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Brief Communication

Capsid protein: Evidences about the partial protective role of neutralizing antibody-independent immunity against dengue in monkeys

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ABSTRACT

The role of cellular immune response in dengue virus infection is not yet fully understood. Only few studies in murine models propose that $CD8^+$ T-cells are associated with protection from infection and disease. At the light of recent reports about the protective role of $CD8^+$ T-cells in humans and the no correlation between neutralizing antibodies and protection observed in several studies, a vaccine based on cell-mediated immunity constitute an attractive approach. Our group has developed a capsid-based vaccine as nucleocpasid-like particles from dengue-2 virus, which induced a protective $CD4^+$ and $CD8^+$ cell-mediated immunity in mice, without the contribution of neutralizing antibodies. Herein we evaluated the immunogenicity and protective efficacy of this molecule in monkeys. Neither IgG antibodies against the whole virus nor neutralizing antibodies were elicited after the antigen inoculation. However, animals developed a cell-mediated immunity, measured by gamma interferon secretion and cytotoxic capacity. Although only one out of three vaccinated animals was fully protected against viral challenge, a viral load reduction was observed in this group compared with the placebo one, suggesting that capsid could be the base on an attractive vaccine against dengue.

Introduction

Dengue virus (DENV) infection is a major emerging disease of tropical and subtropical countries, transmitted by the bite of an infected mosquito, usually *Stegomya aegypti*. Many infections are asymptomatic while the clinical manifestations can range from a self-limiting febrile illness (dengue fever) to a life-threatening disease, characterized by increased vascular permeability, thrombocytopenia, hemorrhagic manifestations and shock (dengue severe) (Kyle and Harris, 2008). It is estimated that nearly half of the world population is at risk of infection and up to 50 million people are infected each year. DENV are positive-stranded RNA viruses belonging to the *Flaviviridae* family. There are four distinct serotypes (DENV-1 to -4), which show 67–75% sequence homology (Fu et al., 1992).

For several years, researchers have associated the generation of neutralizing antibodies as a premise to reach protection against DENV. However, dengue is a non-cytopathic virus that up-regulates the surface expression of MHC-I molecules in the infected cells (Lobigs et al., 2004), thus the cellular immune response should constitute other important mediator of the adaptive immune system against this pathogen. Little is known about the protective role of cell-mediated immunity (CMI) against this pathogen. To our knowledge, only five reports provide evidences about this issue in the mouse model. The first report describes the contribution of $CD8^+$ cells in protecting mice immunized with the Yellow fever-dengue chimeric virus (van der Most et al., 2000). In a second report, our group demonstrated the role of the cellular immune response against DENV-2 after infection with the homologous virus in mice (Gil et al., 2009). The third report has also shown that the immunization of mice with four $CD8^+$ T cell epitopes from DENV-2, which are immunodominant in this animal model, enhances viral clearance (Yauch et al., 2009). Finally our group published new evidence, using recombinant nucleocapsid-like

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particles from DENV-2 (NLPs-2). This antigen induced IFN-γ-secreting and cytolytic $CD4^+$ and $CD8^+$ cells with protective capacity, without the contribution of neutralizing antibodies (Gil et al., 2012). Further studies on CMI to better understand the immunopathology of dengue and the immunogenicity of vaccine candidates are required.

In the present work, the immunogenicity of the recombinant NLPs-2 was evaluated in Vervet monkeys [*Chlorocebus* (formerly *Cercopithecus*) *aethiops sabaeus*] to have another evidence of the protective role of the neutralizing antibodies independent immunity against DENV.

Results

Recombinant NLPs-2 do not induce antiviral and neutralizing antibodies in monkeys

To evaluate the immunogenicity and protective efficacy of NLPs-2 in monkeys, animals were divided in two groups. One group received the NLPs-2 formulation and the second group acted as a negative control. Placebo group was immunized with the same quantity of ODN 39M contained in the formulation of NLPs-2 and alum. All animals received four doses at days 0, 60, 120 and 180.

The kinetics of anti-capsid antibody response was determined. As shown in Fig. 1A, immunized animals developed anti-capsid antibodies after the first dose. Antibody titers increased after the second and third doses to a geometric mean titer (GMT) of >8000. Administration of the fourth dose increased the GMT to 20,000 for NLPs-2-immune animals.

The antibody response against the whole DENV-2 was also measured by an indirect ELISA system. As expected, monkeys receiving the NLPs-2 or placebo did not exhibit anti-virion antibodies at any of the time points analyzed before challenge (Fig. 1B). Also, the kinetics of neutralizing antibodies was measured by PRNT, using the strain SB8553 of DENV-2 and the Vero cell line. None of the animals receiving the NLPs-2 developed detectable neutralizing antibodies at any of the time points tested before challenge (Fig. 1C). However, all animals developed neutralizing antibodies after viral challenge.

Recombinant NLPs-2 induce IFN-γ-secreting and cytotoxic cells against DENV-2 in monkeys

Peripheral blood mononuclear cells (PBMCs) from the immunized monkeys, isolated in four distinct time points, were stimulated with infective DENV-2 SB8553 to measure IFN-γ secretion.

Fifteen days after the fourth dose, PBMCs from all NLPs-2-immune animals secreted the antiviral cytokine (M2072, 80.3 pg/mL; M2126, 367.4 pg/mL and M2048, 126.5 pg/mL). However, on challenge day only two out three monkeys (M2072 and M2126) immunized with NLPs-2 showed a positive response, with concentration of IFN-γ of 123.9 pg/mL and 178.5 pg/mL, respectively (Fig. 2A). Interestingly, PBMCs collected after challenge from all the animals immunized with NLPs-2 secreted high levels of IFN-γ (M2072, 345.8 pg/mL; M2126, 197.7 pg/mL and M2048, 582.7 pg/mL). At the same time-point, no secretion of IFN-γ was detected in the placebo group.

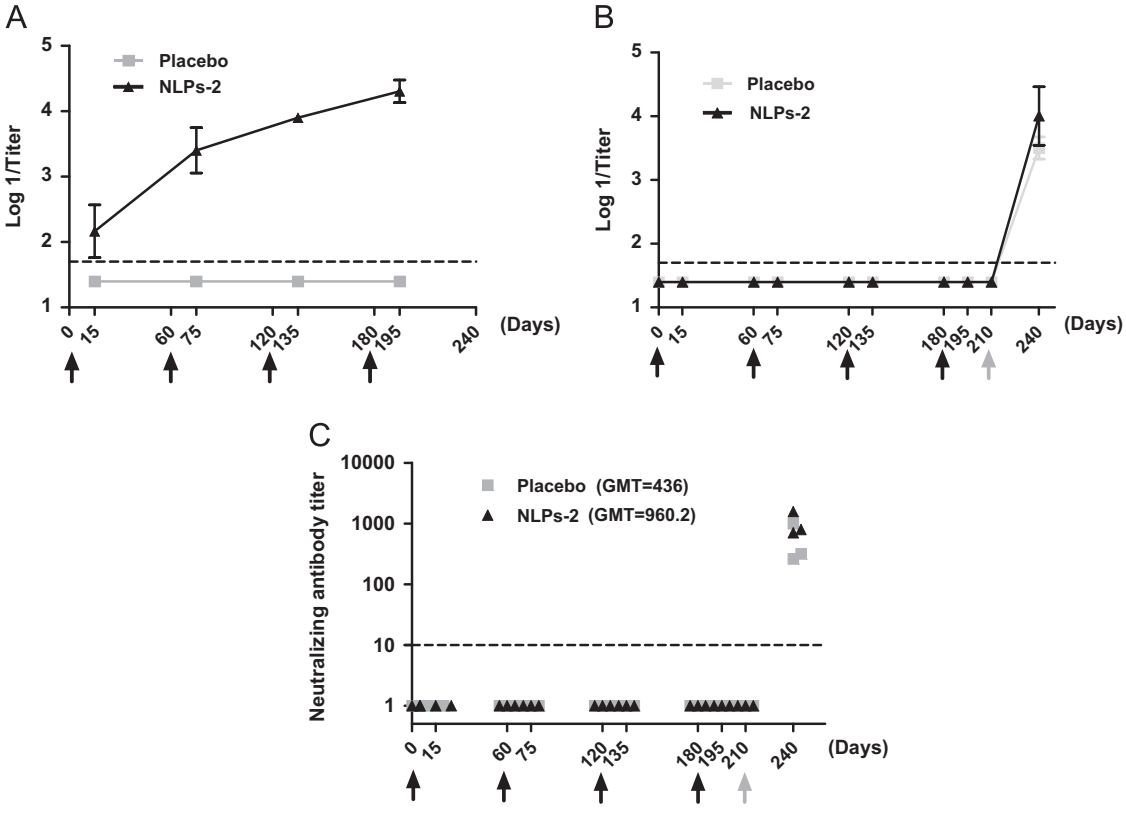

Fig. 1. Kinetics of the humoral immune response induced in monkeys by NLPs-2. (A) IgG antibodies against the recombinant protein, as measured by ELISA. Flat-bottomed 96-well plates were coated with the purified capsid protein (5 μg/mL). Serially diluted samples from sera (starting at 1:50) were assayed and detected with anti-monkey IgG-peroxidase conjugate (B) IgG antibodies against DENV-2, as measured by ELISA. Flat-bottomed 96-well plates were coated with the monoclonal antibody 4G2 (5 μg/mL). Serially diluted samples from sera (starting at 1:50) were assayed and detected with anti-monkey IgG-peroxidase conjugate. In both cases, data represent the mean ± SD of two independent experiments. Animals were considered positive when IgG titers were $>1/50$ (C) Titers of neutralizing antibodies, measured by plaque reduction neutralization test (PRNT), in Vero cells against DENV-2 SB8553 strain. Neutralizing antibody titers are the highest serum dilution which resulted in a 50% reduction in the number of plaques produced by DENV-2. Responders were considered when titers $>1/10$. Data represent the means of two independent experiments. Black arrows indicate days of immunization with the different formulations; the gray arrow indicates day of challenge. The dashed line indicates the cutoff value.

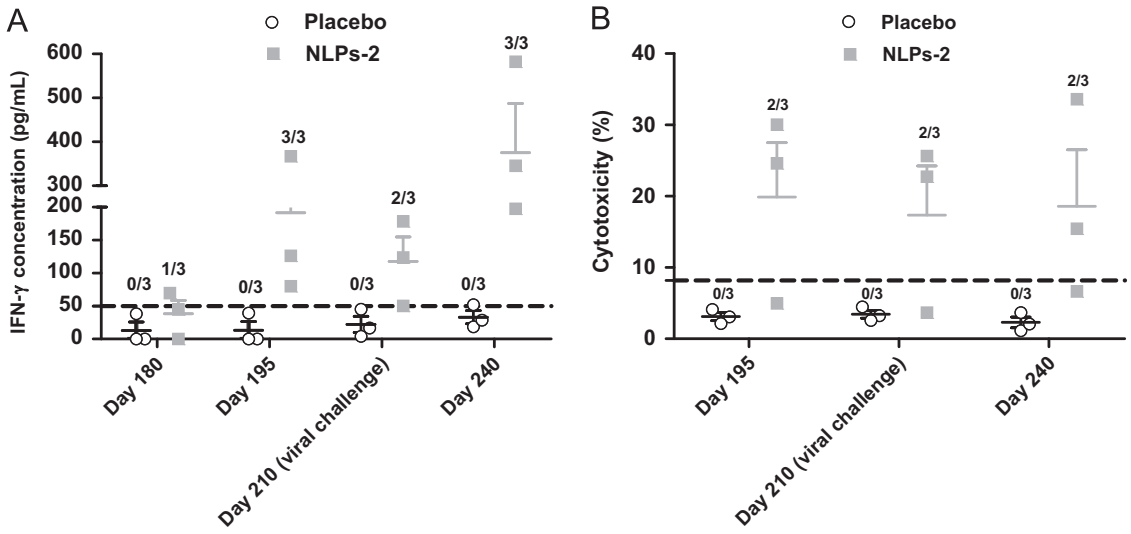

Fig. 2. Cell-mediated immune response against DENV-2 induced in monkeys by NLPs-2. Culture supernatants from mock-treated or DENV-2 SB8553-infected PBMCs from individual animals of all groups were tested on days 180, 195, 210 and 240. (A) Concentration of IFN-γ, as measured by ELISA. (B) Cytotoxicity, as measured by the lactate dehydrogenase assay. In all cases, data represent mean ± SD. Numbers above the plots mean the ratio of responders/total. The dashed line indicates the cutoff value determined as twice the average values of the placebo animals.

The cytotoxic capacity of antigen-induced memory cells was also determined, in 48 h in vitro lactate dehydrogenase (LDH). As a result, significant in vitro cytotoxicity was detected in the PBMCs isolated from two NLPs-2-immune animals (M2126, 22.7% and M2048, 25.6%) on challenge day. No cytotoxic response was detected in the placebo group (M2018, 4.5%; M2122, 3.3%; and M2151, 2.6%) (Fig. 2B).

The cell-mediated immunity induced by NLPs-2 in monkeys reduce significantly the viral load after DENV-2 challenge

To assess the protective efficacy of the recombinant vaccine candidate, all animals were challenged by subcutaneous route with 10^3 pfu of DENV-2 SB8553. Blood was collected daily during 10 days to detect viremia. The presence of virus in serum samples was determined by plaque assay, inoculating 0.13 mL of undiluted serum onto Vero cell. As shown in Fig. 3A, control animals developed viremia with a mean duration of four days and maximum viral loads of $10^{2.6}$ pfu/mL, $10^{1.5}$ pfu/mL and $10^{2.4}$ pfu/mL. In turn, variable levels of viremia were detected in the group immunized with NLPs-2 (Fig. 3B). Animal M2072 had four days of viremia with a maximum virus titer of $10^{1.9}$ pfu/mL, whereas monkey M2126 exhibited three days of viremia with maximum virus titer of $10^{1.6}$ pfu/mL. Monkey M2048 was completely protected. However, our results revealed a significant reduction of the viremia in NLPs-2-immune group on days four, five and six post-challenge ($p=0.049$, $p=0.033$, $p=0.016$, respectively) (Fig. 3C). A summary combining the animal features and viremia after challenge is showed in Table 1. A relation between age, sex, weight and viremia (magnitude and duration) was not found.

Discussion

The capsid protein is an attractive viral region for developing a vaccine candidate against dengue due to three key issues: it contains CTL epitopes for humans (Gagnon et al., 1996, 1999); it can be produced in *Escherichia coli* as a recombinant protein (Jones et al., 2003); and it can act as adjuvant for other viral regions, as previously described for other viral capsids and for the capsid protein of DENV-2 (Lazo et al., 2010, 2012).

This work is a continuation of previously published experiments conducted in mice with the NLPs-2. In the present study, they were produced by addition of 3.4 μg of ODN M39 to the recombinant capsid protein to cause 100% of aggregation. The conditions for aggregation were similar to those previously used in experiments in mice with successful results (Gil et al., 2012). For these experiments, the ODN M39 was selected based on previous evaluations where this specific sequence induced the highest CMI in mice (unpublished data). Theoretically, this ODN contains CpG motifs that activate cells in mice, monkeys and humans (Krug et al., 2001; Verthelyi et al., 2001), suggesting intrinsic adjuvant properties that along with its aggregating function could play a role in vaccine efficacy.

Here, we evaluated the immunogenicity and protective capacity of NLPs-2 in vervet monkeys. Although, the more common species employed for dengue vaccine testing have been rhesus (*Macaca mulatta*) and cynomolgus (*Macaca fascicularis*), green monkeys offer a suitable and less expensive alternative species. Also, they are similar to rhesus macaques in behavior, physiology (Coe et al., 1992; Higley et al., 1996) and proximity to humans (Page and Goodman, 2001; Raaum et al., 2005), and may pose fewer health and safety risks (Baulu et al., 2002).

As expected, NLPs-2 did not induce IgG antibodies against the whole virus neither neutralizing antibodies. The antibodies generated were only directed to the capsid protein. In the virion, it is well known that none of the regions of the viral nucleocapsid is exposed on the surface (Kuhn et al., 2002; Mukhopadhyay et al., 2005) and therefore they are not likely to interact with B cells during the viral infection. This lack of anti-virion antibodies induction upon vaccination with NLPs is a key point for the safety of the vaccine since the ADE phenomenon, associated to antibodies against the Envelope and prM proteins, would not occur.

The evaluation of CMI was the principal arm of the immune system with a potential role in this study. CMI was assessed by two methods: IFN-γ secretion and LDH release (a surrogate of cytotoxic activity) from in vitro-stimulated PBMCs. PBMCs from animals receiving NLPs-2 secreted significant levels of IFN-γ after stimulation with the virus in the time-points tested. Recently, it have been suggested that memory natural killer cells could play a role in protection against secondary DENV infection in mice (Zompi et al., 2012). However, whether T-cell and/or innate lymphoid cells, which include natural killer cells, are involved in the IFN-γ

secretion and cytotoxic activity, induced by NLPs-2 in monkeys, remain as unanswered response.

Interestingly, one month after challenge all animals of the NLPs-2 group were positive with IFN-γ-secreting PBMCs whereas no secretion of IFN-γ was detected in the placebo group. These differences between the two groups indicate a successful priming of the CMI upon immunization with the protein. On the other hand, measurement of LDH release at the day of challenge revealed a functional cytolytic activity upon virus stimulation in two monkeys immunized with NLPs-2.

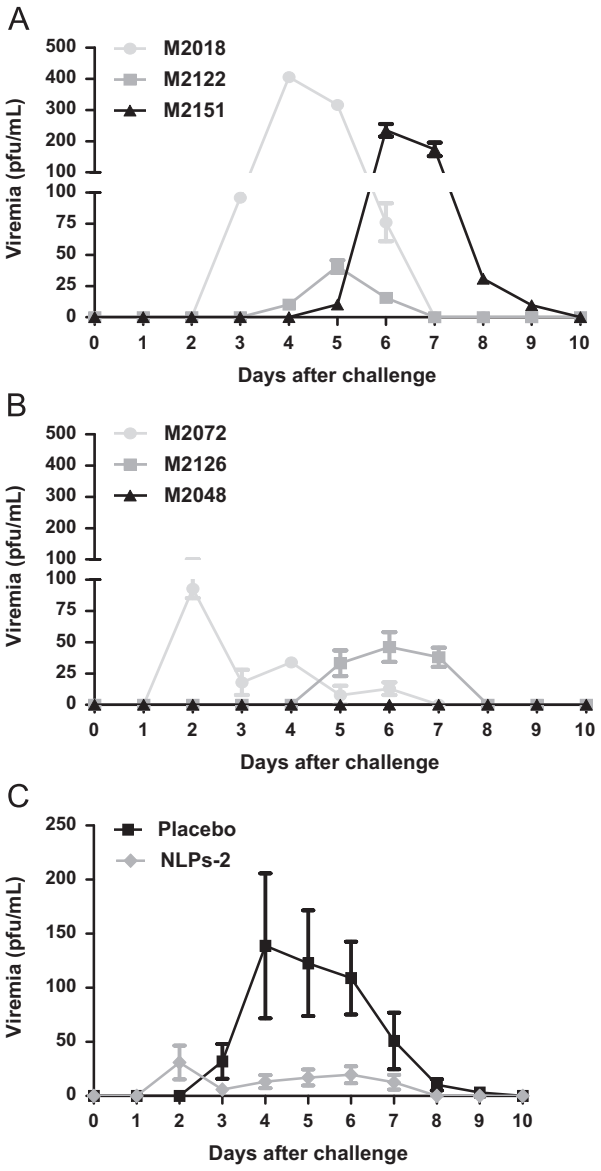

Fig. 3. Viremia levels detected by direct quantification on Vero cell from the monkey sera daily collected after challenge of the immunized animals. (A) Placebo group. (B) NLPs-2 group. (C) Viremia of each group. In all cases, data represent the mean ± SEM of three independent experiments.

The immunogenicity of the protein tested was reflected in the partial protection obtained. It is worth noting the high antibody response detected in monkey M2048 after challenge, as measured both by ELISA and PRNT. The fact that this monekys was completely negative by these test after the administration of NLPs-2, means that some non-detectable priming of the immune system was induced by NLPs-2, which enhanced the novo antibody production upon virus infection. This priming effect was not measured by the IFN-γ secretion tested before challenge probably because of the sensitivity of this technique, but it was detected by LDH test. These results suggest that a Th1 cells induced by NLPs-2 could help to the naive B cells upon challenge, allowing a rapid efficient antibody-response that protected the monkey against challenge.

The results obtained with the NLPs-2-immunized animals are, to our knowledge, the first evidence in monkeys about the role of independent neutralizing antibody immunity in protection against dengue. This is in accordance with evidences from experiments conducted during the last decade, indicating the protective role of CMI against dengue in different mouse models (Gil et al., 2009; Yauch et al., 2010, 2009). Furthermore, published results suggest a protective role for IFN-γ in dengue infected humans previously vaccinated with an experimental vaccine based on attenuated virus. The authors of this work demonstrated solid protection after challenge of one volunteer that had undetectable neutralizing antibodies but a potent CMI, as measured by IFN-γ secretion upon stimulation of PBMCs collected at the day of challenge (Gunther et al., 2011). In addition, a very recent study propose an HLA-linked protective role of $CD8^+$ T-cells response in primary and secondary natural infection (Weiskopf et al., 2013).

The protection obtained in this work was partial since only a reduction of viral load was detected. Although, the qRT-PCR could be another alternative to confirm dengue viremia in monkeys (McGee et al., 2008; Blaney et al., 2008), it has the disadvantage of detection of plasmatic viral genomes from non-infective virus or viral fragments. Because of NLPs-2 potentially induces a cell-mediated immunity, which can only reduce the viral load, qTR-PCR could not detect the differences on viremia between NLPs-2-immune animals and placebo group.

Probably, the capsid protein does not contain a high quantity of CMI epitopes for monkeys. In fact, very recently, a deep study revealed that capsid is not the most immunodominant region for CMI in humans since it contains one region of 4% of responders, with high magnitude of T-cell response (Weiskopf et al., 2013).

Nowdays, the most advances and immunogenic vaccine candidate are based on live attenuated strains, for example Chimerivax that induces full protection against DENV in monkeys (Guy et al., 2010). However, our approach does not have the potentiality to induce an immunopathological antibody response. Subunit vaccines, based on dengue envelope protein have also the same disadvantage. Recent results point out the protective and safety role of CMI against DENV

Table 1
Summary of animal features and viremia after DENV-2 challenge.

Group	Monkey	Age[a]	Sex	Weight (kg)	Peak viremia (pfu/mL)	Duration of viremia (days)
Placebo	M2018	Juvenile	Female	2.2	$10^{2.6}$	4
	M2122	Juvenile	Male	3.1	$10^{1.5}$	3
	M2151	Adult	Female	3.9	$10^{2.4}$	5
NLPs-2	M2072	Juvenile	Female	2.5	$10^{1.9}$	4
	M2126	Adult	Female	3.2	$10^{1.6}$	3
	M2048	Juvenile	Female	2.3	0	0

[a] Juvenil: animals younger than three years old; Adult: animals older than three years old.

(Zellweger et al., 2013; Weiskopf et al., 2013). In fact, a possible explanation for the disappointing results of Sanofi's candidate against DENV-2 in humans (Sabchareon et al., 2012), is the lack of dengue T-cell epitopes in the chimeric virus (Halstead, 2013).

Despite we consider that capsid protein should be enriched with T-cell epitopes, or can be formulated with potent adjuvants to increase the CMI response, the evidences obtained in the present study pointed out about the contribution of independent neutralizing antibody immunity in protection against dengue in monkeys. Additional vaccination schemes would be also assessed to even eliminate the fourth dose administration and measure the long lasting immunity.

A vaccine candidate against dengue based on uniquely CMI is an attractive approach. Because of the antibody-dependent enhancement (ADE) phenomenon associated to dengue infection, a vaccine inducing antibodies should be carefully managed, especially for endemic regions. In previously infected individuals (the typical immunological status of the population in endemic countries), the vaccine may be able to recall the previously humoral immune response established by the primary infection along with the induction of the novo neutralizing antibodies by vaccination. The resultant balance of such interaction is unknown; it would result in protection, non-protection or sensitization against the second infecting serotype. Given this scenario, a vaccine candidate based uniquely in the induction of CMI, will avoid the induction of this “antibody balance” in dengue-positive subjects, and would become in a safer vaccine. Such a candidate would not prevent infection, but could reduce viral load and disease severity without induction of ADE.

In conclusion, we propose that a tetravalent NLPs-based vaccine could be a new and attractive approach to develop a functional vaccine against dengue, which induces neutralizing antibodies independent immunity. Nevertheless, the coverage of NLPs-based vaccine would need to be very high as it may not prevent continual DENV transmission in the population. While the likelihood of transmission to a susceptible mosquito may be lower due to the reduced viremia level, this vaccine would not provide herd immunity that would be conferred by vaccines that prevent infection.

Material and methods

Cells and viruses

African monkey kidney (Vero) cell were received from the National Institute for Biological Standards and Control (NIBSC) (NIBSC accession number: 011038). Cells were grown at 37 °C in Eagle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS).

The following virus strains were used for antibody detection: Hawaii (DENV-1), New Guinea C (DENV-2), H-87 (DENV-3) and H241 (DENV-4) (Clarke and Casals, 1958). DENV-2 strain SB8553 (Kindly provided by Dr. Jane Cardosa) was used for the plaque reduction neutralization test (PRNT). A viral stock for the challenge study in monkeys and for the measurement of CMI was prepared with DENV-2 strain SB8553 in Vero cells (Valdes et al., 2010).

Animals

Healthy adult Vervet monkeys (*Cercopithecus aethiops sabaeus*) were obtained from the Institute of Primate Research of Nairobi (IPR), Kenya. The study was carried out in IPR in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Kenya. The protocol was approved by the Committee on the Ethics of Animal Experiments of IPR (Number: IPR20100908). Animals were maintained throughout the study in individual cages that permitted the evaluation and patterns conduct according to their species, size, age and sex. They were fed with commercial monkey chow, supplemented with fruits and vegetables. Water was available ad libitum. Monkeys were subjected to clinical inspection of lymphatic ganglia, skin, and respiratory, digestive and nervous systems. They did not show any signs of pain or distress at any time during the study, and all results were reported and records maintained in accordance with IPR and Kenyan Guidelines for animal used in research. IPR follows international guidelines for use of animals in biomedical research as it is a World Health Organization Collaborating Centre and has statutory registration with the NIH-Office of Laboratory Animal Welfare, in addition to local and African wide recognition as a Centre of Excellence in preclinical studies. The rectal temperature and body weight in all animals were assessed by the veterinary staff. Clinical biochemistry was performed on animals every month, to avoid additional stress to the animals. All immunizations and blood extractions were performed using ketamine hydrochloride, 10 mg/kg body weight, and all efforts were made to minimize suffering.

All animals were screened for previous exposure to dengue virus by enzyme-linked immunosorbent assay (ELISA) and PRNT. Animals were considered negative when the anti-DENV IgG titers and PRNT titers were less than 1/50 and 1/10, respectively.

Recombinant protein and in vitro aggregation reaction

The design, cloning, expression and purification of the recombinant capsid protein from DENV-2 were previously described (Lazo et al., 2007; Lopez et al., 2009). The parental strain used was DENV-2 Jamaica 1409 American/Asian genotype (Deubel et al., 1988).

NLPs-2 was assembled in vitro as previously described (Gil et al., 2012). Briefly, 20 μg of the capsid protein were incubated with 3.4 μg of single-stranded DNA oligonucleotides of 39 bases (ODN M39) for a protein:nucleic acid molecular ratio of 3:1 in assembly buffer (25 mM HEPES, 100 mM KAc, 1.7 mM MgAc, pH 7.4). The reaction mixture was incubated for 30 min at 30 °C and finally stored at 4 °C.

ODN M39: ATCGACTCTCGAGCGTTCTCGGGGGACGATCGTCGGGGG.

Monkeys immunization and challenge

Six monkeys were ranked by weight, age and sex and then randomly divided in two groups of three animals each. The subcutaneous route was used for the administration of the vaccine formulations.

Group 1: Placebo (8.5 μg of ODN 39 M)
Group 2: 50 μg of NLPs-2

All formulations were prepared using alum at a final concentration of 1.44 mg/mL. The doses were administered in each group at days 0, 60, 120 and 180. Each dose was given in a final volume of 0.5 mL. Blood samples were collected at the time of, and 15 days after each inoculation (days 15, 75, 135 and 195). Serum from clotted blood was stored at −20 °C.

Thirty days after receiving the last dose, monkeys were subcutaneously inoculated in the upper arms with 10^3 pfu of DENV-2 (SB8553). Blood was collected daily for 10 days to detect viremia. The presence of virus in serum samples was determined by direct plaque formation on Vero cells as previously described (Valdes et al., 2009).

Measurement of humoral immune response

The anti-DENV IgG antibodies induced by immunization were monitored by ELISA. Briefly, flat-bottomed 96-well plates (Costar, USA) were coated with the monoclonal antibody 4G2 (5 μg/mL), which recognizes the flavivirus E protein (Kaufman et al., 1987), in coating buffer (0.16% Na_2CO_3, 0.29% NaHCO3, pH 9.5) during 2 h at 37 °C. Three washes with PBS containing 0.05% Tween 20 (v:v) (Merck, Germany) (PBS-T) were completed after each step. Plates were blocked with 2% bovine serum albumin (BSA) during 1 h at 37 °C, and then incubated overnight at 4 °C with a saturating concentration of DENV antigen and mock antigen in separate wells. Serially diluted samples from sera (starting at 1:50) were incubated 1 h at 37 °C with either DEN or mock antigen. Anti-monkey IgG-peroxidase conjugate (Sigma, USA) was added and the plates were incubated 1 h at 37 °C. After washing, 0.04% substrate solution (*O*-phenilendiamine in buffer 2% Na_2HPO_4, 1% citric acid, and 30% H_2O_2, pH 5.0) were added. The plates were kept 30 min at room temperature and the reaction was stopped with 12.5% H_2SO_4. Absorbance was read at 492 nm in a microplate reader (SensIdent Scan; Merck, Germany). Titers were defined as the dilution of serum giving twice the absorbance value of the negative control serum.

To detect the anti-capsid IgG antibodies, flat-bottomed 96-well plates (Costar, USA) were coated 2 h at 37 °C with dengue-2 capsid protein (5 μg/mL) in coating buffer. Three washes with PBS containing 0.05% Tween 20 (v:v) (Merck, Germany) (PBS-T) were completed after each step. Plates were blocked with 2% bovine serum albumin (BSA) during 1 h at 37 °C. Serially diluted samples from sera (starting at 1:50) were incubated 1 h at 37 °C with either DEN or mock antigen. The detection was similar to the assay described above.

The functionality of the antibodies was measured by PRNT (Morens et al., 1985) in Vero cells. The end point neutralization titer was calculated as the highest serum dilution tested that reduced the number of plaques by at least 50% ($PRNT_{50}$). The monoclonal antibody 4G2 was used as positive control (Kaufman et al., 1987).

Measurement of cellular immune response

Monkey blood was obtained at days 180 and 195, 210 and 240. Peripheral blood mononuclear cells (PBMC) were isolated as previously described (Valdes et al., 2010).

Spleen cells were obtained in aseptic conditions and erythrocytes were lysed by adding NH_4Cl 0.83% solution. Cells were washed twice with PBS 2% FBS (PAA Laboratories, Ontario, Canada) and resuspended at 2×10^6 cells/mL in RPMI 1640 medium (Sigma Aldrich) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco, UK), 2 mM glutamine (Gibco, UK), 5×10^{-5} M 2-mercaptoethanol (Sigma St. Louis, MO) and 5% FBS. Finally 2.5×10^5 cells/well were cultured in 96 well round bottom plates with the antigens (2.5×10^4 pfu of DENV-2 antigen or mock preparation). Concanavalin A (Sigma St. Louis, MO) was used as a positive control. In all experiments three wells were plated for each antigen. After 4 days of culture, culture supernatants were collected and stored at −20 °C.

The culture supernatants were analyzed in duplicate for interferon gamma (INF-γ) concentrations by ELISA using monoclonal antibody pairs (Mabtech INF-γ; Sweden) and the protocol recommended by the manufacturer.

in vitro cytotoxicity assay

Cytotoxic activity was determined in a 48 h in vitro lactate dehydrogenase assay (Roche, USA) using 2×10^5 spleen cells, from the immunized animals, co-cultured with 10^3 pfu DENV antigen. Percentage of specific lysis was calculated as [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100. Maximum release was obtained by adding 1% Triton X-100 to cells, and spontaneous release was determined by incubating the cells with medium alone.

Statistical analysis

The analysis of viremia in monkeys was compared using one-tailed unpaired *t* test with Welch's correction. Viremia was compared between both groups per day, using three independent experiments. With this assumption (nine values of viral load for each group per days), the normal distribution of the data was confirmed by the D'Agostino & Pearson omnibus normality test. In both cases, GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA) was used.

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