

# Dengue virus envelope protein domain I/II hinge determines long-lived serotype-specific dengue immunity

William B. Messer<sup>a,b</sup>, Ruklanthi de Alwis<sup>c</sup>, Boyd L. Yount<sup>d</sup>, Scott R. Royal<sup>d</sup>, Jeremy P. Huynh<sup>a,d</sup>, Scott A. Smith<sup>e</sup>, James E. Crowe, Jr.<sup>f,g,h</sup>, Benjamin J. Doranz<sup>i</sup>, Kristen M. Kahle<sup>i</sup>, Jennifer M. Pfaff<sup>j</sup>, Laura J. White<sup>j</sup>, Carlos A. Sario<sup>k,l,m</sup>, Aravinda M. de Silva<sup>c,1</sup>, and Ralph S. Baric<sup>c,d,1</sup>

<sup>a</sup>Department of Molecular Microbiology and Immunology and <sup>b</sup>Division of Infectious Diseases, Department of Medicine, Oregon Health and Sciences University, Portland, OR 97239; <sup>c</sup>Department of Microbiology and Immunology, University of North Carolina School of Medicine, Chapel Hill, NC 27599; <sup>d</sup>Department of Epidemiology, Gillings School of Global Public Health, University of North Carolina, Chapel Hill, NC 27599; Departments of <sup>e</sup>Medicine, <sup>f</sup>Pediatrics, and <sup>g</sup>Pathology and <sup>h</sup>Vanderbilt Vaccine Center, Vanderbilt University, Nashville, TN 37232; <sup>i</sup>Integral Molecular, Philadelphia, PA 19104; <sup>j</sup>Global Vaccines, Inc., Research Triangle Park, NC 27709; and <sup>k</sup>Caribbean Primate Research Center and Departments of <sup>l</sup>Microbiology and Medical Zoology and <sup>m</sup>Internal Medicine, University of Puerto Rico Medical Sciences Campus, San Juan, Puerto Rico 00936

Edited by Michael G. Rossmann, Purdue University, West Lafayette, IN, and approved December 3, 2013 (received for review September 14, 2013)

The four dengue virus (DENV) serotypes, DENV-1, -2, -3, and -4, are endemic throughout tropical and subtropical regions of the world, with an estimated 390 million acute infections annually. Infection confers long-term protective immunity against the infecting serotype, but secondary infection with a different serotype carries a greater risk of potentially fatal severe dengue disease, including dengue hemorrhagic fever and dengue shock syndrome. The single most effective measure to control this threat to global health is a tetravalent DENV vaccine. To date, attempts to develop a protective vaccine have progressed slowly, partly because the targets of type-specific human neutralizing antibodies (NAbs), which are critical for long-term protection, remain poorly defined, impeding our understanding of natural immunity and hindering effective vaccine development. Here, we show that the envelope glycoprotein domain I/II hinge of DENV-3 and DENV-4 is the primary target of the long-term type-specific NAb response in humans. Transplantation of a DENV-4 hinge into a recombinant DENV-3 virus showed that the hinge determines the serotype-specific neutralizing potency of primary human and nonhuman primate DENV immune sera and that the hinge region both induces NAbs and is targeted by protective NAbs in rhesus macaques. These results suggest that the success of live dengue vaccines may depend on their ability to stimulate NAbs that target the envelope glycoprotein domain I/II hinge region. More broadly, this study shows that complex conformational antibody epitopes can be transplanted between live viruses, opening up similar possibilities for improving the breadth and specificity of vaccines for influenza, HIV, hepatitis C virus, and other clinically important viral pathogens.

infectious clone | neutralizing antibody

The four dengue virus serotypes (DENV-1, -2, -3, and -4), transmitted by *Aedes spp.* mosquitoes, are endemic throughout tropical and subtropical regions of the world, with an estimated 390 million new infections annually (1). Primary infection with one serotype confers long-term immunity against that serotype, but repeat infection with a different serotype has an increased risk of potentially fatal severe dengue disease (2), including dengue hemorrhagic fever and dengue shock syndrome. This risk has been attributed, at least in part, to the ability of some cross-reactive antibodies to enhance infection of Fc-receptor bearing cells. The consensus is that, to be safe and effective, any dengue vaccine must simultaneously induce neutralizing antibodies (NAbs) to all four serotypes. However, DENV vaccine development has progressed slowly, highlighted by the disappointing results of the live-attenuated Sanofi Pasteur tetravalent DENV vaccine trial in Thailand (3). Progress is hindered, in part, because the epitopes targeted by the type-specific human NAbs critical for long-term protection

(4, 5) remain poorly defined, limiting our understanding of natural DENV immunity and slowing effective vaccine development.

The DENV envelope glycoprotein (E) (Fig. 1A) is the major surface-exposed DENV antigen and the principle target of NAbs. The E structure consists of three distinct domains: I, II, and III (EDI, EDII, and EDIII) (6, 7); EDIII is a continuous peptide extending from domain I and forming an Ig-like fold, whereas EDI and EDII are discontinuous and connect by four peptide linkers that form the EDI/EDII hinge. We and others have recently described potent human DENV NAbs that bind to epitopes around the EDI/EDII hinge (8, 9). To more fully explore the significance of this antigenic region, we used reverse genetics and synthetic biology to transplant the EDI/EDII antigenic region from DENV-4 into a DENV-3 background and showed by both gain- and loss-of-function assays that the EDI/EDII hinge region is the primary target of the long-lived DENV serotype-specific NAb response.

## Significance

Dengue virus is the most important arthropod-borne viral disease of humans worldwide, with an estimated 390 million acute infections annually. The best means to control this global health threat is a vaccine, but dengue vaccine development has progressed slowly, partly because the antigenic targets required to stimulate long-term immunity are not well-defined. Here, we show a specific region on the viral surface (the envelope domain I/II hinge) that is the target of protective antibodies after primary human infections. These results are critically important for dengue vaccine design, because we hypothesize that a successful dengue vaccine will stimulate antibodies that target this region. More broadly, this study establishes a template for similar approaches for improving vaccines for influenza, HIV, hepatitis C virus, and other clinically important viral pathogens.

Author contributions: W.B.M., B.J.D., K.M.K., J.M.P., C.A.S., A.M.d.S., and R.S.B. designed research; W.B.M., R.d.A., B.L.Y., S.R.R., J.P.H., K.M.K., J.M.P., and C.A.S. performed research; B.L.Y., S.A.S., J.E.C., B.J.D., L.J.W., and C.A.S. contributed new reagents/analytic tools; W.B.M., R.d.A., B.J.D., K.M.K., J.M.P., A.M.d.S., and R.S.B. analyzed data; and W.B.M., R.d.A., S.A.S., B.J.D., L.J.W., C.A.S., A.M.d.S., and R.S.B. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

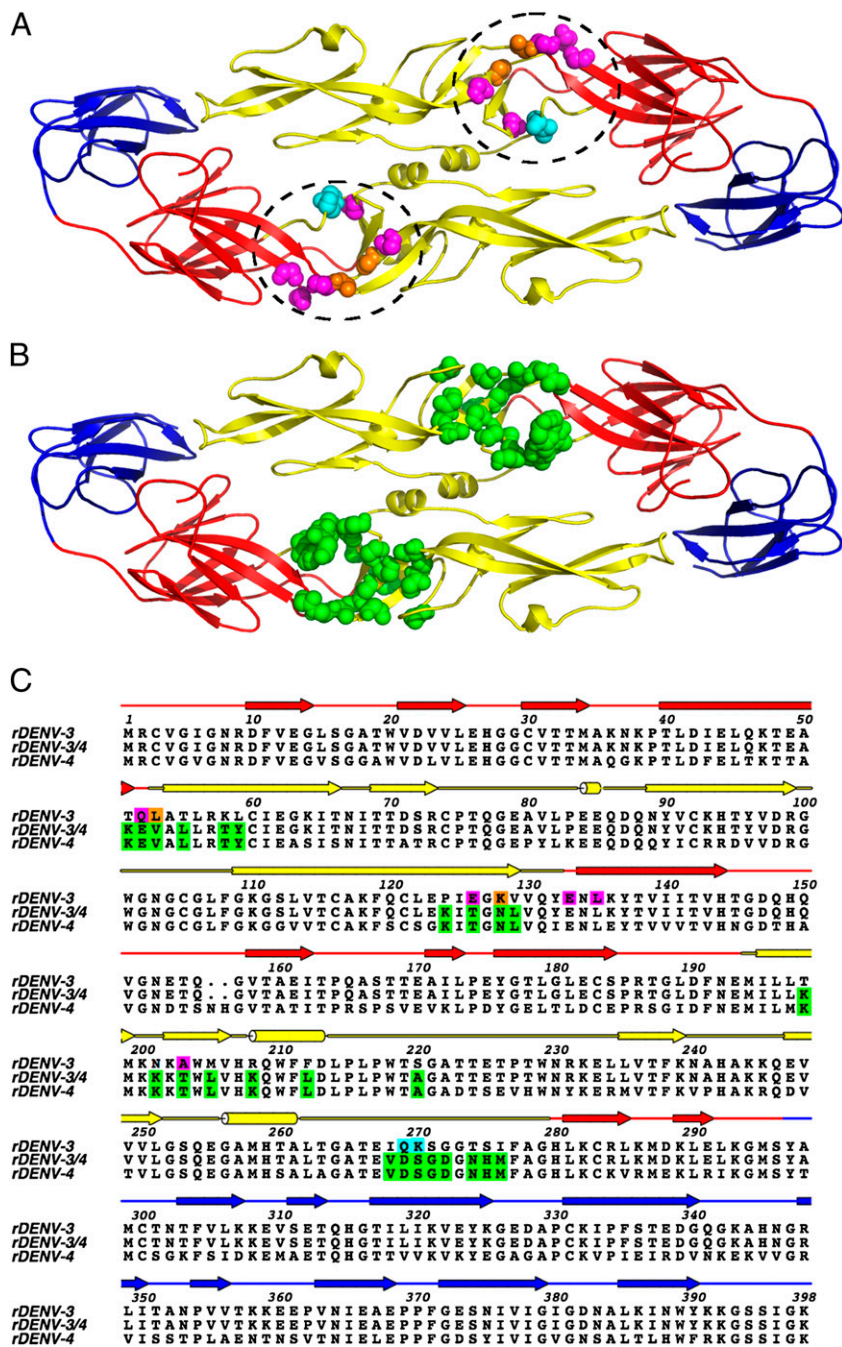
Freely available online through the PNAS open access option.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database, [www.ncbi.nlm.nih.gov/genbank](http://www.ncbi.nlm.nih.gov/genbank).

See Commentary on page 1670.

<sup>1</sup>To whom correspondence may be addressed. E-mail: aravinda\_desilva@med.unc.edu or rbaric@email.unc.edu.

This article contains supporting information online at [www.pnas.org/lookup/suppl/doi:10.1073/pnas.1317350111/-DCSupplemental](http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1317350111/-DCSupplemental).



**Fig. 1.** (A) Cartoon representation of the DENV-3 E dimer with EDI (red), EDII (yellow), and EDIII (blue). The EDI/EDII hinge region is circled. Location of the critical residues associated with escape mutations and mutagenesis-mapped 5J7 residues are shown. Residues critical for 5J7 binding identified by shotgun mutagenesis loss of binding of E glycoprotein expressed in HEK-293T cells are shown in magenta. Mutations associated with both viral escape from 5J7 and loss of binding in HEK-293T cells (L53 and K128) are shown in orange, and the single residue identified by escape mutation alone (Q269K270\_insK) is shown in cyan. All critical residues were individually identified but are shown on a single E dimer for simplicity. (B) Cartoon representation of the DENV E dimer with the locations of variable EDI/EDII hinge residues transplanted between rDENV-3 and rDENV-4 to make rDENV-3/4 shown in green. (C) Primary sequence alignment and secondary structure of rDENV-3 E, rDENV-3/4 E, and rDENV-4 E. Secondary structure is indicated above the primary sequence and color-coded to the corresponding domains on the tertiary structure (A and B). Arrows indicate  $\beta$ -sheets, cylinders indicate helices, and lines indicate spanning loops and strands. Binding, escape, and hinge residues shown in A and B are indicated by corresponding colors in the rDENV-3 sequence. Amino acid residues transplanted between rDENV-3/4 and rDENV-4 are indicated in green for both sequences.

## Results

**Defining the EDI/EDII Hinge.** To more fully explore the significance of the EDI/EDII region, we examined the epitope target of the potentially neutralizing DENV-3-specific human monoclonal NAb 5J7 (8, 10), which was recovered from a donor (donor 105) previously infected with DENV-3. To identify the 5J7 epitope, we generated a comprehensive library of DENV-3 E proteins, in which every residue was separately mutated (1,400 total mutants). Variant E proteins were expressed individually, and the effect of the point mutations on 5J7 binding to E was analyzed, identifying seven critical residues—Q52, L53, E126, K128, E133, L135, and A203 (Fig. 1 A and C)—all localized to the EDI/EDII hinge region. As a complementary approach, we also passaged DENV-3 in the presence of high concentrations of 5J7 to generate viral escape mutants; we identified three escape mutations—Q269K270\_ins\_K

(8), L53P, and K128G (Fig. 1 A and C)—that are also all localized to the EDI/EDII hinge region. When subsequently tested against donor 105 polyclonal serum, the virus containing the Q269K270\_ins\_K mutation showed a statistically significant 78% drop in neutralization titer (from 1:379 to 1:83;  $P < 0.05$ , ANOVA followed by Dunnett test) (SI Appendix, Fig. S1). One potential explanation for the significant drop in titer is that multiple DENV-3 NAb in human polyclonal serum target epitopes within the EDI/EDII hinge region.

**Construction and Characterization of the Recombinant EDI/EDII Hinge Transplant Virus Recombinant DENV-3/4: Long-Lived Neutralization Responses Against Primary DENV Infections Are Specific to the Serotype Of Infection.** To test the hypothesis that the EDI/EDII hinge region is a major target of the human polyclonal neutralization response after primary infection, we first visualized the 5J7

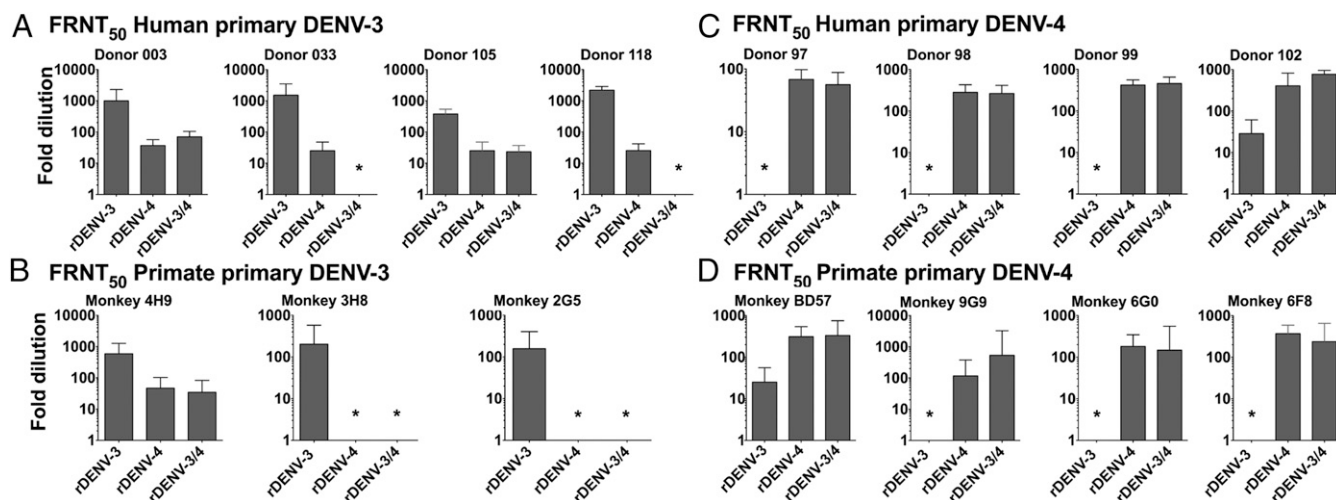


epitope footprint on the DENV-3 E structure (11) by identifying all amino acid residues within 12 Å (i.e., the approximate footprint of an antibody paratope) of the most central escape mutation—K128G. Our intent was to then probe the contribution of this region to polyclonal serum neutralization by extensively mutating it in a DENV-3 infectious clone background (12). Because the EDI/EDII hinge region must remain flexible over the course of DENV infection (13), we replaced the EDI/EDII hinge in a DENV-3 infectious clone with amino acids from the corresponding structures of the DENV-4 EDI/EDII hinge (Fig. 1 *B* and *C*). Our hypothesis was that transplanting DENV-4 EDI/EDII hinge residues into the DENV-3 E protein would maintain short-range residue interactions critical to E-protein hinge function but transfer the DENV-4 EDI/EDII hinge serotype-specific epitopes. The resultant recombinant DENV-3/4 hinge virus (rDENV-3/4) contained 25 amino acid changes, showed similar growth kinetics to parental rDENV-3 in Vero and C6/36 insect cells (*SI Appendix*, Fig. S2), showed similar plaque phenotypes in Vero cells (*SI Appendix*, Fig. S3), and similarly bound E-specific mAbs that target E epitopes outside the EDI/EDII hinge (*SI Appendix*, Fig. S4); collectively, these findings show that EDI/EDII hinge function and E tertiary structure were preserved in the chimeric virus.

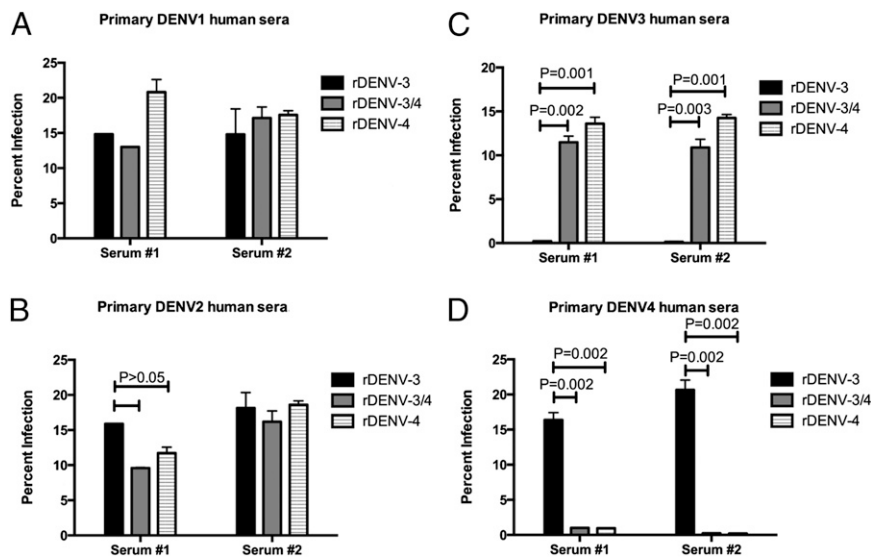
**Transplantation of DENV-4 EDI/EDII Hinge into DENV-3 Leads to Loss of Neutralization by DENV-3 Primary Sera.** To assess the impact of EDI/EDII hinge transplantation on polyclonal antibody neutralization, a panel of human (*SI Appendix*, Table S1) and rhesus macaque (RM) primary DENV-3 convalescent antisera collected >1 y after infection (14) was screened against the parental rDENV-3, rDENV-4, and rDENV-3/4 viruses (Fig. 2). Primary DENV-3 sera potently neutralized DENV-3 and weakly neutralized DENV-4 (Fig. 2 *A* and *B*). Remarkably, rDENV-3/4 hinge was rendered insensitive to neutralization by human and primate primary DENV-3 sera ( $P < 0.05$ ), with a neutralization profile equivalent to DENV-4 sera (Fig. 2 *A* and *B*). This near-complete loss of sensitivity to neutralization by primary DENV-3 sera supports the model that the EDI/EDII hinge region contains most or all of the epitope determinants of serotype specificity after natural DENV infection.

**Transplantation of DENV-4 EDI/EDII Hinge into DENV-3 Leads to Gain of Neutralization by DENV-4 Primary Sera.** If serotype-specific epitopes at the EDI/EDII hinge are the target of human antibodies that neutralize DENV-4 as well, successful transplantation of the DENV-4 EDI/EDII hinge into rDENV-3 should render rDENV-3/4 sensitive to neutralization by primary DENV-4 serum. We then tested rDENV-3, rDENV-4, and rDENV-3/4 virus in neutralization assays against a panel of human (*SI Appendix*, Table S2) and RM primary DENV-4 antisera (14). All eight sera effectively neutralized DENV-4 and had much lower neutralization titers against rDENV-3 (Fig. 2 *C* and *D*). Significantly, rDENV-3/4 was sensitive to neutralization by DENV-4 antisera, gaining >95% of the DENV-4 neutralizing phenotype for all sera tested. This gain of function or sensitivity to neutralization with the EDI/EDII hinge region transplant strongly supports the model that the EDI/EDII region defines the epitopes critical for serotype-specific neutralization after natural DENV infection.

**EDI/EDII Hinge Directs Antibody-Dependent Enhancement in a Serotype-Specific Manner.** In secondary DENV infections, cross-reactive non-NAbs from the first infection are thought to contribute to severe disease pathogenesis by Fc receptor-mediated uptake of antibody-bound virus into target cells, a phenomenon called antibody-dependent enhancement (ADE) (15, 16). To test if EDI/EDII hinge transplantation altered human immune sera enhancement in Fc receptor-bearing cells, rDENV-3, rDENV-4, and rDENV-3/4 viruses were characterized in a K562 cell-based ADE assay using primary human DENV-immune sera (17). As expected, when rDENV-3 or rDENV-4 was incubated with physiologically relevant high concentrations of primary sera from each serotype, each virus was enhanced by heterologous but not homologous immune sera (Fig. 3 and *SI Appendix*, Fig. S5). Thus, at high serum concentrations approximating the levels circulating in people, rDENV-3/4 virus was enhanced by DENV-3 but not DENV-4 immune sera, indicating that the EDI/EDII hinge transplant altered the ADE profile of DENV-3 to a profile similar to DENV-4. When a serum sample had high levels of NAbs to a particular virus (rDENV-3 with primary DENV-3 immune sera), we did not observe enhancement at high serum concentrations (Fig. 3). When NAbs are diluted to subneutralizing levels, they are capable of enhancement (*SI Appendix*, Fig. S5), which is



**Fig. 2.** FRNT<sub>50</sub> values for the parental rDENV-3 infectious clone, parental rDENV-4, and rDENV-3/4 against human and RM primary DENV-3 and DENV-4 immune sera. Each panel shows results for a single donor or RM. The y axis shows serum fold dilution that neutralized 50% of input virus on a log scale. The x axis shows input virus. (A) Four late convalescent primary DENV-4 human primary neutralization titers. Each graph shows one individual. (B) Four convalescent (24 mo postinfection) RM primary DENV-3 neutralization titers. (C) Four late convalescent primary DENV-4 human primary neutralization titers. (D) Four convalescent (24 mo postinfection) RM primary DENV-4 neutralization titers. Error bars show 95% confidence intervals. \*FRNT<sub>50</sub> was <1:20.



**Fig. 3.** Antibody-dependent enhancement of rDENV-3, rDENV-3/4, and rDENV-4 viruses by human primary sera in K562 cells. K562 cells are Fc receptor-bearing cells that are minimally permissive to DENV infection in the absence of antibody, but in the presence of antibody, they become permissive to infection through Fc-mediated endocytosis. The x axis indicates serum tested. The y axis shows percent of K562 cells infected with DENV at 1:20 serum dilution, which was measured by flow cytometry. *P* values were computed for unpaired Student *t* tests using GraphPad Prism, v.6.0b. (A) DENV-1 primary sera, (B) DENV-2 primary sera, (C) DENV-3 primary sera, and (D) DENV-4 primary sera. Error bars show SEM.

unlikely to be of any biological significance at these physiologically irrelevant dilutions of serum.

**EDI/EDII Hinge Directs a Type-Specific Immune Response After Primary Infection.** Primates are the only known vertebrate hosts of DENVs. To study replication and the immunogenic properties of rDENV-3/4 *in vivo*, we inoculated four dengue-naïve RMs with  $5 \times 10^5$  focus forming units (FFUs) virus and followed the animals for viral infection. The RMs did not develop disease but did become viremic for a mean of  $4.25 \pm 0.957$  d (Table 1), similar to days viremia reported for WT DENV at similar doses in RMs (14). At 60 d after rDENV-3/4 inoculation, DENV-4 focus reduction neutralization test (FRNT<sub>50</sub>) titers were approximately four- to sixfold higher than titers against the other three serotypes (Table 1). This early convalescent response is consistent with our hypothesis that the EDI/EDII hinge directs serotype-specific neutralization *in vivo*. It is well-established that RMs exposed to primary DENV infections are protected from repeat infections with the same serotype, while remaining susceptible to secondary infections with new serotypes (18, 19).

**NAbs Targeting the EDI/EDII Are Sufficient to Protect Against DENV Infection.** To test whether NAb targeting the EDI/EDII hinge are sufficient to protect RMs against heterotypic DENV challenge, RMs exposed to primary DENV-4 infections 2 y before (14) were challenged with rDENV-3/4. Heterotypic challenge in

primary immune RMs has been previously shown to result in detectable viremia with a duration of 1–4 d (18, 19). In this challenge, all four animals were protected completely from detectable rDENV-3/4 viremia (Table 1), although serum DENV FRNT<sub>50</sub> titers were significantly boosted (DENV-3 geometric mean titer, 1:1,135; DENV-4 geometric mean titer, 1:5,531) (Table 1). Because the only DENV-4-specific antigen in rDENV-3/4 was the hinge region itself, this result strongly suggests that long-lived NAb that target the EDI/EDII hinge are a major component of protective immunity *in vivo*.

## Discussion

DENV infection typically results in a short-lived broad neutralization response that matures over time into a serotype-specific neutralization response. Results presented here show that the EDI/EDII hinge region is the principle target of long-lived serotype-specific NAb that develop in humans after natural DENV-3 and DENV-4 infections. Moreover, experimental challenge studies in RMs with the rDENV-3/4 chimera show that the hinge region elicits a serotype-specific NAb response *in vivo* and strongly suggest that hinge Abs protect against viremia *in vivo*, although the boosted antibody titers 28 d postchallenge suggest that undetected viral replication may have occurred. The hinge has been shown to play a critical role in the conformational change that E protein undergoes at low pH to fuse with cellular endosomes, allowing viral uncoating and the release of viral RNA into the cellular

**Table 1.** RM prechallenge FRNT<sub>50</sub>, viremia, and postchallenge FRNT<sub>50</sub> following infection with rDENV-3/4

RM identification	DENV exposure history	Prechallenge FRNT <sub>50</sub> titer		Viremia (log FFU/mL) days postchallenge							Days viremia	60-d Postchallenge FRNT <sub>50</sub> titer			
		DENV-3	DENV-4	1	2	3	4	5	6	7		DENV-1	DENV-2	DENV-3	DENV-4
2L7	Naïve	<1:20	<1:20	2.3	2.7	2.8	1.1	1.1	ND	ND	5	1:106	1:160	1:343	1:1,536
2L2	Naïve	<1:20	<1:20	2.7	3.5	1.7	1.1	ND	ND	ND	4	1:269	1:99	1:196	1:1,266
9K8	Naïve	<1:20	<1:20	1.9	2.1	1.8	2.3	1.1	ND	ND	5	1:44	1:95	1:167	1:616
9L4	Naïve	<1:20	<1:20	1.6	2.4	1.1	ND	ND	ND	ND	3	1:107	1:112	1:224	1:1,047
28-d Postchallenge FRNT <sub>50</sub> titer															
BD57	1° DENV-4	<1:20	1:263	ND	ND	ND	ND	ND	ND	ND	0	1:70	1:112	1:1,248	1:10,197
9G9	1° DENV-4	1:35	1:149	ND	ND	ND	ND	ND	ND	ND	0	1:90	1:129	1:734	1:7,147
6G0	1° DENV-4	<1:20	1:146	ND	ND	ND	ND	ND	ND	ND	0	1:36	1:41	1:1,163	1:2,897
6F8	1° DENV-4	<1:20	1:286	ND	ND	ND	ND	ND	ND	ND	0	1:94	1:273	1:1,559	1:5,814

ND, not detected.

cytoplasm (7). We hypothesize that hinge-targeting NAb act through mechanisms that block this structural transition, consistent with what has been reported for West Nile virus (20, 21).

Our data are consistent with models where the EDI/EDII hinge of each serotype contains a single or multiple overlapping epitopes targeted by primate NAb. Indeed, structure studies with human mAbs that bind to the EDI/EDII hinge region of flaviviruses indicate that this region contains overlapping quaternary epitopes (9, 20). Although primary cross-reactive T-cell responses and antibodies do not confer long-term protection against heterologous challenge in primates, it is possible that the EDI/EDII hinge region contains peptides that can be recognized by DENV-specific T cells that also contribute to protection against DENV reinfection. We feel this explanation is less likely given that the most extensive survey of DENV T cell-specific epitopes in humans did not identify any E hinge epitopes among the 25 most immunodominant regions of the DENV proteome (22). Clearly, additional primate challenge and protection studies in different DENV immune backgrounds are needed to more fully decipher the roles of hinge-independent type-specific and cross-protective immunity *in vivo* and the extent to which changes in epitope display outside the EDI/EDII hinge region also impact antibody recognition of the DENV E glycoprotein.

These results have important implications for development of DENV vaccines. The leading DENV vaccines are live-attenuated flaviviruses expressing DENV E proteins (23). Our results indicate that the success of these live flavivirus vaccines may depend on their ability to elicit antibodies that target the EDI/EDII hinge region. Not only will recombinant virus reagents allow us to measure EDI/EDII hinge-specific responses, but our results also provide a basis for designing epitope mimetic peptide vaccines that stimulate serotype-specific NAb and not the cross-reactive antibodies with potential for disease enhancement. More broadly, our demonstration that complex conformational antibody epitopes can be transplanted between live viruses with minimal impacts on growth *in vitro* and *in vivo* opens up possibilities for improving vaccine breadth and specificity in other important human viral pathogens with known complex NAb epitopes, including hepatitis C virus (24), influenza (25), and HIV (26).

## Materials and Methods

**Cells.** Mosquito *Ae. albopictus* C6/36 cells were maintained in MEM (Gibco) media at 32 °C. Human monocyte lymphoma cell line U937 expressing DC-SIGN (U937 DC-SIGN) was maintained in RPMI-1640 (Gibco) at 37 °C supplemented with 50 mM β-mercaptoethanol. Vero-81 cells were maintained in DMEM at 37 °C. All media used were also supplemented with 5% (vol/vol) FBS, 100 U/mL penicillin, 100 mg/mL streptomycin, 0.1 mM nonessential amino acids (Gibco), and 2 mM glutamine, and all cells were incubated in the presence of 5% CO<sub>2</sub>. The 5% FBS was reduced to 2% to make infection media for each cell line.

**AD E Assays.** Antibody-dependent enhance assays were conducted as previously described (17) and adopted for K562 cells. Briefly, polyclonal serum samples were diluted twofold from 1:20 and incubated for 1 h at 37 °C with rDENV-3, rDENV-3/4, or rDENV-4. K562 cells (5 × 10<sup>4</sup> cells/well) were added to the antibody-virus mixture and incubated for an additional 2 h at 37 °C. After the 2-h incubation, cells were washed two times with infection media and incubated overnight at 37 °C and 5% CO<sub>2</sub>. Twenty-four hours after infection, cells were washed, fixed, stained for DENV structural proteins with mAb 4G2, and percent infection-assessed on an EMD Millipore Guava Flow Cytometer.

**Binding ELISA.** Equal virus quantities of DENV-3 and rDENV-3/4 (as previously titrated by ELISA) were captured using a mixture of coated anti-prM and anti-E antibodies. The capture antibodies used were either mouse or human depending on the species of the primary antibody being tested. The primary antibodies, 4G2 (mouse mAb) and 1N5 and 5J7 (human mAbs), were diluted fourfold from 10 to 0.002 μg/mL. Alkaline phosphatase-conjugated secondary antibodies were used to detect binding of primary antibodies

with a P-nitrophenyl phosphate substrate, and color change was quantified with spectrophotometry.

**Shotgun Mutagenesis Epitope Mapping.** A DENV-3 prM/E expression construct (DENV-3 strain CH53489) was subjected to high-throughput mutagenesis (shotgun mutagenesis) to generate a comprehensive mutation library (27). Point mutations were introduced into the DENV-3 prM/E polyprotein by PCR using a Diversity Mutagenesis Kit (Clontech Laboratories, Inc.). In total, 1,400 DENV-3 mutants were generated (>97% coverage of the prM/E ectodomain), sequence confirmed, and arrayed into 384-well plates (one mutation per well). Each E mutant was individually transfected into HEK-293T cells and allowed to express for 22 h. Cells were fixed in 4% (wt/vol) paraformaldehyde (Electron Microscopy Sciences) and permeabilized with 0.1% (wt/vol) saponin (Sigma-Aldrich) in PBS plus calcium and magnesium (PBS++). Cells were stained with purified 5J7 antibody (0.2 μg/mL) diluted in 10% normal goat serum (NGS) (Sigma)/0.1% saponin (pH 9). The optimal primary antibody concentration was determined using an independent immunofluorescence titration curve against WT prM/E to ensure that signals were within the linear range of detection and that signal exceeded background by at least fivefold. Antibody binding was detected using 3.75 μg/mL AlexaFluor488-conjugated secondary antibody (Jackson ImmunoResearch Laboratories) in 10% NGS/0.1% saponin. Cells were washed three times, and mean cellular fluorescence was detected using an Intellicyt High-Throughput Flow Cytometer (Intellicyt). Antibody reactivities against each mutant Env clone were calculated relative to WT Env protein reactivity by subtracting the signal from mock-transfected controls and normalizing to the signal from WT Env-transfected controls for the serotype tested. Mutations within critical clones were identified as critical to the MAb epitope if they did not support reactivity of the test MAb but did support reactivity of other antibodies. This counterscreen strategy facilitates the exclusion of Env mutants that are locally misfolded or have an expression defect (28). Critical amino acids required for antibody binding were visualized on the DENV Env crystal structure (Protein Data Bank ID code 1UZG) (11).

**DENV Infectious Clones.** The four-fragment cloning strategy for the recombinant parental rDENV-3 clone was recently described (12). rDENV-4 [submitted to GenBank (submission no. 1683917)] was constructed in a similar manner. In brief, cDNAs were transcribed from a clinical DENV-3 and DENV-4 isolated and subcloned as four separate DNA fragments into stable plasmids (A–D). Primers were used to introduce a T7 promoter at the 5' end of the A fragment and unique type IIS restriction sites at the 5' and 3' ends of each of the other fragments as follows: rDENV-3: A fragment, SpeI + BsmBI; B fragment, BsmBI + BglI; C fragment, BglI + BglI; D fragment, BglI + BsmBI; rDENV-4: A fragment, SpeI + PflmI; B fragment, PflmI + DralI; C fragment, DralI + PflmI; D fragment, PflmI\_BsmBI. The rDENV-4 A–B fragment junction is at genomic nucleotide positions 3185/3186, the B–C junction is at positions 5458/5459, and the C–D junction is at positions 8832/8833. Plasmids were propagated in *Escherichia coli*, purified, restriction enzyme-digested with type IIS restriction enzymes, and directionally ligated with T4 DNA ligase to create a full-length cDNA of the dengue viral genome. The cDNAs were subsequently transcribed with T7 polymerase. This RNA produced infectious dengue virus when electroporated into Vero E6 cells.

The EDI/EDII hinge residues are encoded exclusively on the A fragment. To generate rDENV-3/4, the nucleotides encoding the DENV-3 EDI/EDII hinge amino acids were replaced with nucleotides encoding the DENV-4 hinge, and a new A fragment containing the nucleotide substitutions was synthesized and inserted into plasmid pUC-57 (BioBasic). Plasmid was propagated in *E. coli*, purified, digested, ligated, and transcribed as previously described (12). Electroporation of recombinant RNA into Vero E6 cells led to recovery of viable recombinant rDENV-3/4. All infectious clones were subsequently passaged one or two times in C6/36 cells, and cell culture supernatants were clarified, supplemented with 20% FBS, and stored at –80 °C.

**DENV Immune Sera.** Human DENV immune sera were collected from either adult volunteers with histories of DENV infection (29) or anonymous blood donors, or they were provided from a pool of previously characterized DENV immune sera. Sera were initially characterized by flow cytometry at the University of North Carolina (30), FRNT<sub>50</sub> at the University of North Carolina, plaque reduction neutralization test (PRNT<sub>60</sub>) at the National Institutes of Health, or PRNT<sub>90</sub> at the Centers for Disease Control and Prevention San Juan to confirm past DENV exposure and also, identify the serotype responsible for primary infections.

**Virus Titration and FRNT.** The FRNT procedure is based on a method previously described by Durbin et al. (31). Briefly, 24-well plates were seeded with 5 × 10<sup>4</sup>



Vero cells in MEM supplemented with 5% FBS and grown for 24 h. Growth media were removed. For virus titration, virus stocks were diluted serially 10-fold and added to individual wells. Cells were overlaid with 1 mL 0.8% methylcellulose in OptiMEM (Gibco) supplemented with 2% FBS (Cellgro) and antibiotic mix (Gibco Anti-Anti), and they were incubated for 5 d at 37 °C and 5% CO<sub>2</sub>. On day 5, overlay was removed, and cells were washed with PBS, fixed in 80% methanol, and developed. To develop plates, fixed monolayers were blocked with 5% instant milk PBS followed by incubation with anti-flavivirus MAb 4G2 diluted 1:1,000. Wells were washed with PBS and incubated with HRP-conjugated goat anti-mouse Ab (Sigma) diluted 1:500 in blocking buffer for 1 h at 37 °C. Plates were washed one time in PBS, and foci were developed by the addition of 100 μL TrueBlue HRP substrate (KPL). Foci were counted on a light box, and viral titers were calculated by standard methods. For FRNT, mAbs or human sera were serially diluted fourfold from starting dilutions of 1:10 or 1:20. Each dilution was mixed with ~50 FFUs virus to a final volume of 200 μL, incubated for 1 h at 37 °C and 5% CO<sub>2</sub>, added in triplicate to 24-wells plates, and processed as above. Mean focus diameter was calculated from ≥20 foci/clone measured at 5× magnification.

**RM Infection and Challenge.** All animals were housed at the Caribbean Primate Research Center facilities. Four young adult RMs (2–4 y of age) seronegative for dengue virus were used as controls (2L7, 2L2, 9K8, and 9L4), and four animals previously exposed to DENV-4 24 mo before (14) were used as the experimental group (6G0, 9G9, BD57, and 6F0). All animals were challenged s.c. with 5 × 10<sup>5</sup> FFUs rDENV-3/4, and then, they were bled every day for 10 d postchallenge and days 30 and 60 after challenge. All animals' procedures were reviewed and approved by the Animal Care and Use Committee at the Medical Sciences Campus, University of Puerto Rico and performed in a facility fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (Animal Welfare Assurance Number: A3421,

protocol number: 7890113). In addition, steps were taken to ameliorate suffering in accordance with the recommendations of the Weatherall report, "The Use of Non-Human Primates in Research." During the time of the protocol, animals were under the Environmental Enrichment Program of the facility, which was also approved by the University of Puerto Rico Institutional Animal Care and Use Committee.

**Software and Statistics.** Multiple alignments were performed using ClustalX version 1.83 (32). The structural model of the hinge epitope region was generated using MacPymol (Delano Scientific) and the crystal structure of DENV-3 envelope (Protein Data Bank ID code 1UZG) (6). Alignments and secondary structure figures were generated using ALINE (33). FRNT counts were entered into Graphpad Prism (Version 5.00 for OSX; GraphPad Software). FRNT<sub>50</sub> values were calculated by sigmoid dose–response curve fitting, with upper and lower limits of 100 and 0, respectively. All error bars show 95% confidence intervals unless otherwise specified.

**ACKNOWLEDGMENTS.** We thank Dr. Fred Sparling for his long-term support and leadership that brought these studies to fruition. We thank Anne Broadwater, Nurgun Kose, Frances House, Teresa Arana Santiago, and Petraleigh Pantoja for excellent technical support. We also thank Dr. Idia V. Rodriguez for excellent veterinarian support of the nonhuman primate studies. This research was supported by National Institutes of Health National Institute of Allergy and Infectious Diseases Grant U54 AI057157 from the Southeast Region Center of Excellence for Emerging Infections and Biodefense (to W.B.M., S.A.S., J.E.C., A.M.d.S., and R.S.B.), National Institute of Allergy and Infectious Diseases Contract HHSN272200900055C (to B.J.D.), and National Institutes of Health National Institute of Allergy and Infectious Diseases Grants P40 OD012217 (to C.A.S.), U24 OD010421 (to C.A.S.), U42OD011128 (to C.A.S.), 1 R56 AI097560-01 (to A.M.d.S., R.S.B., and J.E.C.), and 1 RO1 AI 107731-01 (to A.M.d.S., R.S.B., and J.E.C.).

- Bhatt S, et al. (2013) The global distribution and burden of dengue. *Nature* 496(7446): 504–507.
- World Health Organization (2009) *Dengue: Guidelines for Diagnosis, Treatment, Prevention and Control* (World Health Organization, Geneva).
- Sabchareon A, et al. (2012) Protective efficacy of the recombinant, live-attenuated, CYD tetravalent dengue vaccine in Thai schoolchildren: A randomised, controlled phase 2b trial. *Lancet* 380(9853):1559–1567.
- Rothman AL (2011) Immunity to dengue virus: A tale of original antigenic sin and tropical cytokine storms. *Nat Rev Immunol* 11(8):532–543.
- Dowd KA, Pierson TC (2011) Antibody-mediated neutralization of flaviviruses: A reductionist view. *Virology* 411(2):306–315.
- Modis Y, Ogata S, Clements D, Harrison SC (2003) A ligand-binding pocket in the dengue virus envelope glycoprotein. *Proc Natl Acad Sci USA* 100(12):6986–6991.
- Zhang Y, et al. (2004) Conformational changes of the flavivirus E glycoprotein. *Structure* 12(9):1607–1618.
- de Alwis R, et al. (2012) Identification of human neutralizing antibodies that bind to complex epitopes on dengue virions. *Proc Natl Acad Sci USA* 109(19):7439–7444.
- Teoh EP, et al. (2012) The structural basis for serotype-specific neutralization of dengue virus by a human antibody. *Sci Transl Med* 4(139):139ra83.
- Smith SA, et al. (2012) Persistence of circulating memory B cell clones with potential for dengue virus disease enhancement for decades following infection. *J Virol* 86(5): 2665–2675.
- Modis Y, Ogata S, Clements D, Harrison SC (2005) Variable surface epitopes in the crystal structure of dengue virus type 3 envelope glycoprotein. *J Virol* 79(2): 1223–1231.
- Messer WB, et al. (2012) Development and characterization of a reverse genetic system for studying dengue virus serotype 3 strain variation and neutralization. *PLoS Negl Trop Dis* 6(2):e1486.
- Modis Y, Ogata S, Clements D, Harrison SC (2004) Structure of the dengue virus envelope protein after membrane fusion. *Nature* 427(6972):313–319.
- White LJ, et al. (2013) An alphavirus vector-based tetravalent dengue vaccine induces a rapid and protective immune response in macaques that differs qualitatively from immunity induced by live virus infection. *J Virol* 87(6):3409–3424.
- Halstead SB, Chow JS, Marchette NJ (1973) Immunological enhancement of dengue virus replication. *Nat New Biol* 243(122):24–26.
- Halstead SB (1988) Pathogenesis of dengue: Challenges to molecular biology. *Science* 239(4839):476–481.
- Smith SA, et al. (2013) Human monoclonal antibodies derived from memory B cells following live attenuated dengue virus vaccination or natural infection exhibit similar characteristics. *J Infect Dis* 207(12):1898–1908.
- Halstead SB, Casals J, Shotwell H, Palumbo N (1973) Studies on the immunization of monkeys against dengue. I. protection derived from single and sequential virus infections. *Am J Trop Med Hyg* 22(3):365–374.
- Koraka P, Benton S, van Amerongen G, Stittelaar KJ, Osterhaus AD (2007) Characterization of humoral and cellular immune responses in cynomolgus macaques upon primary and subsequent heterologous infections with dengue viruses. *Microbes Infect* 9(8):940–946.
- Kaufmann B, et al. (2010) Neutralization of west nile virus by cross-linking of its surface proteins with fab fragments of the human monoclonal antibody CR4354. *Proc Natl Acad Sci USA* 107(44):18950–18955.
- Vogt MR, et al. (2009) Human monoclonal antibodies against west nile virus induced by natural infection neutralize at a postattachment step. *J Virol* 83(13):6494–6507.
- Weiskopf D, et al. (2013) Comprehensive analysis of dengue virus-specific responses supports an HLA-linked protective role for CD8+ T cells. *Proc Natl Acad Sci USA* 110(22):E2046–E2053.
- Durbin AP, Whitehead SS (2011) Next-generation dengue vaccines: Novel strategies currently under development. *Viruses* 3(10):1800–1814.
- Keck ZY, et al. (2012) Human monoclonal antibodies to a novel cluster of conformational epitopes on HCV E2 with resistance to neutralization escape in a genotype 2a isolate. *PLoS Pathog* 8(4):e1002653.
- Tsibane T, et al. (2012) Influenza human monoclonal antibody 1F1 interacts with three major antigenic sites and residues mediating human receptor specificity in H1N1 viruses. *PLoS Pathog* 8(12):e1003067.
- Julien JP, et al. (2013) Asymmetric recognition of the HIV-1 trimer by broadly neutralizing antibody PG9. *Proc Natl Acad Sci USA* 110(11):4351–4356.
- Christian EA, et al. (2013) Atomic-level functional model of dengue virus Envelope protein infectivity. *Proc Natl Acad Sci USA* 110(46):18662–18667.
- Paes C, et al. (2013) Atomic-level mapping of antibody epitopes on a GPCR. *JACS* 131(20):6952–6954.
- Wahala WM, Kraus AA, Haymore LB, Accavitti-Loper MA, de Silva AM (2009) Dengue virus neutralization by human immune sera: Role of envelope protein domain III-reactive antibody. *Virology* 392(1):103–113.
- Kraus AA, Messer W, Haymore LB, de Silva AM (2007) Comparison of plaque- and flow cytometry-based methods for measuring dengue virus neutralization. *J Clin Microbiol* 45(11):3777–3780.
- Durbin AP, et al. (2001) Attenuation and immunogenicity in humans of a live dengue virus type-4 vaccine candidate with a 30 nucleotide deletion in its 3'-untranslated region. *Am J Trop Med Hyg* 65(5):405–413.
- Chenna R, et al. (2003) Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Res* 31(13):3497–3500.
- Bond CS, Schuttelkopf AW (2009) ALINE: A WYSIWYG protein-sequence alignment editor for publication-quality alignments. *Acta Crystallogr D Biol Crystallogr* 65(Pt 5): 510–512.