5-specific MAb (GT361) in Western blot assays (Fig. 1c and d). The immunoassay results were supported by the finding that NS5-predicted human B cell epitopes are conserved among all four DENV serotypes (Table 2). Collectively, these results indicated that the obtained DENV2 NS5 preserved immunological features of the native protein.

Immunization with purified NS5 elicits specific IgG antibodies. Immunization of mice with nonadjuvanted NS5 resulted in serum NS5-specific IgG titers (Fig. 4). Addition of alum to the purified NS5 enhanced the serum anti-NS5 antibody titers in immunized mice, but the differences did not reach statistical significance. Determination of IgG subclass responses showed that mice immunized with NS5 adjuvanted with alum produced a higher IgG1-to-IgG2a ratio (9.8) than mice immunized only with NS5 (IgG1-to-IgG2a ratio, 1.5) (Fig. 4b). These results indicated that NS5 is highly immunogenic and induced a balanced serum IgG subclass response in mice.

Induction of cellular immune responses in mice immunized with NS5. Two weeks after the last dose, IFN- γ and TNF- α ELISPOT assays were carried out with spleen cells collected from mice immunized with nonadjuvanted NS5. As shown in Fig. 5a, mice immunized with NS5 accumulated TNF- α -secreting cells, but not IFN- γ -secreting cells, in spleens after *in vitro* stimulation with synthetic peptides corresponding to the predicted MHC-I-restricted epitopes. When the experiment was repeated with synthetic peptides corresponding to MHC-II-restricted epitopes, both TNF- α -secreting and IFN- γ -secreting cells were detected in spleens of immunized mice (Fig. 5b). These results indicate that immunization with NS5 induced specific cellular immune responses, leading to the production

and secretion of TNF- α by CD8⁺ T cells and TNF- α and IFN- γ by CD4⁺ T cells.

Partial protective immunity induced in mice immunized with recombinant NS5. Protective immunity induced after immunization with recombinant NS5 was determined after administration of a lethal *i.c.* challenge with the DENV2 NGC strain and the JHA1 clinical isolate. Immunization with NS5 conferred a protective immunity to the two tested DENV2 strains. Mice immunized with NS5 reached 80% protection after challenge with the NGC strain and 60% protection after challenge with the JHA1 isolate (Fig. 6). No morbidity signs were detected among mice surviving the lethal challenge. In contrast, all mice inoculated with PBS died after challenge with JHA1, and 70% of them died after challenge with the NGC strain; all survivors showed morbidity signs (e.g., paralysis in the hind legs).

DISCUSSION

Dengue fever is presently the main human arboviral infection in several countries. There are no effective treatments or vaccines in clinical use. The development of anti-DENV vaccine candidates historically has been focused on DENV structural proteins (inactivated, attenuated, or chimeric viruses), and, despite enormous efforts, the most advanced approved vaccine formulation so far tested did not confer high levels of protection against the four DENV serotypes (26–28). In contrast, DENV nonstructural proteins have been shown to represent good antigen targets for T cell-based immune responses in DENV-infected subjects (35–37, 54), but their potential use as vaccine antigens has not been fully evaluated. In the present study, we reported the generation of a recombinant DENV2 NS5 at high purity and

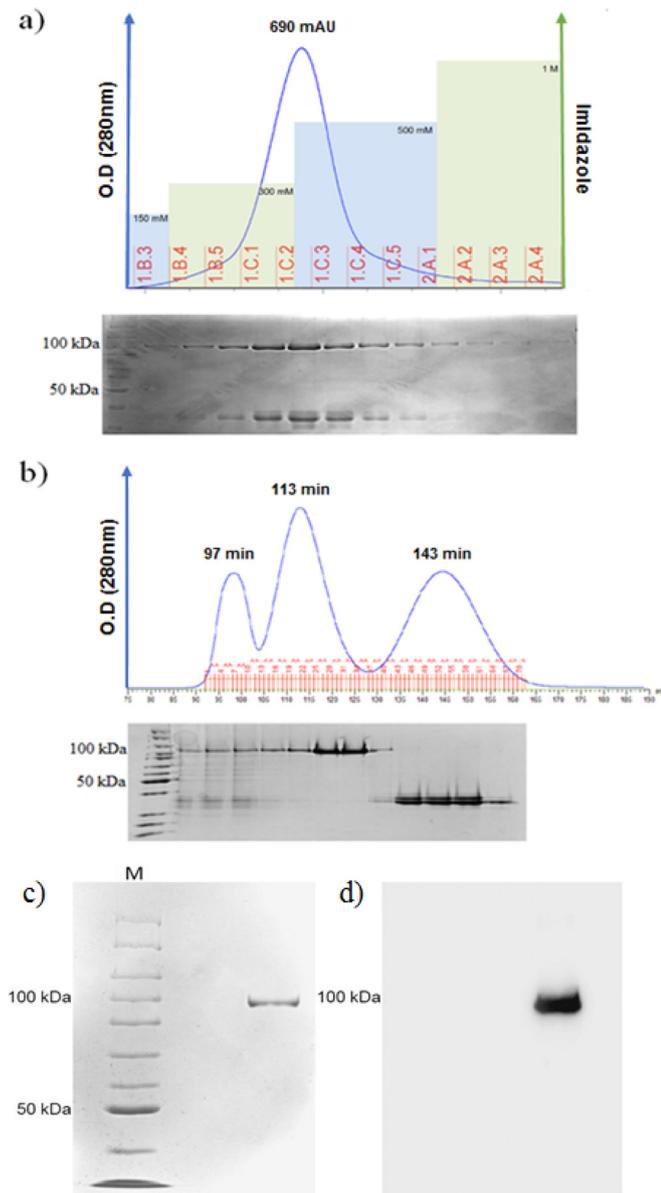


FIG 2 Purification of the recombinant NS5. The soluble NS5 was subjected to two sequential purification steps. (a) Following binding to the nickel column, the protein was eluted with increasing imidazole concentrations: 150 mM, 300 mM, 500 mM, and 1 M. The eluted fractions were pooled and dialyzed with Tris-NaCl buffer (50 mM Tris, 500 mM NaCl, 10% glycerol; pH 8.0). (b) The dialyzed material was subjected to gel filtration, resulting in elution of three peaks at 97, 113, and 143.74 min. (c and d) Comassie blue-stained gel (c) and Western blot (d) of the eluted fractions (1 μ g of purified protein).

recovery yields, and we demonstrated that the recombinant protein retains both antigenic and immunogenic features of the viral protein. Finally, we showed that mice immunized with the recombinant NS5 mounted protective immunity to a lethal challenge with DENV2.

The established conditions for expression and purification of the DENV2 NS5 resulted in the recovery of large amounts and purity of the protein. Furthermore, the purification procedure resulted in low levels of contamination with endotoxin com-

patible with human use (55, 56). The average recovery yield of the protein (approximately 8 mg per liter of induced culture) was 2- to 14-fold higher than those previously reported (51, 52). A previous study showed that NS5 is toxic to *E. coli* cells (57). To overcome this problem, we modified the expression and purification protocol based on previously reported conditions (38). The delay in the induction phase and incubation at a lower temperature resulted in less degradation of the recombinant protein and higher protein yields. The diminished protein degradation seems to have been critical for recovery. Modifications of culture conditions and induction time may favor parameters such as reduced molecular hydrophobic interactions, inhibition inclusion body formation, and lower protein synthesis rate, which in turn favor the correct folding and reduce the activity of proteases (58). Thus, the protocol presented in this work for purification of DENV NS5 results in the highest recovery values so far reported.

So far, there is no report on the use of the DENV NS5 protein as a vaccine antigen. Nonetheless, previous evidence indicates that this protein is a promising DENV antigen. It is the most conserved protein found among all four serotypes and, thus, can potentially induce cross-protective immunity to all four serotypes. DENV NS5 is highly immunogenic in humans, and a recently published study reports that subjects immunized with a tetravalent vaccine formulation based on live attenuated viruses elicited cellular immune responses targeting highly conserved NS5 epitopes (34). In this study, we demonstrated that antibodies from patients infected with different DENV serotypes recognized the purified NS5. These results demonstrated that the recombinant NS5 preserves the antigenicity of the native protein. In addition, heat denaturation of the protein significantly reduced the reaction of the protein with the tested human sera, which indicates that the recombinant protein maintains structural features of the native viral protein. Recognition of NS5 by antibodies from DENV-infected patients has been reported previously (59). Nonetheless, our results demonstrated that antibodies in DENV-infected subjects recognized both linear and conformational epitopes of the protein. In addition, these results demonstrated that NS5 epitopes are immunologically conserved among the different DENV serotypes, as previously inferred by sequence analyses of this protein (60).

The immunogenicity of the recombinant NS5 was tested after s.c. immunization of BALB/c mice. The protein was capable of

TABLE 1 Recovery yields of the different NS5 purification steps^a

Variable	Value ^b (mg/liter)	Yield (%)
After affinity chromatography ^c	20.23	
After dialysis ^d	19.68	97.28
After SEC ^e	7.64	37.80
NS5 ^f	0.5	
NS5 ^g	3.0	

^a The recovery yields refer to 1 liter of IPTG-induced culture.

^b Value represents the NS5 yield relative to 1 liter of IPTG-induced culture, determined by the BCA method (Pierce BCA protein assay kit, Thermo Scientific).

^c All NS5-containing samples obtained after affinity chromatography.

^d Sample obtained after dialysis of the pooled fractions recovered from affinity chromatography.

^e Sample obtained after the size exclusion chromatography (SEC) step.

^f Previously reported yield for recombinant DENV1 GST-NS5 (51).

^g Previously reported yield for recombinant DENV3 NS5 polymerase domain (52).

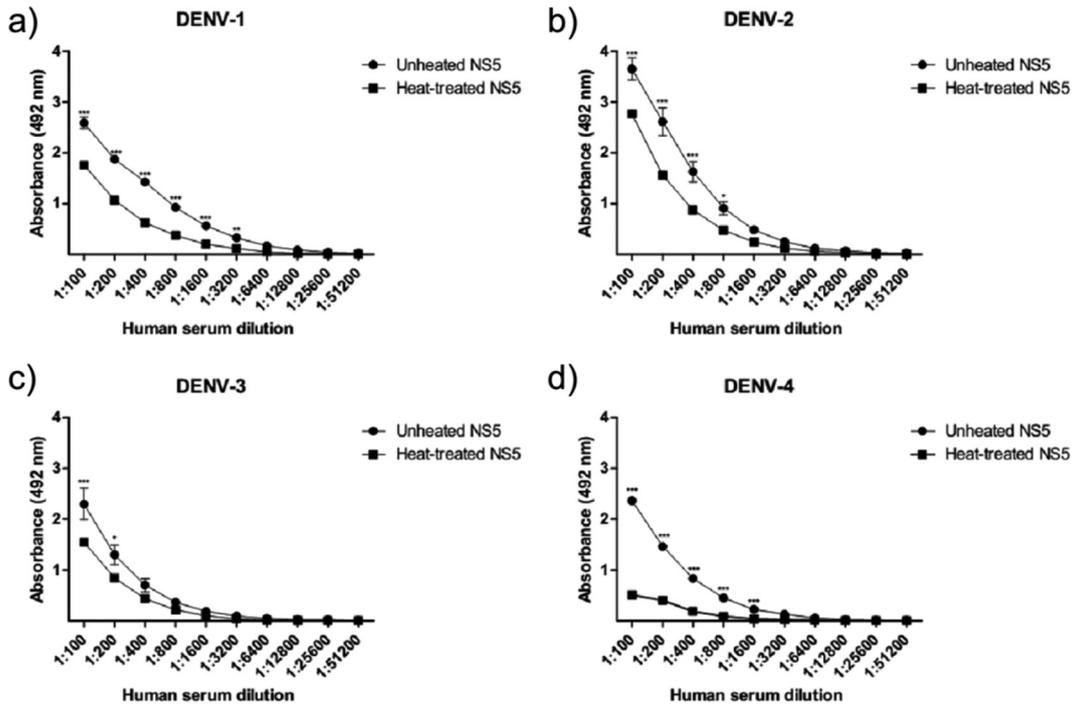


FIG 3 Reactivity of DENV-infected human sera with the recombinant NS5. Sera collected from subjects infected with different DENV serotypes were reacted with purified NS5 (●) and with NS5 subjected to a heat denaturation step (100°C for 20 min) (■) by ELISA. Serum samples were collected from convalescent patients infected with DENV1 (a), DENV2 (b), DENV3 (c), or DENV4 (d). Serum samples were serially diluted (log2), with an initial dilution of 1/100. Each well was adsorbed with 400 ng of recombinant NS5. Statistical analyses were performed by two-way variance analysis tests followed by Bonferroni's posttest. ***, $P < 0.01$; **, $P < 0.1$; *, $P < 0.5$.

generating high NS5-specific IgG antibody levels, and addition of alum did not increase the specific antibody response but did affect the quality of the antibodies. The detected IgG subclass responses in mice immunized with the NS5 showed a more balanced IgG1-to-IgG2a profile, while mice immunized with NS5 admixed with alum showed a prevailing IgG1 response. The

high immunogenicity of NS5 may be ascribed to specific features of the protein, such as the high molecular weight, the chemical complexity, solubility, and the lack of similarity of the protein with self proteins (61, 62).

A significant production of TNF- α and IFN- γ has also been observed after *in vitro* stimulation of spleen cells collected from

TABLE 2 Conservancy analysis of predicted human B cell epitopes *in silico* along the DENV2 NS5 amino acid sequence

Sequence of predicted epitope ^a	Epitope length (aa) ^b	% (fraction) of protein sequence matches at identity $\geq 60\%$ ^c	Minimum identity (%) ^d	Maximum identity (%) ^e
GTGNIGETLGEK	12	100.00 (18/18)	83.33	100.00
GIKRGETDHHAVSR	14	72.22 (13/18)	50.00	100.00
GLTKGGPGHEEIPMS	16	100.00 (18/18)	81.25	100.00
GESSPSPTVEAG	12	100.00 (18/18)	66.67	100.00
KATYEPDVDLGSQTRNIGIESETPNL	26	50.00 (9/18)	53.85	100.00
KQEHETSWHYDQDHPYKWAYHGSYETKQTGSASS	35	100.00 (18/18)	68.57	100.00
VDTRTQEPKEGTK	13	88.89 (16/18)	53.85	100.00
SKKEGGAMYADDTAGWD	17	100.00 (18/18)	70.59	100.00
IQQWEPSRGWNDW	13	100.00 (18/18)	69.23	100.00
PWMEDKTPVESWEE	14	100.00 (18/18)	64.29	100.00

^a Human B cell epitopes were predicted *in silico* along the DENV2 JHA1 NS5 amino acid sequence using the Bepipred linear epitope prediction method (45). Predicted epitopes with higher scores (cutoff of 1.0 for each amino acid) were selected for conservancy analysis.

^b Length of predicted epitopes, in amino acids (aa).

^c The conservancy of human B cell epitopes predicted in DENV2 NS5 was compared among the four serotypes of DENV, using four samples of NS5 amino acid sequences representative of DENV1 and DENV3 and five representatives of DENV2 and DENV4. The minimum identity for selection was set to $\geq 60\%$. Numbers of protein sequences matching a minimal identity of 60% with DENV2 JHA1 strain NS5 protein are given as percent and as fraction. Conservancy analysis was carried out using the epitope conservancy analysis method of the Immune Epitope Database and Analysis Resource (46).

^d Minimum identity found for each epitope predicted for the DENV2 JHA1 NS5 protein compared to its counterparts found in NS5 amino acid sequences from other DENV strains or serotypes.

^e Maximum identity found for each epitope predicted for the DENV2 JHA1 NS5 protein compared to its counterparts found in NS5 amino acid sequences from other DENV strains or serotypes.

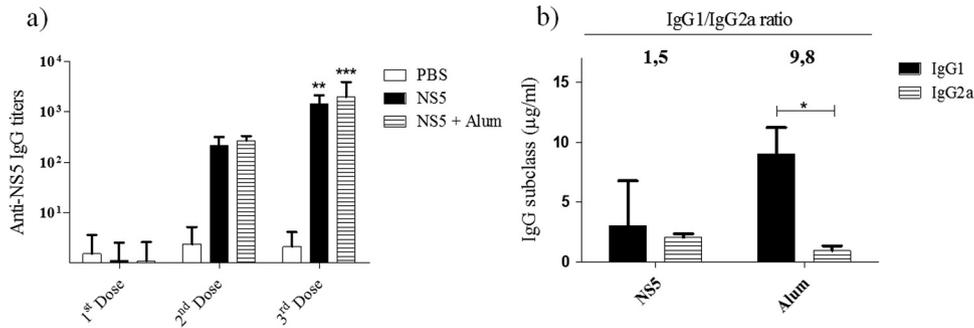


FIG 4 Evaluation of the serum antibody responses generated in mice immunized with NS5. BALB/c mice were s.c. immunized with only 10 µg of NS5 (NS5) or NS5 admixed with alum (NS5 + Alum) in three doses at intervals of 2 weeks. (a) Two weeks after each immunization, sera from mice were subjected to ELISA for detection of anti-NS5 serum IgG titers. Values are expressed as mean ± standard deviation. (b) NS5-specific IgG subclass responses after the third vaccine dose. The value at the top represents the IgG1-to-IgG2a subclass ratio. Background values detected in sham-treated mice (PBS) were deduced from values determined in other immunization groups. Data are representative of at least two independent experiments with similar results. Statistical analyses were performed by two-way variance analysis tests followed by Bonferroni's posttest (a) and Student's *t* test followed by the Mann-Whitney posttest (b). ***, *P* < 0.01; **, *P* < 0.1; *, *P* < 0.5.

mice immunized only with NS5. The production of TNF-α is associated with hemorrhagic phenomena in C57BL/6 mice in a DENV2 infection model, and treatment with anti-TNF-α in such a model led to increased survival (63). However, in contrast with C57BL/6 mice, BALB/c mice have a Th-2 bias of innate immune response, which may explain, at least in part, the results observed after immunization with NS5 (64). The responses were associated with both MHC-I- and MHC-II-restricted epitopes, recognized by CD8⁺ and CD4⁺ T cells, respectively. Such T cell-based response patterns are usually observed after endogenous protein expression and are not expected to be found in animals immunized with nonadjuvanted protein (43). Further studies will address the inherent high immunogenicity of the NS5 and its capability to trigger cross-presentation to CD8⁺ T cells by anti-gen-presenting cells.

Immunization of the BALB/c mice with the nonadjuvanted recombinant NS5 conferred partial protective immunity to lethal challenges carried out with two DENV2 strains. As far we know, this is the first report in which DENV NS5 has been tested as a vaccine antigen candidate, and it further supports the potential role of this protein in the generation of protective immunity to DENV. Recently, NS5 was recognized as the most prominent

DENV antigen, based on the activation of multifunctional CD8⁺ T cells in subjects immunized with a live trivalent vaccine (35). In addition, induction of TNF-α and IFN-γ expression after NS5 immunization may also play a role in T-cell antiviral inactivation mechanisms that do not depend on T-cell cytotoxic activity (65). Indeed, enhanced TNF-α and IFN-γ production induced by nonstructural proteins has been repeatedly detected in DENV-infected primates and humans (31, 37). Although the relevance of the T cell responses in the generation of protective immunity to DENV by vaccines in humans is still a matter of debate, we have recently demonstrated that induction of T cell-based responses is more relevant than antibody responses in mice subjected to the i.c. lethal-challenge DENV model (66).

It is worth mentioning that the partial protection achieved in mice immunized with NS5 was observed without the addition of any vaccine adjuvant. It is well known that the immunogenicity, regarding activation of T cells and production of antibodies, induced by vaccines containing recombinant purified proteins can be significantly improved after addition of different adjuvants. Similarly, the conserved nature of the NS5 protein encoded by the four different DENV types is expected to confer at least partial protection to DENV types other than DENV2. Thus, the testing of

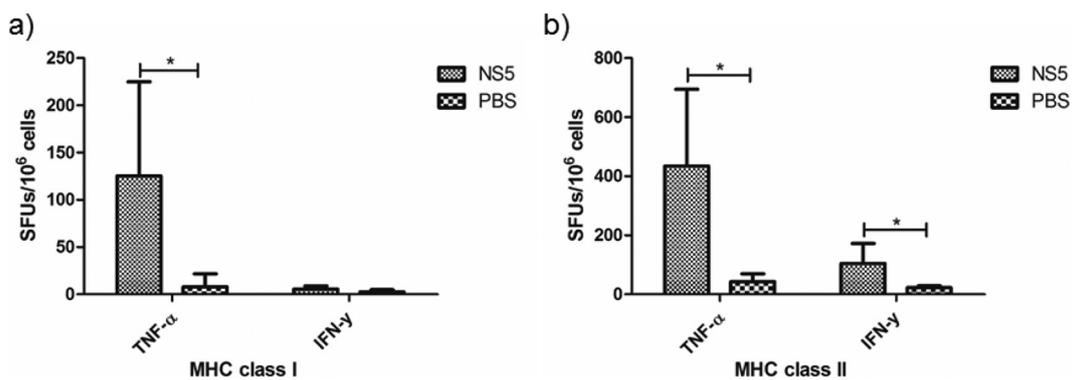


FIG 5 Detection of IFN-γ- and TNF-α-secreting cells in spleens of mice immunized with NS5. Two weeks after the last dose, the numbers of IFN-γ- and TNF-α-secreting cells in spleens of immunized mice were determined in ELISPOT assays using pools of NS5-specific MHC-I-restricted (a) or MHC-II-restricted (b) synthetic peptides. Data are representative of at least two independent experiments with similar results. The *t* test, followed by the Mann-Whitney test, was used to compare means of cytokine-secreting cells. SFU, spot-forming units. *, *P* < 0.05.

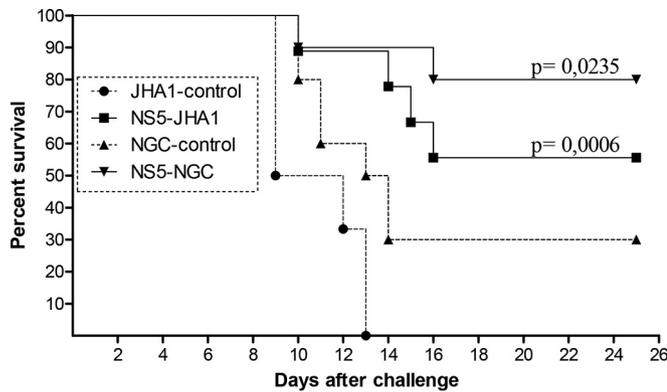


FIG 6 Protective anti-DENV2 immunity induced in mice immunized with NS5. Two weeks after the last immunization, mice were i.c. challenged with 5×10^5 PFU of the DENV2 NGC strain or 1.5×10^2 PFU of the JHA1 clinical isolate. Survival was monitored for 25 days. Each test survival curve was compared to the respective control using the log-rank test, and the *P* value is represented.

NS5-based vaccine formulations containing different adjuvants and measurement of immune responses and protective immunity induced in appropriate mouse models represent priorities presently under study by the team.

In conclusion, these results demonstrated that generation of a recombinant form of the DENV2 NS5 represents an important tool in the evaluation of cellular and molecular mechanisms involved in the generation of a protective immunological status to DENV and may contribute to the development of vaccines and antiviral drugs.

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