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Use of a Recombinant Gamma-2 Herpesvirus Vaccine Vector against Dengue Virus in Rhesus Monkeys

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ABSTRACT Research on vaccine approaches that can provide long-term protection against dengue virus infection is needed. Here we describe the construction, immunogenicity, and preliminary information on the protective capacity of recombinant, replication-competent rhesus monkey rhadinovirus (RRV), a persisting herpesvirus. One RRV construct expressed nonstructural protein 5 (NS5), while a second recombinant expressed a soluble variant of the E protein (E85) of dengue virus 2 (DENV2). Four rhesus macaques received a single vaccination with a mixture of both recombinant RRVs and were subsequently challenged 19 weeks later with 1×10^5 PFU of DENV2. During the vaccine phase, plasma of all vaccinated monkeys showed neutralizing activity against DENV2. Cellular immune responses against NS5 were also elicited, as evidenced by major histocompatibility complex class I (MHC-I) tetramer staining in the one vaccinated monkey that was *Mamu-A*01* positive. Unlike two of two unvaccinated controls, two of the four vaccinated monkeys showed no detectable viral RNA sequences in plasma after challenge. One of these two monkeys also showed no anamnestic increases in antibody levels following challenge and thus appeared to be protected against the acquisition of DENV2 following high-dose challenge. Continued study will be needed to evaluate the performance of herpesviral and other persisting vectors for achieving long-term protection against dengue virus infection.

IMPORTANCE Continuing studies of vaccine approaches against dengue virus (DENV) infection are warranted, particularly ones that may provide long-term immunity against all four serotypes. Here we investigated whether recombinant rhesus monkey rhadinovirus (RRV) could be used as a vaccine against DENV2 infection in rhesus monkeys. Upon vaccination, all animals generated antibodies capable of neutralizing DENV2. Two of four vaccinated monkeys showed no detectable viral RNA after subsequent high-dose DENV2 challenge at 19 weeks postvaccination. Furthermore, one of these vaccinated monkeys appeared to be protected against the acquisition of DENV2 infection on the basis of undetectable viral loads and the lack of an anamnestic antibody response. These findings underscore the potential utility of recombinant herpesviruses as vaccine vectors.

KEYWORDS DENV vaccine, herpesviral vector, live-vector vaccines, recombinant vaccine

The mosquito-borne dengue virus (DENV) exists as four different subtypes, denoted DENV1 to DENV4. Together, all four subtypes account for approximately 390 million infections in over 120 countries annually. Globally, DENV has expanded drastically within the last 5 decades. Four billion people are now estimated to be at risk each year

due to increased international travel, increases in urban populations, and insufficient mosquito control (1–3).

The majority of primary DENV infections occur without the emergence of symptoms or with very mild symptoms. However, it is estimated that more than 2 million DENV infections annually result in severe clinical outcomes, such as dengue hemorrhagic fever and dengue shock syndrome, which may be fatal (1, 4). One potential risk factor for severe DENV infection is antibody-dependent enhancement (ADE) due to preexisting immunity against heterotypic DENV serotypes. During ADE, cross-reactive yet nonneutralizing antibodies against previously encountered DENV serotypes bind to virus particles and deliver them to cells of the myeloid lineage, thereby enhancing infection (5–8). Infants are also prone to ADE from the passive transfer of maternal antibodies against DENV (9, 10). Due to an increasing fraction of the world's population being at risk of contracting DENV, the potential severity of infection, and the lack of dengue-specific antiviral drugs, the development of optimal DENV vaccines is an important goal.

Currently, the only licensed DENV vaccine is Dengvaxia (chimeric yellow fever-tetravalent dengue vaccine [CYD-TDV]) from Sanofi Pasteur. CYD-TDV is a tetravalent live attenuated vaccine made by replacing the premembrane (prM) and envelope (E) genes of the yellow fever live attenuated vaccine strain with the prM and E genes of DENV serotypes 1 to 4. The vaccine has been approved in only a few countries (11, 12). The efficacy of CYD-TDV is heavily dependent on the serotype, with the poorest protection against DENV2 (13). In addition, longer-term assessments have raised concerns because the 3-year postvaccination risk for hospitalization upon DENV infection was elevated in CYD-TDV recipients under the age of 9 years compared to the placebo group (14).

The Laboratory of Infectious Disease at the National Institute of Allergy and Infectious Disease (NIAID) is pursuing a different approach toward a live attenuated replicating DENV vaccine. Their approach utilizes a mixture of attenuated strains of all four DENV serotypes. The DENV1, DENV3, and DENV4 strains used in this vaccine harbor deletions in the 3' untranslated region, and the attenuated strain of DENV4 was employed as a backbone for the remaining dengue virus serotype 2 (15). One formulation (TV003) elicited full protection against DENV infection in small-scale testing using a human challenge model (16). Results of human clinical trials have yet to be reported.

Several strategies for the development of nonreplicating viral vaccines focus on the DENV E protein, which is the main target for neutralizing antibodies. DENV E consists of a membrane-spanning region and an N-terminal ectodomain. The ectodomain is divided into three domains, namely, envelope domain I (EDI) to EDIII (17, 18). All three EDs play an important role in the evocation and specificity of humoral responses (19, 20). Adjuvanted recombinant EDIII constructs have been shown to generate anti-DENV responses in nonhuman primates (21). Additionally, a Venezuelan equine encephalitis virus replicon vector-based vaccine containing a C-terminally truncated soluble form of the DENV E protein (E85) elicited serotype-specific antibodies capable of neutralizing DENV in rhesus macaques (22).

In this report, we describe the use of a replication-competent, persisting herpesvirus expressing DENV2 E85 and nonstructural protein 5 (NS5) in rhesus monkeys as an experimental vaccine against DENV2.

RESULTS

Expression of DENV2 proteins in recombinant RRV-infected rhesus fibroblasts. Replication-competent RRVs encoding either a codon-optimized soluble variant of the E protein (E85) or NS5 of DENV2 were generated (Fig. 1). Rhesus fibroblasts (RFs) permissive for lytic RRV replication were infected with recombinant RRVs, and cell lysates and cell culture supernatants were prepared and analyzed for the presence of the desired DENV2 gene products by immunoblotting. DENV2 E85 was readily detected in the RF cell lysate as well as in the cell culture supernatant (Fig. 2A). Similarly, the
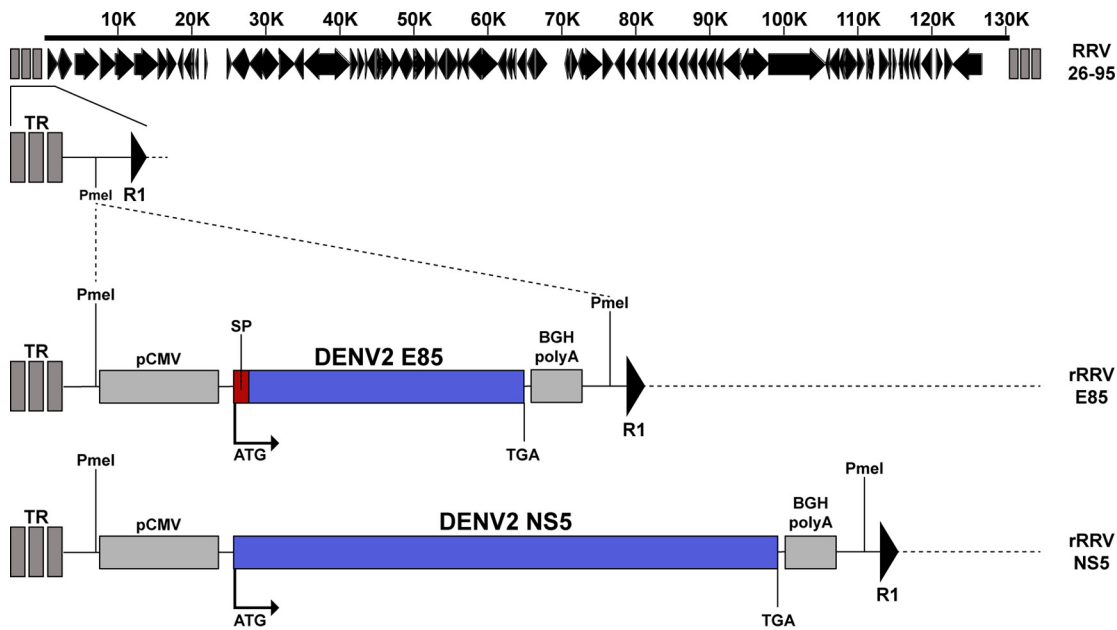
FIG 1 Positions of DENV2 E85 and NS5 transgene cassettes in the recombinant RRV constructs. The expression cassette for codon-optimized DENV2 E85, preceded by the PrM-derived signal peptide (SP), or codon-optimized NS5 was inserted between the left terminal repeats (TR) and the first open reading frame R1 of RRV. The expression cassette consists of the cytomegalovirus promoter (pCMV), the transgene, and the bovine growth hormone (BGH) poly(A) signal.

expression of DENV2 NS5 was detected in the cell lysate of RRV-NS5-infected RF cells (Fig. 2B).

Recombinant RRV vaccination phase. Recombinant RRV was evaluated as a vaccine against DENV2 infection in RRV-negative rhesus macaques (Table 1). A mixture of two recombinant RRVs, one expressing E85 and the other expressing NS5, was given to four rhesus macaques intravenously, whereas two additional monkeys served as unvaccinated controls. Two antigens were included in the vaccine that each test monkey received in order to enhance the chances of seeing protective effects.

To determine the ability of recombinant RRV expressing codon-optimized E85 to elicit antibodies capable of neutralizing DENV2, a flow cytometry-based neutralization assay was performed. Dilutions of plasma samples from the vaccinated animals at two distinct time points were used for analyses. At both time points, plasma of all vaccinated animals showed neutralizing activity against DENV2. Overall, the $NEUT_{50}$ titers (the plasma dilution at which 50% of the virus was neutralized) ranged from 1:17 to 1:337, 54 days after recombinant RRV administration (Fig. 3). Additional testing at 89 and 129 days post-recombinant RRV administration yielded similar results, with $NEUT_{50}$ titers of between 1:75 and 1:200 and between 1:52 and 1:153, respectively (Fig. 3 and see Table 3).

The emergence of DENV2-specific cellular responses upon vaccination was also examined. TV9 MHC-I tetramer staining was used to evaluate the frequency of post-vaccination DENV2 NS5-specific CD8^{+} T cell responses in *Mamu-A*01*-positive animal r12078. The measurements performed revealed an expansion of DENV2 NS5-specific cellular responses over time, with relatively steady levels 38 to 68 days after immunization (Fig. 4). At day 68 after recombinant RRV administration, 0.0346% of the CD8^{+} T cells showed NS5-specific staining. In comparison, 0.198% of the CD8^{+} T cells in *Mamu-A*01*-positive control animal rh2313 showed positive NS5-specific tetramer staining, 31 days after infection with DENV2 (Fig. 4).

DENV2 challenge phase. Nineteen weeks into the study, the four immunized and two control animals were challenged subcutaneously with 1×10^5 PFU of a DENV2 challenge stock.

In order to evaluate the protective ability of our vaccine regimen against DENV2 infection, plasma samples from all the monkeys were analyzed for the presence of
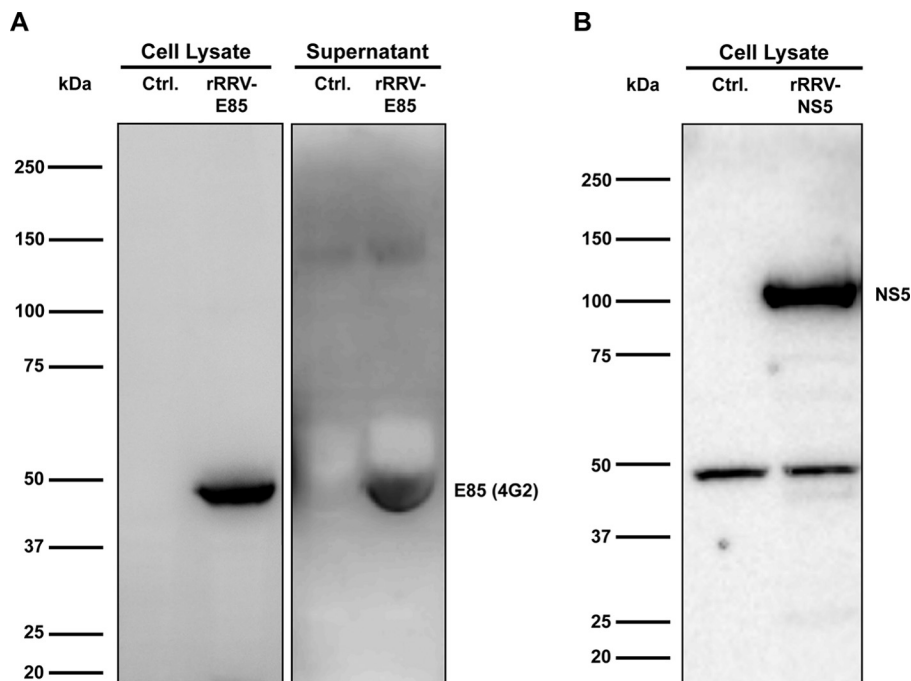
FIG 2 Expression of the desired DENV2 proteins in rhesus fibroblast cultures after infection with RRV-DENV2 recombinants. RF cell lysates were prepared, and cell culture supernatants were harvested. Proteins were separated by SDS-polyacrylamide gel electrophoresis, and the separated proteins were transferred onto a PVDF membrane and subsequently tested with DENV2-specific antibodies. (A) Recombinant RRV-E85 (rRRV-E85) represents either the cell lysate or the cell culture supernatant from RF cells infected with RRV-E85. Blots were probed with an antibody specific for the E protein of DENV2 (4G2). (B) Recombinant RRV-NS5 represents cell lysate from RF cells infected with RRV-NS5. Blots were reacted with an antibody specific for NS5 of DENV2. kDa, atomic mass (in thousands).

DENV2 by using real-time quantitative reverse transcriptase PCR. Two of the four vaccinated animals, namely, animals r09084 and r12078, showed no sign of viral RNA in plasma over 10 days of postchallenge measurements, whereas DENV2 was readily detectable in all of the remaining animals, including both control animals (Table 2). Control group macaque r08037 had the highest peak viral load, with 5.95×10^6 DENV2 copies/ml at day 10 postchallenge. One vaccinated animal (r10019) showed overall lower viral loads than those in the other infected monkeys and with a peak viral load of 3.36×10^3 DENV2 copies/ml at day 10.

We also evaluated neutralizing antibody titers pre- versus post-DENV2 challenge (Table 3 and Fig. 5). Three of the four vaccinated monkeys showed a marked, anamnestic response in anti-DENV2 neutralization titers following challenge. $NEUT_{50}$ titers in these animals went from the range of 1:52 to 1:112 prior to challenge to 1:2,591 to 1:3,094 by day 37 postchallenge (Table 3). These titers were much higher than those that were observed in the two control monkeys at this day 37 time point ($NEUT_{50}$ titers

TABLE 1 Characteristics of research animals

Monkey	Inoculum	Sex[a]	Age (yr)[b]	Mamu-A*01 status[c]	RRV status PV[c,d]
r09084	rRRV-E85/NS5	F	5.33	−	+
r10019	rRRV-E85/NS5	F	4.75	−	+
r12078	rRRV-E85/NS5	F	2.17	+	+
rhBE94	rRRV-E85/NS5	M	5.83	−	+
rh2313	None	M	10.75	+	−
r08037	None	M	6.42	−	−

[a]F, female; M, male.
[b]Age at day of vaccination.
[c]+, positive; −, negative.
[d]PV, postvaccination. All monkeys were negative for RRV on the day of vaccination.
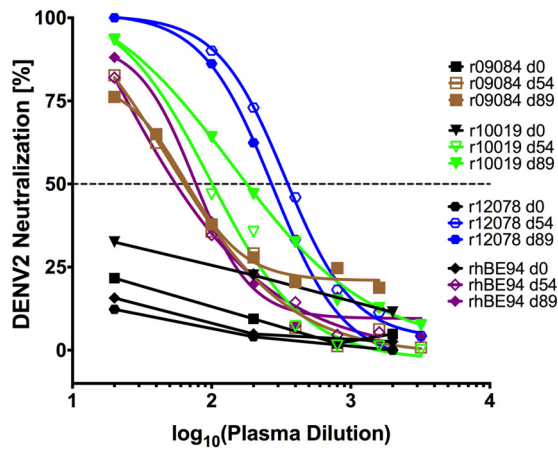
FIG 3 DENV2 plasma neutralization titers prior to challenge. DENV2 neutralization titers in EDTA-treated plasma from four vaccinated research animals were analyzed by using a flow cytometry-based neutralization assay on day 0, day 54, and day 89 after inoculation with the RRV-DENV2 recombinants.

of 1:192 and 1:311). The one vaccinated monkey without a significant increase in neutralizing titers following challenge (r12078) was also one of the monkeys without measurable DENV2 RNA in plasma following challenge. Interestingly, this monkey also had the highest postvaccination neutralizing antibody response (Fig. 3 and Table 3).

DISCUSSION

Vaccination with persistent, replication-competent, recombinant RRV expressing a soluble variant of the DENV2 E protein (E85) generated neutralizing antibodies against DENV2 in all four vaccinated monkeys (Fig. 3). The $NEUT_{50}$ titers in these animals were comparable to the titers observed in control monkeys after primary DENV2 infection (Table 3). Overall, the observed $NEUT_{50}$ titers were higher than those observed in a
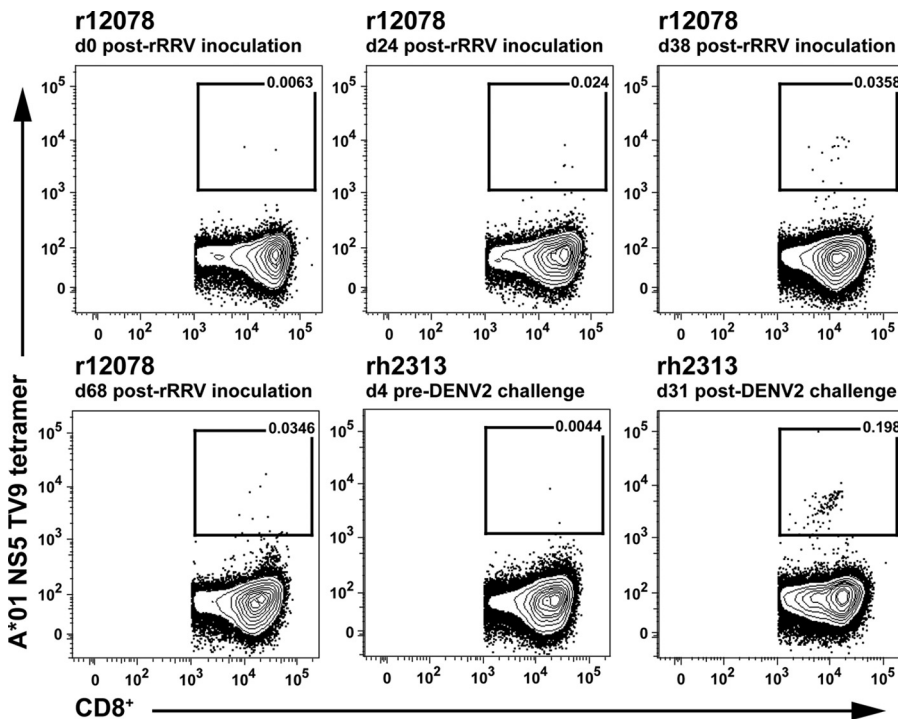

FIG 4 Frequency of *Mamu-A*01* NS5 TV9 tetramer binding T cells. PBMCs were obtained from vaccinated animal r12078 and from DENV2-naive control animal rh2313 at the indicated time points. The frequencies of NS5 TV9 tetramer binding cells in $CD3^+$ $CD8^+$ lymphocytes are shown.

TABLE 2 Viral loads after DENV2 challenge

Group	Monkey	DENV2 load (RNA copies/ml) on postchallenge day[a]:
		2	3	4	5	6	10
Vaccinated	r09084	<20	<20	<20	<20	<20	<20
	r10019	<400	<400	<400	1,120	<400	3,360
	r12078	<20	<20	<20	<20	<20	ND
	rhBE94	15,900	17,700	33,300	88,400	18,200	<20
Control	rh2313	1,560	760	730	3,230	14,300	28,600
	r08037	520	610	<400	<400	4,410	5,950,000

[a]Viral loads were quantified via quantitative real-time PCR. Monkeys were challenged 19 weeks after inoculation of recombinant RRV. <20 indicates that the viral load was below the limit of detection of 20 DENV2 RNA copies/ml plasma; <400 indicates that the monkeys tested positive but that the viral load was below the limit of quantitation; ND, not done.

previous study using a live attenuated virus in rhesus macaques (23). Although showing some variability, the $NEUT_{50}$ titers in our study remained rather steady until the day of challenge 19 weeks after the single time of vaccine administration. In contrast, a study utilizing an alpha vector-based vaccine containing E85 showed a decline of $NEUT_{50}$ titers over time after the second immunization (22).

The protective effects of recombinant RRV against high-dose DENV2 challenge were moderate. Two of the four vaccinated monkeys (animals r12078 and r09084) had no detectable viral RNA in plasma during the challenge phase (Table 2); one of these two monkeys exhibited no significant boost in neutralizing titers against DENV2 following challenge, indicating the absence of an anamnestic response (Table 3 and Fig. 5). The lack of an anamnestic response in animal r12078 further supports the claim that it was protected against DENV2 acquisition. Noteworthy, all aviremic monkeys enrolled in a study utilizing CYD-TDV showed anamnestic responses post-DENV challenge (24). Although viral RNA was not detected in the plasma of animal r09084 following challenge, the presence of an anamnestic antibody response indicates that this animal indeed became infected by DENV2 but that the level of replication was below the level of detection with our viral RNA load assay.

Animal r12078 had the highest neutralizing titers of the four vaccinated monkeys, and it was the one monkey apparently protected fully against acquisition. These results suggest an approximate neutralization titer that can protect against the acquisition of infection with the DENV2 challenge dose that was used. Although clinical parameters were not monitored in this experiment, the very low viral loads in two of the remaining vaccinated monkeys would likely translate into protective effects against clinical manifestations of disease.

Our data show that the recombinant RRV regimen was able to induce a specific T cell response against NS5. However, the frequency of these T cells was about 1 order of magnitude lower than the frequencies observed in an unvaccinated rhesus monkey infected by DENV2 (Fig. 4). Although NS5 is among the main targets for CD8^{+} T cells in naturally occurring DENV infections, it may be worth considering additional non-

TABLE 3 DENV2 $NEUT_{50}$ titers pre- and post-DENV2 challenge

Group	Monkey	\multicolumn{2}{c}{$NEUT_{50}$ titer on day relative to day of challenge[a]}
		−4	37
Vaccinated	r09084	1:52	1:2,591
	r10019	1:63	1:2,682
	r12078	1:153	1:192
	rhBE94	1:112	1:3,094
Control	rh2313	1:<5	1:311
	r08037	1:<5	1:192

[a]The $NEUT_{50}$ titer is defined as the highest dilution of plasma that reduced DENV2 infectivity by 50%.
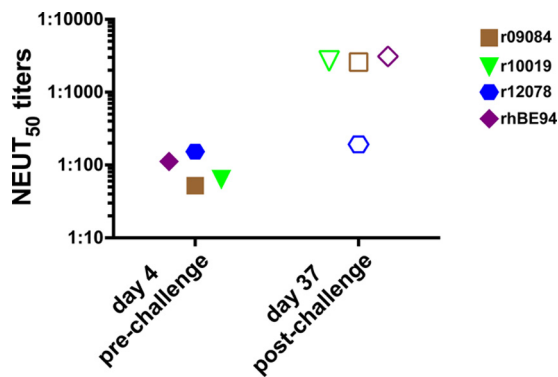
FIG 5 Comparison of DENV2 plasma neutralization titers pre- and post-DENV2 challenge. DENV2 neutralization titers of plasma of the four vaccinated animals were analyzed by using a flow cytometry neutralization assay 4 days prior to and 37 days after DENV2 challenge.

structural genes as a part of the vaccine regimen. In particular, NS3 has been shown to be the most immunodominant target of cellular responses (25, 26).

Unfortunately, no plasma samples were collected beyond day 10 in the study described here. Plasma sample collection dates and the duration of collection were chosen based on a thorough meta-analysis of available data on DENV infections in nonhuman primates (27). However, the overall kinetics of viremia in all infected animals differed from the expected outcome. The reasons for this are not clear.

Given that this is the first attempt at using a recombinant herpesvirus vector to protect against DENV in an animal model, we are encouraged by these early findings. A variety of variations in vaccine design can be envisioned to increase the potency and breadth of immune responses in future experiments.

MATERIALS AND METHODS

Cell culture. Rhesus fibroblast (RF) cells were cultured and maintained in Dulbecco's modified Eagle medium (DMEM; Life Technologies) supplemented with 20% fetal bovine serum (FBS; Life Technologies) and Primocin (InvivoGen). Vero cells (ATCC CCL-81) were cultured and maintained in 199 medium with Earle's balanced salt solution (EBSS; GE Healthcare Life Sciences) supplemented with 5% FBS (Life Technologies) and a 1% penicillin-streptomycin-amphotericin B solution (Millipore).

Generation of dengue virus 2 E85 and NS5 polynucleotides. A pUC plasmid containing the sequence for a codon-optimized soluble variant of the DENV2 New Guinea C strain (DENV2_NGC) (GenBank accession number AF038403.1) E protein (E85) was made to order (GenScript). E85 is made up of 85% of a C-terminally truncated soluble form of the DENV2 E protein. It contains the E protein signal peptide (SP) derived from the last 18 residues of the premembrane protein (PrM), E domains I to III, the first alpha helix (H1), and part of the conserved sequence (CS). It lacks the second alpha helix (H2) and both membrane-spanning regions. Similarly, a pUC plasmid containing the sequence for codon-optimized NS5 of DENV2 was made (GenScript).

Generation of recombinant rhesus monkey rhadinovirus. Recombinant RRVs expressing either codon-optimized E85 or codon-optimized NS5 were made by overlapping cosmid transfection as previously described (28). Recombinant RRV-infected RF culture supernatants were harvested and spun down twice at 2,000 × g for 5 min to remove any cell debris, and resulting virus titers in the culture supernatants of infected rhesus fibroblast cells were measured via quantitative real-time PCR using an RRV latency-associated nuclear antigen (LANA)-specific primer set. Quantification was performed by using the TaqMan Fast Virus 1-Step master mix (Life Technologies) in a real-time PCR thermocycler (Applied Biosystems 7500 fast and 7500 real-time PCR system) with forward primer ACCGCCTGTTGCGT GTTA, reverse primer CAATCGCCAACGCCTCAA, and the reporter FAM (6-carboxyfluorescein)-CAGGCCC CATCCCC.

Preparation of dengue virus 2 challenge virus. Vero cells were seeded at a density of 1 × 10^6 cells in a T-175 flask. The following day, the cells were incubated with DENV2_NGC (GenBank accession number AF038403.1) at a multiplicity of infection (MOI) of 0.01 at 37°C for 1 h. Afterwards, the cells were kept in serum-free HyClone 199 medium (GE Healthcare Life Sciences) supplemented with 1% L-glutamine (Life Technologies) and a 1% penicillin-streptomycin-amphotericin B solution (Millipore). The supernatant was harvested at 6 days postinoculation, spun down at 800 × g, aliquoted, and subsequently analyzed with a plaque assay.

Research animals. Six rhesus macaques of Indian origin (*Macaca mulatta*) were utilized for this study, all of which were housed at the Wisconsin National Primate Research Center (WNPRC). Animals were cared for in accordance with the guidelines of the Weatherall Report (30) under a protocol

approved by the University of Wisconsin Graduate School Animal Care and Use Committee. All animals were under anesthesia during recombinant RRV vaccinations and DENV2 challenge.

Recombinant RRV vaccination. Recombinant RRV stocks expressing codon-optimized DENV2 E85 or NS5 were diluted to a total of 1×10^9 genome copies/ml in $1\times$ phosphate-buffered saline (PBS). One milliliter of each dilution was administered to four rhesus macaques intravenously. Two animals served as unvaccinated controls.

DENV2 challenge. The DENV2 challenge stock was diluted to a total of 1×10^5 PFU/ml in PBS, 1 ml of which was administered to all six rhesus macaques subcutaneously.

***In vitro* expression of DENV2 proteins.** A total of 250,000 RF cells were seeded into each well of a six-well plate. The following day, cells were infected with 50 μl of a stock of recombinant RRV (1×10^9 genome copies/ml) expressing codon-optimized DENV2 E85 or NS5. Cells were kept in culture 6 days before harvesting of the cells or cell culture supernatants when cells exhibited advanced cytopathic effect.

Antibodies and reagents. The following antibodies were used in this study: mouse anti-DENV2-E (4G2; Millipore), rabbit anti-DENV2-NS5 (Thermo Fisher Scientific), a goat anti-mouse–horseradish peroxidase (HRP) conjugate (SouthernBiotech), a goat anti-rabbit-HRP conjugate (SouthernBiotech), a rat anti-mouse-IgG2a-allophycocyanin (APC) conjugate (BioLegend), a mouse anti-CD3-peridinin chlorophyll protein (PerCP)-Cy5.5-conjugate (BD Bioscience), a mouse anti-CD8-Brilliant Violet 421 (BV421) conjugate (BioLegend), a mouse anti-CD14-Brilliant Violet 510 (BV510) conjugate (BioLegend), a mouse anti-CD16-BV510 conjugate (BioLegend), a mouse anti-CD20-BV510 conjugate (BioLegend), and a mouse anti-granzyme B-phycoerythrin (PE) conjugate (Life Technologies). In addition, the Live/Dead Fixable Aqua Dead Cell Stain kit (Life Technologies) was used in this study. The MHC-I A*01/NS5 TV9 tetramer conjugated to APC was produced at the NIH Tetramer Core Facility (task order 20448).

Immunoblotting. Cells were harvested, resuspended, and lysed with an NP-40-based lysis buffer containing a protease inhibitor (Roche). After centrifugation, supernatants were transferred into new tubes, and their protein levels were measured and normalized by using a bicinchoninic acid protein assay kit (Pierce). Each lysate was mixed with an equal volume of $5\times$ nonreducing sample buffer (Thermo Fisher Scientific) or $2\times$ sodium dodecyl sulfate (SDS) Laemmli sample buffer (Sigma-Aldrich). Additionally, cell culture supernatants were harvested and mixed with an equal volume of $5\times$ nonreducing sample buffer. Subsequently, samples were incubated at 97°C for 5 min and separated by SDS-polyacrylamide gel electrophoresis (PAGE) under either reducing or nonreducing conditions. The proteins from the gels were transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) and subjected to immunoblotting. Briefly, the membranes were blocked with $1\times$ PBS containing 5% skim milk and incubated with the appropriate primary antibody overnight. Three wash steps with PBS containing Tween were followed by incubation with the appropriate HRP-conjugated secondary antibodies. After a final wash, specific signals were detected with a LAS4000 Minisystem (GE Healthcare Systems) by using the SuperSignal West Pico chemiluminescent substrate (Pierce).

Flow cytometry neutralization assay. Plasma samples from each research animal were serially diluted, and 110 μl of each dilution was added to a 1.5-ml Eppendorf tube together with 110 μl of a 1:5 dilution of the DENV2 challenge stock (2×10^4 PFU/ml). The samples were placed into a cell culture incubator at 37°C for 1 h. After incubation, 100 μl of each sample was added to a 100% confluent Vero cell layer in 24-well plates. The plates were incubated at 37°C for 1 h and gently agitated every 15 min. Afterwards, the wells were aspirated, and the cells were washed with 500 μl of cell culture medium. Finally, 400 μl of fresh cell culture medium was added to each well, and the cells were incubated at 37°C for 24 h. Subsequently, cell culture medium was aspirated, and the cells were detached via trypsinization and distributed into 96-well cluster tubes. The cells were then centrifuged at $530 \times g$ for 10 min, and the medium was decanted. The cells contained in approximately 100 μl residual liquid were vortexed and fixated with 250 μl of a 2% paraformaldehyde solution at 4°C for 15 min. Afterwards, cells were washed twice with 500 μl of a saponin-based permeabilization buffer. Following aspiration of permeabilization buffer and subsequent vortexing, 100 μl of a 1:1,000 dilution of a mouse anti-DENV2 envelope antibody (4G2) was added to the cluster tubes, and the tubes were incubated at room temperature for 1 h. After an additional wash step, 100 μl of a 1:200 dilution of an anti-mouse IgG2a-APC-conjugated antibody was added each tube, which was then incubated in the dark at room temperature for 1 h. Finally, cells were washed, centrifuged, vortexed, and analyzed by flow cytometry. The NEUT$_{50}$ titer is defined as the highest dilution of plasma that reduced binding of the dengue virus-infected Vero cells by 50% and was calculated by using a sigmoidal model and nonlinear regression analysis (GraphPad Prism 7).

Staining of MHC-I tetramer-positive CD8$^+$ T cells. Peripheral blood mononuclear cells (PMBCs) of the research animals were isolated by using Ficoll density gradient centrifugation. Up to 8×10^5 cells in a volume of 100 μl per flow cytometry tube were incubated with 25 μl of a master mix containing the fluorochrome-conjugated MHC-I A*01/NS5 TV9 tetramer at 37°C for 45 min in the dark. Subsequently, 50 μl of a master mix containing fluorochrome-labeled monoclonal antibodies (MAbs) against the surface molecule CD8 was added to each tube. In order to create a "dump gate," cells were also stained with anti-CD14, anti-CD16, and anti-CD20 antibodies labeled with appropriate fluorochromes as well as an amine reactive dye. The samples were then incubated at room temperature for 20 min in the dark. Afterwards, cells were washed with a PBS-based wash buffer containing 0.1% bovine serum albumin (BSA) and 0.45 g/liter NaN$_3$ and pelleted at $530 \times g$, the supernatant was decanted, and cells contained in the residual liquid were vortexed and fixated with 250 μl of a 2% paraformaldehyde solution at 4°C for 20 min in the dark. Subsequently, cells were treated with 500 μl of a saponin-based permeabilization buffer, vortexed, and incubated at room temperature for 10 min in the dark. After an additional wash step, 50 μl of a master mix containing fluorochrome-labeled MAbs against CD3 and granzyme B was

added to each tube, and the tubes were incubated at room temperature for 30 min in the dark. Finally, cells were washed, pelleted, vortexed, and analyzed by flow cytometry.

DENV2 load measurement. RNA was extracted from frozen, EDTA-anticoagulated plasma by using the QIAamp circulating nucleic acid kit according to the manufacturer's protocols (Qiagen). Extractions were performed by using 1 ml of the sample and eluted in 80 μl (2 × 40 μl) for maximum nucleic acid recovery. The isolated viral RNA was reverse transcribed in triplicate reaction mixtures containing 20 μl of the eluted RNA each and amplified by using TaqMan Fast Virus 1-Step master mix (Life Technologies) in a real-time PCR thermocycler (Applied Biosystems 7500 fast and 7500 real-time PCR system). The reverse transcription reaction was performed at 50°C for 30 min. An activation temperature of 95°C for 10 min was followed by 40 amplification cycles of 95°C for 15 s and 60°C for 1 min. A double-quenched (ZEN/Iowa Black FQ) FAM dye qPCR probe was used (500 nM primers and 250 nM probe; IDT Technologies). The probe and primer sequences were modified to match the DENV2_NGC sequence (GenBank accession number AF038403.1). The oligonucleotide sequences are as follows: forward primer CAGGCT ATGGCACTGTCACGAT, reverse primer CCATTTGCAGCAACACCATCTC, and the reporter FAM-CTCTCCGA GAACGGGCCTCGACTTCAA (29). Quality control was performed at several levels, and every RNA extraction batch contained a positive plasma sample and an uninfected plasma sample. Quantitation took into account the extraction and elution volumes. Standard curves were generated in every run, using 3-fold serial dilutions of commercially available total DENV2 RNA standards (Amplirun; Vircell).

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