Evolution of a Functionally Intact but Antigenically Distinct DENV Fusion Loop

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ABSTRACT

A hallmark of Dengue virus (DENV) pathogenesis is the potential for antibody-dependent enhancement, which is associated with deadly DENV secondary infection, complicates the identification of correlates of protection, and negatively impacts the safety and efficacy of DENV vaccines. Antibody-dependent enhancement is linked to antibodies targeting the fusion loop (FL) motif of the envelope protein, which is completely conserved in mosquito-borne flaviviruses and required for viral entry and fusion. In the current study, we utilized saturation mutagenesis and directed evolution to engineer a functional variant with a mutated fusion-loop (D2-FL) which is not neutralized by fusion-loop-targeting monoclonal antibodies. The fusion-loop mutations were combined with our previously evolved pre-membrane cleavage site to create a mature version of D2-FL (D2-FLM), which evades both pre-membrane and fusion-loop antibodies but retains sensitivity to other type-specific and quaternary cross-reactive antibodies. Cross-reactive serum from heterotypic (DENV4) infected non-human primates showed lower neutralization titers against D2-FL and D2-FLM than isogenic wildtype DENV2 while similar neutralization titers were observed in serum from homotypic (DENV2) infected non-human primates. We propose D2-FL and D2-FLM as valuable tools to delineate cross-reactive antibody subtypes in serum as well as an exciting platform for safer live attenuated DENV vaccines suitable for naïve individuals and children.

INTRODUCTION

Dengue virus (DENV) is a member of the *Flavivirus* genus and is a major global public health threat, with four major serotypes of DENV found worldwide. Dengue causes ~400 million infections each year, of which ~20% of cases present clinically, a subset of which may progress to severe Dengue Hemorrhagic Fever/Dengue Shock Syndrome (DHF/DSS).[1,2] DENV is transmitted through *Aedes* mosquito vectors, and globalization and global warming are increasing the endemic range of Dengue worldwide.[3,4] The pathogenesis of Dengue is complex, as first-time infections are rarely severe and lead to serotype-specific immunity. However, re-infection with a different serotype increases the risk of developing DHF/DSS.[5] This is thought to be due to the phenomenon of antibody-dependent enhancement, in which poorly neutralizing cross-reactive antibodies lead to enhanced viral uptake and infection of unique cell populations in an Fcγ-receptor-mediated manner.[6]

Antibody-dependent enhancement remains a major challenge for DENV vaccine development.[7] The leading DENV vaccine platforms in clinical testing are tetravalent live attenuated virus mixtures of all four serotypes. However, creating formulations that elicit a balanced response has proven challenging.[8] Additionally, lab-grown strains differ from patient-derived DENVs in both maturation status and antigenicity.[9] In particular, antibodies targeting the fusion loop have been reported to neutralize lab and patient strains with differing strengths and have been observed to facilitate Fcγ-receptor uptake *in vitro* and therefore antibody-dependent enhancement.[10–12] Currently, there is a single approved DENV vaccine, Dengvaxia. However, it is only FDA-approved for use in individuals aged 9-16 with previous DENV infection living in endemic areas and is

54 contraindicated for use in naïve individuals and younger children. In naïve children,
55 vaccination stimulated non-neutralizing cross-reactive antibodies that increased the risk
56 of severe disease after DENV infection.[13,14] Other DENV vaccines have been tested or
57 are currently undergoing clinical trial, but thus far none have been approved for use.[15]

58 The DENV fusion loop is located in Envelope protein domain II (EDII) and is
59 involved in monomer-monomer contacts with EDIII.[16] During the DENV infection cycle,
60 low pH triggers a conformational change in the E protein.[17] The structure of the virion
61 rearranges, and individual monomers form a trimer with all three fusion loops in the same
62 orientation, ready to initiate membrane fusion.[16,17] The core fusion-loop motif
63 (DRGWGNGCGLFGK) is highly conserved, with 100% amino acid conservation in all
64 DENV serotypes and other mosquito-borne flaviviruses, including Yellow fever virus
65 (YFV), Zika virus (ZIKV), West Nile virus (WNV), Kunjin virus (KUNV), Murray Valley
66 encephalitis virus (MVEV), Japanese encephalitis virus (JEV), Usutu virus (USUV), and
67 Saint Louis encephalitis virus (SLEV; Figure 1A). Although the extreme conservation and
68 critical role in entry have led to it being traditionally considered impossible to change the
69 fusion loop, we successfully tested the hypothesis that massively parallel directed
70 evolution could produce viable DENV fusion-loop mutants that were still capable of fusion
71 and entry, while altering the antigenic footprint. The fusion-loop mutations, in combination
72 with optimized pre-membrane cleavage site mutations, ablate neutralization by the pre-
73 membrane and fusion-loop antibodies, retain sensitivity to other protective antibodies,
74 and provide a novel vaccine strategy for DENV.

75

RESULTS

To engineer a virus with a novel antigenic footprint at the fusion loop, we targeted the core conserved fusion-loop motif. We generated two different saturation mutagenesis libraries, each with 5 randomized amino acids: DRG**X**G**X**G**XXX**FGK (Library 1) and DRG**XXXXX**GLFGK (Library 2). Library 1 was designed to mutate known residues targeted by fusion-loop antibodies while Library 2 focused on a continuous linear peptide that is the epitope for fusion-loop antibodies to maximally alter antigenicity.[18] Saturation mutagenesis plasmid libraries were used to produce viral libraries in either C6/36 (*Aedes albopictus* mosquito) or Vero 81 (African green monkey) cells and passaged three times in their respective cell types. Following directed evolution, viral genomes were extracted and subjected to deep sequencing to identify surviving and enriched variants (Figure 1B). Due to the high level of conservation, it was not surprising that most mutational combinations failed to yield viable progeny. In fact, evolutions carried out on Library 2 only yielded wild-type sequences. For Library 1, wild-type sequences dominated in Vero 81 evolved libraries. However, a novel variant emerged in C6/36 cells with two amino acid changes: DRGWG**S**GC**L**LFGK. Bulk Sanger sequencing revealed an additional Env-T171A mutation outside of the fusion-loop region. This major variant comprised ~95% of the population, while the next most populous variant comprised only 0.25% (Figure 1C). Residues W101, C105, and L107 were preserved in our final sequence, supporting the structural importance of these residues.[16] When modeled on the pre-fusion DENV2 structure, the N103S and G106L mutations are located at the interface with the neighboring monomer EDIII domain, protected from the aqueous environment. In the post-fusion form, the two residues are located between W101 and F108 and form the

99 bowl concavity above the chlorine ion in the post-fusion trimer (Figure 1D). We used
100 reverse genetics to re-derive the fusion-loop N103S/G106L mutant, which we term D2-
101 FL. As enhancing antibodies also target pre-membrane,[19] we also created a mature
102 version of D2-FL termed D2-FLM, containing both the evolved fusion-loop motif and our
103 previously published evolved pre-membrane furin cleavage site, which results in a more
104 mature virion like those found in infected patients (Figure 1E).[9,20]

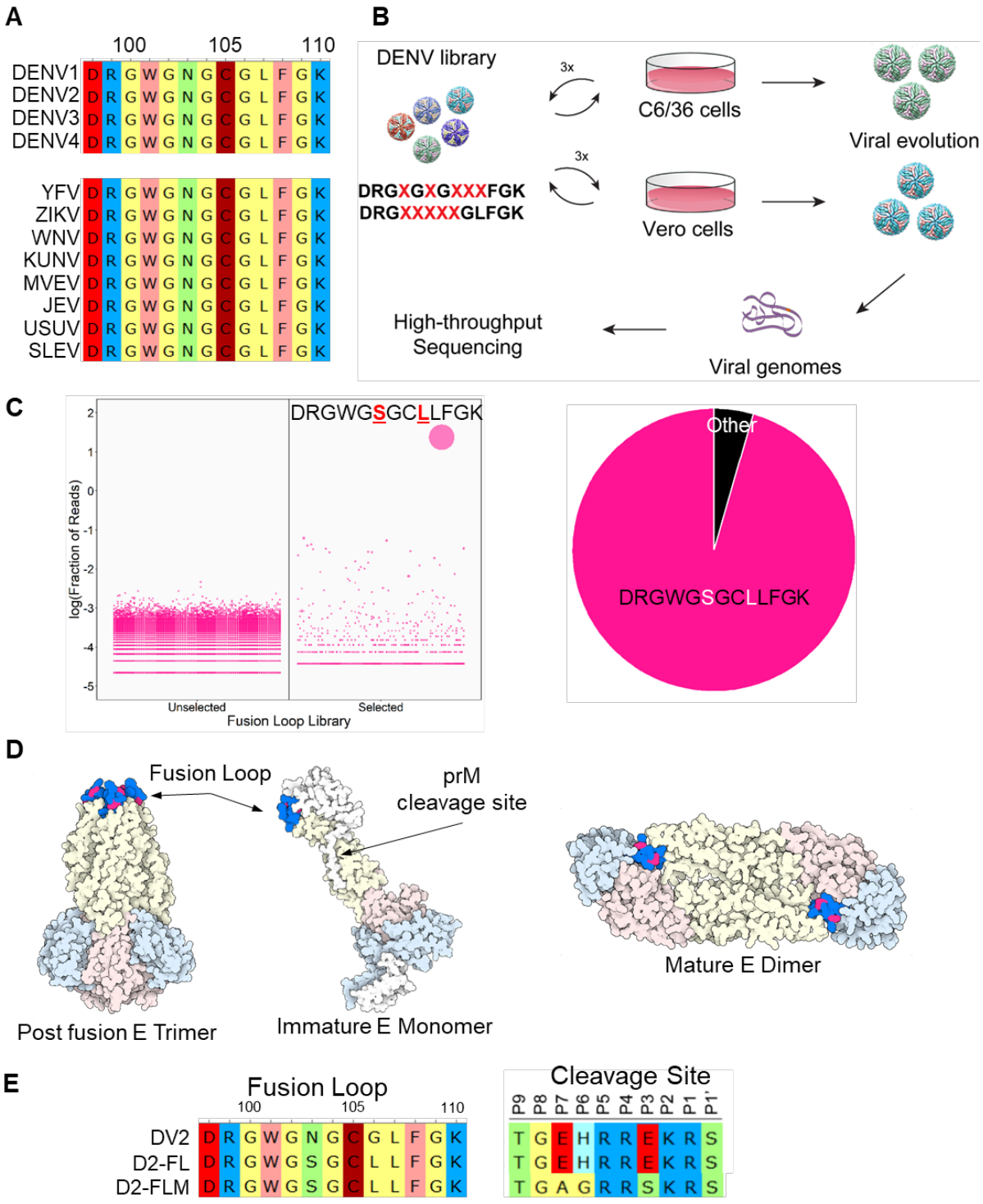


Figure 1: Generation of DENV2 fusion loop mutants via directed evolution. A) Alignment of Top: Dengue virus fusion loops; Bottom: Mosquito-borne flavivirus fusion loops, including Yellow Fever virus (YFV), Zika virus (ZIKV), West Nile virus (WNV), Kunjin virus (KUNV), Murray Valley Encephalitis virus (MVEV), Japanese Encephalitis virus (JEV), Usutu virus (USUV), and Saint Louis Encephalitis virus (SLEV). Amino acids are colored

111 by functional groups: negatively charged (red), positively charged (blue), nonpolar
112 (yellow), polar (green), aromatic (pink), and sulfide (dark red). B) Schematic of directed
113 evolution procedure. Saturation mutagenesis libraries were used to produce viral libraries,
114 which were passaged three times in either C6/36 or Vero 81 cells. At the end of the
115 selection, viral genomes were isolated, and mutations were identified by high-throughput
116 sequencing. C) Left: Bubble plot of the sequences identified from either the unselected
117 or selected (passage 3) C6/36 DENV libraries. Right: Pie chart of the sequences from
118 passage 3 C6/36 DENV libraries. D) Structure of the DENV envelope with the fusion loop
119 mutations highlighted in red. E) Sequences of the fusion loop and furin cleavage site of
120 DENV2, D2-FL, and D2-FLM.
121

122 We performed growth curves comparing DENV2, D2-FL, and D2-FLM in both
123 C6/36 and Vero 81 cells. In C6/36 cells, the growth of all three viruses was comparable,
124 reaching high titers of 10^6-10^7 FFU/mL. However, in Vero 81 cells, both fusion-loop
125 mutant viruses were highly attenuated, with a 2-2.5 log reduction in titer (Figure 2A). The
126 species-specific phenotype in culture involved a change from insect to mammalian cells,
127 as well as a change in growth temperature. To investigate if the mutant viruses were more
128 unstable at higher temperatures, we performed a thermostability assay, comparing
129 viruses incubated at temperatures ranging from 4-55°C before infection. The three viruses
130 had comparable thermostabilities, indicating that this does not explain the attenuation of
131 the fusion-loop mutants (Figure 2B). Because the D2-FLM virus contains mutations that
132 increase pre-membrane cleavage frequency, we also assayed the maturation status of
133 the three viruses by western blot. D2-FL had a comparable pre-membrane-to-envelope
134 ratio to the isogenic wildtype DENV2 (DV2-WT), while, as expected, D2-FLM had a
135 reduced pre-membrane-to-envelope ratio, indicating a higher degree of maturation
136 (Figure 2C).

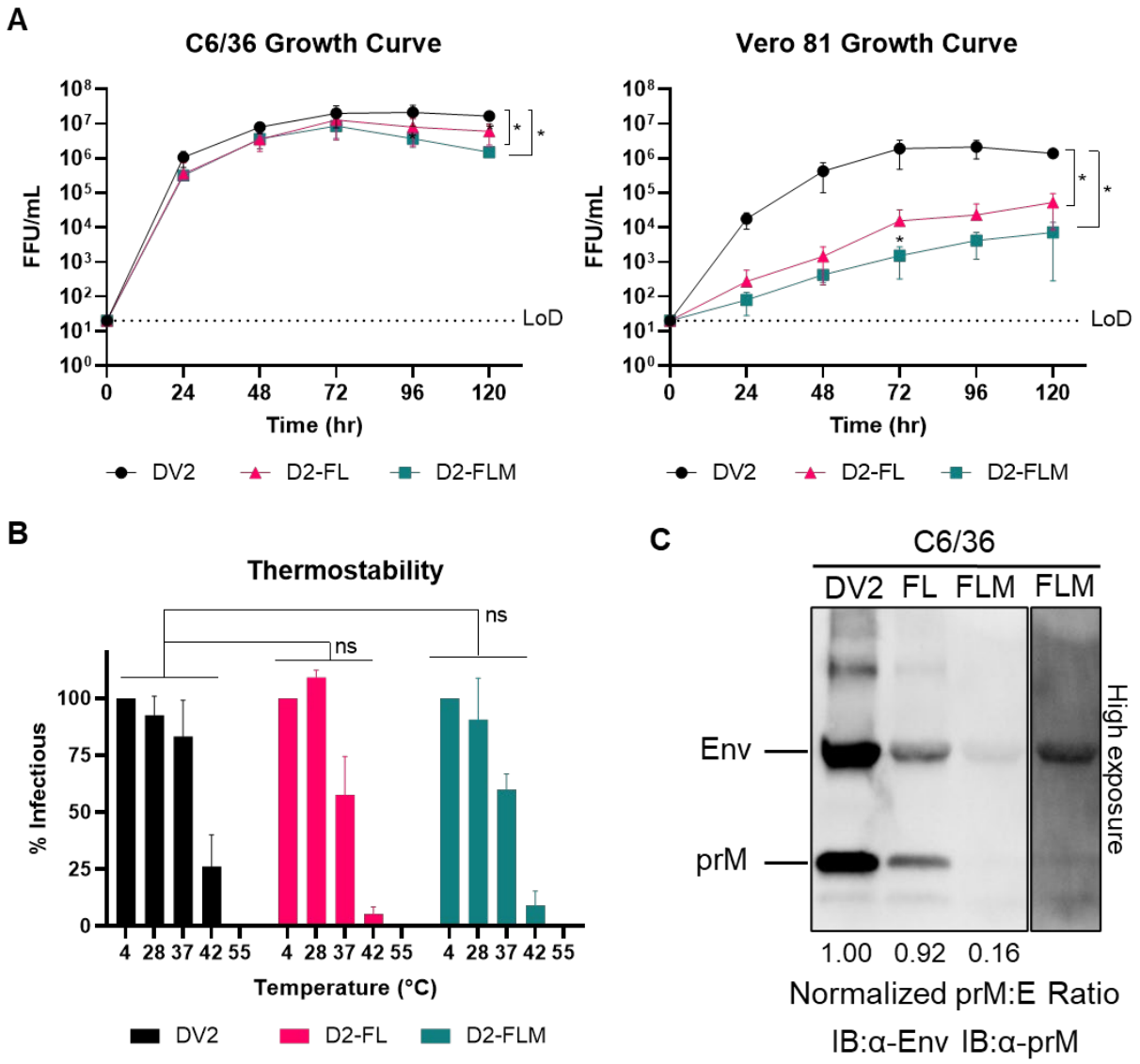


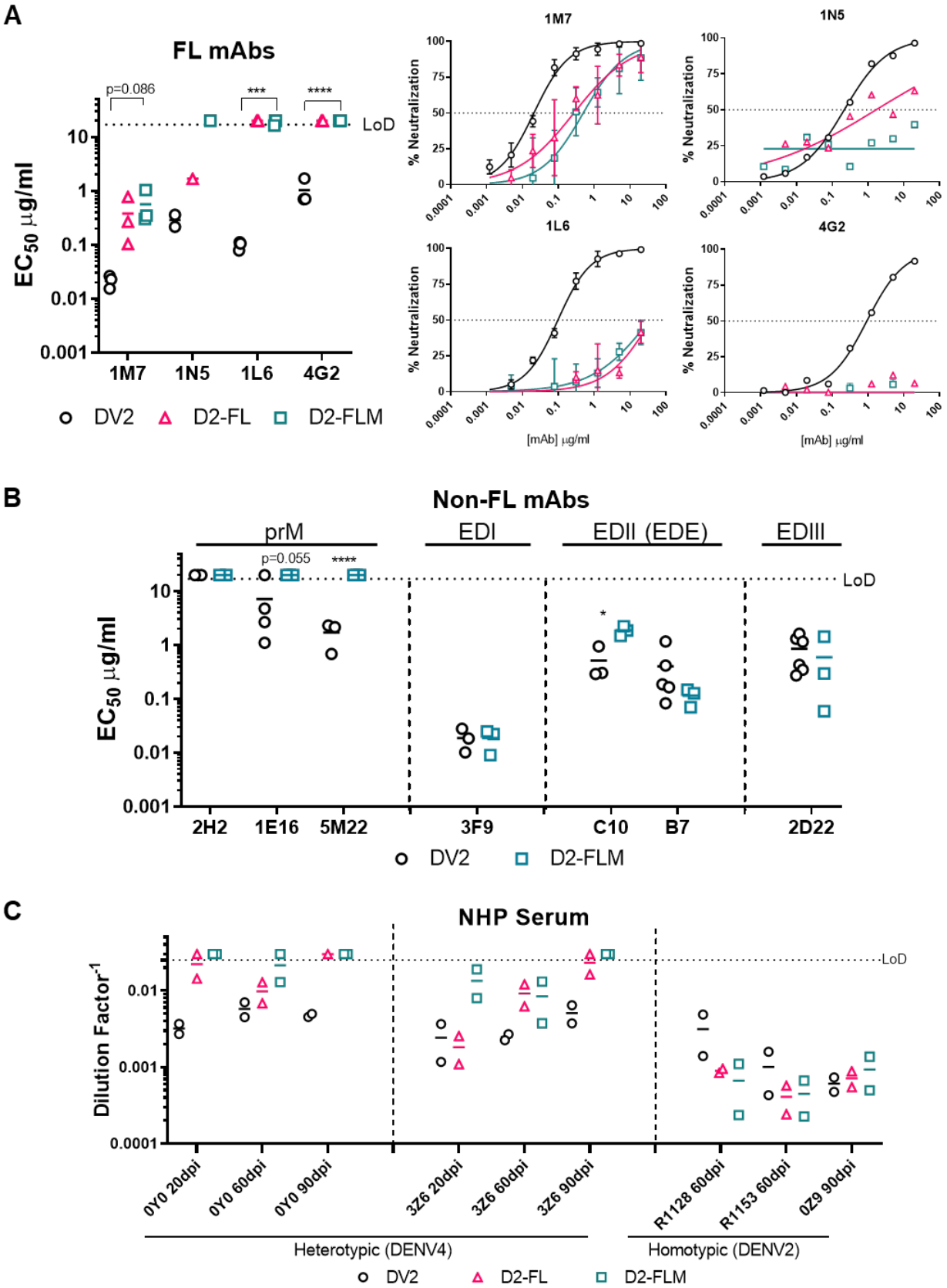
Figure 2: Biological and physical properties of mature DENV2 fusion loop mutants. A) Multistep growth curves (MOI = 0.05-0.1) of DV2-WT, D2-FL, and D2-FLM on C6/36 cells (left) or Vero 81 cells (right). B) Thermostability assay on DV2-WT, D2-FL, and D2-FLM. C) Western blot of virions, blotted against envelopes and pre-membrane proteins. The pre-membrane-to-envelope ratio was determined and normalized to the DV2-WT ratio. Averages of 3 biological replicates are shown. Two-way ANOVA was used for statistical comparison of growth curves and thermostability: ns = not significant; * < 0.05; ** < 0.005; *** < 0.0005.

Next, we characterized the ability of antibodies targeting the fusion loop to recognize DV2-WT, D2-FL, and D2-FLM with a panel of monoclonal antibodies.

149 Importantly, D2-FL and D2-FLM were resistant to antibodies targeting the fusion loop.
150 While neutralization by 1M7 is reduced by ~2-logs, no neutralization was observed for
151 1N5, 1L6, and 4G2 for either variant (Figure 3 A).[18] Focusing on the D2-FLM virus
152 containing both evolved motifs, we then characterized the antigenicity of the whole virion
153 with a panel of antibodies. As expected, D2-FLM was unable to be neutralized by the pre-
154 membrane antibodies 1E16 and 5M22; the antibody 2H2 does not neutralize either DV2-
155 WT or D2-FLM (Figure 3B). For antibodies targeting epitopes in non-mutated regions,
156 including the ED1 and envelop-dimer-epitope that target EDII and EDIII, $FRNT_{50}$ values
157 were generally comparable, although EDE1-C10 shows a moderate but statistically
158 significant reduction between DV2-WT and D2-FLM, indicating that the overall virion
159 structural integrity was intact (Figure 3B).

160 Next, we analyzed neutralization of the D2-FLM virus using serum derived from
161 convalescent humans and experimental infected non-human primates. Overall, we tested
162 serum from 6 humans and 9 non-human primates at different time points with a total of
163 27 samples. Serum from a homotypic infected non-human primate (n=3) did not display
164 a difference in neutralization between DV2-WT, D2-FL, and D2-FLM, confirming that pre-
165 membrane and fusion-loop epitopes are not significant contributors to the homotypic type-
166 specific neutralizing antibody response in primates (Figure 3C). In heterotypic vaccination
167 and infection, most of the serum (18/24) did not cross-neutralize ($FRNT_{50}$ < 1:40) DV2-
168 WT, confirming the serotypic difference of DENVs (Table 1). However, in two non-human
169 primates infected with DENV4, strong neutralization potency ($FRNT_{50}$ between 1: 100 –
170 1:1,000) was demonstrated against DV2-WT (Figure 3C). Heterologous cross-
171 neutralization was significantly reduced to background levels ($FRNT_{50}$ < 1:40) against the

172 D2-FLM virus at 90 days post-infection (dpi). Of note, one DENV4 animal (3Z6) showed
173 low levels of neutralization against D2-FLM at early time points (20- and 60-days post-
174 infection), which was eventually lost at later time points. Neutralization observed against
175 D2-FL, in general, fell between DV2-WT and D2-FLM. Interestingly, in animal 3Z6, at 20
176 dpi, neutralization against DENV-fusion-loop was comparable to DV2-WT, while D2-FLM
177 was greatly reduced, indicating that antibodies in the sera targeting the immature virion
178 formed a large portion of the cross-reactive response. In contrast, animal 0Y0 displayed
179 less difference in neutralization between D2-FL and D2-FLM, suggesting that fusion-loop
180 antibodies were more prominent in this animal. These data suggest that after a single
181 infection, much of the cross-reactive antibody responses target the pre-membrane and
182 the fusion loop and tend to wane over time (Figure 3C). The collection of fusion-loop,
183 mature, and fusion-loop-mature variants provides new opportunities to delineate antibody
184 composition in complex polyclonal serum from DENV natural infection and vaccination.


A
FL mAbs
p=0.086
***
****
LoD
EC50 µg/ml
1M7
1N5
1L6
4G2
DV2
D2-FL
D2-FLM
% Neutralization
[mAb] µg/ml
B
Non-FL mAbs
prM
EDI
EDII (EDE)
EDIII
p=0.055
2H2
1E16
5M22
3F9
C10
B7
2D22
C
NHP Serum
Dilution Factor-1
0Y0 20dpi
0Y0 60dpi
0Y0 90dpi
3Z6 20dpi
3Z6 60dpi
3Z6 90dpi
R1128 60dpi
R1153 60dpi
0Z9 90dpi
Heterotypic (DENV4)
Homotypic (DENV2)

185

186 **Figure 3**: Fusion loop mutant is insensitive to fusion loop antibodies, the major target for
187 cross-reactive antibodies in non-human primates. A) Left: $FRNT_{50}$ values for
188 neutralization of DV2-WT, D2-FL, and D2-FLM with antibodies against the fusion-loop
189 (1M7, 1N5, 1L6, 4G2). All antibodies were tested in at least n=3 independent
190 experiments, except 1N5 due to limited antibodies. Right: Average neutralization curves
191 for neutralization of DV2-WT, D2-FL, and D2-FLM with antibodies against the DENV2
192 fusion loop. B) $FRNT_{50}$ values for neutralization of DV2-WT and D2-FLM with antibodies
193 against DENV2 pre-membrane (2H2, 1E16, 5M22), EDI (3F9), envelop-dimer-epitope
194 (C10, B7), and EDIII (2D22). All antibodies were tested in at least n=3 independent
195 experiments. C) Neutralization of DV2-WT, D2-FL, and D2-FLM with sera from non-
196 human primates s infected with either DENV4 or DENV2. $FRNT_{50}$s were compared using
197 Student's t-test. Significant symbols are as follows: *, $P < 0.05$; **, $P < 0.005$; ***,
198 $P < 0.0005$; ****, $P < 0.00005$. The data are graphed as means ± standard deviations.
199

Human Convalescent Serum	DV2	D2-FL	D2-FLM
DS1500 (DENV1)	0.013	n/a	0.019
DS2499 (DENV1)	0.012	n/a	0.019
DS1136 (DENV3)	0.008	n/a	0.022
DS1660 (DENV3)	0.012	n/a	0.005
DS0275 (DENV4)	0.011	n/a	0.011
DS2239 (DENV4)	0.006	n/a	0.024
* Limit of detection (1:40; 0.25)			
Primate Infection Serum	**DV2**	**D2-FL**	**D2-FLM**
R628 30dpi (DENV4)	0.025	0.025	0.025
R628 60dpi (DENV4)	0.025	0.025	0.025
R628 90dpi (DENV4)	0.024	0.025	0.025
R737 30dpi (DENV4)	0.025	0.025	0.025
R737 60dpi (DENV4)	0.025	0.025	0.025
R737 90dpi (DENV4)	0.025	0.025	0.025
R1132 30dpi (DENV4)	0.025	0.025	0.025
R1132 60dpi (DENV4)	0.025	0.025	0.025
R1132 90dpi (DENV4)	0.025	0.025	0.025
R1160 30dpi (DENV4)	0.017	0.025	0.025
R1160 60dpi (DENV4)	0.017	0.020	0.025
R1160 90dpi (DENV4)	0.025	0.017	0.011

200

201 **Table 1**: Summary of $FRNT_{50}$s of human convalescent serum and non-human primate
202 infection serum against DV2, D2-FL, and D2-FLM.

203 **DISCUSSION**

204 Mechanistic understanding of vaccine protection and identification of correlates of
205 protection are immensely important for DENV vaccine development. The dual protective
206 and enhancing properties of DENV antibodies create major challenges for dissecting the
207 role of various antibody populations in disease protection. Cross-reactive weakly
208 neutralizing pre-membrane and fusion loop antibodies are often immunodominant after
209 primary DENV infection,[10,21–24] and can lead to overestimation of the levels of heterotypic
210 protection in traditional neutralization assays. Since these same antibodies are also
211 associated with antibody-dependent enhancement,[19] inaccurate conclusions could have
212 dire consequences if protection *in vitro* translates to the enhancement of disease in
213 human vaccinees. Unfortunately, antibody profiling in polyclonal serum is mainly
214 performed by ELISA, and a neutralization assay that can discriminate antibodies does not
215 exist. The D2-FLM variant is not neutralized by fusion-loop and pre-membrane antibodies
216 and appears insensitive to neutralization by these antibodies in polyclonal serum. Of note,
217 EDE1-C10 neutralization potency was also reduced in our FLM variant, further indicating
218 our mutations are affecting the tip of the EDII region which partially overlaps with the
219 EDE1 epitope[25]. In combination with other chimeric DENVs,[26–28] D2-FLM provides a
220 reagent to distinguish between type-specific, protective cross-reactive (e.g. envelop-
221 dimer-epitope),[29,30] and antibody-dependent enhancement-prone cross-reactive (e.g.
222 fusion-loop and pre-membrane)[24] antibody subclasses in neutralization assays after
223 infection and vaccination.

224 Due to the antibody-dependent enhancement properties of DENV antibodies,
225 studies to understand and eliminate antibody-dependent enhancement phenotypes are

226 under active investigation. For example, antibodies can be engineered to eliminate
227 binding to the Fcγ receptor, abolishing antibody-dependent enhancement.[31] While this
228 methodology holds potential for antibody therapeutic development and passive
229 immunization strategies, it is not relevant for vaccination. As fusion-loop and pre-
230 membrane targeting antibodies are the major species demonstrated to cause antibody-
231 dependent enhancement *in vitro* and are thought to be responsible for antibody-
232 dependent enhancement-driven negative outcomes after primary infection and
233 vaccination,[10–12,32] we propose that genetic ablation of the fusion-loop and pre-membrane
234 epitopes in vaccine strains will minimize the production of these subclasses of antibodies
235 responsible for undesirable vaccine responses. Indeed, efforts have been made to
236 reduce the availability of the fusion-loop or reduce the ability of fusion-loop antibodies to
237 drive antibody-dependent enhancement. Covalently locked envelope-dimers and
238 envelope-dimers with fusion-loop mutations have been engineered as subunit vaccines
239 that reduce the availability of the fusion-loop, thereby reducing the production of fusion-
240 loop antibodies.[33–36] DENV subunit vaccines are an area of active study;[37] however,
241 monomer/dimer subunits can also expose additional, interior-facing epitopes not normally
242 exposed to the cell. Furthermore, dimer subunits are not a complete representation of the
243 DENV virion which presents other structurally important interfaces such as the 3-fold and
244 5-fold symmetries. Concerns about balanced immunity to all four serotypes also apply to
245 subunit vaccine platforms. Given the complexity of the immune response to DENV, live
246 virus vaccine platforms have thus far been more successful. Previously, a single study on
247 WNV successfully generated a viable virus with a single mutation at the fusion loop,
248 although it severely attenuated neurovirulence.[38] Otherwise, it has not been generated in

249 DENV or other mosquito-borne flaviviruses. Using directed-evolution, we successfully
250 generated our D2-FLM variant that combines viability with the desired antibody
251 responses. Therefore, the D2-FLM variant is a novel candidate for a vaccine strain which
252 presents all the native structures and complex symmetries of DENV necessary for T-cell
253 mediated responses and which can elicit more optimal protective antibody responses.[39–
254 41]

255 Other considerations of high importance when designing a live DENV vaccine
256 include strain selection and serotype balance.[42] In the current study we used DENV2
257 S16803, a prototype for DENV2.[43] However, S16803 was isolated several decades ago,
258 and it may be beneficial to utilize more contemporaneous strains.[8,44] Work is currently
259 ongoing to demonstrate the portability of the evolved fusion-loop motif on additional
260 DENV2 strains and other serotypes, which is essential for tetravalent vaccine production.
261 D2-FLM was highly attenuated in Vero cells, creating a challenge for vaccine production.
262 Therefore, further adaptation of this strain to grow efficiently in mammalian cells while
263 retaining its antigenic properties is needed. Taken together, the FLM variant holds
264 exciting new possibilities for a new generation of DENV vaccines, as well as a platform
265 to readily measure type-specific and cross-reactive antibody-dependent enhancement-
266 type responses and thereby assess the true protective potential of any DENV vaccine
267 trials and safeguard approval of DENV vaccines for human use.

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AUTHOR CONTRIBUTION

R.M.M. and L.V.T. designed the study. R.M.M. performed high-throughput sequencing preparation and analysis. R.M.M., D.Z., S.D., L.J.S., Y.D., and L.V.T. performed experiments. D.T., L.J.W., A.M.D.S., and R.S.B. provided reagents. L.V.T. and R.S.B. provided oversight of the project and funding. R.M.M. wrote the manuscript. L.V.T. reviewed and revised the final version. All authors approved the final version of the manuscript.

CONFLICT DISCLOSURE

R.M.M., R.S.B., and L.V.T. are inventors on a patent application filed on the subject matter of the manuscript.

284 **MATERIALS AND METHODS**

285 Cells and viruses

286 C6/36 (ATCC cross-reactiveL-1660) were grown in MEM (Gibco) with 5% FBS (HyClone),
287 1% penicillin/streptomycin (Gibco), 0.1mM nonessential amino acids (Gibco), 1% HEPES
288 (Gibco), and 2mM GlutaMAX (Gibco), cultured at 32°C with 5% CO_2. Vero 81 cells (ATCC
289 CCL-81) were grown in DMEM/F12 (Gibco) with 10% FBS, 1% penicillin/streptomycin
290 (Gibco), 0.1mM nonessential amino acids (Gibco), and 1% HEPES (Gibco), cultured at
291 37°C with 5% CO_2. DENV viruses were grown in C6/36 or Vero 81 cells maintained in
292 infection media. C6/36 infection media consists of Opti-MEM (Gibco) with 2% FBS
293 (HyClone), 1% penicillin/streptomycin (Gibco), 0.1mM nonessential amino acids (Gibco),
294 1% HEPES (Gibco), and 2mM GlutaMAX (Gibco). Vero 81 infection media consists of
295 DMEM/F12 (Gibco) with 2% FBS, 1% penicillin/streptomycin (Gibco), 0.1mM
296 nonessential amino acids (Gibco), and 1% HEPES (Gibco). DENV2 strain S16803 was
297 used in this study.[43] Sequences used for the alignments include DENV1 WestPac-74
298 (U88535.1), DENV2 S-16803 (GU289914.1), DENV3 3001 (JQ411814.1), DENV4 Sri
299 Lanka-92 (KJ160504.1), YFV 17D (NC_002031.1), SLEV Kern217 (NC_007580.2), JEV
300 (NC_001437.1), USUV Vienna-2001 (NC_006551.1), MVEV (NC_000943.1), WNV-1
301 NY99 (NC_009942.1), and ZIKV MR-766 (NC_012532.1).

302 DENV Reverse Genetics

303 DENV2 S16803 was used in this study. Recombinant viruses were created using a four-
304 plasmid system as previously described,[45] consisting of the DENV genome split into four
305 segments, each cloned into a separate plasmid. The DENV plasmids were digested and
306 ligated to form a single template for *in vitro* transcription. The resulting RNA was

307 electroporated into either C6/36 or Vero cells. Virus-containing supernatant was
308 harvested at 4-5 days post electroporation and passaged. DENV variants were created
309 through site-directed mutagenesis of the DENV plasmids.

310 Library Generation and Directed Evolution

311 DENV fusion loop libraries were generated through saturation mutagenesis of the
312 indicated resides, based on a previously published protocol.[20,46] Degenerate NNK
313 oligonucleotides were used to amplify the region, generating a library of mutated DNA
314 fragments. Q5 DNA Polymerase was used with less than 18 cycles to maintain accuracy.
315 The resulting library was cloned into the DENV reverse genetics system. The ligated
316 plasmids were electroporated into DH10B ElectroMax cells (Invitrogen) and directly
317 plated on 5,245mm^2 dishes (Corning) to avoid bias from the suspension culture. Colonies
318 were pooled and purified using a Maxiprep kit (Qiagen), and the plasmid library was used
319 for DENV reverse genetics (above). Viral libraries were passaged three times in the
320 corresponding cell type.

321 High-throughput Sequencing and Analysis

322 Viral RNA was isolated with a QIAamp viral RNA kit (Qiagen), and cDNA was produced
323 using the Superscript IV Reverse Transcriptase (Invitrogen). Amplicons were prepared
324 for sequencing using the Illumina TruSeq system with two rounds of PCR using Q5 Hot
325 Start DNA polymerase (NEB). For the first round of PCR, primers were specific to the
326 DENV2 E sequence surrounding the fusion loop motif with overhangs for the Illumina
327 adapters. After purification, this product was used as the template for the second round
328 of PCR using Illumina P5 and P7 primers containing 8-nucleotide indexes. Purified PCR
329 products were analyzed on a Bioanalyzer (Agilent Technologies) and quantified on a

330 Qubit 4 fluorometer (Invitrogen). Amplicon libraries were run on a MiSeq system with
331 2x150bp reads. Plasmid and P0 libraries were sequenced at a depth of ~4.5 million reads;
332 later passages were sequenced at a depth of ~750,000 reads. Custom perl and R scripts
333 were used to analyze and plot the data as previously published.[20]

334 DENV Growth Kinetics

335 One day before infection, $5x10^5$ cells were seeded in every well of a 6-well plate. Cells
336 with infected with an MOI of 0.05-0.1, estimating $1x10^6$ cells on the day of infection.
337 Infection was carried out for one hour in the incubator, followed by 3x washes with PBS
338 and replenishment with fresh infection medium. 300 uL of viral supernatant was collected
339 at 0, 24, 48, 72, 96, and 120 hours and stored at -80°C. All experiments were performed
340 independently at least three times.

341 DENV Focus-Forming Assay

342 Titers of viral supernatant were determined using a standard DENV focus-forming assay.
343 In brief, cells were seeded at $2x10^4$ cells per well of a 96-well plate one day before
344 infection. The next day, 50 uL of 10-fold serial dilution of viral supernatant were added to
345 each well for 1 hour in the incubator. After, 125uL of overlay (Opti-MEM, 2% FBS, NEAA,
346 P/S, and methylcellulose) was added to each well. Infection was allowed to continue for
347 48 hours in the incubator. Overlay was removed, and each well rinsed 3x with PBS
348 followed by a 30-minute fixation with 10% formalin in PBS. Cells were blocked in
349 permeabilization buffer (eBioscience) with 5% nonfat dried milk. Primary antibodies anti-
350 pre-membrane 2H2 and anti-E 4G2 from non-purified hybridoma supernatant were used
351 at a 1:500 dilution in blocking buffer. Goat anti-mouse HRP secondary (SeraCare KPL)
352 was used at a 1:1000 dilution in a blocking buffer. Following washing, foci were developed

353 using TrueBlue HRP substrate (SeraCare) and counted using an automated Immunospot
354 analyzer (Cellular Technology).

355 Thermal Stability Assay

356 The indicated viruses were thawed and incubated at temperatures ranging from 4°C to
357 55°C for one hour. Following, viral titers were determined by focus-forming assay as
358 described above.

359 Western Blotting

360 Viral supernatants were combined with 4X Laemmli Sample Buffer (Bio-Rad) and boiled
361 at 95°C for 5 minutes. After SDS-PAGE electrophoresis, samples were transferred to
362 PVDF membrane and blocked in 3% nonfat milk in PBS-T. A polyclonal rabbit anti-pre-
363 membrane (1:1000; Invitrogen PA5-34966) and polyclonal rabbit anti-Env (Invitrogen
364 PA5-32246) in 2% BSA in PBS-T were incubated on the blot for 1 hour at 37C. Goat anti-
365 rabbit HRP (1:10,000 Jackson ImmunoLab) in 3% milk in PBS-T was incubated on the
366 blot for 1 hour at room temperature. Blots were developed by SuperSignal West Pico Plus
367 chemiluminescent substrate (ThermoFisher). Blots were imaged on an iBright fusion-
368 loop1500 imaging system (Invitrogen).

369 FRNT Assay

370 Focus reduction neuralization titer (FRNT) assays were performed as described
371 previously with C6/36 cells.[20] $1x10^5$ cells were seeded in a 96-well plate the day prior to
372 infection. antibodies or sera were serially diluted and mixed with virus (~100 FFU/well) at
373 a 1:1 volume and incubated for 1 hour in the incubator. The mixture was added onto the
374 plate with cells and incubated for 1 hour in the incubator, then overlay was added (see
375 Focus-Forming Assay) and plates were incubated for 48 hours. Viral foci were stained

376 and counted as described above (Focus-Forming Assay). A variable slope sigmoidal
377 dose-response curve was fitted to the data, and values were calculated with top or bottom
378 restraints of 100 and 0 using GraphPad Prism version 9.0. All experiments were
379 performed independently at least two times, due to limited amounts of human serum.

380 Statistical Analysis

381 GraphPad Prism version 9.0 was used for statistical analysis. Titer and % infection of D2-
382 FL and D2-FLM were compared to the DV2 using two-way ANOVA. $FRNT_{50}$s were
383 compared using Student's t-test. Significant symbols are as follows: *, $P < 0.05$; **, $P <$
384 0.005; ***, $P < 0.0005$; ****, $P < 0.00005$. The data are graphed as means ± standard
385 deviations.

386 **REFERENCES**

387 1. Bhatt, S. *et al.* The global distribution and burden of dengue. *Nature* **496**, (2013).
388 2. Brady, O. J. *et al.* Refining the Global Spatial Limits of Dengue Virus
389 Transmission by Evidence-Based Consensus. doi:10.1371/journal.pntd.0001760.
390 3. Ebi, K. L. & Nealon, J. Dengue in a changing climate. *Environ Res* **151**, 115–123
391 (2016).
392 4. Messina, J. P. *et al.* The current and future global distribution and population at
393 risk of dengue. *Nat Microbiol* doi:10.1038/s41564-019-0476-8.
394 5. Halstead, S. B. Pathogenesis of Dengue: Challenges to Molecular Biology.
395 *Science (1979)* **239**, 476–481 (1988).
396 6. Katzelnick, L. C. *et al.* Antibody-dependent enhancement of severe dengue
397 disease in humans. *Science (1979)* **358**, 929–932 (2017).
398 7. Rey, F. A., Stiasny, K., Vaney, M., Dellarole, M. & Heinz, F. X. The bright and the
399 dark side of human antibody responses to flaviviruses: lessons for vaccine
400 design. *EMBO Rep* **19**, 206–224 (2018).
401 8. Rabaa, M. A. *et al.* Genetic epidemiology of dengue viruses in phase III trials of
402 the CYD tetravalent dengue vaccine and implications for efficacy. *Elife* **6**, 1–22
403 (2017).
404 9. Raut, R. *et al.* Dengue type 1 viruses circulating in humans are highly infectious
405 and poorly neutralized by human antibodies. *Proc Natl Acad Sci U S A* **116**, 227–
406 232 (2019).
407 10. Lai, C. Y. *et al.* Analysis of Cross-Reactive Antibodies Recognizing the Fusion
408 Loop of Envelope Protein and Correlation with Neutralizing Antibody Titers in
409 Nicaraguan Dengue Cases. *PLoS Negl Trop Dis* **7**, 1–11 (2013).
410 11. Beltramello, M. *et al.* The human immune response to dengue virus is dominated
411 by highly cross-reactive antibodies endowed with neutralizing and enhancing
412 activity. *Cell Host Microbe* **8**, 271–283 (2010).
413 12. Costin, J. M. *et al.* Mechanistic Study of Broadly Neutralizing Human Monoclonal
414 Antibodies against Dengue Virus That Target the Fusion Loop. *J Virol* **87**, 52–66
415 (2013).
416 13. Wilder-Smith, A. *et al.* Personal View Deliberations of the Strategic Advisory
417 Group of Experts on Immunization on the use of CYD-TDV dengue vaccine The
418 Strategic Advisory Group of Experts (SAGE) on Immunization advises WHO on
419 global policies for vaccines. In. *Lancet Infect Dis* **19**, e31–e38 (2019).
420 14. Dengue vaccine: WHO position paper, September 2018 – Recommendations.
421 *Vaccine* **37**, 4848–4849 (2019).
422 15. Izmirly, A. M., Alturki, S. O., Alturki, S. O., Connors, J. & Haddad, E. K.
423 Challenges in Dengue Vaccines Development: Pre-existing Infections and Cross-
424 Reactivity. *Front Immunol* **11**, 1055 (2020).
425 16. Modis, Y., Ogata, S., Clements, D. & Harrison, S. C. Structure of the dengue virus
426 envelope protein after membrane fusion. *Nature* **427**, 313–319 (2004).
427 17. Klein, D. E., Choi, J. L. & Harrison, S. C. Structure of a Dengue Virus Envelope
428 Protein Late-Stage Fusion Intermediate. *J Virol* **87**, 2287–2293 (2013).

429 18. Smith, S. A. *et al.* The potent and broadly neutralizing human dengue virus-
430 specific monoclonal antibody 1C19 reveals a unique cross-reactive epitope on the
431 bc loop of domain II of the envelope protein. *mBio* **4**, (2013).
432 19. Rodenhuis-Zybert, I. A. *et al.* Immature Dengue Virus: A Veiled Pathogen? *PLoS*
433 *Pathog* **6**, e1000718 (2010).
434 20. Tse, L. v. *et al.* Generation of Mature DENVs via Genetic Modification and
435 Directed Evolution. *mBio* **13**, 1–18 (2022).
436 21. Smith, S. A. *et al.* Persistence of Circulating Memory B Cell Clones with Potential
437 for Dengue Virus Disease Enhancement for Decades following Infection. *J Virol*
438 **86**, 2665–2675 (2012).
439 22. Oliphant, T. *et al.* Antibody Recognition and Neutralization Determinants on
440 Domains I and II of West Nile Virus Envelope Protein. *J Virol* **80**, 12149–12159
441 (2006).
442 23. Lai, C.-Y. *et al.* Antibodies to Envelope Glycoprotein of Dengue Virus during the
443 Natural Course of Infection Are Predominantly Cross-Reactive and Recognize
444 Epitopes Containing Highly Conserved Residues at the Fusion Loop of Domain II.
445 *J Virol* **82**, 6631–6643 (2008).
446 24. Dejnirattisai, W. *et al.* Cross-Reacting Antibodies Enhance Dengue Virus Infection
447 in Humans. *Science (1979)* **328**, 745–748 (2010).
448 25. Sharma, A. *et al.* The epitope arrangement on flavivirus particles contributes to
449 Mab C10's extraordinary neutralization breadth across Zika and dengue viruses.
450 *Cell* **184**, 6052-6066.e18 (2021).
451 26. Andrade, D. v. *et al.* Tracking the polyclonal neutralizing antibody response to a
452 dengue virus serotype 1 type-specific epitope across two populations in Asia and
453 the Americas. *Scientific Reports 2019 9:1* **9**, 1–12 (2019).
454 27. Young, E. *et al.* Identification of Dengue Virus Serotype 3 Specific Antigenic Sites
455 Targeted by Neutralizing Human Antibodies. (2020)
456 doi:10.1016/j.chom.2020.04.007.
457 28. Gallichotte, E. N. *et al.* A new quaternary structure epitope on dengue virus
458 serotype 2 is the target of durable type-specific neutralizing antibodies. *mBio* **6**,
459 (2015).
460 29. Barba-Spaeth, G. *et al.* Structural basis of potent Zika–dengue virus antibody
461 cross-neutralization. *Nature 2016 536:7614* **536**, 48–53 (2016).
462 30. Dejnirattisai, W. *et al.* A new class of highly potent, broadly neutralizing antibodies
463 isolated from viremic patients infected with dengue virus. *Nature Immunology*
464 *2014 16:2* **16**, 170–177 (2014).
465 31. Kotaki, T. *et al.* An affinity-matured human monoclonal antibody targeting fusion
466 loop epitope of dengue virus with in vivo therapeutic potency. *Sci Rep* **11**, 1–14
467 (2021).
468 32. de Alwis, R. *et al.* Dengue Viruses Are Enhanced by Distinct Populations of
469 Serotype Cross-Reactive Antibodies in Human Immune Sera. *PLoS Pathog* **10**,
470 e1004386 (2014).
471 33. Rouvinski, A. *et al.* Covalently linked dengue virus envelope glycoprotein dimers
472 reduce exposure of the immunodominant fusion loop epitope. *Nat Commun* **8**,
473 (2017).

34. Rockstroh, A. *et al.* Recombinant Envelope-Proteins with Mutations in the Conserved Fusion Loop Allow Specific Serological Diagnosis of Dengue-Infections. *PLoS Negl Trop Dis* **9**, 1–12 (2015).
35. Slon-Campos, J. L. *et al.* A protective Zika virus E-dimer-based subunit vaccine engineered to abrogate antibody-dependent enhancement of dengue infection. *Nature Immunology 2019 20:10* **20**, 1291–1298 (2019).
36. Metz, S. W. *et al.* In Vitro Assembly and Stabilization of Dengue and Zika Virus Envelope Protein Homo-Dimers. *Scientific Reports 2017 7:1* **7**, 1–8 (2017).
37. Kudlacek, S. T. *et al.* Designed, highly expressing, thermostable dengue virus 2 envelope protein dimers elicit quaternary epitope antibodies. *Sci Adv* **7**, (2021).
38. Zhang, S. *et al.* A mutation in the envelope protein fusion loop attenuates mouse neuroinvasiveness of the NY99 strain of West Nile virus. *Virology* **353**, 35–40 (2006).
39. Tian, Y., Grifoni, A., Sette, A. & Weiskopf, D. Human T Cell Response to Dengue Virus Infection. *Front Immunol* **10**, 2125 (2019).
40. Ngono, A. E. & Shresta, S. Cross-reactive T Cell immunity to dengue and zika viruses: New insights into vaccine development. *Front Immunol* **10**, 1316 (2019).
41. Waickman, A. T. *et al.* Dissecting the heterogeneity of DENV vaccine-elicited cellular immunity using single-cell RNA sequencing and metabolic profiling. *Nature Communications 2019 10:1* **10**, 1–16 (2019).
42. Gallichotte, E. N. *et al.* Genetic Variation between Dengue Virus Type 4 Strains Impacts Human Antibody Binding and Neutralization. *CellReports* **25**, 1214–1224 (2018).
43. Halstead, S. B. & Marchette, N. J. BIOLOGIC PROPERTIES OF DENGUE VIRUSES FOLLOWING SERIAL PASSAGE IN PRIMARY DOG KIDNEY CELLS: STUDIES AT THE UNIVERSITY OF HAWAII. *Am J Trop Med Hyg* **69**, 5–11 (2003).
44. Juraska, M. *et al.* Viral genetic diversity and protective efficacy of a tetravalent dengue vaccine in two phase 3 trials. *Proc Natl Acad Sci U S A* **115**, E8378–E8387 (2018).
45. Messer, W. B. *et al.* Development and characterization of a reverse genetic system for studying dengue virus serotype 3 strain variation and neutralization. *PLoS Negl Trop Dis* **6**, (2012).
46. Tse, L. V. *et al.* Structure-guided evolution of antigenically distinct adeno-associated virus variants for immune evasion. *Proc Natl Acad Sci U S A* **114**, E4812–E4821 (2017).