Stabilized dengue virus 2 envelope subunit vaccine redirects the neutralizing antibody response to all E-domains

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Abstract

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The four dengue virus (DENV) serotypes cause several hundred million infections annually. Several live-attenuated tetravalent dengue vaccines (LAVs) are at different stages of clinical testing and regulatory approval. A major hurdle faced by the two leading LAVs is uneven replication of vaccine serotypes stimulating a dominant response to one serotype at the expense of the other three which could lead to an increased likelihood of severe dengue. Protein subunit vaccines are a promising alternative since antigen dosing can be precisely controlled. However, DENV envelope (E) protein subunit vaccines have not performed well to date, possibly due to differences between the monomeric structure of soluble E and the E homodimer of the viral surface. Previously, we have combined structure-guided computational and experimental approaches to design and produce DENV2 E antigens that are stable homodimers at 37°C and stimulate higher levels of neutralizing antibodies (NAbs) than the WT E antigen in mice. The goal of the current study was to evaluate if DENV2 E homodimers stimulate NAbs that target different epitopes on E protein compared to the WT E monomer. Using DENV4/2 chimeric viruses and Ab depletion methods, we mapped the WT E-elicited NAbs to simple epitopes on domain III of E. In contrast, the stable E homodimer stimulated a more complex response towards all three surface-exposed domains of the E protein. Our findings highlight the impact of DENV2 E oligomeric state on the quality and specificity of DENV NAbs, and the promise of DENV E homodimers as subunit vaccines.

Importance

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The ideal dengue virus vaccine should elicit a balanced and highly protective immune response against all 4 DENV serotypes. Current tetravalent live dengue virus vaccines have faced challenges due to uneven replication of vaccine virus strains, stimulating a strong immune response to one serotype and weak responses to the other three. Protein subunit vaccines provide novel opportunities to stimulate a balanced response because dosing can be precisely controlled and independent of virus replication used in the vaccine. Here, we compare immune responses elicited by a new dengue serotype 2 protein vaccine designed to match the structure of proteins on the viral surface versus the previously tested suboptimal protein vaccine. We find that proteins designed to match the viral surface stimulate better immune responses targeting multiple sites on the viral surface compared to previous protein vaccines. Our results justify further testing and development of these second-generation dengue protein subunit vaccines.

Introduction

The four dengue virus (DENV) serotypes are mosquito-borne flaviviruses that are estimated to infect several hundred million people per year living in tropical and sub-tropical regions of the world(1). From January to June 2024, there has been around 10 million suspected dengue cases in the Americas alone(2). Clinically, DENV infections can be asymptomatic or present as dengue fever or dengue hemorrhagic fever and shock syndrome. Vaccination is one of the most effective methods for preventing flavivirus infections and effective vaccines have been developed against other deadly flaviviruses, such as yellow fever virus and Japanese encephalitis virus. DENV vaccines must be tetravalent because immunity to a single serotype has been linked to antibody-enhanced replication of heterologous serotypes, associated with more severe clinical disease(3). Protein subunit vaccination is a well-established technology for developing safe and effective monovalent and multivalent vaccines. However, DENV subunit vaccines based on the viral envelope (E) protein have performed poorly in pre-clinical and early clinical studies. One plausible explanation is the structure of soluble E protein disrupts key quaternary epitopes that are presented on the infectious virions. Consequently, soluble wild-type (WT) E protein fails to properly display key E epitopes that likely elicit protective human neutralizing antibodies (NAb)(4, 5). Using structure-guided computational methods and experimentation, we have produced stable DENV E homodimers that display quaternary structure epitopes recognized by human NAbs(6, 7). Here, we sought to map the specificity of NAbs elicited by DENV2 WT and dimer-stabilized DENV2 recombinant E protein (rE) in mice.

The most advanced DENV vaccines are based on tetravalent live attenuated virus formulations. While the two leading candidates (Dengvaxia and Qdenga) have been approved

77 for use by some countries in restricted populations, both candidates suffer from uneven
78 replication of attenuated vaccine viruses that frequently elicit an effective immunity against one
79 or two serotypes only(8–11). In clinical trials, children with no pre-existing immunity to DENVs
80 who received Dengvaxia were more likely to be hospitalized than unvaccinated children, and
81 this vaccine is currently only approved for use in DENV-immune children(12). Qdenga clinical
82 trials have been underpowered to fully assess safety against serotypes not covered by the
83 vaccine in children with no previous dengue immunity(13). Given the challenges faced by live
84 vaccine developers, we are focusing on subunit vaccines, which are safer and easier to
85 formulate as a balanced tetravalent vaccine since immunogenicity is independent of viral
86 replication.

87 The E protein is a membrane-anchored structural protein covering the surface of
88 flaviviruses and the main target of neutralizing and protective Ab. On the viral surface, 90 E
89 homodimers are tightly packed to create a smooth protein coat with pseudo-icosahedral
90 symmetry(14, 15). Many human Abs that neutralize DENVs and other flaviviruses recognize
91 quaternary structure epitopes that span E homodimers or higher-order assemblies on the
92 virion(16–19). However, soluble DENV rE at physiological temperature (37℃) is mostly
93 monomeric and does not display the native homodimer form(4). The abundance of monomers
94 is potentially a major contributor to the poor performance of DENV subunit vaccines to date.

95 We previously identified specific amino acid substitutions that greatly stabilized the rE
96 homodimer at 37℃, while preserving the overall structure of the native homodimer, including
97 quaternary epitopes recognized by human NAb(6). We also showed that mice immunized with
98 stabilized DENV2 rE dimer (SD*FL) had higher levels of DENV2 NAb than animals immunized

99 with WT rE. In this study, we determined the specificity and epitope targets of the NAbs elicited
100 by the DENV2 SD*FL rE in comparison to the traditional WT rE when they are used as vaccine
101 antigens in mice.

102 ## Results

103 **Immunogenicity of stabilized DENV2 recombinant E homodimers: 2 dose study**

104 We performed experiments to characterize the binding and functional properties of Abs
105 stimulated by WT and stable homodimer E protein subunit vaccines. Groups of Balb/C mice
106 were primed and boosted (week 3) with WT rE and a stabilized E homodimer (SD*FL) in the
107 presence or absence of an adjuvant (Alum) (**Fig. 1B**). The SD*FL antigen has 2 mutations in the
108 E-domain(ED) II at the dimer interface (A259W, T262R), 2 mutations in the hinge between EDI
109 and EDIII (F279W, T280P), and a point mutation in the fusion loop (FL, G106D) that
110 independently contribute to dimer stabilization(6) (**Fig. 1A**). At 12 weeks after vaccination, mice
111 immunized with SD*FL had more DENV2 binding Abs compared to animals immunized with WT
112 rE (**Fig. 1C**). We compared the binding of vaccine-induced antibodies to rE monomer (M2) and
113 stable homodimer (SD) (**Fig. S1A-B**). SD*FL immunized animals had higher levels of Abs to both
114 E monomer and dimer antigens compared to WT E immunized animals (**Fig. 1D, E**). When Alum
115 was included in the formulation, SD*FL immunized animals also had a response that was
116 skewed in favor of the dimer over monomer compared to WT E immunized animals (**Fig. 1F**).
117 The SD*FL rE antigen also stimulated higher levels of NAbs compared to WT rE (**Fig. 1G**).
118 Collectively, these results indicate that the stable dimer antigen stimulates higher levels of
119 binding Abs (skewed in favor of the E homodimer over the monomer in the presence of Alum)
120 and NAbs compared to the WT E antigen.

121 **Immunogenicity of stabilized DENV2 recombinant E homodimers: 3 dose study**

122 Next, we performed a 3-dose immunogenicity study to characterize Ab levels at different
123 times after vaccination and to map the binding sites of vaccine-induced NAbs (**Fig. 2A**). To focus
124 on the impact of antigen structure alone on immunogenicity, we did not include the Alum
125 adjuvant, which could alter protein structure(20). DENV2 binding Ab levels were boosted after
126 the second (week 3) and third (week 8) vaccine doses (**Fig. 2B**). In general, the SD*FL rE elicited
127 higher levels of DENV2 binding Ab compared to WT rE. At week 12, animals immunized with
128 SD*FL had higher levels of binding Ab to the E dimer than WT E-immunized animals (**Fig. 2C, Fig.
129 S1C**). The DENV2 NAb levels steadily increased over time and were similar in both groups (**Fig.
130 2D**). Mice immunized with WT and SD*FL rE did not cross-neutralize the other three DENV
131 serotypes demonstrating the dominance of DENV2 type-specific NAbs in both vaccine
132 constructs (**Fig. 2E**).

133 **Mapping DENV2 E domains targeted by NAb using DENV4/2 chimeric viruses**

134 To map the E domains targets of NAb elicited by WT and SD*FL, we used DENV4/2
135 chimera viruses which have a DENV4 backbone with transplanted domain (EDI/EDII/EDIII) from
136 DENV2(21) (**Fig. 3A**). Mice immunized with WT and SD*FL rE antigens had DENV2 NAb (**Fig. 3B**),
137 and little to no cross-neutralization of DENV4 (**Fig. 3C**), enabling the use of the DV4/2 chimeras
138 for mapping domain specific responses. Both antigens induced Abs that neutralized the
139 DENV4/2 EDIII chimera demonstrating the presence of NAb targeting DENV2 EDIII (**Fig. 3D**).
140 SD*FL rE elicited Abs that neutralized the DENV4/2 EDI and EDII chimeric viruses, whereas WT
141 rE stimulated lower levels of NAbs that tracked with EDI (**Fig. 3F**) and no NAbs to EDII (**Fig. 3E**).

142 These results indicate that WT rE mainly induced EDIII targeting NAbs, while SD*FL rE
143 stimulated a broader response directed to all three domains.

144 **Characterizing EDIII-Ab elicited by WT and stable dimer immunized mice**

145 Antibodies that neutralize DENV2 have been mapped to simple epitopes contained
146 within a domain or more complex epitopes that span two different domains on the same or
147 different E molecules. For example, DENV2 type-specific and neutralizing mouse monoclonal Ab
148 (mAb) 3H5 binds to a simple epitope on the lateral ridge of EDIII(22). In contrast, DENV2 type-
149 specific and neutralizing human mAb 2D22 recognizes epitope centered on EDIII that expands
150 into conserved amino acids on EDII of the second E protomer forming the homodimer. This
151 cross-dimer interaction is critical for 2D22 binding to DENV2(16, 18). Consequently, unlike 3H5,
152 2D22 cannot bind to and depleted by soluble EDIII rE (**Fig. S2**). We performed Ab depletion
153 studies with recombinant DENV2 EDIII to measure the contribution of simple EDIII binding Abs
154 to neutralization following vaccination with WT and SD*FL rE antigens (**Fig. 4A**). Sera were
155 incubated with DENV2 EDIII coated beads and effective removal of EDIII binding antibodies was
156 confirmed by ELISA (**Fig. 4B**). Removal of EDIII binding Ab resulted in a large reduction of SD
157 binding Ab in mice immunized with WT E and a modest reduction of Ab in SD*FL rE immunized
158 mice (**Fig. 4C)**.

159 Next, we assessed how the removal of EDIII-binding Ab impacted DENV2 neutralization
160 (**Fig. 4D**). In WT E immune sera, the removal of EDIII-binding Abs completely ablated DENV2
161 neutralization. While the removal of EDIII-binding Abs also lowered NAb levels in SD*FL rE
162 immunized animals, a sub-population of NAbs (~30-40% of total response) was resistant to EDIII

163 depletion (**Fig. 4E**). This suggests that SD*FL drive response that also includes non-simple EDIII
164 epitopes, which may include 2D22-like Ab, EDI- and EDII-targeting Ab.

165 ## Discussion

166 DENV protein subunit vaccines based on the ectodomain of E protein have not
167 performed well in the past(4, 5, 23–25). While investigators had assumed that recombinant E
168 protein produced as a soluble, secreted antigen formed homodimers similar to the protein on
169 the viral surface, recent studies have demonstrated that the antigen is mostly present as a
170 monomer at 37℃, which is a plausible explanation for the overall poor performance of DENV
171 subunit vaccines(4, 5). The main goal of our study was to compare the properties of DENV2
172 NAbs induced by soluble E protein antigens that are monomers (WT) or homodimers (SD*FL) at
173 37℃. Our results demonstrate that stable homodimers stimulated DENV2 NAbs targeting
174 multiple regions of the viral envelope, whereas the WT E antigen stimulated NAbs response that
175 mainly target simple EDIII epitopes. Together, our results demonstrate that by altering the
176 oligomeric state of DENV2 E protein to better match the antigen on the surface of the infectious
177 virus, it is possible to elicit Abs against all three domains of the E protein.

178 Currently, the leading DENV vaccine candidates are based on tetravalent live attenuated
179 flavivirus platforms. These vaccines have been challenging to develop because of inherent
180 replication differences between vaccine viruses resulting in unbalanced immunity skewed to a
181 single serotype and/or a single genotype within a serotype(11, 26, 27). In clinical trials,
182 immunodominance of a single vaccine serotype over the other three serotypes has been linked
183 to poor outcomes, including vaccine-enhanced WT DENV infections(12). Given these challenges

184 with LAVs, protein subunit vaccine that mimic the architecture of the viral envelope is a
185 promising alternative strategy that is not dependent on balanced replication of each vaccine
186 component.

187 Investigators have relied on DENV neutralization assays both to characterize vaccine
188 responses and as a correlate of protection. However, in clinical trials, the presence of NAbs to
189 each serotype alone was not a reliable predictor of protection. Recent studies have established
190 that DENVs produced for laboratory studies are inefficiently processed and mostly consist of
191 partially mature virions. These partially mature virions are hypersensitive to neutralization by
192 some low-affinity antibodies, and unlikely to be protective against mature DENVs circulating in
193 humans(28). To control for virus maturation state and selectively measure vaccine responses
194 that are effective against fully mature DENV2 virions, we used fully mature DENV2 stocks
195 produced on a cell line overexpressing a protease necessary for DENV processing and
196 maturation(29). Under these conditions, we observed higher levels of maturation-insensitive
197 NAb stimulated by the stable homodimer as compared to WT E protein.

198 Neutralizing Abs that target different regions of the E-protein would be able to block
199 viral entry at different stages (attachment, internalization, membrane fusion) and minimize
200 vaccine escape. The different domains of the DENV E-protein play different roles in various
201 stages of the virus lifecycle. EDIII is involved in the initial virus attachment with host cell
202 receptors(30). EDII plays a role in the dimerization of the E protein, and contains the fusion loop
203 region that is used by DENV to fuse to the host's endosome(30, 31). Linking the EDIII and EDII is
204 EDI that helps with low pH triggered E protein conformational changes required for viral fusion
205 with endosome membranes(30). The WT E antigen stimulated a much limited NAb response

206 that was mainly directed to EDIII. Previous studies have demonstrated that the lateral ridge
207 region of EDIII contains epitopes targeted by type-specific NAbs(32, 33). However, this region of
208 E protein is highly variable between DENV genotypes belonging to the same serotype and
209 responsible for the variable neutralization potency including complete escape from EDIII
210 antibodies(32, 34, 35). Furthermore, previous mapping study done with the DENV4/2 chimeric
211 viruses showed that in individuals who had DENV infection, their NAb target EDII or EDII/EDIII
212 regions. In contrast, sera from attenuated DENV2 human challenge target mostly EDIII and
213 EDI/EDIII Ab to a certain extent(21). Our studies showed that the SD*FL-elicited sera response
214 tracks better with natural DENV infection. All these suggested that a broader Ab response to
215 multiple domains is more beneficial and potentially lead to a better vaccine.

216 In conclusion, our studies support further development and evaluation of E dimer-based
217 subunit vaccines, demonstrating that DENV2 stable homodimers stimulate high levels of NAbs
218 targeting multiple domains on E protein. We have found that the mutations used for stabilizing
219 DENV2 homodimers also work for the other three serotypes and Zika virus with minor virus-
220 specific modifications(7). Subunit vaccine studies are in progress with DENV1, DENV3 and
221 DENV4 because DENV vaccines must be tetravalent to minimize the risk of ADE. Moreover, we
222 are evaluating different vaccine delivery platforms (nanoparticles, liposomes, nucleic acid) and
223 adjuvants to increase the immunogenicity and durability of vaccine responses.

224 Methods

225 Cell lines and viruses

226 EXPI293F (Thermo Fisher Scientific) cells were maintained in EXPI293 expression medium at
227 37°C with 8% CO_2 at 250 rpm.

228 Vero-81 (ATCC CCL-81) cells were maintained at 37°C with 5% CO_2 in DMEM-F12 (Gibco)
229 supplemented with 5% of heat-inactivated fetal bovine serum (HI FBS, Gibco), 1% of Penicillin-
230 Streptomycin (Gibco), 1% L-Glutamine (Gibco), and 1% MEM non-essential amino acids (MEM
231 NEAA, Gibco).

232 Vero-furin (VF) cells were given by the Baric lab at UNC Chapel Hill(29). The cells were
233 maintained at 37°C with 5% CO_2 in DMEM-F12 + 5% HI FBS + 1% Anti-anti (Gibco) + 1% MEM
234 NEAA + 1% L-Glutamine.

235 Viruses. All DENV2 S16681 used in the experiments were grown in VF cells, except for DENV2
236 S16681 used in the 2-dose study ELISA which was grown in Vero cells. For 3-dose study ELISA,
237 DENV2 S16681 VF-grown virus was concentrated using Centricon® Plus-70 Centrifugal Filter
238 (Millipore) per manufacturer instruction to obtain enough titer required for ELISA.

239 Cross-neutralization assessment was done using DENV1 WestPac-74, DENV3 CH53489, DENV4
240 Sri Lanka 92. Chimera viruses and EDIII depletion experiments used the following viruses:
241 DENV2 16803, DENV4 Sri Lanka 92, DENV4/2 EDIII, DENV4/2 EDII and DENV4/2 EDI that were
242 grown using a previously described method(21). All viruses were grown in VF cells.

243 **DENV2 sE plasmid generation**

244 Cloning method for various DENV2 S16681 variants sE into PαH mammalian expression vector
245 was described previously(6). For the HisAvi-variants, after the C-terminal His8-tag, glycine
246 linkers (GGCAGCTCTGGCGGCAGC) and AviTag

247 (GGCCTGAACGATATTTTCGAGGCCCAGAAAATCGAGTGGCACGAGTAG) were added. The DENV2 sE
248 cloned plasmids were heat-shock transformed into Escherichia coli DH5α cells for amplification,
249 miniprepped or midiprepped using DNA endotoxin-free kits, and stored in endotoxin-free H_2O
250 at −20°C (Macherey-Nagel).

251 **DENV2 sE protein large-scale expression**

252 For DENV2 WT-His: Stable line was grown up in Expi293 supplemented with 250 µg/ml
253 geneticin, with geneticin removed during the final amplification step. The cultures were
254 supplemented with 5 ml of 100X GlutaMAX (Thermo 35050061) and 50 ml of 6% HyClone Cell
255 Boost Boost I (Cytiva SH30584.02) in OptiMEM (Thermo 31985070) per liter of culture when the
256 cells reached a density of approximately $1.5x10^6$ cells/ml (day 4 after reaching the final culture
257 scale). The cultures were harvested when the cells dropped below 90% viability on day 9 after
258 reaching the final scale, with the centrifugation, filtering, and purifications done the same as for
259 the proteins made in Expi293 transfection cultures.

260 For other DENV2 variants-His: Expi293 cells were transfected per manufacturer instructions
261 (Thermo Scientific), using 1mg of DNA/1L of culture volume for each flask. Enhancers 1 and 2
262 were added on day 1 post-transfection, then the cultures were harvested on day 3 at 12,000 x g
263 for 1 hour at 4°C. The supernatants were sterile-filtered (0.22 µm), then stored at 4°C until
264 purification.

265 For biotinylated SD-HisAvi: Expi293 cells were co-transfected per manufacturer instructions
266 (Thermo Scientific) in 1L volume with 900 µg of SD-HisAvi (I2-U6-HisAvi) and 100 µg of
267 mammalian BirA ligase expression plasmids. Culture media was supplemented with 100 µM

268 biotin before transfection to aid I2-U6-His-Avi biotinylation by BirA ligase. Downstream
269 processes (enhancers and harvest timeline) are the same as DENV2 variants-His.

270 **DENV2 sE protein purification and analysis**

271 Filtered supernatants were buffer-exchanged via tangential-flow filtration (10 kDa MWCO) into
272 cobalt-binding buffer (50 mM sodium phosphate pH 7.4, 500 mM NaCl, 5 mM imidazole, 0.02%
273 sodium azide, 10% glycerol), with 4 total buffer exchanges. The supernatant was then loaded at
274 1.5 ml/min recirculating overnight with a peristaltic pump and flow adapter onto a hand-packed
275 TALON column. The next day, the column was switched to flow-through, and once completely
276 loaded, the column was washed with 10 CV binding buffer. After a 10 CV second wash with
277 binding buffer supplemented with 10 mM imidazole, bound proteins were eluted in two steps
278 (one of 5 CV, and one of 2.5 CV) with binding buffer supplemented with 150 mM imidazole.
279 Column fractions were assessed by SDS-PAGE and fractions containing target protein were
280 pooled, filtered, and concentrated before loading onto a Superdex 200 16/600 column
281 equilibrated in 1x PBS + 10% glycerol. The sizing column fractions were assessed by SDS-PAGE,
282 and DENV2 protein-containing fractions were pooled, filtered, and concentrated before
283 aliquoting and flash-freezing in liquid nitrogen. The endotoxin level in the final stock was
284 measured using Endosafe-PTS cartridges (#PTS20F; Charles River) on a nexgen-PTS reader
285 between 1:20 - 1:40 dilution in endotoxin-free water.

286 <u>For biotinylated SD-HisAvi:</u> To assess the success of the biotinylation reaction, column fractions
287 were assessed by SDS-PAGE, with elution fractions assessed for biotinylated protein by mixing
288 with a 2x molar excess of avidin. Downstream processes are the same as above.

289 **DENV2 EDIII- & HaloTag-His production**

290 A Halo-tag fused DENV-2 EDIII (ADA00411·1, E protein aa 297-394) was expressed in Expi293
291 with an N-terminal human serum albumin secretion signal peptide and a C-terminal His6-tag as
292 previously described(36). A Halo-tag construct, lacking DENV-2 EDIII, was also produced
293 similarly with a C-terminal His6-tag. The DENV2 EDIII and Halo-tag proteins were purified using
294 Ni-NTA agarose (Qiagen) from the cell culture medium. Purified DENV2 EDIII was biotinylated
295 by incubating 100 µg of DENV2 EDIII with 5 nmol of the HaloTag PEG biotin ligand (Promega) at
296 room temperature (RT) for 2 hours. Excess biotin ligand was removed by desalting column.
297 Biotinylated DENV2 EDIII was assessed by comparing the mobility of the biotinylated EDIII in the
298 presence and absence of streptavidin in SDS-PAGE.

299 **Mouse immunizations**

300 Female Balb/c mice were purchased from Jackson Laboratory and used in immunization studies
301 at 6 weeks of age. Mice were injected subcutaneously in the flank with 5 µg DENV2 rE subunit
302 vaccine antigen in 100 µL PBS. Vaccine groups (n = 5) included placebo (PBS), WT rE, WT rE +
303 Alum, SD*FL rE, SD*FL rE + Alum. Adjuvant Alum (Alhydrogel, InvivoGen) was included at 125
304 µg per dose. Mice were immunized twice with the same antigen formulation and dose level at
305 days 0 and 21. In another experiment, a third dose was given at day 56. Serum samples were
306 collected at various time points as indicated for antibody analysis.

307 **Ethics statement**

308 All experiments involving mice were performed according to the animal use protocol approved
309 by the University of North Carolina Animal Care and Use Committee. The animal care and use

310 related to this work complied with federal regulations: the Public Health Service Policy on
311 Humane Care and Use of Laboratory Animals, Animal Welfare Act, and followed the Guide for
312 the Care and Use of Laboratory Animals.

313 **Enzyme-linked immunosorbent assay (ELISA)**

314 Whole virus ELISA. DENV2 IgG binding titer was measured by DENV2 antigen capture ELISA. A
315 high-binding 96-well plate (Greiner bio-one) was coated using 0.2 µg/ml of 1M7 (DENV fusion
316 loop mAb) in 0.1M bicarbonate buffer (pH 9.6) overnight at 4°C, and washed with wash buffer
317 (1X Tris-buffered saline (TBS) + 0.2% Tween-20). The plate was blocked with blocking buffer (3%
318 non-fat milk in 1X TBS + 0.05% Tween20), before DENV2 was loaded to each well. Next, serially
319 diluted mouse sera were added onto the plate. Plates were washed in between each step, and
320 each incubation was done for 1 hour at 37°C. Bound antibodies were detected by alkaline
321 phosphatase (AP)-conjugated anti-mouse IgG (1:1000, Sigma-Aldrich) for 45 min at 37°C, and
322 washed. Plates were developed using AP substrate (SIGMAFAST p-Nitrophenyl phosphate tablet,
323 Sigma-Aldrich) and measured at 405 nm. IgG titer was determined by area under the curve
324 (AUC) in GraphPad Prism using nonlinear fit – sigmoidal dose-response (variable slope).

325 Soluble E-coated ELISA. Nickel-coated ELISA plates (Thermo Fisher Scientific, Pierce 15142) were
326 used to capture 0.2 µg/ml of rE protein in 1X TBS for 1 hour at 37°C. The plate was washed with
327 wash buffer, and serially diluted sera in blocking buffer were added for 1 hour at 37°C and
328 subsequently washed with wash buffer. Downstream processes are the same as above for whole
329 virus ELISA.

330 Biotinylated-protein ELISA. High-binding 96-well plate was coated using 0.2 µg/ml of
331 Streptavidin (Invitrogen) in 1X TBS overnight at 4°C or 1 hour at 37°C. Supernatant was
332 removed, and 0.2 µg/ml of biotinylated antigens (DENV2 EDIII or SD) was added in 1X TBS, and
333 incubated for 1 hour at 37°C. The plate was washed using wash buffer and then blocked with
334 blocking buffer for 1 hour at 37°C. The plate was washed, and serially diluted sera in 1X TBS
335 were added for 1 hour at 37°C and subsequently washed with wash buffer. Bound antibodies
336 were detected by AP-conjugated anti-mouse IgG (1:1000 in 1X TBS) for 45 min at 37°C, and
337 washed. Plates were developed using AP substrate and measured at 405 nm. IgG titer was
338 determined by area under the curve (AUC) in GraphPad Prism using nonlinear fit – Sigmoidal,
339 4PL.

340 **Focus reduction neutralization (FRNT) assay**

341 *Cell seeding.* Vero cells were seeded in 96-well plate at 2x10^{5}cells/ml in 100ul of cell culture
342 media per well. Plates were incubated overnight at 37°C.

343 *Cell infection.* Sera were serially diluted in infection media (similar to cell culture media, only
344 with 2% HI FBS) and incubated with a fixed amount of virus at 1:1 v/v ratio for 1 hour at 37°C.
345 The amount of virus had been previously determined to give a minimum of 30 foci/well. Culture
346 media on cells were removed, and serum/virus complex was added onto cells for 1 hour at
347 37°C. Serum/virus complex was removed from cells, and overlay media (OptiMEM (Gibco) + 2%
348 HI FBS + 1% Anti-anti + 1% (w/v) carboxymethylcellulose (Sigma-Aldrich)) was added to cells.
349 Cells were left to incubate for 42-48 hour (depending on the viruses used).

350 *Cell harvest and staining.* Cells were washed with 1X phosphate-buffered saline (PBS), and fixed
351 with 4% paraformaldehyde. Next, cells were permeabilized with 1X permeabilization (Perm)
352 buffer (10X Perm buffer was diluted in MilliQ water. 10X Perm: 1% of bovine serum albumin
353 (BSA, Sigma), 1% of Saponin (Sigma), 0.1% of sodium azide (Sigma) in 10X PBS), and blocked
354 using blocking buffer (5% non-fat dry milk in 1X Perm) at RT for 10 min. Blocking buffer was
355 removed, and cells were stained using 1M7 at $1.2x10^{-3}$mg/ml for 1 hour at 37°C. Cells were
356 washed with 1X PBS, and stained with mouse anti-human IgG Fc HRP (Southern biotech) at
357 1:4000 for 1 hour at 37°C. Cells were washed and stained with KPL TrueBlue Peroxidase
358 Substrate (SeraCare). Cells were then rinsed with MilliQ water and plates were left to dry prior
359 to imaging with ImmunoSpot S6 analyzer (Cellular Technology Limited Analyzer). Foci were
360 counted using Viridot on RStudio, and checked for accuracy(37). Neutralization curves were
361 generated by plotting sera dilution factor (log) vs % neutralization. Curves were fitted using
362 nonlinear fit – sigmoidal dose-response (variable slope) with constraints (top = 100, bottom =
363 0). Curves were acceptable when R^2 ≥0.75 and Hill Slope >0.5. $Neut_{50}$ value is the dilution factor
364 at which sera neutralize 50% of the virus. Analysis was done using Graphpad Prism

365 <u>DENVs chimera neutralization.</u> All steps are identical to FRNT assay explained above, with the
366 exception of serum/virus complex wasn't removed prior to the addition of overlay media.

367 **EDIII Depletion**

368 *Protein-beads conjugation.* First, Magne HaloTag beads (Promega) were transferred into Lo-
369 bind tubes (Eppendorf), and beads were washed with 1X TBS for a total of 3 times. To separate
370 beads from supernatant, magnetic stand (DynaMag2, Invitrogen) was used. After the third
371 wash, pre-determined amount of biotinylated protein was added to the beads. Ratio of initial

372 beads volume (µl) to antigen amount (µg) is 1:2. Beads and biotinylated protein were left to
373 incubate for 2 hour at RT, mixing at 1300rpm in ThermoMixer C. Once the conjugation was
374 done, beads were washed 3 times using blocking buffer (5% BSA in 1X TBS). Beads were further
375 blocked using blocking buffer for 1 hour at RT, mixing at 1300rpm. After blocking step, beads
376 were washed 3 times with 1X TBS and re-suspend in 1X TBS. The final resuspension volume is
377 equal to the total volume of all diluted sera that is going to be depleted; the final protein
378 concentration in this resuspension is 48 µg/ml. Beads were stored at 4°C in lo-bind tubes if not
379 being used immediately.

380 *Sera depletion.* Depletion was done using non-binding 96-well plate (Corning). Volume of
381 conjugated beads/well is equal to volume of depleted sera/well. Conjugated beads were added
382 to each well, and the supernatant was discarded by placing the plate on 96-well plate magnetic
383 separator (Millipore Sigma). Next, diluted sera (1:60, in 1X TBS) was added per well and plate
384 was covered with adhesive film (Thermo). The plate was incubated at 37°C for 1 hour at
385 800rpm. Depletion was done for 2-3 rounds. Sera depletion was considered complete when
386 EDIII-depleted sera (1:1200) against EDIII antigen on an ELISA reached background level
387 compared to undepleted sera. Depleted sera were stored in untreated polypropylene 96-well
388 plates (Corning) at 4°C.

389 BioRender was used to make schemas for mouse vaccination study and EDIII depletion.

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391 depletion experiments.

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Figure 1

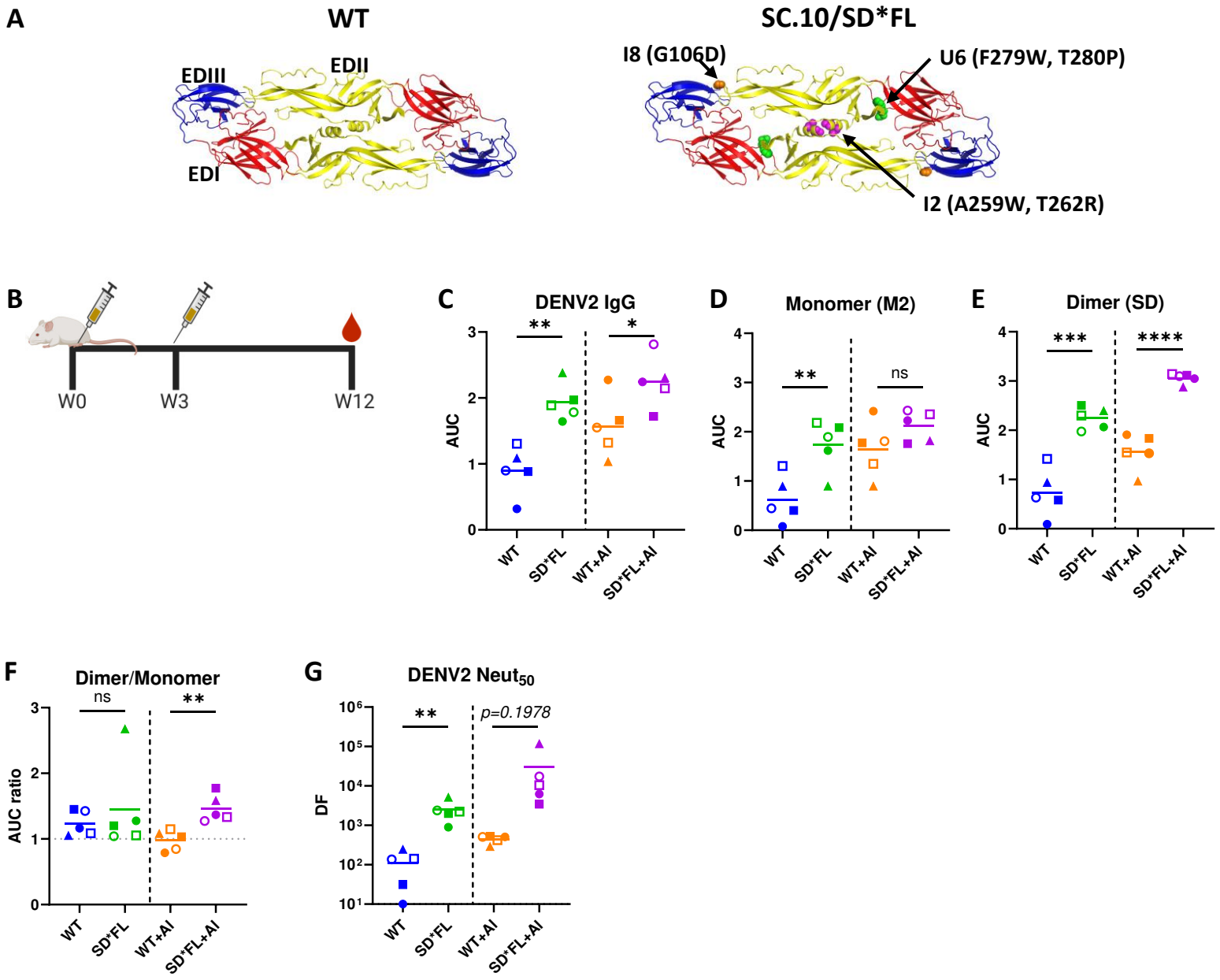


Figure 1. Immunogenicity of recombinant DENV2 E protein vaccines: 2 dose study. (**A**) DENV2 soluble E structure (PDB 1OAN) of the vaccine antigens: wild-type (WT) and SD*FL with stabilizing amino acid mutations shown. (**B**) 6-week old female Balb/c mice were immunized with 5µg of WT or SD*FL rE ±Alum (125ug) at week 0 (W0), and boosted with the same antigen ±Alum at week 3 (W3). (**C**) DENV2 IgG binding titer over time is reported as the area under the curve (AUC). IgG binding against DENV2 (**D**) Monomer (M2), and (**E**) stabilized dimer (SD) rE were measured on week 12 by serially diluting the mouse sera, and the resulting area under the curve (AUC) was determined. (**F**) To determine the ratio of dimer:monomer binding Ab, AUC value from dimer- and monomer- binding was used. (**G**) DENV2 neutralizing Ab titer at week 12 was measured by FRNT, whereby mouse serum was serially diluted and incubated with a fixed amount of DENV2 prior to cell infection. The dilution factor (DF) at which the serum can neutralize 50% of the DENV2 is reported as $Neut_{50}$. Each data point is the average of each sample's duplicates. The line represents the mean of all mice within the group. Statistical differences were determined by unpaired t-test (two-tailed, $p<0.05$).

Figure 2

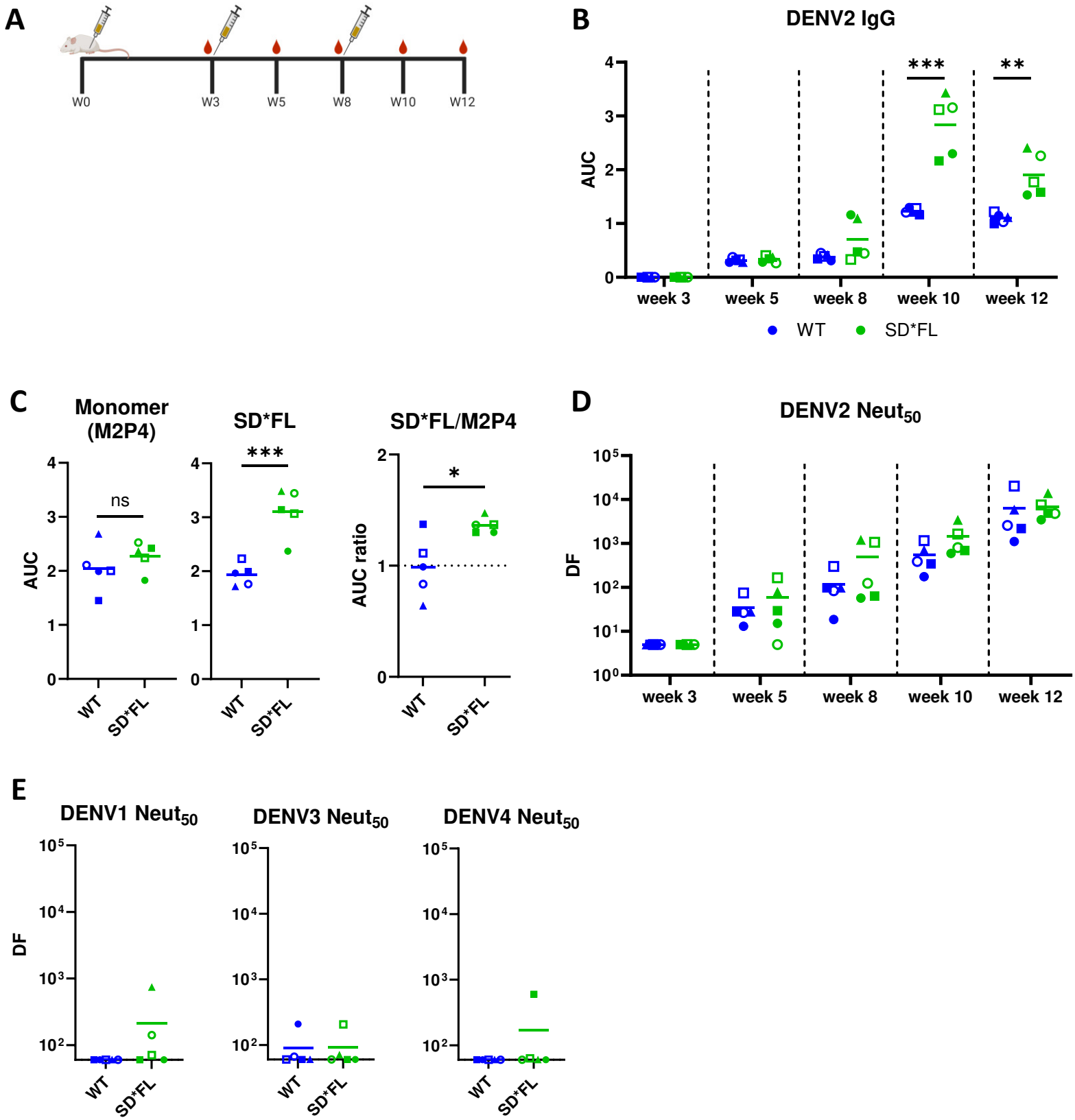


Figure 2. Immunogenicity of recombinant DENV2 E protein vaccines: 3 dose study. (**A**) 6-week old female Balb/c mice were immunized with 5µg of WT or SD*FL rE at W0, and boosted twice with the same antigen at W3 and W8. Serum samples from each animal were collected pre- (W3, W8) and post-boost (W5, W10, W12). (**B**) DENV2 IgG binding titer over time is reported as AUC. (**C**) IgG binding against DENV2 rE monomer (M2P4) and dimer with mutated fusion loop (SD*FL) were measured on W12 sera by serially diluting the mouse sera and the resulting AUC was determined. We took the AUC value of the monomer and dimer binding to quantify the dimer:monomer Ab ratio. (**D**) DENV2 $Neut_{50}$ value of mouse sera over time. (**E**) Cross-neutralization to DENV1, DENV3 and DENV4. Each data point is the average of each sample's duplicates. The line represents the mean of all mice within the group. Statistical differences were determined by unpaired t-test (two-tailed, $p<0.05$).

Figure 3

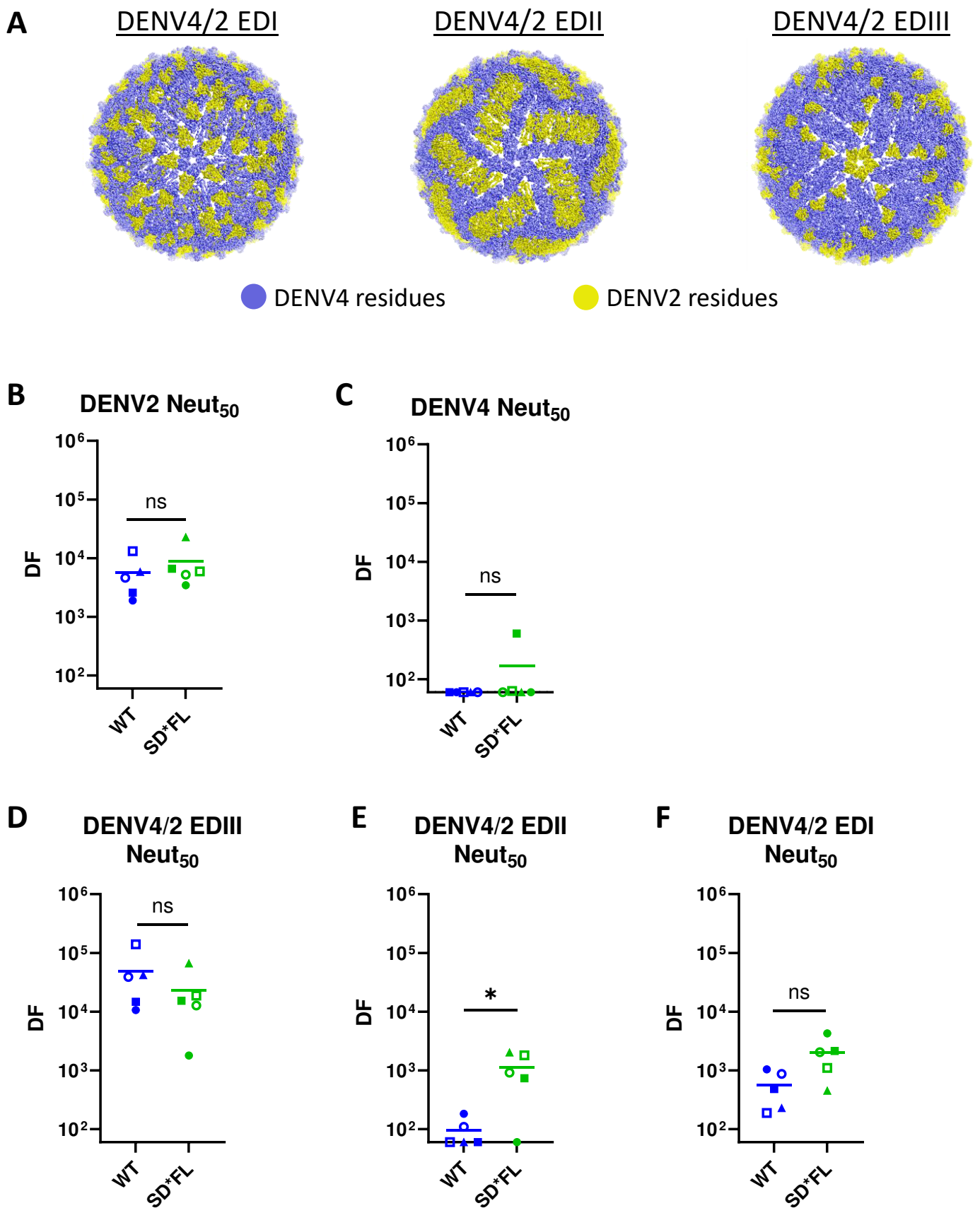


Figure 3. Mapping DENV2 E-domains targeted by neutralizing Ab using DENV4/2 chimeric viruses. (A) Schematic of DENV4/2 chimera (PBD 4CBF) with DENV2 domain substitution highlighted in yellow. The neutralizing IgG titer elicited by the different rE is reported as the $Neut_{50}$ value against the parental viruses (**B**) DENV2, (**C**) DENV4, and against the various domains of DENV2 on a DENV4 backbone (**D**) DENV4/2 EDIII, (**E**) DENV4/2 EDII, (**F**) DENV4/2 EDI. Each data point is the average of each sample's duplicates. The line represents the mean of all mice within the group. Statistical differences were determined by unpaired t-test (two-tailed, $p<0.05$).

Figure 4

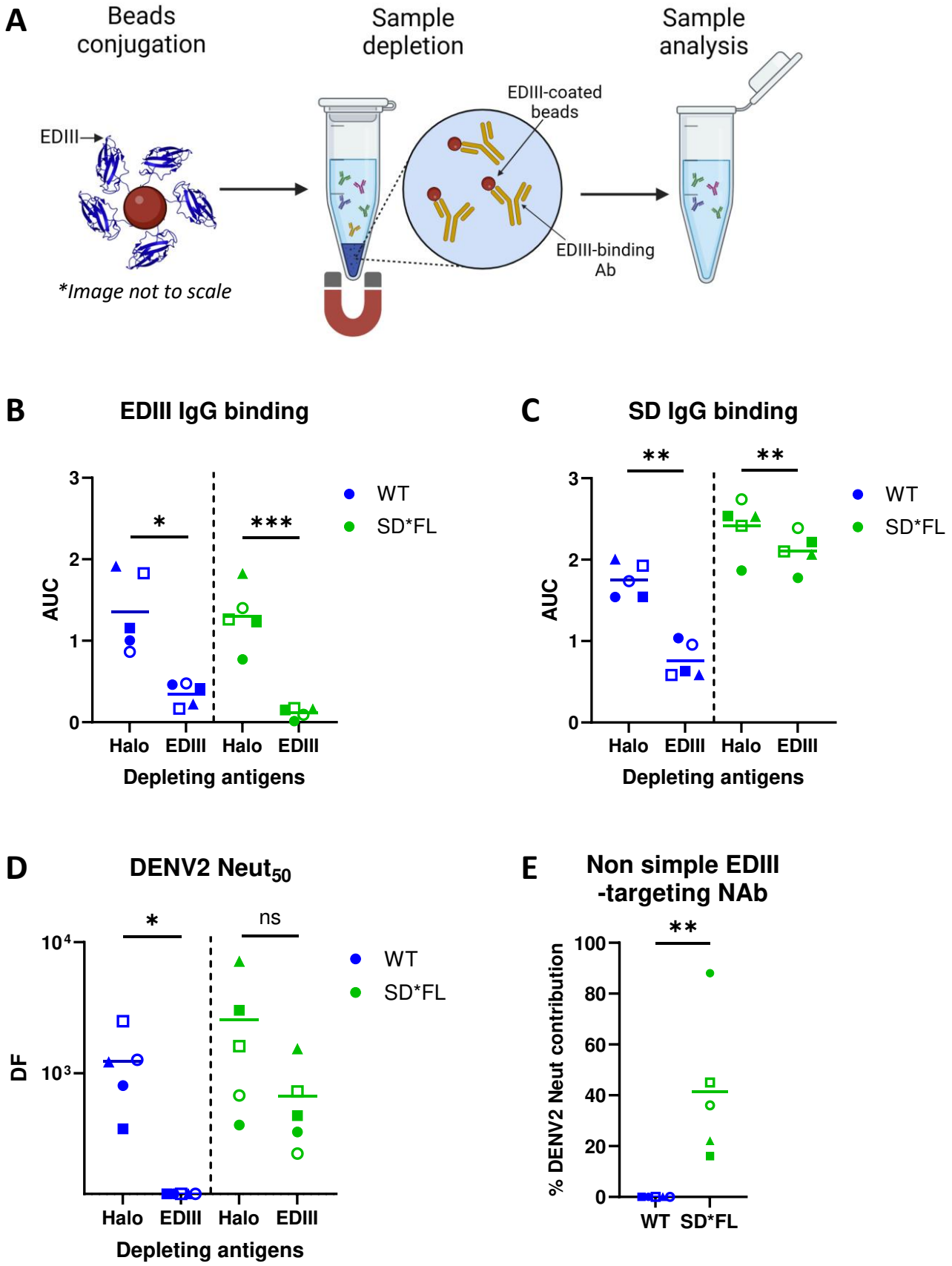
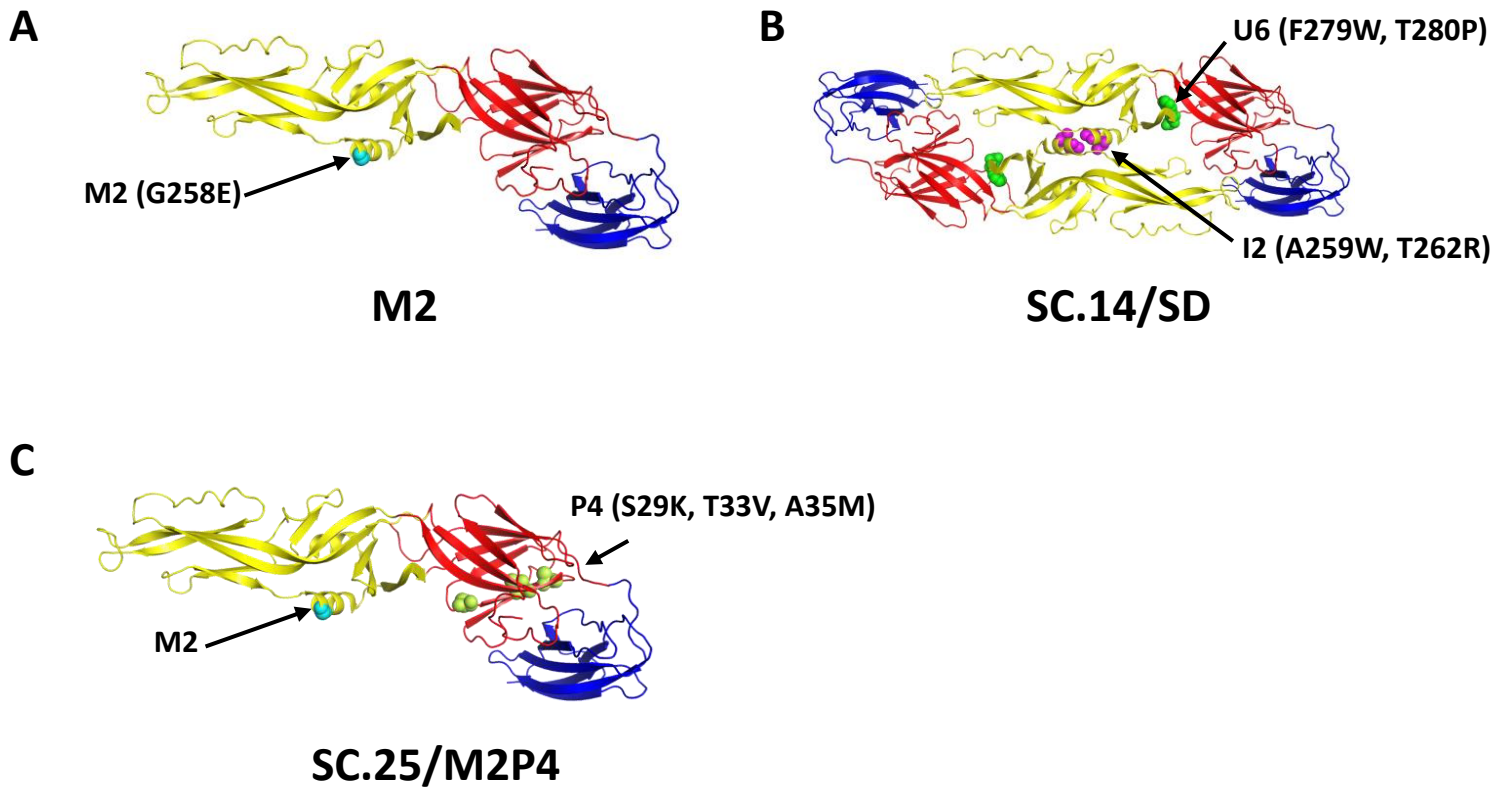


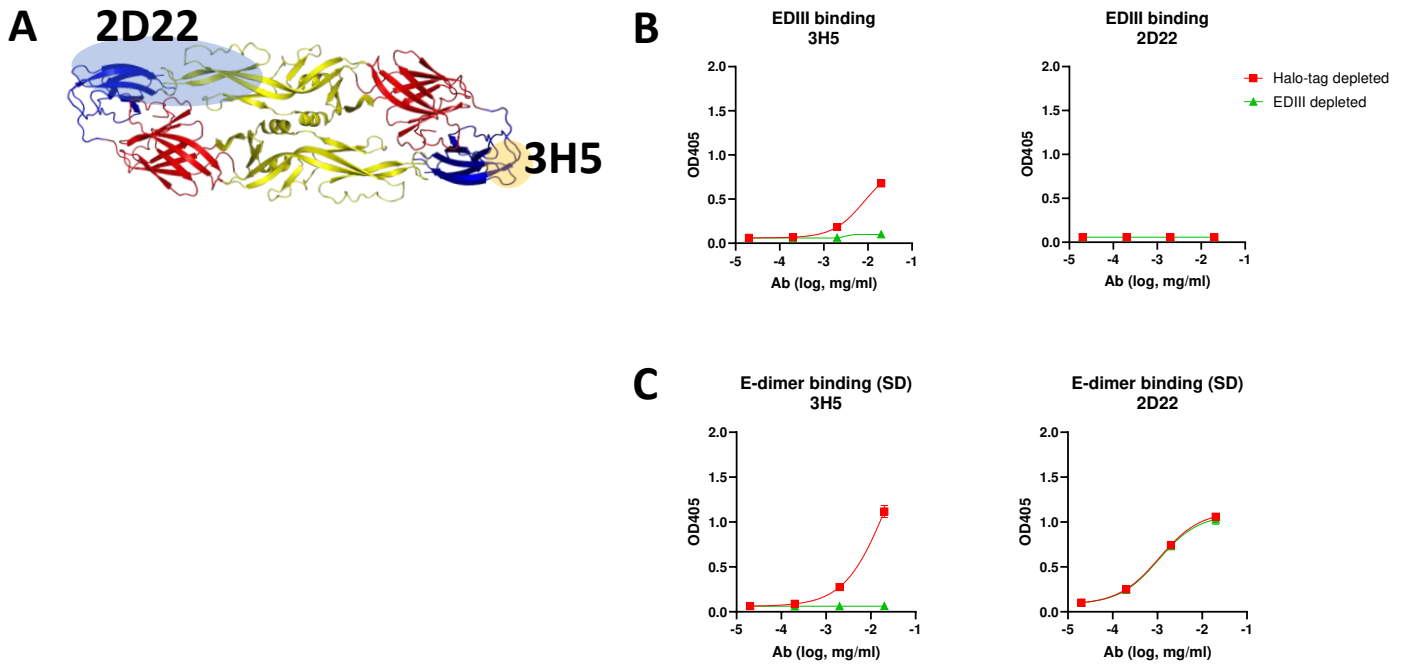
Figure 4. Characterization of EDIII binding antibody. (**A**) A schema for DENV2 EDIII depletion. First, we conjugated Magne HaloTag beads with either EDIII or HaloTag. Next, we treated the mouse sera with either EDIII- or HaloTag (control)-coated beads. Magnetic pulldown will separate any antibodies that bind to the coated beads, leaving behind unbound antibodies (non-simple EDIII or non-HaloTag antibodies) in the sera. Downstream depleted samples analysis include DENV2 (**B**) EDIII IgG binding, (**C**) SD (full-length dimer stabilized rE) IgG binding, and (**D**) DENV2 neutralization. (**E**) Percentage of NAb that target other areas outside of simple EDIII region. Each data point is the average of each sample's duplicates. The line represents the mean of all mice within the group. Statistical differences between Halo- vs EDIII-depleted samples (**B-D**) were determined by paired t-test (one-tailed, $p<0.05$). Unpaired t-test (one-tailed, $p<0.05$) was used to determine the statistical differences for (**E**).

Supplementary Figure 1



Supplementary Figure 1. DENV2 Soluble E structure (PDB: 1OAN) used in various ELISA with their amino acid mutations (**A**) M2 (**B**) SD (previously known as SC.14[6]), (**C**) M2P4 (SC.25)

Supplementary Figure 2



Supplementary Figure 2. Properties of Ab that can bind and depleted by soluble EDIII. (**A**) Epitope location of simple EDIII-targeting Ab 3H5 and quaternary-targeting Ab 2D22. (**B**) EDIII binding of 3H5 vs 2D22 (**C**) E-dimer binding (SD) of 3H5 vs 2D22.