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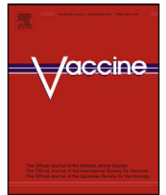

Protective immunity to DENV2 after immunization with a recombinant NS1 protein using a genetically detoxified heat-labile toxin as an adjuvant

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ABSTRACT

The dengue virus non-structural 1 (NS1) protein contributes to evasion of host immune defenses and represents a target for immune responses. Evidences generated in experimental models, as well as the immune responses elicited by infected individuals, showed that induction of anti-NS1 immunity correlates with protective immunity but may also result in the generation of cross-reactive antibodies that recognize platelets and proteins involved in the coagulation cascade. In the present work, we evaluated the immune responses, protection to type 2 dengue virus (DENV2) challenges and safety parameters in BALB/c mice vaccinated with a recombinant NS1 protein in combination with three different adjuvants: aluminum hydroxide (alum), Freund's adjuvant (FA) or a genetically detoxified derivative of the heat-labile toxin (LT_{G33D}), originally produced by some enterotoxigenic *Escherichia coli* (ETEC) strains. Mice were subcutaneously (s.c.) immunized with different vaccine formulations and the induced NS1-specific responses, including serum antibodies and T cell responses, were measured. Mice were also subjected to lethal challenges with the DENV2 NGC strain. The results showed that maximal protective immunity (50%) was achieved in mice vaccinated with NS1 in combination with LT_{G33D}. Analyses of the NS1-specific immune responses showed that the anti-virus protection correlated mainly with the serum anti-NS1 antibody responses including higher avidity to the target antigen. Mice immunized with LT_{G33D} elicited a prevailing IgG2a subclass response and generated antibodies with stronger affinity to the antigen than those generated in mice immunized with the other vaccine formulations. The vaccine formulations were also evaluated regarding induction of deleterious side effects and, in contrast to mice immunized with the FA-adjuvanted vaccine, no significant hepatic damage or enhanced C-reactive protein levels were detected in mice immunized with NS1 and LT_{G33D}. Similarly, no detectable alterations in bleeding time and hematological parameters were detected in mice vaccinated with NS1 and LT_{G33D}. Altogether, these results indicate that the combination of a purified recombinant NS1 and a nontoxic LT derivative is a promising alternative for the generation of safe and effective protein-based anti-dengue vaccine.

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1. Introduction

Dengue fever is a common mosquito-borne viral disease that represents a major worldwide public health concern, particularly for those living in tropical countries and people traveling to these zones. Globally, more than 2.5 billion people are exposed to dengue virus (DENV) infection in endemic areas, and thousands of them die each year [1]. The spread of the virus observed over the last 25 years and the growing numbers of the more serious clinical cases, the dengue hemorrhagic fever (DHF) and the dengue shock syndrome (DSS), underscore the need for an effective anti-dengue vaccine [2,3]. Efforts to develop a DENV vaccine have mainly focused on attenuated or inactivated virus-based vaccine formulations. Despite the success of similar vaccine approaches in controlling other Flaviviruses, such as the yellow fever virus and the Japanese encephalitis virus, and several clinical trials conducted using most promising formulations, an effective dengue vaccine is still not available for human use [4–6]. Inefficient induction of protective immunity to the four viral types (DENV1, 2, 3 and 4),

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and safety concerns involving induction of antibody dependent enhancement (ADE), a mechanism believed to be involved in DHF and DSS occurrence, and deleterious cross-reactive reactions are the most relevant obstacles for the development of an effective dengue vaccine based on live virus particles [7].

DENV subunit vaccine formulation, based either on DNA or purified recombinant proteins represent safer alternatives to attenuated or recombinant viruses [3]. The most studied subunit vaccine approaches for dengue virus are based on either the complete envelope glycoprotein or fragments of this protein [1,8–11]. Immunization of mice with the DENV non-structural protein 1 (NS1), either as purified protein or encoded by DNA vaccines, have also shown promising results [12–16]. The DENV NS1 is a highly immunogenic 46–50 kDa glycoprotein expressed by infected cells both as a secreted oligomeric form and as a membrane-associated protein [17,18]. Although the precise functions of NS1 in the infection cycle remains unclear, it is accepted that this protein has an important role in the viral pathogenesis interfering with the complement activation cascade [19].

Mice immunized with NS1-based vaccines, particularly those encoded by DNA vaccines, develop protective immunity that involves both antibody and T cell responses [14–16]. In contrast, the protective immunity generated in mice immunized with purified NS1 protein alone seems to be based mainly on the generation of antigen-specific serum antibodies [12,13,20,21]. However, further studies have raised concern regarding the safety of NS1 as a vaccine antigen. Anti-NS1 antibodies detected in infected subjects or elicited in vaccinated mice may cross-react with proteins exposed on the surface of platelets, endothelial cells and proteins involved in the blood coagulation cascade, which may lead to vascular damages, thrombocytopenia and hemorrhage [22–27].

Adjuvants are key components of most vaccine formulations, particularly those based on purified proteins. Besides reducing the amount of antigen and number of doses required to achieve a specific immune response, adjuvants are modulators of the adaptive immunity but may lead to deleterious inflammatory reactions [28]. During decades aluminum hydroxide (alum) has been the only adjuvant alternative for human use. Nonetheless, new adjuvant alternatives became recently available for human use, such as MF59 and ASO4 [28,29]. Clearly, the identification of safe and effective adjuvants represents a key step on the development of new vaccine formulations.

The heat-labile enterotoxins (LT) are AB-type toxins produced by some enterotoxigenic *Escherichia coli* (ETEC) endowed with powerful adjuvant effects on both humoral and cellular immune responses to co-administered antigens [30,31]. Due to the intrinsic toxic effects of mucosal-delivered LT, attenuated or nontoxic LT mutants with preserved adjuvanticity have been generated by site-directed mutagenesis [31]. LT_{K63}, LT_{R72} and LT_{R192G}, with amino acid changes in the A subunit, and LT_{G33D} with a single point mutation at the B subunit, are the best characterized LT derivatives regarding both biological effects and immunological activities [32–35]. Replacing the glycine at position 33 of the B subunit with aspartate (G33D) abolishes LT binding to the GM1 ganglioside receptor and, consequently, reduces the toxin adjuvanticity following delivery via oral route [33]. Nonetheless, parenteral administration of LT_{G33D} has been shown to preserve the adjuvant properties of the protein for both B and T cell responses against co-administered antigens without induction of deleterious inflammatory reactions [35].

In this study, we evaluated the efficacy of anti-DENV vaccines based on a recombinant NS1 protein derived from type 2 DENV (DENV2) generated in a prokaryotic expression system with preserved structural and immunological features [36]. Vaccine formulations based on the recombinant NS1 protein admixed with three different adjuvants, alum, Freund's adjuvant [FA] and LT_{G33D}, were tested in mice trough parenteral administration. The results demonstrated that the adjuvant choice strongly affects both the immunogenicity and, more relevantly, the induction of protective immune responses in vaccinated mice. The results also indicate that the combination of recombinant NS1 and LT_{G33D} generates protective antibody responses without the induction of significant deleterious side effects.

2. Materials and methods

2.1. Ethics statement

All handling procedures and experiments involving mice were approved by the committee on the ethical use of laboratory animals from the Institute of Biomedical Sciences of São Paulo University, in accordance with the recommendations in the guidelines for the care and use of laboratory animals of the National Committee on the Ethics of Research (CONEP).

2.2. Virus and cell lines

The dengue 2 virus (DENV-2) strain New Guinea C (NGC) was used in the challenge assays [16,37,38]. DENV-2 NGC strain propagation was carried out in Vero cells cultured in medium 199 with Earle salts (E199) buffered with sodium bicarbonate (Sigma, USA), supplemented with 10% fetal bovine serum (FBS).

2.3. Generation of the mutated elt_{G33D} gene and purification of the recombinant NS1 and LT_{G33D}

The etl_{G33D} gene, mutated at amino acid position 33 of the B subunit, was generated by overlap extension splicing using the *elt* gene sequence of the ETEC H10407 strain [39,40]. The external primers used were 5′-CACGGTACCTCTTTCTTTATCG-3′ (*KpnI* restriction site underlined) and 5′-GGTTCTCTGCAGAGACATGC-3′ (*PstI* restriction site underlined). The internal primers responsible for introducing the mutation leading to the amino acid replacement G33D were 5′-GAATCGATGGCAGATAAAAG-3′ and 5′-CTCTTTTATCTGCCATCGAT-3′. The amplification reactions were performed as described previously [39]. The resulting fragment was purified using a gel purification kit (Ilustra™ GFX™ PCR DNA and Gel Band Purification Kit, GE Healthcare), digested with restriction enzymes and then ligated into the corresponding *KpnI* and *PstI* sites of the linearized pBSPKS (−) vector [41], generating the recombinant plasmid $pKSLT_{G33D}$. The $pKSLT_{G33D}$ plasmid was subsequently introduced into chemically competent *E. coli* DH5α bacteria. One bacterial clone carrying the correct plasmid was named $LDVLT_{G33D}$. The correct sequence of the etx_{G33D} gene was confirmed by DNA sequencing. LT_{G33D} was purified by galactose-affinity chromatography following a standard LT purification procedure [40]. Briefly, the $LDVLT_{G33D}$ lineage was cultivated in Terrific Broth (TB) [42], containing 200 μg/ml of ampicillin, overnight at 37 °C in an orbital shaker set at 200 rpm. Cells were suspended at a 10% (w/v) concentration in TEAN buffer (50 mM Tris; 1 mM EDTA; 3 mM azide-Na and 200 mM NaCl; pH 7.5) and lysed by mechanical shearing in an APLAB-10 homogenizer (ARTEPEÇAS, Brazil). The soluble extract was applied into a XK 16/20 column (GE Amershan Biosciences) containing immobilized D-galactose gel (Pierce), extensively washed with TEAN buffer prepared with pyrogen-free water, and subsequently eluted with TEAN buffer containing 0.3 M galactose. The final amount of LT_{G33D} was determined in GeneQuant spectrophotometer (GE Amershan Biosciences). The purification of the DENV2 NS1 recombinant protein was achieved after denaturation/refolding steps of the protein expressed in bacterial cells and affinity chromatography, as previously reported

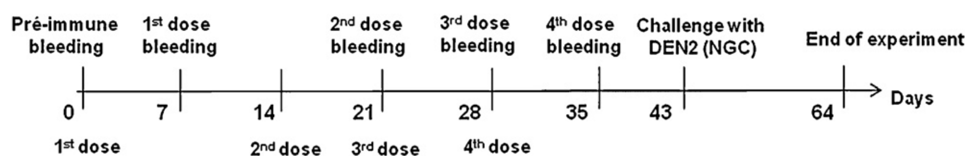

Fig. 1. Immunization regimen and virus challenge with the tested NS1-based vaccine formulations.

[36]. Endotoxin levels in LT_{G33D} and NS1 preparations were determined with the Chromogenic Limulus Amebocyte Lysate assay (Cambrex Bio Science) [43].

2.4. ELISA and immunoblot analyses of NS1 and LT_{G33D}

The recombinant NS1 and LT_{G33D} proteins were analyzed for purity and antigenicity by SDS-PAGE and Western blot. Protein aliquots (2 μg) were sorted in 15% polyacrylamide gels after heat treatment (100 °C for 10 min) or kept at room temperature with sample buffer [36,44]. Standard ELISA assays were performed as previously described [36,45]. The recombinant NS1 protein was tested in the non-heated or in heat-denatured state with serum samples collected from a DENV2-infected individual (kindly supplied by Dr. Bergman M. Ribeiro, Brasília University, FD, Brazil). A serum sample generated after immunization of mice with heat-denatured (100 °C for 10 min) NS1 in FA after the same immunization regimen described bellow (Fig. 1), was used in order to demonstrate that heat denaturation of the recombinant NS1 did not affect binding to ELISA plates. GM1-ELISAs using purified LT_{G33D} and parenteral LT derived from ETEC H10407 strain were carried out as reported previously [40].

2.5. Immunization regimens

BALB/c mice, 4–6 weeks old, were divided into groups (n = 6 for immune response monitoring and n = 10 for the virus challenges) and submitted to an immunization regimen comprising four doses of the tested vaccine formulations administered via the subcutaneous (s.c.) route on days 0, 14, 21 and 28 (Fig. 1). Mice were inoculated with 10 μg of NS1 alone or the same amount of NS1 combined with: 1.25 μg of alum (Rehydragel from Reheis), according to a standard procedure [46] that results in 99.7% binding of the protein to the solid matrix, Freund's adjuvant (50%, v/v), with the complete adjuvant in the first dose and the incomplete formulation in the subsequent injections; or 1 μg of LT_{G33D}. The amount of LT_{G33D} used in the vaccine formulations was based on previously reported results [36]. Sham-treated mice were injected with phosphate buffered saline (PBS). Mice were bled at the retro-orbital plexus before each vaccine dose and one week after the last administration. Serum samples were individually tested for reactivity to NS1, pooled and stored at −20 °C for subsequent analyses.

2.6. Determination of anti-NS1 serum antibody responses

Mouse sera were tested individually for the presence of NS1-specific antibodies by ELISA, as previously described [45]. Briefly, MaxiSorp plates (Nunc) were coated with 0.2 μg per well of the recombinant NS1 protein in 100 μL PBS and blocked for 1 h at 37 °C with 5% skim milk in 0.05% Tween-20–PBS (PBST). Serum samples were serially diluted and added to wells previously washed with PBST. After 1 h at room temperature, plates were washed with PBST and incubated with goat anti-mouse immunoglobulin (whole IgG isotype, IgG1 or IgG2a subclasses) conjugated with horseradish peroxidase (Southern Biotechnology) for 1 h at room temperature. Reactions were measured at $A_{490\,nm}$ with *ortho*-phenylenediamine dihydrochloride (Sigma) and H_2O_2 as substrate and with a 2 N H_2SO_4 stopping solution. Titers were established as the reciprocal of serum dilution which gave an absorbance two-fold higher than the SD values of the respective non-immunized samples.

2.7. Determination of cytokine-secretion patterns

One week after the last immunization, mice were euthanized and their spleens were harvested. Splenocytes were pooled and seeded (5×10^5 cells per well) in 12-well plates (Nunc) in RPMI supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 2 mM nonessential amino acids, 10 mM HEPES buffer and 50 units/ml of penicillin–streptomycin. Cells were then incubated with purified NS1 at 37 °C with 5% CO_2 for 48 h. Culture supernatants were collected and tested individually for IFN-γ and IL-5 by ELISA, according to the manufacturer's instructions (BD Bioscience), as markers for activation of type 1 and type 2 Th responses, respectively.

2.8. Lethal challenges with DENV2

Two weeks after the final vaccine dose, mice were challenged with the DENV2 NGC strain, a mouse-adapted virus strain, as previously described [16,37,38]. Animals were anesthetized with a mixture of ketamine and xylazine [47] and intra cranially (i.c.) challenged with 30 μl of E199 medium supplemented with 5% FBS containing 4.32 $\log_{10}$ PFU of DENV-2, which corresponds to approximately 3.8 LD_{50}. Animals were monitored for 21 days, and mortality and morbidity rates were recorded.

2.9. ELISPOT

The IFN-γ ELISPOT assay was performed as previously described [40]. Two weeks after the immunization regimen, cells derived from spleens of vaccinated mice were placed (2×10^5 cells/well) in a 96-well micro titer plate (MultiScreen, Millipore) previously coated with 10 μg/ml of rat anti-mouse INF-γ monoclonal antibody (mAb) (BD Pharmingen). Cells were cultured at 37 °C with 5% CO_2 for 18 h in the presence or absence of 5 μg of the H-2d-restricted $CD8^+$ T cell-specific epitope AGPWHLGKL ($NS1_{265–273}$), a highly conserved epitope among the DENV serotypes [48]. As a positive control, cells from all groups were pooled and cultured in the presence of concanavalin A, as previously described [49]. After incubation, cells were washed away, and plates were incubated with a biotinylated anti-mouse INF-γ mAb (BD Pharmingen) at a final concentration of 2 μg/ml at 4 °C. After 16–18 h, the plates were incubated with diluted peroxidase-conjugated streptavidin (Sigma–Aldrich). The spots were developed using diaminobenzidine (DAB) substrate (Sigma–Aldrich) and counted with a stereo microscope (model SMZ645, Nikon).

2.10. In vivo evaluation of NS1-specific cytotoxic $CD8^+$ T lymphocytes

The in vivo assessment of the cytotoxic activity of $CD8^+$ T cells induced in the different immunization groups was carried out as previously described [40]. Splenocytes from naive mice were stained with 0.5 μM or 5 μM carboxyfluorescein diacetate

succinimidyl ester (CFSE) (Invitrogen) for 15 min at 37 °C. The cells labeled with 5 μM of CFSE were then pulsed with the $NS1_{265–273}$ oligopeptide (AGPWHLGKL) [48,50]. Both CFSE-labeled cell populations, $NS1_{265–273}$ pulsed or not, were transferred intravenously to vaccinated mice (2×10^7 cells of each population). One day later, the inoculated animals were euthanized and individual spleens were isolated to identify the two CFSE-labeled cell populations by multivariant FACScan analyses (FACSCalibur from BD Biosciences). The percentages of specific target cell killing were calculated for each individual by comparing the reduction of peptide-pulsed cells relative to that of the non-pulsed cells.

2.11. NS1-antibody affinity determination

The affinity of anti-NS1 antibodies was assessed by the ammonium thiocyanate elution-ELISA method, as previously described [51]. The procedure was similar to that of the standard ELISA with the inclusion of an extra step. After incubation with the pooled sera diluted according to titers obtained by ELISA, the plates were washed and ammonium thiocyanate, diluted in PBS, was added to the wells in concentrations ranging from 0 to 8 M. Plates were maintained at room temperature for 15 min. The percentage of antibody binding was calculated as the OD_{490} in the presence of ammonium thiocyanate × 100, divided by the OD_{490} in the absence of ammonium thiocyanate.

2.12. Monitoring tissue damages in vaccinated mice

Individual serum samples were used to determine glutamic oxalacetic transaminase (GOT), glutamic pyruvic transaminase (GPT) and C-reactive protein (CRP) levels, using analytical kits as recommended by the supplier (Bioclin, Brazil).

2.13. Bleeding time and blood cell analyses

Bleeding time was measured at day seven following the fourth vaccine dose by creating a 3 mm incision at the tail tip. Blood droplets were collected on filter paper every 30 s for the first 3 min, and every 10 s thereafter. Bleeding was considered to be finished when the collected blood spot's diameter was less than 0.1 mm [22]. Complete blood cell counts were also taken at this time. Whole blood samples were collected in micro tubes containing 0.37 M EDTA. For hematocrit determination, micro capillaries were filled with blood samples, centrifuged at 5000 rpm for 5 min and properly positioned in a packed cell volume table for hematocrit scoring [52]. Red blood cell (RBC) and white blood cell (WBC) counts were carried out using a Neubauer chamber. Platelet numbers were determined according to the Fonio's method and neutrophil and lymphocyte differentiation was performed visually using a phase contrast microscope [52], (Eclipse E200 model, Nikon).

2.14. Statistical analyses

Statistical analyses were carried out using ANOVA and a subsequent Bonferroni's Multiple Comparison test. For survival and morbidity rates, Mantel–Cox and Gehan–Breslow–Wilcoxon tests were performed. Statistical significance was set as $p < 0.05$.

3. Results

3.1. Generation of purified NS1 and LT_{G33D}

Both NS1 and LT_{G33D} were produced by recombinant *E. coli* cells and tested for antigenicity and/or biological activity. The recombinant DENV2 NS1 protein was obtained mainly as dimers, as demonstrated after sorting in polyacrylamide gels (Fig. 2A). As demonstrated previously [36], the recombinant NS1 preserved, at least partially, some features of the native virus protein. In addition, the recombinant NS1 retained, at least in part, the antigenicity of the native protein as demonstrated by the reactivity of the recombinant protein with a serum sample collected from a DENV2 infected patient (Fig. 2B). The reactivity of the anti-NS1 serum sample was drastically reduced after heat denaturation of the recombinant protein, which indicates that conformational epitopes of the protein were lost. To demonstrate that the heat-denaturation treatment did not interfered with the binding of protein to the ELISA plates, the protein samples were reacted with a mouse serum raised in mice immunized with a heat-denatured NS1 (Fig. 2B). In contrast to antibodies raised in the DENV2 infected subject, this serum sample did not show any reduction in the recognition of the heat-denatured NS1 in ELISA, which indicated that denaturation of the recombinant protein did not affect the binding of the protein to the plate. The purified recombinant LT_{G33D} protein encompassed both the A and B subunits, as detected in polyacrylamide gels (Fig. 2C). As expected, the recombinant protein showed reduced binding to the GM1 ganglioside when compared to the parental toxin (Fig. 2D), as previously reported [35]. Both NS1 and LT_{G33D} preparations had low residual LPS concentrations (50 EU/mg and 82 EU/mg, respectively). The amount of endotoxin administered in each mice was 0.5 endotoxin units/dose and 0.582 endotoxin units/dose in samples containing NS1 alone or NS1 and LT_{G33D}, respectively, which did not interfere with the induced immune response of vaccinated mice (data not shown) [43].

3.2. Immune responses in mice immunized with NS1-containing vaccines

To determine the immunogenicity of the recombinant NS1 protein, BALB/c mice were s.c. immunized with the purified protein admixed with one of three different adjuvants (alum, FA or LT_{G33D}) using a four-dose vaccine regimen (Fig. 1). Under the testing conditions, 99.7% of the NS1 protein remained bound to the alum salts, while vaccines adjuvanted with FA or LT_{G33D} were prepared according to previously reported conditions [35,46]. Measurement of the serum anti-NS1 IgG responses showed that mice immunized with three or four doses of NS1 admixed with LT_{G33D} elicited stronger responses than those immunized with vaccines containing alum or FA ($p < 0.001$). In addition, assessment of the serum IgG subclass responses showed that mice immunized with NS1 and alum produced low IgG2a levels (IgG1/IgG2a ratios of 83) while those immunized with NS1 in combination with FA or LT_{G33D} elicited more balanced subclass responses with IgG1/IgG2a ratios of 4.3 and 1.8, respectively. A similar response profile was observed when assessing IFN-γ and IL-5 secretion in the culture supernatants of NS1-stimulated spleen cells collected from mice immunized with the three different vaccine formulations. As demonstrated in Fig. 3C, the IFN-γ/IL-5 ratio (5.74) detected in mice immunized with NS1 and LT_{G33D} was higher than the ratios detected in mice immunized with NS1 combined with alum or FA (0.32 and 3.52, respectively). Interestingly, mice immunized with LT_{G33D} and NS1 generated serum antibodies with enhanced avidity to the NS1 protein (Fig. 3D). The concentration of ammonium thiocyanate required to dissociate 50% of the antibodies bound to NS1 in sera collected from mice immunized with LT_{G33D} was approximately two and four-fold higher than the amounts of the reagent required to dissociate anti-NS1 antibodies generated in mice treated with FA and alum, respectively.

We also measured the induced T cell responses in mice immunized with the different NS1-based vaccine formulations. As shown in Fig. 3E and F, the tested vaccine formulations induced low anti-NS1 $CD8^+$ T cell responses in mice, as measured by the numbers of

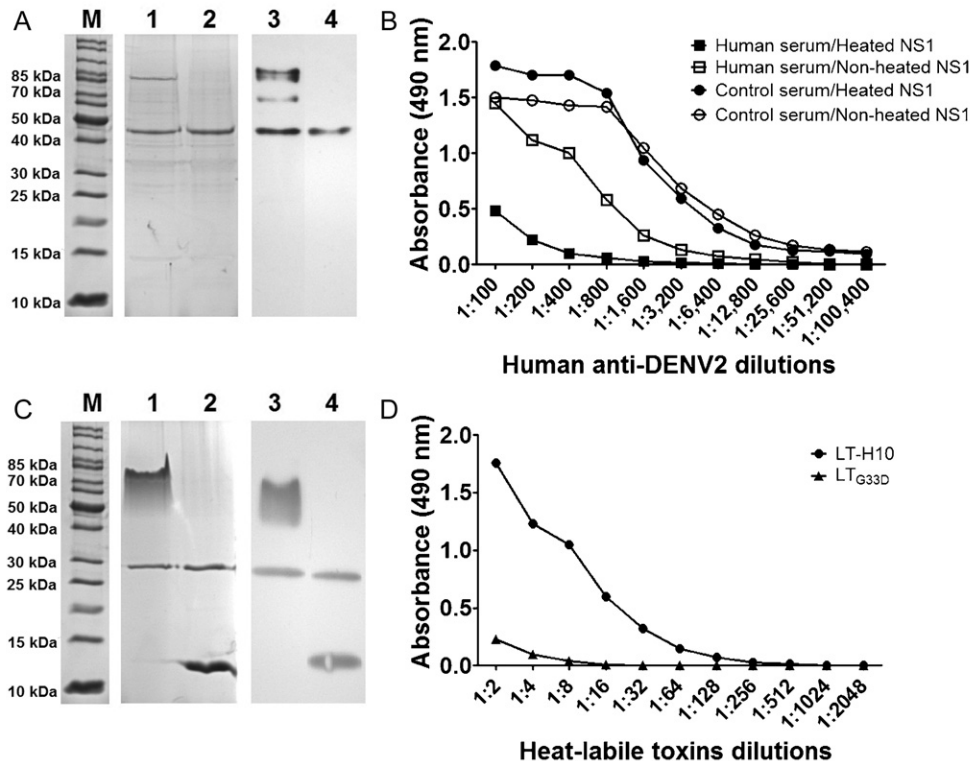

Fig. 2. Electrophoretic patterns and biological activities of the recombinant NS1 and LT_{G33D}. (A) Purified refolded NS1 protein detected in a SDS-polyacrylamide gel (lanes 1 and 2) and immunoblot (lanes 3 and 4). Aliquots of the purified protein (1 μg) were sorted in a 15% polyacrylamide gel stained with Coomassie brilliant blue (lanes 1 and 2) or transferred to a nitrocellulose membrane and reacted with serum collected from mice immunized with a DNA vaccine encoding the DENV2 NS1 protein (lanes 3 and 4). Heat-denatured samples (lanes 2 and 4) were obtained following protein incubation at 100 °C for 10 min M, molecular weight markers (band sizes indicated at the left side). (B) Reactivity of the intact recombinant NS1 protein with a serum sample collected from a DENV2-infected patient (open squares). Heat treatment (100 °C for 10 min) abolished the antigenicity of the protein as demonstrated by the lack of reactivity with the serum antibodies (closed squares). The control serum generated in mice against the heat-denatured form of the recombinant NS1 reacted in the same degree with the non-heated (closed circles) and the heated (open circles) forms of the antigen, showing that heat treatment did not affect NS1 binding to the plate. (C) Purification of the LT_{G33D} by galactose-affinity chromatography. Protein samples (1 μg) were sorted in 15% polyacrylamide gels and stained with Coomassie brilliant blue (lanes 1 and 2) or reacted with an anti-LT mouse serum in a Western blot (lanes 3 and 4). Heat-denatured samples (lanes 2 and 4) were obtained following incubation of the protein at 100 °C for 10 min M, molecular weight markers (band sizes indicated at the left of the figure). (D) GM1-ELISA of the purified non-toxic LT_{G33D} and the parental non-mutated LT1. GM1-binding mediated by the B subunit was ablated by the G33D B subunit mutation in LT_{G33D}.

NS1-specific IFN-γ secreting cells. In addition, experiments carried out with mouse splenocytes labeled with CFSE and pulsed with the MHC-I-restricted $CD8^+$ T cell specific AGPWHLGKL peptide showed that the induced antigen-specific cytotoxic responses induced in animals submitted to the different immunization regimen were low but with higher responses detected in mice immunized with NS1 admixed with FA or LT_{G33D} regarding mice immunized with NS1 adjuvanted with alum.

3.3. Protective responses induced in mice immunized with NS1-based vaccines

Protective anti-DENV2 responses were measured in mice immunized with the different vaccination formulations following administration of a lethal i.c. challenge with the DENV2 NGC virus strain. As demonstrated in Fig. 4A, mice vaccinated with NS1 and LT_{G33D} showed a 50% protection level. A lower but not statistically different result was observed in mice immunized with NS1 and FA (40% protection). In contrast, no protection was observed in mice immunized with NS1 combined with alum, non-adjuvanted NS1 or sham-treated animals. We also monitored the DENV2-associated morbidity and, as indicated in Fig. 4B, and mice immunized with NS1 combined with LT_{G33D} or FA showed similar degree of partial limb paralysis (80% and 70% of the vaccinated mice, respectively). As expected, all mice immunized with NS1 and alum, NS1 or sham-treated animals showed severe limb paralysis before death by virus encephalitis.

3.4. Safety evaluation of the NS1-based vaccines

Previous studies indicated that anti-NS1 antibodies may recognize cross-reacting epitopes on platelets and endothelial cells, as well as proteins involved in the coagulation pathway, provoking hematological disturbances [22–26]. As a first step to investigate the safety of the NS1-based vaccine formulations, we measured biochemical markers of hepatic function and nonspecific tissue inflammatory reactions in vaccinated mice. As shown in Fig. 5A and B, GOT and GPT enzyme markers were significantly increased in mice immunized with NS1 admixed with FA but not in mice immunized with NS1 and LT_{G33D}. Similarly, C-reactive protein levels were, on average, higher in mice immunized with NS1 and FA than in mice immunized with NS1 and LT_{G33D} or in sham-treated mice. These results indicate that incorporation of FA, but not LT_{G33D}, could induce mild inflammatory reactions among the vaccinated mice. In a second step, we determined hematological parameters that could indicate disturbances induced by the vaccine formulations adjuvanted with LT_{G33D}. For that purpose mice immunized with NS1 and LT_{G33D} were monitored for hematocrit values, bleeding time,

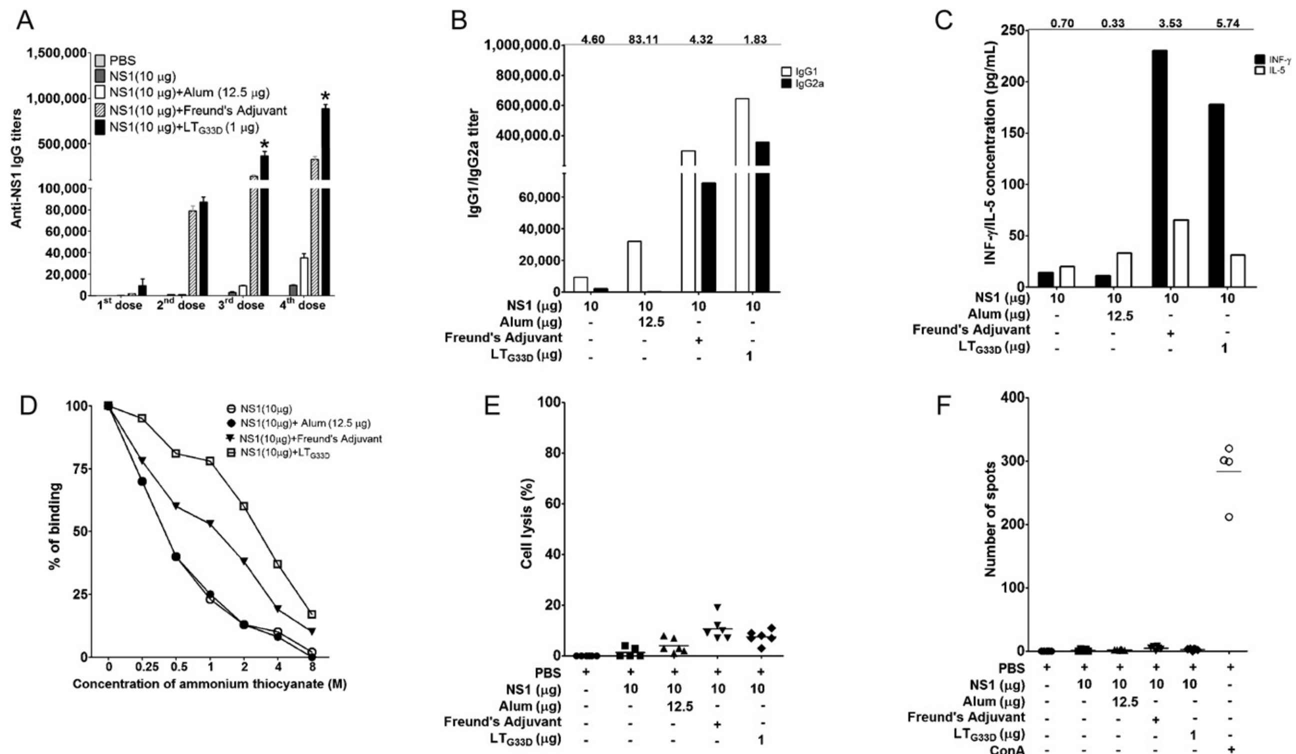

Fig. 3. Immune responses in mice immunized with NS-1-based vaccine formulations. (A) Serum IgG anti-NS1 responses detected in BALB/c mice immunized with purified NS1 in combination with different adjuvants. Mouse groups ($n = 6$) were s.c. immunized with four doses of 10 μg of purified NS1 admixed with alum (1.25 μg), FA (1:1, v/v), or LT_{G33D} (1 μg). The anti-NS1 antibody responses were measured one week after each vaccine dose. Mouse groups immunized with non-adjuvanted NS1 and sham-treated mice were also included as controls to determine the adjuvant effects and non-specific reactions, respectively. Anti-NS1 titers were represented as reverse values of the maximal dilutions yielding $A_{490\,nm} > 0.1$. Values are based on individual responses and expressed as means ± SD. Mice immunized with three or four doses of NS1 admixed with LT_{G33D} elicited stronger responses than those immunized with vaccines containing alum or FA (*$p < 0.001$). (B) Anti-NS1 serum IgG subclasses responses in mice treated with the different vaccine regimens. Anti-NS1 IgG1 and IgG2a titers were represented as reverse values of the maximal dilutions yielding $A_{490\,nm} > 0.1$. The IgG1/IgG2a ratios of each vaccination group are indicated on the top of the figure. Values were determined using serum pools of each immunization groups. (C) Secreted cytokine responses measured in culture supernatants of spleen cells collected from mice vaccinated with the different immunization regimens. The INF-γ and IL-5 values were determined after stimulation with purified NS1. INF-γ/IL-5 ratios are indicated at the top of the figure. (D) Antigen affinity determination of anti-NS1 antibodies raised in mice treated with the different vaccination regimens. Antigen affinity was determined as the ammonium thiocyanate concentration (M) required to dissociate 50% of antibodies bound to NS1 on ELISA plates. (E) In vivo NS1-specific cytotoxic $CD8^+$ T cell activity in mice immunized with different vaccine formulations. Spleen cells collected from naïve mice were labeled with CFSE and pulsed with the H-2d-restricted $CD8^+$ specific NS1 epitope AGPWHLGKL ($NS1_{265–273}$) and subsequently i.v. inoculated in mice immunized with the vaccine regimens. Results are expressed as cell count reduction percentages with regard to cells labeled only with CFSE, 18 h after administration of the labeled cells in the tested mice. (F) Numbers of INF-γ-secreting $CD8^+$ T lymphocytes measured in ELISPOT following *in vitro* stimulation with the $NS1_{265–273}$ peptide. Concanavalin A was used as a stimulatory positive control. Data presented in this figure represent one of two independent experiments.

platelet counts and leukocyte counting, including neutrophils and lymphocytes. As indicated in Table 1, no evidence of hematological disturbance or hemorrhage was observed in mice immunized with NS1 and LT_{G33D} up to seven days after immunization.

4. Discussion

In this study, we tested NS1-based vaccine formulations using a purified recombinant protein co-administered with different

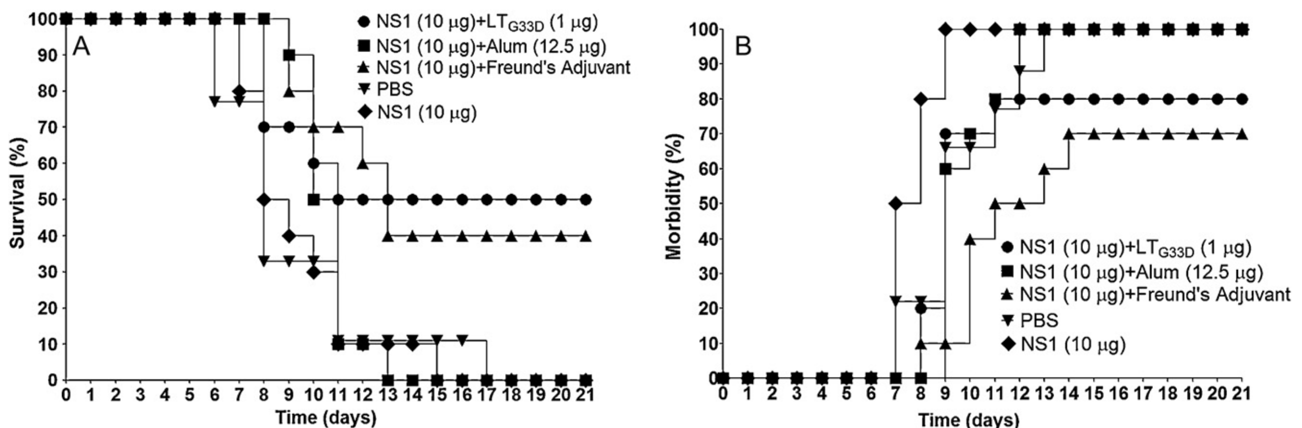

Fig. 4. Anti-DENV2 protection conferred to mice immunized with NS1-based vaccine formulations. Mice were i.c. challenged with 4.32 $\log_{10}$ PFU of the NGC DENV2 strain two weeks after the last vaccine dose. Survival (A) and morbidity (B) values were monitored for 30 days after the virus challenge.

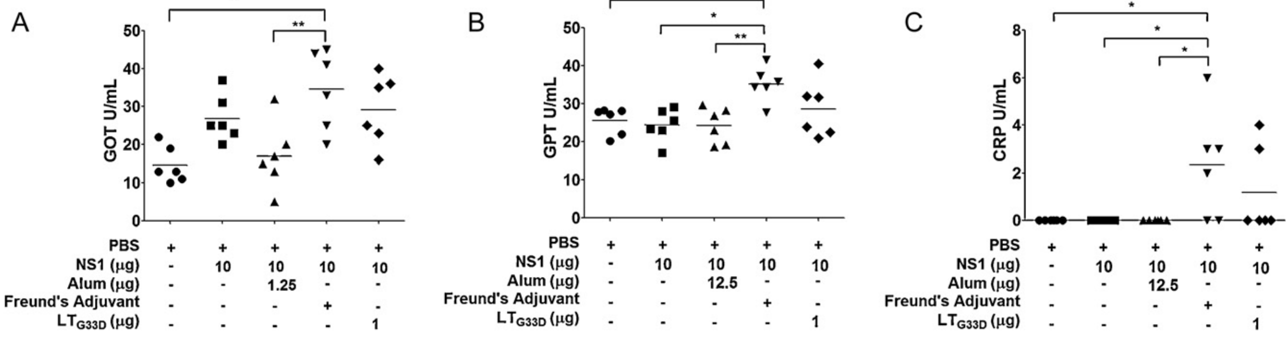

Fig. 5. Safety evaluation of the tested NS1-based vaccine formulations. (A) GOT, (B) GPT and (C) CRP levels were measured in serum samples collected from mice submitted to the different immunization regimens. Values are expressed as units/ml of blood. $^{*}p < 0.05$; $^{**}p < 0.01$. Data presented in this figure represent one representative result of two independently performed experiments.

adjuvants as an attempt to develop a safe and effective alternative for the control of dengue virus infection. The recombinant NS1 protein, despite production in bacterial cells, preserved important immunological features of the native protein, including specific reactivity with antibodies generated in a DENV-2 infected subject. In addition to alum and FA, we tested a nontoxic LT derivative, LT_{G33D}, as parenterally delivered adjuvants. Although devoid of the natural toxicity, the LT derivative showed strong adjuvant effects regarding induction of anti-NS1 serum antibodies. Mice immunized with vaccine formulations adjuvanted with LT_{G33D} showed partial protection to lethal encephalitis after challenge with a mouse-adapted DENV2 strain, similar to that achieved in mice immunized with NS1 adjuvanted with FA. However, in contrast to mice immunized with FA, mice immunized with NS1 and LT_{G33D} did not show any significant side effects regarding altered hepatic function and unspecific inflammatory reactions. In addition, mice immunized with NS1 and LT_{G33D} did not show any altered hematological parameters, such as neutropenia, and bleeding tendency. Altogether, these results demonstrated that the combination of NS1 and LT_{G33D} represents a promising alternative for the development of potentially safe and effective protein-based anti-dengue vaccines.

Parenteral administration of the recombinant NS1 protein admixed with one of three tested vaccine adjuvants (alum, FA and non-toxic LT derivative) had distinct effects regarding the induction of antigen-specific immune responses. Mice immunized with NS1 in combination with LT_{G33D} showed higher NS1-specific IgG titers compared to mice immunized with vaccines adjuvanted with alum or FA. These results were particularly relevant since alum still represents the first adjuvant choice for human vaccines. The rather low anti-NS1 antibody responses elicited in mice immunized with alum was not attributed to a defective binding of NS1 to the salt matrix and may reflect an inherent feature of the antigen. Although mice immunized with FA and NS1 elicited strong anti-NS1 antibody responses the use of this adjuvant is not acceptable for a potential human vaccine due to its reactogenicity. Thus, the demonstration that the administration of a non-toxic LT derivative induces elevated anti-NS1 IgG levels without exacerbated inflammatory reactions represents a relevant contribution for the development of new protein-based anti-dengue vaccines. Of particular interest was the observation that anti-NS1 antibodies elicited in mice immunized with LT_{G33D} have shown a clear increase in the avidity to the viral antigen. Previous studies based on immunization of rhesus monkeys with inactivated, live attenuated virus or DNA vaccines encoding the envelope protein showed that protective antibody responses correlated both with the serum antibody titers and avidity to the target antigen [10]. The finding that co-administration of LT_{G33D} may increase the affinity of the anti-NS1 antibodies to the target antigen may, therefore, represent an important feature of an adjuvant incorporated into a subunit-based anti-dengue vaccine.

Protection induced by NS1-encoding DNA vaccines to the DENV mouse encephalitis challenge model indicated that both antigen-specific B and T cells are important for the mounting of a protective immune response [14–16]. Under our experimental conditions, immunization with purified NS1 in combination with FA or LT_{G33D} resulted in the activation of B lymphocytes, as evaluated by the serum anti-NS1 levels, but less efficient activation of cytotoxic NS1-specific $CD8^{+}$ T cell-mediated responses. Activation of $CD4^{+}$ T helper lymphocytes was inferred indirectly both by the IgG subclass response as well as by the production of cytokines by NS1-stimulated splenocytes (IFN-γ for a Th1-biased pattern and IL5 for a Th2-biased response). Although IgG subclass response does not seem to be a particularly relevant parameter regarding DENV protection, INF-γ is known to interfere with viral replication and positively correlates with development of protective immunity [16,53]. In these two aspects both FA and LT_{G33D} showed similar behavior after s.c. administration to mice with a more balanced Th1/Th2 immune response pattern regarding animals immunized with NS1 and alum. It is conceivable that the partial protective immunity induced in mice immunized with FA or LT_{G33D} vaccine formulations is closely related to the circulating NS1-specific antibodies, in accordance to previous observations [12,13,20,21]. More proper evaluation of the protective role of anti-NS1 T cell responses, particularly those involving activation of cytotoxic responses, will

Table 1
Hematological analyses of mice immunized with purified NS1 and LT_{G33D}.

Hematological parameters[b]	Immunization groups[a]		
	PBS	NS1 (10 µg)	NS1 (10 µg) + LT_{G33D} (1 µg)
WBC	7.5 ± 1.28	6.1 ± 2.07	7.47 ± 2.92
NEU	1.5 ± 0.33	1.22 ± 0.37	1.42 ± 0.72
LYM	5.32 ± 0.99	4.52 ± 1.79	5.75 ± 2.24
RBC	6.53 ± 1.72	6.32 ± 2.13	5.48 ± 0.57
HCT[c]	36.83 ± 0.98	37.17 ± 0.98	37.5 ± 1.05
PLT	1.55 ± 0.35	1.57 ± 0.45	1.38 ± 0.32
BT[d]	188 ± 46	220 ± 151	195 ± 75

[a] Six Balb/c mice per immunization group were bled 7 days following the final dose.
[b] Blood samples were processed to determine white blood cells (WBC), neutrophils (NEU), lymphocytes (LYM), red blood cells (RBC), and platelets (PLT) numbers. WBC, NEU, LYM and PLT counts are given in 10^3 cells/µl. RBC count are expressed in 10^6 cells/µl.
[c] Hematocrit (HCT) values are given in percentages (%).
[d] The bleeding times (BT) are expressed in seconds. Data are expressed as the mean ± SD of individual measurements. Differences among groups were not seen after ANOVA and Bonferroni's Multiple Comparison test. Results contained in this table represent one of two independent experiments.

require the development of protein-based vaccines with improved effect on the induction of $CD8^{+}$ T cell-dependent responses or the testing of more complex vaccine regimens, such as those involving priming with NS1-encoding DNA vaccines.

The safety of the vaccine formulation is a major issue for those working on the development of anti-dengue vaccines. Although protein-based subunit vaccines tend to be safer than vaccines based on live attenuated or recombinant viruses [3], incorporation of an adjuvant required for induction of better immune response may result in undesirable side effects, including strong inflammatory reactions. In addition, previous studies showed that NS1-specific antibodies generated during DENV infection may cross-react with different host proteins including proteins exposed on the surface of platelets and endothelial cells [22–24,54]. In our experimental conditions, no hepatic damage, exacerbated inflammatory reactions and, more relevantly, altered hematological parameters have been detected in mice immunized with NS1 admixed with LT_{G33D}. These results further confirm that LT_{G33D} represents an effective and safe vaccine adjuvant, particularly following administrative via parenteral routes. Further experiments should address the question of deleterious effects induced in vaccinated mice following challenge with other DENV types.

Collectively the present results demonstrated that anti-DENV vaccines based on purified recombinant NS1 protein adjuvanted with a non-toxic LT derivative represent a new and promising alternative for the development of acellular-based dengue vaccines. The partial protection observed in the mouse lethal encephalitis model was achieved in a more restrictive condition if compared to previously reported studies [14–16], where the sham group had a significant survival rate, reflecting the protective potential of the tested vaccine formulation. Moreover, the incorporation of additional antigens to the vaccine preparation, such as the envelope protein or immunogenic domains derived from it, may improve the protective immunity induced in vaccinated subjects. Such ideas are presently under investigation and shall contribute for a better understanding of the immunological features of an effective protein-based anti-dengue vaccine.

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