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Functional Transplant of a Dengue Virus Serotype 3 (DENV3)-Specific Human Monoclonal Antibody Epitope into DENV1

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ABSTRACT

The four dengue virus (DENV) serotypes, DENV1 through 4, are endemic throughout tropical and subtropical regions of the world. While first infection confers long-term protective immunity against viruses of the infecting serotype, a second infection with virus of a different serotype carries a greater risk of severe dengue disease, including dengue hemorrhagic fever and dengue shock syndrome. Recent studies demonstrate that humans exposed to DENV infections develop neutralizing antibodies that bind to quaternary epitopes formed by the viral envelope (E) protein dimers or higher-order assemblies required for the formation of the icosahedral viral envelope. Here we show that the quaternary epitope target of the human DENV3-specific neutralizing monoclonal antibody (MAb) 5J7 can be partially transplanted into a DENV1 strain by changing the core residues of the epitope contained within a single monomeric E molecule. MAb 5J7 neutralized the recombinant DENV1/3 strain in cell culture and was protective in a mouse model of infection with the DENV1/3 strain. However, the 5J7 epitope was only partially recreated by transplantation of the core residues because MAb 5J7 bound and neutralized wild-type (WT) DENV3 better than the DENV1/3 recombinant. Our studies demonstrate that it is possible to transplant a large number of discontinuous residues between DENV serotypes and partially recreate a complex antibody epitope, while retaining virus viability. Further refinement of this approach may lead to new tools for measuring epitope-specific antibody responses and new vaccine platforms.

IMPORTANCE

Dengue virus is the most important mosquito-borne pathogen of humans worldwide, with approximately one-half the world's population living in regions where dengue is endemic. Dengue immunity following infection is robust and thought to be conferred by antibodies raised against the infecting virus. However, the specific viral components that these antibodies recognize and how they neutralize the virus have been incompletely described. Here we map a region on dengue virus serotype 3 recognized by the human neutralizing antibody 5J7 and then test the functional significance of this region by transplanting it into a serotype 1 virus. Our studies demonstrate a region on dengue virus necessary for 5J7 binding and neutralization. Our work also demonstrates the technical feasibility of engineering dengue viruses to display targets of protective antibodies. This technology can be used to develop new dengue vaccines and diagnostic assays.

The four dengue virus serotypes (DENV1 to DENV4), transmitted by *Aedes* species mosquitoes, are endemic throughout tropical and subtropical regions of the world (1). Primary infection with virus of one serotype confers long-term immunity against viruses of that serotype, but subsequent infection with virus of a different serotype results in an increased risk of potentially fatal severe dengue disease, including dengue hemorrhagic fever and dengue shock syndrome (2–4). This risk has been attributed, at least in part, to the ability of some cross-reactive antibodies to enhance DENV infection of Fc receptor-bearing cells (5, 6). DENV vaccines are being developed to induce neutralizing and protective antibodies (Abs) to all four serotypes. Both the promise and the challenge of this approach were highlighted by the most advanced live-attenuated vaccine (LAV) candidate (7–9), which has demonstrated varying efficacy across serotypes and age groups. To further advance vaccine development, a better under-

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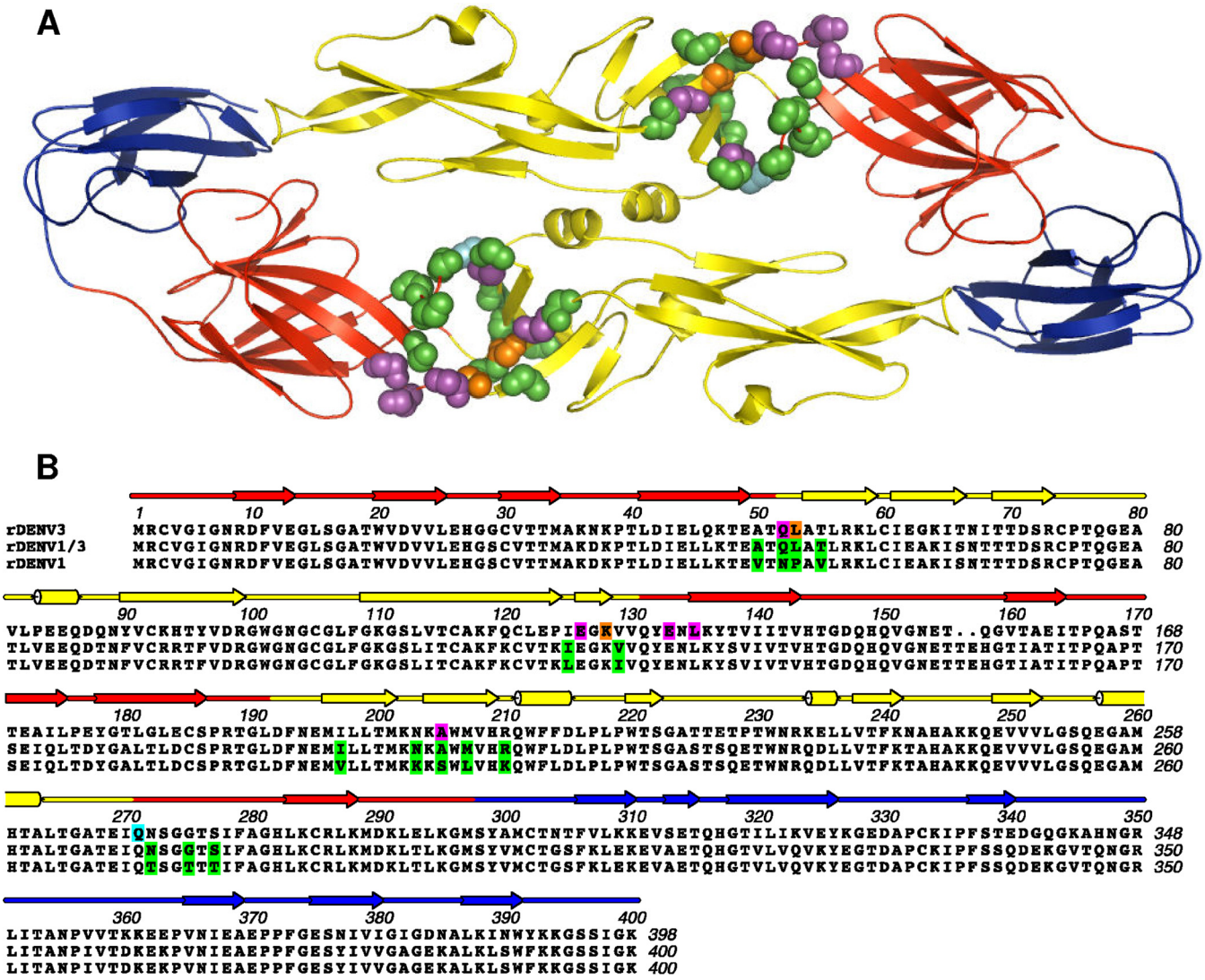

FIG 1 Transplantation of DENV3 MAb 5J7 epitope into DENV1. (A) Structure of DENV3 E dimer with E domains (ED) I (red), II (yellow), and III (blue). The colored spheres represent the putative 5J7 epitope (see below for details). (B) Primary sequence alignment and secondary structure of DENV3, DENV1/3, and DENV1 E protein ectodomains. The secondary structure is indicated above the primary sequence and color coded to the corresponding domains on the tertiary structure. Arrows indicate beta-sheets, cylinders indicate helices, and lines indicate spanning loops and strands. In the DENV3 E sequence, residues critical for human MAb 5J7 binding identified by shotgun mutagenesis of E glycoprotein expressed in HEK-293T cells are shown in magenta. Mutations associated with both viral neutralization escape from MAb 5J7 and loss of binding in HEK-293T cells (L53, K128) are shown in orange, and the single residue identified by escape mutation alone (Q269K270_insK) is shown in cyan. The EDI/II hinge residues not conserved between DENV1 and DENV3 that were mutated to create the rDENV1/3 virus are shown in green.

standing of the properties of natural infection-induced DENV type-specific neutralizing Abs (NAbs), which appear to be critical for long-term protection, is needed (10, 11).

The DENV envelope glycoprotein (E) (Fig. 1A) is the major surface-exposed DENV antigen and the principal target of NAbs. The E protein structure consists of three distinct domains: I, II, and III (EDI to EDIII) (12, 13). EDIII is a continuous peptide extending from EDI and forming an immunoglobulin-like fold, while EDI and EDII are discontinuous and connect via four peptide linkers that form the EDI/II hinge. The EDI/II hinge is thought to play a critical role in the DENV life cycle by facilitating E conformational changes that allow fusion between the viral and endosomal membranes during viral entry (14). Several groups have recently described potent human DENV NAbs that bind to quaternary epitopes centered on the EDI/II hinge (15–18) and EDIII (17, 19–21). We recently reported a cryo-electron microscopy (cryo-EM) structure of monoclonal antibody (MAb) 5J7 bound to DENV3 (17). The cryo-structure revealed a complex, quaternary epitope that spanned three different E protein molecules on the surface of the virion. The cryo-structure raised new questions about the functionally important regions of the 5J7 footprint, in particular regarding the functional role of quaternary interaction residues at the margins of the footprint. Here, using structure-guided design and DENV molecular clones, we test the hypothesis that a neutralizing antibody epitope can be transplanted from one DENV serotype into a virus of another serotype.

Specifically, we demonstrate a partial functional transfer of the complex 5J7 epitope from DENV3 into DENV1 to generate a recombinant DENV1/3 virus (rDENV1/3).

MATERIALS AND METHODS

Viruses and cell lines. All viruses were propagated in *Aedes albopictus* C6/36 cells as previously described (22). *Aedes albopictus* C6/36 cells were maintained in minimal essential medium (MEM; Gibco) at 32°C. All media used were supplemented with 5% fetal bovine serum (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_2. The 5% FBS was reduced to 2% to make infection media for each cell line. rDENV3 is an infectious clone based on the DENV3 clinical isolate UNC3001 (22, 23), and the DENV3 strain used in the mouse infections was the closely related DENV3 clinical isolate UNC3009 (23). Viruses used for AG129 mouse infections were propagated as previously described (24) and concentrated by centrifugation using 100,000 MWCO Amicon filters (Millipore). Titration for mouse infection was performed via plaque assay on baby hamster kidney cells (BHK21, clone 15) as previously described (25).

Shotgun mutagenesis epitope mapping. A DENV3 prM/E expression construct (DENV3 strain CH53489) was subjected to high-throughput mutagenesis ("shotgun mutagenesis") to generate a comprehensive mutation library (26). Point mutations were introduced into the DENV3 prM/E polyprotein by PCR using a Diversity Mutagenesis kit (Clontech Laboratories, Inc.). In total, 1,400 DENV3 mutants were generated (>97% coverage of the prM/E ectodomain), sequence confirmed, and arrayed into 384-well plates (one mutant per well). Each E mutant was individually transfected into HEK-293T cells and allowed to express for 22 h. Cells were fixed in 4% (vol/vol) paraformaldehyde (Electron Microscopy Sciences) and permeabilized with 0.1% (wt/vol) saponin (Sigma-Aldrich) in phosphate-buffered saline (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 wild-type prM/E to ensure that signals were within the linear range of detection and that signal exceeded background at least 5-fold. Antibody binding was detected using 3.75 μg/ml Alexa Fluor 488-conjugated secondary antibody (Jackson ImmunoResearch Laboratories) in 10% NGS–0.1% saponin. Cells were washed 3 times, and mean cellular fluorescence was determined using an Intellicyt high-throughput flow cytometer (Intellicyt). Antibody reactivity against each mutant E clone was calculated relative to wild-type E protein reactivity by subtracting the signal from mock-transfected controls and normalizing to the signal from wild-type E-transfected controls. 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 E mutants that are locally misfolded or have an expression defect (27). Critical amino acids required for antibody binding were visualized on the DENV3 E crystal structure (PDB ID 1UZG) (12).

Binding ELISA. Equal virus quantities of DENV1, DENV3, and rDENV1/3 (as previously titrated by enzyme-linked immunosorbent assay [ELISA]) were captured using a mixture of coated anti-prM and anti-E antibodies. The capture antibodies utilized were either mouse or human depending on the species of the primary antibody being tested. The primary antibodies were diluted 2-fold from starting concentrations of 10 μg/ml with the exception of MAb 14A4 (5 μg/ml). Alkaline phosphatase-conjugated secondary antibodies were utilized to detect binding of primary antibodies with a P-nitrophenyl phosphate substrate and color change quantified with spectrophotometry.

DENV1 infectious clone. The recombinant DENV1 infectious clone is derived from the DENV1 reference strain West Pac '74 (28, 29) and was constructed in a manner similar to that recently described for DENV3 (22). In brief, cDNAs were transcribed from DENV1 RNA, isolated, and subcloned as four separate DNA fragments into stable plasmids (A to D) (see Fig. S1A in the supplemental material). The A fragment was PCR amplified using primers DENV3:1+ (5′-NNNNNNGCGGCCG CTAATACGACTCACTATAGAGTTGTTAGTCTACGTGGACC-3′) and DENV1:2(−) (5′-TGCTCCTACCACAATGTAGC-3′). In primer DENV3:1(+), the T7 RNA polymerase promoter is underlined. The approximately 2.05-kb amplicon was cloned into the TOPO XL PCR cloning vector (Invitrogen). The B fragment was amplified using DENV1:1+ (5′-ACAGCCAACCCCATAGTCAC-3′) and DENV1:4− (5′CATGCCTCC AGCTATTAGTG-3′). This approximately 2.16-kb PCR product was cloned into the TOPO XL plasmid. The C fragment was amplified with DENV1:3+ (5′-TCTCAAGAATGATGTGCCAC-3′) and DENV1:6− (5′-CAGTCATGGCTATTTGTGTG-3′). This approximately 4.35-kb fragment was cloned into the pSMART LC (Kan^r) plasmid (Lucigen). The C fragment has an internal PflMI site (approximately position 6239), which is blocked by overlapping *dcm* methylation. The D fragment was amplified using DENV1:5+ (5′-CAATGGTGTGGTGAGACTGC-3′) and 3DEN-SAP (5′-GCTCTTCNNNNAGAACCTGTTGATTCAACAG CACC-3′). This approximately 2.18-kb fragment was cloned into the TOP0 XL plasmid. A PflMI site (at approximately 10705) was removed by changing an A at position 10700 to a G using standard techniques. Plasmids were propagated in *Escherichia coli*, purified, restriction enzyme digested, and directionally ligated with T4 DNA ligase to create a full-length cDNA of the DENV1 genome. The cDNA was transcribed with T7 polymerase (Ambion) to produce DENV1 full-length genomic RNA. This RNA produced infectious DENV1 when electroporated into Vero E6 cells.

Construction of a recombinant DENV1 with DENV3 EDI/II hinge residues implicated in MAb 5J7 binding. The EDI/II hinge residues are encoded exclusively on the A fragment. To generate rDENV1/3, the nucleotides encoding the DENV1 EDI/II hinge amino acids were replaced with nucleotides encoding the DENV3 hinge and a new subclone 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 (22). Electroporation of recombinant RNA into Vero E6 cells led to recovery of viable recombinant rDENV1/3. All infectious clones were subsequently passaged 2 times in C6/36 cells, and cell culture supernatants were clarified, supplemented with 20% FBS, and stored at −80°C. The presence of specific mutations in virus stocks was confirmed by Sanger sequencing, and the purity of virus stocks was confirmed by serotype type-specific PCR (see Fig. S1B and C in the supplemental material) and restriction enzyme fragment length polymorphism analysis using enzymes that cut parental rDENV1 but not chimeric rDENV1/3 E gene DNA amplified from RNA isolated from working virus stocks of rDENV1/3 (see Fig. S1D in the supplemental material).

Virus titration and FRNT. The focus reduction neutralization test (FRNT) procedure is based on a method previously described by Whitehead (30). Briefly, 24-well plates were seeded with C6/36 or Vero cells in MEM supplemented with 5% FBS and grown for 24 h to 90% confluence, and growth medium was removed. For FRNT, MAb 5J7 was serially diluted 4-fold from starting concentration of 32 μg/ml (a single dilution of 200 μg/ml was also tested against DENV1). Each dilution was mixed with virus titrated to provide ~30 focus-forming units (FFU) of virus/well and applied to Vero cells in triplicate. For titration of virus stocks, tissue culture supernatants were diluted serially 10-fold and added to individual wells in triplicate. Cells were overlaid with 1 ml 0.8% methylcellulose in Opti-MEM (Gibco) supplemented with 2% FBS (Cellgro) and antibiotic mix (Gibco Antibiotic-Antimycotic) and incubated 5 days at 37°C, 5% CO_2. 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 horseradish peroxidase (HRP)-conjugated goat anti-mouse Ab (Sigma) diluted 1:500 in blocking buffer for 1 h at 37°C. Plates were washed once in PBS, and foci were developed by the addition of 100

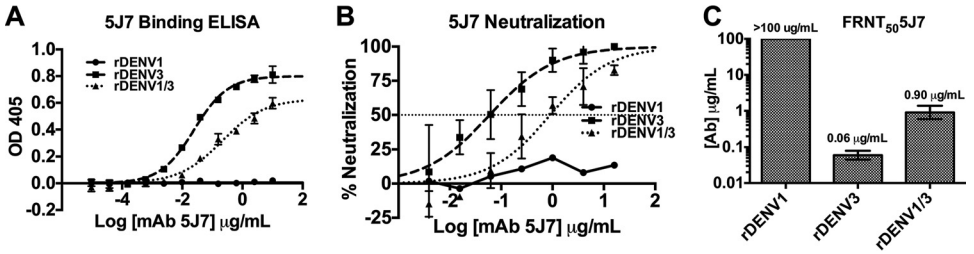

FIG 2 Human MAb 5J7 binds and neutralizes rDENV-1/3. (A) Enzyme-linked immunosorbent assay (ELISA) binding of MAb 5J7 to rDENV-3, rDENV-1, and rDENV-1/3. Results are from 2 technical replicates. Whiskers show the standard deviation for each point estimate. (B) $FRNT_{50}$ curves for MAb 5J7 against rDENV-1, rDENV-3, and rDENV-1/3. The highest MAb 5J7 Ab concentration tested was 100 μg/ml. Results are from 3 technical replicates. Whiskers show 95% confidence intervals for each point estimate. (C) $FRNT_{50}$ values for MAb 5J7 against rDENV-1, rDENV-3, and rDENV-1/3. Results are from 3 technical replicates. $FRNT_{50}$ values are shown above each bar. Whiskers show 95% confidence intervals.

μl of TrueBlue HRP substrate (KPL). Foci were counted on a light box, and viral titers were calculated by standard methods.

Mouse infections. This study was carried out in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health. All procedures were approved by the UC Berkeley Animal Care and Use Committee guidelines. AG129 mice (31) were bred in the Animal Facilities at UC Berkeley. Mice 6 to 8 weeks of age were administered 50 μg of MAb 5J7 or an isotype control antibody (IgG1) intraperitoneally (i.p.) in a total volume of 200 μl, 24 h prior to DENV inoculation. A sublethal dose (1×10^6 PFU) of rDENV1, DENV3, or rDENV1/3 was administered intravenously (i.v.) in a total volume of 100 μl. Three days postinoculation, mice were sacrificed. Serum was obtained from whole blood by centrifugation and stored at −80°C.

Software and statistics. Multiple alignments were performed using ClustalX version 1.83 (32). The structural model of the hinge epitope region was generated using a DENV1 E crystal structure predicted by SWISS-MODEL (33–36) using the rDENV1 primary amino acid sequence threaded through the crystal structure of DENV3 E (PDB 1UZG) (12). Ribbon structure figures were generated using MacPyMOL (The PyMOL Molecular Graphics System, version 1.7.4; Schrödinger, LLC.). Alignments and secondary structure figures were generated using ALINE (37). FRNT counts were entered into GraphPad Prism (version 5.00 for OSX; GraphPad Software, San Diego, CA, USA). Fifty percent FRNT ($FRNT_{50}$) 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.

RESULTS

Design and construction of a recombinant DENV1/3 strain with the DENV3 5J7 epitope. DENV3-specific neutralizing human MAb 5J7 was isolated from a donor with a history of primary DENV3 infection (15, 38). We used two approaches to identify the functionally important residues critical for MAb 5J7 binding and neutralization of DENV3. The first approach, shotgun mutagenesis, used a library of DENV3 E proteins in which every residue was separately mutated. 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. 1A and B), all localized to the EDI/II hinge region. As a second approach, we passaged DENV3 in the presence of increasing concentrations MAb 5J7 to generate viral escape mutants, identifying three escape mutations, Q269K270ins_K (15), L53P, and K128G (Fig. 1A and B), which also localized to the EDI/II hinge region. Taken together, these sites constitute amino acids required for MAb 5J7 binding and subsequent neutralization of DENV3 in these assays.

To test if MAb 5J7 binding and neutralization could be conferred to a heterotypic DENV serotype, we used the DENV3 E structure to identify all amino acid residues within 12 Å (i.e., the approximate size of a typical footprint of an antibody paratope) of the most central escape mutation, K128G. Using this putative epitope footprint, we next aligned DENV3 (donor) (22) and DENV1 (recipient) E glycoprotein primary amino acid sequences and identified the amino acids within that 12-Å footprint that varied between donor and recipient serotypes (Fig. 1A and B). These residues define positions to mutate in order to "transplant" the DENV3 5J7 epitope into DENV1. Though not captured by the 12-Å footprint, we also elected to include variant residues (positions 270, 273, and 275) next to Q269, as mutation in this position conferred neutralization escape. A total of 14 amino acids (Fig. 1, green highlighted residues) in DENV1 were selected for mutating into the corresponding DENV3 amino acids to generate the rDENV1/3 virus displaying the 5J7 epitope.

To carry out the transplant, we first constructed a full-length molecular clone of DENV1 strain WestPac'74. To maximize stability, the molecular clone was designed as four subclones, maintained on separate plasmids, with DNA carrying the DENV genome flanked by unique class II restriction endonucleases, which allow for systematic assembly of a full-length molecular clone (see Fig. S1A in the supplemental material) (19, 22). Using this DENV1 clone as the transplant recipient, we next introduced 14 nucleotide mutations into DNA carrying the DENV1 E gene of a DENV1 infectious clone to create the rDENV1/3 chimeric protein. The resultant recombinant DENV1/3 virus (rDENV1/3) was sequence verified, was shown to be free from contaminating viruses (see Fig. S1B, C, and D in the supplemental material), and showed growth curves and peak titers similar to those of parental rDENV1 in Vero (mammalian) or C6/36 (insect) cells, albeit at early times the recombinant virus grew more slowly than the parental virus (see Fig. S2 in the supplemental material).

The rDENV1/3 virus displays a functional 5J7 epitope. To determine if the 5J7 epitope was displayed and functional on the rDENV1/3 strain, we tested the ability of MAb 5J7 to bind and neutralize rDENV1/3. MAb 5J7 bound to rDENV1/3, confirming the transplantation of amino acids required for antibody binding (Fig. 2A). However, binding to the rDENV1/3 virus was weaker than binding to wild-type (WT) DENV3 (Fig. 2A). We further characterized the neutralization of DENV1, DENV3, and rDENV1/3 by MAb 5J7 using a focus reduction neutralization test (FRNT) (Fig. 2B). The transplanted epitope was functional, as 5J7

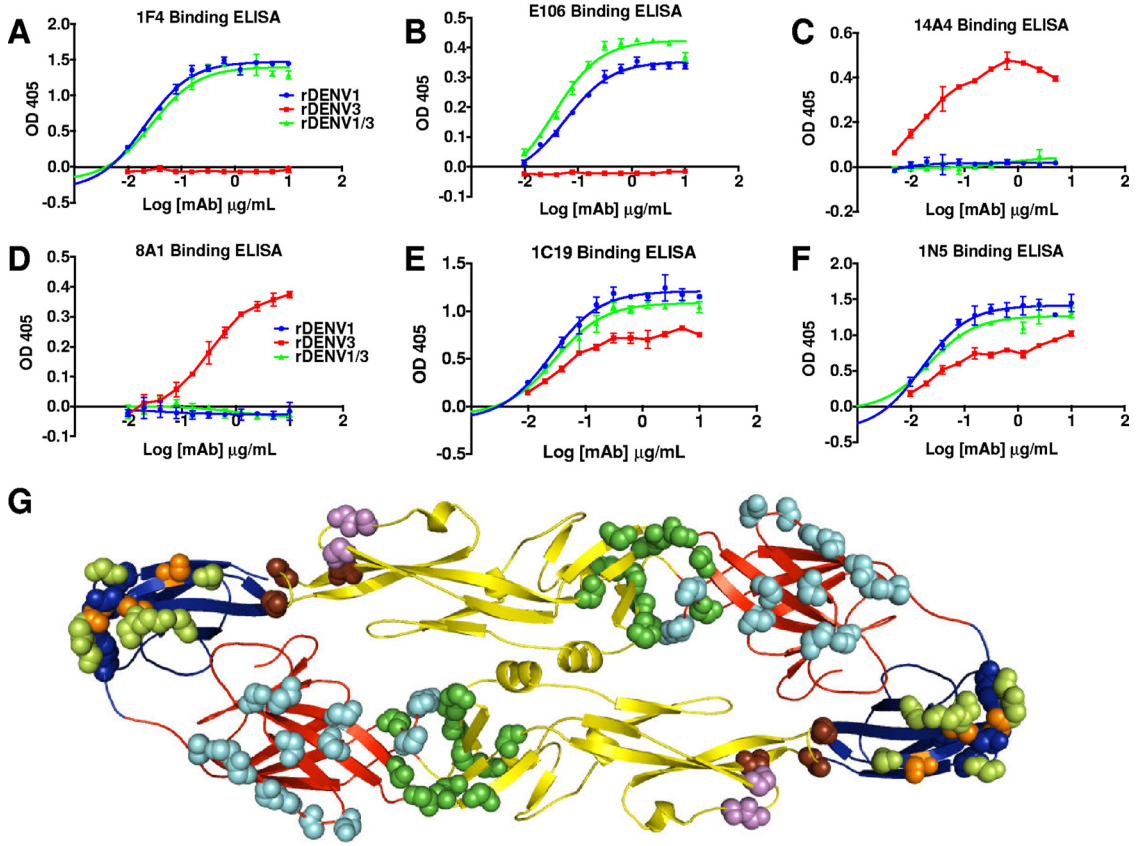

FIG 3 Binding of DENV type-specific and cross-reactive MAbs to rDENV1/3 virus. (A to F) the binding of DENV-1 type-specific (1F4 and E106), DENV-3 type-specific (14A4 and 8A1), and DENV cross-reactive MAbs to rDENV-1, rDENV-3, and rDENV1/3. (G) DENV E protein ribbon structure indicating residues known to be associated with binding for each of the MAbs tested. Cyan, DENV-1 type-specific human MAb 1F4; light purple, DENV cross-reactive human MAb 1C19; lime green, DENV-1 type-specific mouse MAb E106; brown, DENV cross-reactive human MAb 1N5; orange, DENV-3 type-specific mouse MAb 14A4; dark blue, DENV-3 type-specific mouse MAb 8A1; green, DENV-3 type-specific human MAb 5J7 (see Table S1 in the supplemental material for a full description of MAbs used). Results are from 2 technical replicates. Whiskers show the standard deviation for each point estimate.

neutralized the rDENV1/3. However, the WT DENV3 was neutralized approximately 15-fold better than rDENV1/3 (Fig. 2B and C). These data confirmed transplantation of a significant portion of the functional 5J7 antibody epitope into a heterotypic DENV1 background.

To assess whether 5J7 epitope transplantation into DENV1 disrupted or altered the overall structure of DENV1 E protein, we tested the binding of a panel of previously mapped DENV-specific MAbs (see Table S1 in the supplemental material) to rDENV1/3 (Fig. 3A to F). The DENV1 type-specific and neutralizing human MAb 1F4 binds a quaternary epitope that is immediately adjacent to, and potentially disrupted by, the transplanted 5J7 epitope (16) (Fig. 3G). 5J7 epitope transplantation preserved 1F4 binding (Fig. 3A) to rDENV1/3, indicating that the introduction of the 5J7 epitope did not disrupt an immediately adjacent tertiary DENV1 E epitope structure. DENV1 type-specific MAb E106 (39) binds to EDIII. MAb E106 bound to rDENV1/3 (Fig. 3C), demonstrating proper folding of EDIII. DENV cross-reactive MAbs 1C19 and 1N5 bind to conserved residues on the BC loop and near the fusion loop of EDII (40) (Fig. 3G). Both the 1C19 and 1N5 MAb bound to rDENV1/3 (Fig. 3B and D), supporting preservation of more distal EDII tertiary structures. Finally, as a negative control, rDENV1/3 was probed with two DENV3-specific MAbs, 8A1 and 14A4 (41), that bind the DENV3 EDIII lateral ridge and A strand, respectively (Fig. 3G). Neither MAb bound to rDENV1/3 (Fig. 3E and F). Taken together, these results demonstrate that the tertiary structure and antigen terrain of the DENV1 E glycoprotein, including the EDI/II hinge adjacent 1F4 epitope, are preserved in the rDENV1/3 chimera with the 5J7 DENV3 epitope.

The rDENV1/3 virus is sensitive to MAb 5J7 in a mouse model of DENV infection. The alpha/beta and gamma interferon receptor-deficient AG129 mouse strain is a well-established model for DENV viremia (42, 43). We used the AG129 mouse model to test if human MAb 5J7 was protective *in vivo* against DENV3 and rDENV1/3 displaying the 5J7 epitope. Three groups of AG129 mice passively administered 50 μg of 5J7 or an IgG1 isotype control were challenged 24 h later with 10^6 PFU of DENV1, DENV3, or rDENV1/3, and viremia was measured by infectious focus assay 3 days postchallenge. While no animals challenged with rDENV1 were protected from viremia by 5J7, when challenged with either DENV3 or rDENV1/3 the animals treated with 5J7 displayed significantly lower viremia than did those treated with IgG1 alone (Fig. 4), demonstrating that the transplanted epitope was a target of protective 5J7 antibodies in a mouse model of dengue infection.

DISCUSSION

The DENV EDI/II hinge region has been recently identified as a target for several potent, serotype-specific human MAbs (15–18),

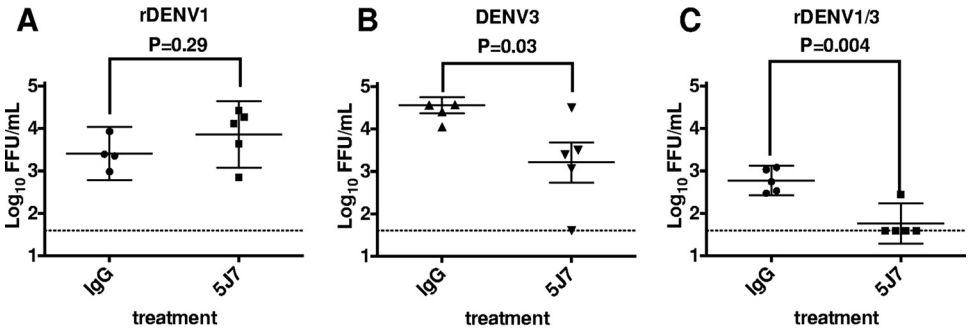

FIG 4 rDENV-1/3 is sensitive to MAb 5J7 in a mouse model of dengue viremia. Mice were passively transfused with 50 μg of MAb 5J7 or nonspecific IgG and challenged 24 h later with 10^6 PFU rDENV-1, rDENV-3, or rDENV-1/3. Three days postinoculation, mice were terminally bled. Viremia in $\log_{10}$ focus-forming units (FFU) per milliliter serum as measured by direct immunofocus assay is shown on the *y* axis, and challenge virus and pretreatment are indicated on the *x* axis. rDENV-1/3 and DENV-3 viremia was significantly lower in animals treated with 5J7 than in animals treated with control IgG. MAb 5J7 treatment did not have an effect on rDENV-1 viremia. Whiskers show 95% confidence intervals (Mann-Whitney U test followed by Dunn's multiple-comparison *post hoc* test, $P < 0.05$).

resulting in increased scrutiny of this region of E protein as a target of human neutralizing and protective antibodies. The results presented here demonstrate that the core epitope of the potently neutralizing human DENV3-specific MAb 5J7, located within the EDI/II hinge, can be both structurally and functionally transplanted between DENV serotypes.

The structure of MAb 5J7 bound to whole DENV3 has recently been resolved at 9 Å by cryo-electron microscopy (cryo-EM) (17) (Fig. 5) (17). The 5J7 MAb footprint, which is mainly contained within the EDI/II hinge region of a single E protein monomer, extends into EDIII and EDII of the adjacent E dimer (Fig. 5). In other words, three E proteins are required to complete the epitope. In the cryo-EM modeled structure of 5J7 bound to DENV3, 24 amino acids distributed across three E protein molecules come into contact with the 5J7 heavy chain. Twenty-three of the 24 heavy-chain contact residues were present on rDENV1/3 (Fig. 5). The single heavy-chain contact residue that was not matched was at position 307 on EDIII of molecule B. Of the 7 light-chain contact residues, three residues at positions 123, 223, and 227 were not accurately matched in rDENV1/3 (Fig. 5).

Although our empirically defined 5J7 epitope showed good agreement with the cryo-EM structure within the EDI/II core

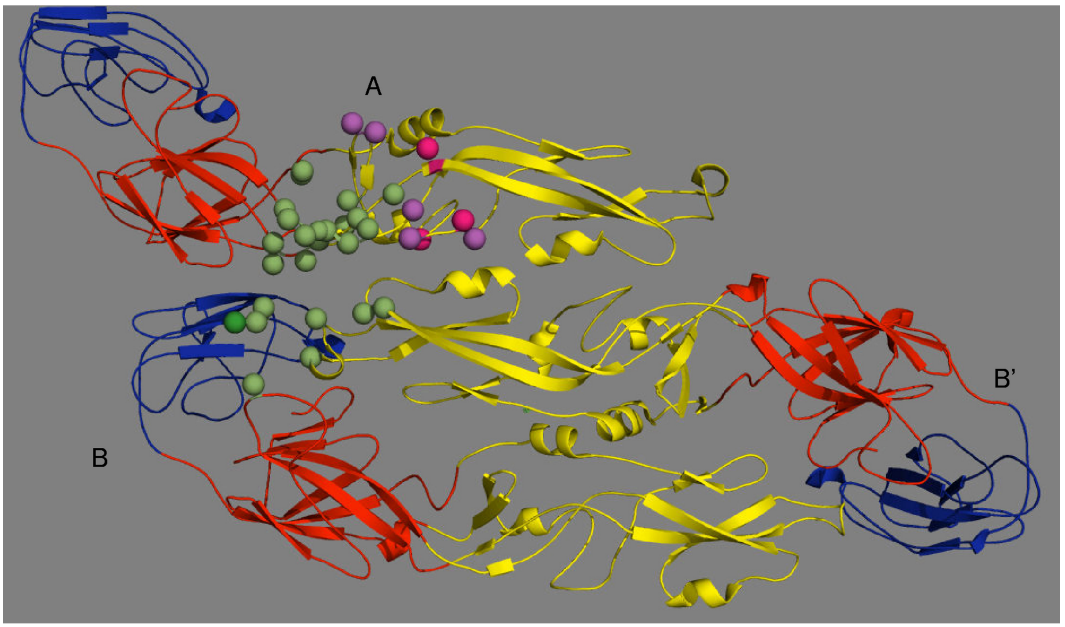

FIG 5 Relationship of MAb 5J7 epitope on rDENV1/3 to the complete 5J7 epitope footprint on DENV3. Fibriansah et al. (17) used cryo-EM to solve a 9-Å resolution structure of the Fab 5J7 DENV3 complex. The 5J7 footprint spanned three different E proteins assembled on the surface of the virus. Each E protein raft on the viral surface contains six E protein monomers (3 dimers). The figure depicts 3 of the 6 monomers on a raft and likely antibody heavy-chain (green circles) and light-chain (pink circles) contact residues. Note that the heavy-chain contact residues are centered on the EDI/II hinge region of monomer A and a few residues on EDIII (monomer B) and EDII (monomer B') of the adjacent dimer. All the light-chain contact residues are contained within the EDII of molecule A. The figure also depicts 5J7 contact residues preserved on rDENV1/3. From the 24 potential heavy-chain contact residues on DENV3, 23 were present on rDENV1/3 because the residues were included in the transplant or conserved between DENV1 and DENV3. The heavy-chain contact residue at position 307 on molecule B (dark green circle) was not matched in the rDENV1/3 chimera. Of the potential 7 light-chain contact residues, 3 amino acids at positions 123, 223, and 227 on molecule A (dark pink) were not matched in the rDENV1/3 chimera.

epitope, the shotgun mutagenesis and antibody escape approaches did not capture the full quaternary epitope. As the shotgun mutagenesis approach evaluated 5J7 binding to E expressed on 293 cells, it is unlikely that the quaternary epitope was displayed, but it is notable that 5J7 bound E expressed on 293 cells, indicating the importance of EDI/II core epitope to 5J7 binding. And while the escape mutant approach did make the full quaternary epitope available, mutations in the EDI/II core were most strongly selected for under neutralizing antibody pressure. Taken together, these two approaches demonstrate the critical role that the EDI/II core plays in 5J7 activity, most likely due to the preservation of nearly all the DENV3 residues contacted by the heavy chain. The reduced binding and neutralization of rDENV1/3 compared to those of WT DENV3 are most likely due to the poor display of 5J7 light-chain binding residues and, possibly, the single heavy-chain mismatch at position 307 on molecule B (Fig. 5). Our observation that transplantation of just 14 residues on the EDI/II hinge region from DENV3 into DENV1 leads to gain of 5J7 binding and neutralization indicates that the transplanted region defines the core of the epitope and that the adjacent quaternary E residues, likely, play a supportive role and contribute to overall Ab affinity.

Our demonstration that a complex conformational antibody epitope can be transplanted between DENV serotypes while maintaining infectivity indicates a hitherto-underappreciated flexibility in the flavivirus E glycoprotein. Of note, the rDENV1/3 virus has "bivalent" properties in that it displays both the DENV1-specific 1F4 and DENV3-specific 5J7 MAb epitopes. The approach of epitope transplantation, while maintaining virus infectivity, sets the stage for developing new diagnostic tools to track epitope-specific Ab responses and new recombinant flavivirus vaccines.

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