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

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Abstract

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, leading to the potential for vaccine antibody (Ab) enhanced more severe infections by wild type DENV serotypes that fail to replicate in the vaccine. 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℃ and stimulate higher levels of neutralizing Abs (NAbs) than the WT E antigen in mice. The goal of this 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.

41 ## Importance

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

53

54 ## Introduction

55 The four dengue virus (DENV) serotypes are mosquito-borne flaviviruses that are
56 estimated to infect several hundred million people per year living in tropical and sub-tropical
57 regions of the world(1). From January to June 2024, there has been around 10 million suspected
58 dengue cases in the Americas alone(2). Clinically, DENV infections can be asymptomatic or
59 present as dengue fever, dengue hemorrhagic fever and shock syndrome. Vaccination is one of
60 the most effective methods for preventing flavivirus infections and effective vaccines have been
61 developed against other deadly flaviviruses, such as yellow fever virus and Japanese
62 encephalitis virus. DENV vaccines must be tetravalent because immunity to a single serotype
63 has been linked to antibody-enhanced replication of heterologous serotypes, associated with
64 more severe clinical disease(3). Protein subunit vaccination is a well-established technology for
65 developing safe and effective monovalent and multivalent vaccines. However, DENV subunit
66 vaccines based on the viral envelope (E) protein have performed poorly in pre-clinical and early
67 clinical studies(4–8). One plausible explanation lies in the structure of soluble E protein under
68 physiological conditions. While soluble E protein is in equilibrium between monomer and dimer
69 states, at 37°C, the equilibrium favors the monomer that does not display key dimer dependent
70 antibody epitopes on the viral surface recognized by human neutralizing antibodies (NAbs)(8,
71 9). Using structure-guided computational methods and experimentation, we have produced
72 stable DENV E homodimers that display quaternary structure epitopes recognized by human
73 NAbs(10, 11). Here, we sought to map the specificity of NAbs elicited by DENV2 WT and dimer-
74 stabilized recombinant E protein (rE) in mice.

75 The most advanced DENV vaccines are based on tetravalent live attenuated virus
76 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(12–15). 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(16). In seronegative
82 children, Qdenga was efficacious against DENV1 and 2 but not DENV3(17). The number of
83 DENV4 cases was insufficient to assess efficacy against this serotype. Given the challenges faced
84 in developing tetravalent live DENV vaccines, we are focusing on subunit vaccines, which are
85 safer and easier to formulate as a balanced tetravalent vaccine since immunogenicity is
86 independent of viral 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 antibodies (Ab). On the viral
89 surface, 90 E homodimers are tightly packed to create a smooth protein coat with pseudo-
90 icosahedral symmetry(18, 19). Many human Abs that neutralize DENVs and other flaviviruses
91 recognize quaternary structure epitopes that span E homodimers or higher-order assemblies on
92 the virion(20–23). However, soluble DENV rE at physiological temperature (37°C) is mostly
93 monomeric and does not display the native homodimer form(9). 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 stabilized the rE
96 homodimer at 37°C, while preserving the overall structure of the native homodimer, including

97 quaternary epitopes recognized by human NAb(10). We also showed that mice immunized with
98 stabilized DENV2 rE dimer had higher levels of DENV2 NAb than animals immunized with WT rE.
99 In this study, we determined the specificity and epitope targets of the NAbs elicited by the
100 DENV2 rE dimer in comparison to the traditional WT rE when they are used as vaccine antigens
101 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. All the recombinant DENV2
106 E proteins used in the current study have been previously described(10). The protein
107 nomenclature used in the current study and the location of specific mutations are summarized
108 in **Table S1**. Groups of Balb/c mice were primed and boosted (week 3) with WT rE and a
109 stabilized E homodimer (SD*FL) in the presence or absence of an adjuvant (Alum) (**Fig. 1A, B**).
110 The SD*FL antigen has 2 mutations (A259W, T262R) in the E-domain(ED) II at the dimer
111 interface (I2), 2 mutations (F279W, T280P) in the hinge between EDI and EDIII (U6), and a point
112 mutation (G106D) in the fusion loop (I8) that independently contribute to dimer stabilization
113 (10)(**Fig. 1A, Table S1**). At 12 weeks after vaccination, mice immunized with SD*FL had more
114 DENV2 binding Abs compared to animals immunized with WT rE (**Fig. 1C**). We compared the
115 binding of vaccine-induced antibodies to DENV2 rE monomer (M2P4) and SD*FL. As previously
116 described(10), the M2P4 protein has a mutation (G258E) at the dimer interface (M2) that
117 destabilizes the dimer and 3 mutations (S29K, T33V, A35M) in EDI (P4) that increase stability
118 and secretion of the monomer (**Table S1**). SD*FL immunized animals had higher levels of Abs to

119 both E monomer and dimer antigens compared to WT E immunized animals (**Fig. 1D, E**). To
120 compare relative levels of monomer and dimer binding Abs in each group, we calculated the
121 ratio of dimer/monomer binding. SD*FL immunized animals had a response that was skewed in
122 favor of the dimer over monomer compared to WT E immunized animals (**Fig. 1F**). The SD*FL rE
123 antigen also stimulated higher levels of NAbs compared to WT rE (**Fig. 1G**). Collectively, these
124 results indicate that the stable dimer antigen stimulates higher levels of binding Abs (skewed in
125 favor of the E homodimer over the monomer) and NAbs compared to the WT E antigen.

126 **Immunogenicity of stabilized DENV2 recombinant E homodimers: 2 vs. 3-dose study**

127 Next, we compared the immunogenicity of 2-dose versus 3-dose vaccination schedules
128 (**Fig. 2A**) in the absence of any adjuvant. Our goal was to obtain moderate to high titer sera
129 required for mapping NAbs stimulated by WT and SD*FL rE antigens. To understand the impact
130 of antigen structure alone on immunogenicity, we did not include the Alum adjuvant, which
131 could alter protein structure(24).

132 The 2-dose component of this study confirmed our previous result (**Fig. 1**) that SD*FL
133 stimulated binding Abs skewed in favor of the dimer and higher levels of NAbs compared to WT
134 rE (**Fig. 2B-E**). As expected, animals in 3-dose groups had higher levels of binding and NAbs than
135 the matched 2-dose groups. In the 3-dose regiment, the SD*FL rE stimulated binding Abs
136 skewed in favor of the dimer over the monomer (**Fig. 2E**). The NAb responses at week 12 were
137 similar for WT and SD*FL rE in the 3-dose study (**Fig. 2F**). Our results demonstrate that in the 2-
138 dose regiment, SD*FL stimulates higher levels of NAbs (compared to WT rE) that are maintained
139 through week 12 (**Fig. 2F**). A third vaccine dose at week 8 preferentially increases the low WT rE

140 response such that both WT and SD*FL leading to comparable levels of NAb for both groups at
141 week 12.

142 **Mapping DENV NAbs stimulated by WT and SD*FL rE vaccines**

143 We used the week 12 high titer sera from the 3-dose study to further characterize NAbs
144 stimulated by WT and SD*FL vaccine antigens. When the sera were tested for neutralization of
145 the four DENV serotypes, we observed strong neutralization of DENV2 and no detectable
146 neutralization of other serotypes demonstrating the dominance of DENV2 type-specific NAbs
147 for both vaccine constructs (**Fig. 3A**).

148 To further map vaccine sera, we used an existing panel of DENV4/2 E domain transplant
149 viruses that have been previously used to map the epitopes of Abs from people exposed to WT
150 DENV2 infections (**Fig. 3B**)(25). The chimeric viruses have DENV4 backbone with transplanted
151 domain from DENV2 as indicated (EDI or EDII or EDIII) (**Fig. 3B**). Both WT and SD*FL antigens
152 induced Abs that neutralize the DENV4/2 EDIII chimera demonstrating the presence of NAb
153 targeting DENV2 EDIII, as well as Ab that spans EDII/EDIII such as 2D22(26) (**Fig. 3E**). SD*FL rE
154 also elicited Abs that neutralized the DENV4/2 EDI and EDII chimeric viruses, whereas WT rE
155 stimulated lower levels of NAbs that tracked with EDI (**Fig. 3C**) and no detectable NAbs to EDII
156 (**Fig. 3D**). These results indicate that WT rE mainly induced EDIII targeting NAbs, while SD*FL rE
157 stimulated a broader response directed to all three domains.

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

159 Antibodies that neutralize DENV2 have been mapped to simple epitopes contained within a
160 domain or more complex epitopes that span two different domains on the same or different E

161 molecules. For example, DENV2 type-specific and neutralizing mouse monoclonal Ab (mAb) 3H5
162 binds to a simple epitope on the lateral ridge of EDIII(27). In contrast, DENV2 type-specific and
163 neutralizing human mAb 2D22 recognizes epitope centered on EDIII that expands into
164 conserved amino acids on EDII of the second E protomer forming the homodimer. This cross-
165 dimer interaction is critical for 2D22 binding to DENV2(20, 22). Consequently, unlike 3H5, 2D22
166 binds poorly to soluble EDIII (**Fig. S1**). We performed Ab depletion studies with recombinant
167 DENV2 EDIII to measure the contribution of simple EDIII binding Abs to neutralization following
168 vaccination with WT and SD*FL rE antigens (**Fig. 4A**). Sera were incubated with DENV2 EDIII
169 coated beads and effective removal of EDIII binding antibodies was confirmed by ELISA (**Fig. 4B**).
170 As expected, removal of simple EDIII binding Abs also led to a reduction in binding to E dimers
171 (SD), which include simple EDIII epitopes (**Fig. 4C**). Next, we assessed how the removal of EDIII-
172 binding Ab impacted DENV2 neutralization (**Fig. 4D**). In WT E immune sera, the removal of EDIII-
173 binding Abs dropped DENV2 neutralization to a level below the detection limit of the assay.
174 While the removal of EDIII-binding Abs also lowered NAb levels in SD*FL rE immunized animals,
175 a sub-population of NAbs (~30-40% of total response) was resistant to EDIII depletion (**Fig. 4E**).
176 This result together with results obtained with the DENV4/2 chimeric E viruses suggests that
177 SD*FL stimulates NAbs targeting all three domains on E protein.

178 ## Discussion

179 DENV protein subunit vaccines based on the ectodomain of E protein have not
180 performed well in the past in non-human primates (NHPs) and human clinical studies(4, 5, 8, 9,
181 28). Vaccine stimulated NAb levels declined steeply over the first 6-12 months after vaccination
182 (5–7). When vaccinated NHPs were challenged with DENV, some animals were protected, while

183 others experienced breakthrough viremia(6, 7). While investigators had assumed that
184 recombinant E protein produced as a soluble, secreted antigen formed homodimers similar to
185 the protein on the viral surface, recent studies have demonstrated that the antigen is mostly
186 present as a monomer at 37℃, which is a plausible explanation for the overall poor
187 performance of DENV subunit vaccines(8, 9). The main goal of our study was to compare the
188 properties of DENV2 NAbs induced by soluble E protein antigens that are monomers (WT) or
189 homodimers (SD*FL) at 37℃. Our results demonstrate that stable homodimers stimulated
190 DENV2 NAbs targeting multiple regions of the viral envelope, whereas the WT E antigen
191 stimulated NAbs that mainly target simple EDIII epitopes. Together, our results demonstrate
192 that by altering the oligomeric state of DENV2 E protein to better match the antigen on the
193 surface of the infectious virus, it is possible to elicit Abs against all three domains of the E
194 protein.

195 Currently, the leading DENV vaccine candidates are based on tetravalent live attenuated
196 flavivirus platforms. These vaccines have been challenging to develop because of inherent
197 replication differences between vaccine viruses resulting in unbalanced immunity skewed to a
198 single serotype and/or a single genotype within a serotype(15, 29, 30). In clinical trials,
199 immunodominance of a single vaccine serotype over the other three serotypes has been linked
200 to poor outcomes, including vaccine-enhanced WT DENV infections(16). Given these challenges
201 with LAVs, a protein subunit vaccine that mimics the architecture of the viral envelope is a
202 promising alternative strategy that is not dependent on balanced replication of each vaccine
203 component.

204 Investigators have relied on DENV neutralization assays both to characterize vaccine
205 responses and as a correlate of protection. However, in clinical trials, the presence of NAbs to
206 each serotype alone was not a reliable predictor of protection. Recent studies have indicate that
207 DENVs produced for laboratory studies are inefficiently processed and mostly consist of partially
208 mature virions(31). These partially mature virions are hypersensitive to neutralization by some
209 low-affinity antibodies, which are unlikely to be protective against mature DENVs circulating in
210 humans(31). To control for virus maturation state and selectively measure vaccine responses
211 that are effective against fully mature DENV2 virions, we used fully mature DENV2 stocks
212 produced in a cell line overexpressing a protease necessary for DENV processing and
213 maturation(32). Under these conditions, we observed that 2 doses of the stabilized E
214 homodimer stimulated higher levels of mature DENV2 NAb compared to WT E protein. These
215 results are consistent with our previous work(10) demonstrating that 2 doses of the stabilized E
216 homodimer stimulates higher levels of NAb than WT rE in mice, although the original study did
217 not control for DENV maturation state. In the current study, DENV2 NAb titers were comparable
218 in the 3-dose study indicating that the low response stimulated by 2 doses of WT rE is
219 preferentially boosted by the third dose. The animals were only monitored for 4 weeks after the
220 third dose and we have not addressed durability of NAbs in this study.

221 Neutralizing Abs that target different regions of the E-protein would be able to block
222 viral entry at different stages (attachment, internalization, membrane fusion) and minimize
223 vaccine escape. The different domains of the DENV E-protein play different roles in various
224 stages of the virus lifecycle. EDIII is involved in the initial virus attachment with host cell
225 receptors(33). EDII is critical for the dimerization of E protein, and contains the fusion loop

226 required for viral and host cell membrane fusion during viral entry into cells(33, 34). People
227 exposed to DENV infections develop NAbs to multiple epitopes on different E protein domains,
228 and these responses that are broadly effective against variant genotypes belong to a
229 serotype(22, 25, 39, 40). In contrast, live attenuated DENV vaccines appear to stimulate a more
230 focused NAb response with a narrow breadth across genotypes within a serotype, with the
231 potential for breakthrough infections with vaccine mispatched genotypes(29, 30, 39, 41). In the
232 current study, the WT E vaccine stimulated a focused NAb response that was mainly directed to
233 EDIII. Previous studies have demonstrated that the lateral ridge region of EDIII contains epitopes
234 targeted by some type-specific NAbs (35, 36). However, these epitopes are variable between
235 DENV genotypes belonging to the same serotype and responsible for variable neutralization
236 potency including escape from EDIII antibodies(35, 37, 38). Our studies showed that the SD*FL
237 vaccine elicited a NAb response that targeted epitopes on all three domains. While a NAb
238 response to multiple targets on E protein is likely to have more breadth than a response focused
239 to a single site, follow up studies are needed to directly assess efficacy of WT and SD*FL rE
240 vaccines against vaccine matched and variant DENV2 genotypes.

241 In conclusion, our studies support further development and evaluation of E dimer-based
242 subunit vaccines, demonstrating that DENV2 stable homodimers stimulate high levels of NAbs
243 targeting multiple domains on E protein. We have found that the mutations used for stabilizing
244 DENV2 homodimers also work for the other three serotypes and Zika virus with minor virus-
245 specific modifications(11). Subunit vaccine studies are in progress with DENV1, DENV3 and
246 DENV4 because DENV vaccines must be tetravalent to minimize the risk of ADE. Moreover, we

247 are evaluating different vaccine delivery platforms (nanoparticles, liposomes, nucleic acid) and
248 adjuvants to increase the immunogenicity and durability of vaccine responses.

249 Methods

250 Cell lines and viruses

251 EXPI293F (Thermo Fisher Scientific) cells were maintained in EXPI293 expression medium at
252 37°C with 8% CO_2 at 250 rpm.

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

257 Vero-furin (VF) cells were given by the Baric lab at UNC Chapel Hill(32). The cells were
258 maintained at 37°C with 5% CO_2 in DMEM-F12 + 5% HI FBS + 1% Anti-anti (Gibco) + 1% MEM
259 NEAA + 1% L-Glutamine.

260 Viruses. All DENV2 S16681 used in the experiments were grown in VF cells, except for DENV2
261 S16681 used in the 2-dose study ELISA which was grown in Vero cells. For 3-dose study ELISA,
262 DENV2 S16681 VF-grown virus was concentrated using Centricon® Plus-70 Centrifugal Filter
263 (Millipore) per manufacturer instruction to obtain the amount and concentration of virus
264 required for ELISA.

265 Cross-neutralization assessment was done using DENV1 WestPac-74, DENV3 CH53489, DENV4
266 Sri Lanka 92. All viruses were grown in VF cells.

267 Chimera viruses and EDIII depletion experiments used the following viruses: DENV2 16803,
268 DENV4 Sri Lanka 92, DENV4/2 EDIII, DENV4/2 EDII and DENV4/2 EDI that were grown using a
269 previously described method(25). All viruses were grown in VF cells. As the chimeric panel is
270 based on DENV2 strain S16803, we used DENV2 S16803 for our mapping study. DENV2 strains
271 16681 and 16803 E protein sequences differ at 5 positions (M6I, R120T, I141V, I164V, and
272 E383D) – all are outside of the SD*FL mutations.

273 **DENV2 sE plasmid generation**

274 Cloning method for various DENV2 S16681 variants sE into PαH mammalian expression vector
275 was described previously(10). For the HisAvi-variants, after the C-terminal His8-tag, glycine
276 linkers (GGCAGCTCTGGCGGCAGC) and AviTag
277 (GGCCTGAACGATATTTTCGAGGCCCAGAAAATCGAGTGGCACGAGTAG) were added. The DENV2 sE
278 cloned plasmids were heat-shock transformed into *Escherichia coli* DH5α cells for amplification,
279 miniprepped or midiprepped using DNA endotoxin-free kits, and stored in endotoxin-free H_2O
280 at −20°C (Macherey-Nagel).

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

282 <u>For DENV2 WT-His:</u> Stable line was grown up in Expi293 supplemented with 250 µg/ml
283 geneticin, with geneticin removed during the final amplification step. The cultures were
284 supplemented with 5 ml of 100X GlutaMAX (Thermo 35050061) and 50 ml of 6% HyClone Cell
285 Boost Boost I (Cytiva SH30584.02) in OptiMEM (Thermo 31985070) per liter of culture when the
286 cells reached a density of approximately $1.5x10^6$ cells/ml (day 4 after reaching the final culture
287 scale). The cultures were harvested when the cells dropped below 90% viability on day 9 after

288 reaching the final scale, with the centrifugation, filtering, and purifications done the same as for
289 the proteins made in Expi293 transfection cultures.

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

294 For biotinylated SD-HisAvi: Expi293 cells were co-transfected per manufacturer instructions
295 (Thermo Scientific) in 1L volume with 900 µg of a plasmid with SD-HisAvi (UNC Protein Core)
296 and 100 µg of mammalian BirA ligase expression plasmids (UNC Protein Core). Culture media
297 was supplemented with 100 µM biotin before transfection to aid SD-HisAvi biotinylation by BirA
298 ligase. Downstream processes (enhancers and harvest timeline) are the same as DENV2
299 variants-His.

300 **DENV2 sE protein purification and analysis**

301 Filtered supernatants were buffer-exchanged via tangential-flow filtration (10 kDa MWCO) into
302 cobalt-binding buffer (50 mM sodium phosphate pH 7.4, 500 mM NaCl, 5 mM imidazole, 0.02%
303 sodium azide, 10% glycerol), with 4 total buffer exchanges. The supernatant was then loaded at
304 1.5 ml/min recirculating overnight with a peristaltic pump and flow adapter onto a hand-packed
305 TALON column. The next day, the column was switched to flow-through, and once completely
306 loaded, the column was washed with 10 Column Volume (CV) binding buffer. After a 10 CV
307 second wash with binding buffer supplemented with 10 mM imidazole, bound proteins were
308 eluted in two steps (one of 5 CV, and one of 2.5 CV) with binding buffer supplemented with 150

309 mM imidazole. Column fractions were assessed by SDS-PAGE and fractions containing target
310 protein were pooled, filtered, and concentrated before loading onto a Superdex 200 16/600
311 column equilibrated in 1x PBS + 10% glycerol. The sizing column fractions were assessed by SDS-
312 PAGE, and DENV2 protein-containing fractions were pooled, filtered, and concentrated before
313 aliquoting and flash-freezing in liquid nitrogen. The endotoxin level in the final stock was
314 measured using Endosafe-PTS cartridges (#PTS20F; Charles River) on a nexgen-PTS reader
315 between 1:20 - 1:40 dilution in endotoxin-free water.

316 For biotinylated SD-HisAvi: To assess the success of the biotinylation reaction, column fractions
317 were assessed by SDS-PAGE, with elution fractions assessed for biotinylated protein by mixing
318 with a 2x molar excess of avidin. Downstream processes are the same as above.

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

320 A Halo-tag fused DENV-2 EDIII (ADA00411·1, E protein aa 297-394) was expressed in Expi293
321 with an N-terminal human serum albumin secretion signal peptide and a C-terminal His6-tag as
322 previously described(42). A Halo-tag construct, lacking DENV-2 EDIII, was also produced
323 similarly with a C-terminal His6-tag. The DENV2 EDIII and Halo-tag proteins were purified using
324 Ni-NTA agarose (Qiagen) from the cell culture medium. Purified DENV2 EDIII was biotinylated
325 by incubating 100 µg of DENV2 EDIII with 5 nmol of the HaloTag PEG biotin ligand (Promega) at
326 room temperature (RT) for 2 hours. Excess biotin ligand was removed by desalting column.
327 Biotinylated DENV2 EDIII was assessed by comparing the mobility of the biotinylated EDIII in the
328 presence and absence of streptavidin in SDS-PAGE.

329 **Mouse immunizations**

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

337 **Ethics statement**

338 All experiments involving mice were performed according to the animal use protocol approved
339 by the University of North Carolina Animal Care and Use Committee. The animal care and use
340 related to this work complied with federal regulations: the Public Health Service Policy on
341 Humane Care and Use of Laboratory Animals, Animal Welfare Act, and followed the Guide for
342 the Care and Use of Laboratory Animals.

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

344 Whole virus ELISA. DENV2 IgG binding titer was measured by DENV2 antigen capture ELISA. A
345 high-binding 96-well plate (Greiner bio-one) was coated using 0.2 µg/ml of 1M7 (DENV fusion
346 loop human mAb) in 0.1M bicarbonate buffer (pH 9.6) overnight at 4°C, and washed with wash
347 buffer (1X Tris-buffered saline (TBS) + 0.2% Tween-20). The plate was blocked with blocking
348 buffer (3% non-fat dried milk in 1X TBS + 0.05% Tween20), before DENV2 was loaded to each
349 well. Next, serially diluted mouse sera were added onto the plate. Plates were washed in

350 between each step, and each incubation was done for 1 hour at 37°C. Bound antibodies were
351 detected by alkaline phosphatase (AP)-conjugated anti-mouse IgG (1:1000, Sigma-Aldrich) for
352 45 min at 37°C, and washed. Plates were developed using AP substrate (SIGMAFAST p-
353 Nitrophenyl phosphate tablet, Sigma-Aldrich) and measured at 405 nm. IgG titer was
354 determined by area under the curve (AUC) in GraphPad Prism using nonlinear fit – sigmoidal
355 dose-response (variable slope).

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

361 Biotinylated-protein ELISA. High-binding 96-well plate was coated using 0.2 µg/ml of
362 Streptavidin (Invitrogen) in 1X TBS overnight at 4°C or 1 hour at 37°C. Supernatant was
363 removed, and 0.2 µg/ml of biotinylated antigens (DENV2 EDIII or SD) was added in 1X TBS, and
364 incubated for 1 hour at 37°C. The plate was washed using wash buffer and then blocked with
365 blocking buffer for 1 hour at 37°C. The plate was washed, and serially diluted sera in 1X TBS
366 were added for 1 hour at 37°C and subsequently washed with wash buffer. Bound antibodies
367 were detected by AP-conjugated anti-mouse IgG (1:1000 in 1X TBS) for 45 min at 37°C, and
368 washed. Plates were developed using AP substrate and measured at 405 nm. IgG titer was
369 determined by area under the curve (AUC) in GraphPad Prism using nonlinear fit – Sigmoidal,
370 4PL.

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

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

374 *Cell infection.* Sera were serially diluted in infection media (similar to cell culture media, only
375 with 2% HI FBS) and incubated with a fixed amount of virus at 1:1 v/v ratio for 1 hour at 37°C.
376 The amount of virus had been previously determined to give a minimum of 30 foci/well. In each
377 experiment, the starting serum dilution was different and based on the NAb titer of the
378 specimen and the quantity of sample remaining. The limit of detection (starting dilution) for Fig.
379 1G and 2F is 1:10, Fig. 3 is 1:60, Fig. 4D is 1:120. Culture media on cells were removed, and
380 serum/virus complex was added onto cells for 1 hour at 37°C. Serum/virus complex was
381 removed from cells, and overlay media (OptiMEM (Gibco) + 2% HI FBS + 1% anti-anti + 1% (w/v)
382 carboxymethylcellulose (Sigma-Aldrich)) was added to cells. Cells were left to incubate for 42-48
383 hours (depending on the viruses used).

384 *Cell harvest and staining.* Cells were washed with 1X phosphate-buffered saline (PBS), and fixed
385 with 4% paraformaldehyde. Next, cells were permeabilized with 1X permeabilization (Perm)
386 buffer (10X Perm buffer was diluted in MilliQ water. 10X Perm: 1% of bovine serum albumin
387 (BSA, Sigma), 1% of Saponin (Sigma), 0.1% of sodium azide (Sigma) in 10X PBS), and blocked
388 using blocking buffer (5% non-fat dry milk in 1X Perm) at RT for 10 min. Blocking buffer was
389 removed, and cells were stained using 1M7 at $1.2x10^{-3}$mg/ml for 1 hour at 37°C. Cells were
390 washed with 1X PBS, and stained with mouse anti-human IgG Fc HRP (Southern biotech) at
391 1:4000 for 1 hour at 37°C. Cells were washed and stained with KPL TrueBlue Peroxidase
392 Substrate (SeraCare). Cells were then rinsed with MilliQ water and plates were left to dry prior

393 to imaging with ImmunoSpot S6 analyzer (Cellular Technology Limited). Foci were counted using
394 Viridot on RStudio, and checked for accuracy(43). Neutralization curves were generated by
395 plotting sera dilution factor (log) vs % neutralization. Curves were fitted using nonlinear fit –
396 sigmoidal dose-response (variable slope) with constraints (top = 100, bottom = 0). Curves were
397 acceptable when R^2 ≥0.75 and Hill Slope >0.5. $Neut_{50}$ value is the dilution factor at which sera
398 neutralize 50% of the virus. Analysis was done using Graphpad Prism

399 DENVs chimera neutralization. All steps are identical to FRNT assay explained above, with the
400 exception of serum/virus complex was not removed prior to the addition of overlay media.

401 **EDIII Depletion**

402 *Protein-beads conjugation.* First, Magne HaloTag beads (Promega) were transferred into Lo-
403 bind tubes (Eppendorf), and beads were washed with 1X TBS for a total of 3 times. To separate
404 beads from supernatant, magnetic stand (DynaMag2, Invitrogen) was used. After the third
405 wash, pre-determined amount of biotinylated protein was added to the beads. Ratio of initial
406 beads volume (µl) to antigen amount (µg) is 1:2. Beads and biotinylated protein were left to
407 incubate for 2 hour at RT, mixing at 1300rpm in ThermoMixer C. Once the conjugation was
408 done, beads were washed 3 times using blocking buffer (5% BSA in 1X TBS). Beads were further
409 blocked using blocking buffer for 1 hour at RT, mixing at 1300rpm. After blocking step, beads
410 were washed 3 times with 1X TBS and re-suspend in 1X TBS. The final resuspension volume is
411 equal to the total volume of all diluted sera that is going to be depleted; the final protein
412 concentration in this resuspension is 48 µg/ml. Beads were stored at 4°C in lo-bind tubes if not
413 being used immediately.

414 *Sera depletion.* Depletion was done using non-binding 96-well plate (Corning). Volume of
415 conjugated beads/well is equal to volume of depleted sera/well. Conjugated beads were added
416 to each well, and the supernatant was discarded by placing the plate on 96-well plate magnetic
417 separator (Millipore Sigma). Next, diluted sera (1:60, in 1X TBS) was added per well and plate
418 was covered with adhesive film (Thermo). The plate was incubated at 37°C for 1 hour at
419 800rpm. Depletion was done for 2-3 rounds. Sera depletion was considered complete when
420 EDIII-depleted sera (1:1200) against EDIII antigen on an ELISA reached background level
421 compared to undepleted sera. Depleted sera were stored in untreated polypropylene 96-well
422 plates (Corning) at 4°C.

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

424 **Data availability:** not applicable

425 **Acknowledgment:** We thank Edwing D. C. Cuadra for discussions and suggestions with the EDIII
426 depletion experiments.

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428 References

429 1. Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL, Drake JM, Brownstein
430 JS, Hoen AG, Sankoh O, Myers MF, George DB, Jaenisch T, Wint GRW, Simmons CP, Scott
431 TW, Farrar JJ, Hay SI. 2013. The global distribution and burden of dengue. Nature
432 496:504–507.

433 2. Situation Report No 24 - Dengue Epidemiological Situation in the Region of the Americas

434 - Epidemiological Week 24, 2024 - PAHO/WHO | Pan American Health Organization.
435 https://www.paho.org/en/documents/situation-report-no-24-dengue-epidemiological-
436 situation-region-americas-epidemiological. Retrieved 9 July 2024.

437 3. Halstead S. 2019. Recent advances in understanding dengue. F1000Research. F1000
438 Research Ltd https://doi.org/10.12688/f1000research.19197.1.

439 4. Manoff SB, George SL, Bett AJ, Yelmene ML, Dhanasekaran G, Eggemeyer L, Sausser ML,
440 Dubey SA, Casimiro DR, Clements DE, Martyak T, Pai V, Parks DE, Coller BAG. 2015.
441 Preclinical and clinical development of a dengue recombinant subunit vaccine. Vaccine
442 33:7126–7134.

443 5. Manoff SB, Sausser M, Falk Russell A, Martin J, Radley D, Hyatt D, Roberts CC, Lickliter J,
444 Krishnarajah J, Bett A, Dubey S, Finn T, Coller BA. 2019. Immunogenicity and safety of an
445 investigational tetravalent recombinant subunit vaccine for dengue: results of a Phase I
446 randomized clinical trial in flavivirus-naïve adults. Hum Vaccines Immunother 15:2195–
447 2204.

448 6. Clements DE, Coller BAG, Lieberman MM, Ogata S, Wang G, Harada KE, Putnak JR, Ivy JM,
449 McDonell M, Bignami GS, Peters ID, Leung J, Weeks-Levy C, Nakano ET, Humphreys T.
450 2010. Development of a recombinant tetravalent dengue virus vaccine: Immunogenicity
451 and efficacy studies in mice and monkeys. Vaccine 28:2705–2715.

452 7. Govindarajan D, Meschino S, Guan L, Clements DE, ter Meulen JH, Casimiro DR, Coller
453 BAG, Bett AJ. 2015. Preclinical development of a dengue tetravalent recombinant subunit
454 vaccine: Immunogenicity and protective efficacy in nonhuman primates. Vaccine

455 33:4105–4116.

456 8. Kudlacek ST, Metz SW. 2019. Focused dengue vaccine development: Outwitting nature's
457 design. Pathog Dis 77:3.

458 9. Kudlacek ST, Premkumar L, Metz SW, Tripathy A, Bobkov AA, Payne AM, Graham S,
459 Brackbill JA, Miley MJ, de Silva AM, Kuhlman B. 2018. Physiological temperatures reduce
460 dimerization of dengue and Zika virus recombinant envelope proteins. J Biol Chem
461 293:8922–8933.

462 10. Kudlacek ST, Metz S, Thiono D, Payne AM, Phan TTN, Tian S, Forsberg LJ, Maguire J, Seim
463 I, Zhang S, Tripathy A, Harrison J, Nicely NI, Soman S, McCracken MK, Gromowski GD,
464 Jarman RG, Premkumar L, De Silva AM, Kuhlman B. 2021. Designed, highly expressing,
465 thermostable dengue virus 2 envelope protein dimers elicit quaternary epitope
466 antibodies. Sci Adv 7:4084–4099.

467 11. Phan TTN, Hvasta MG, Kudlacek ST, Thiono DJ, Tripathy A, Nicely NI, de Silva AM,
468 Kuhlman B. 2022. A conserved set of mutations for stabilizing soluble envelope protein
469 dimers from dengue and Zika viruses to advance the development of subunit vaccines. J
470 Biol Chem 298:102079.

471 12. Henein S, Swanstrom J, Byers AM, Moser JM, Shaik SF, Bonaparte M, Jackson N, Guy B,
472 Baric R, De Silva AM. 2017. Dissecting antibodies induced by a chimeric yellow fever-
473 dengue, live-attenuated, tetravalent dengue vaccine (CYD-TDV) in naive and dengue-
474 exposed individuals. J Infect Dis 215:351–358.

475 13. White LJ, Young EF, Stoops MJ, Henein SR, Adams EC, Baric RS, de Silva AM. 2021.
476 Defining levels of dengue virus serotype-specific neutralizing antibodies induced by a live
477 attenuated tetravalent dengue vaccine (Tak-003). PLoS Negl Trop Dis 15:1–15.

478 14. Swanstrom JA, Henein S, Plante JA, Yount BL, Widman DG, Gallichotte EN, Dean HJ,
479 Osorio JE, Partidos CD, De Silva AM, Baric RS. 2018. Analyzing the Human Serum
480 Antibody Responses to a Live Attenuated Tetravalent Dengue Vaccine Candidate. J Infect
481 Dis 217:1932–1941.

482 15. Dayan GH, Thakur M, Boaz M, Johnson C. 2013. Safety and immunogenicity of three
483 tetravalent dengue vaccine formulations in healthy adults in the USA. Vaccine 31:5047–
484 5054.

485 16. Sridhar S, Luedtke A, Langevin E, Zhu M, Bonaparte M, Machabert T, Savarino S,
486 Zambrano B, Moureau A, Khromava A, Moodie Z, Westling T, Mascareñas C, Frago C,
487 Cortés M, Chansinghakul D, Noriega F, Bouckenooghe A, Chen J, Ng S-P, Gilbert PB,
488 Gurunathan S, DiazGranados CA. 2018. Effect of Dengue Serostatus on Dengue Vaccine
489 Safety and Efficacy. N Engl J Med 327–340.

490 17. Rivera L, Biswal S, Sáez-Llorens X, Reynales H, López-Medina E, Borja-Tabora C, Bravo L,
491 Sirivichayakul C, Kosalaraksa P, Vargas LM, Yu D, Watanaveeradej V, Espinoza F, Dietze R,
492 Fernando LK, Wickramasinghe P, Moreira ED, Fernando AD, Gunasekera D, Luz K, Da
493 Cunha RV, Rauscher M, Zent O, Liu M, Hoffman E, LeFevre I, Tricou V, Wallace D, Alera
494 MT, Borkowski A. 2022. Three-year Efficacy and Safety of Takeda's Dengue Vaccine
495 Candidate (TAK-003). Clin Infect Dis 75:107–117.

496 18. Zhang X, Ge P, Yu X, Brannan JM, Bi G, Zhang Q, Schein S, Hong Zhou Z. 2012. Cryo-EM
497 structure of the mature dengue virus at 3.5-Å resolution. Nat Struct Mol Biol 2012 201
498 20:105–110.

499 19. Kuhn RJ, Zhang W, Rossmann MG, Pletnev S V., Corver J, Lenches E, Jones CT,
500 Mukhopadhyay S, Chipman PR, Strauss EG, Baker TS, Strauss JH. 2002. Structure of
501 dengue virus: Implications for flavivirus organization, maturation, and fusion. Cell
502 108:717–725.

503 20. Fibriansah G, Ibarra KD, Ng TS, Smith SA, Tan JL, Lim XN, Ooi JSG, Kostyuchenko VA,
504 Wang J, De Silva AM, Harris E, Crowe JE, Lok SM. 2015. Cryo-EM structure of an antibody
505 that neutralizes dengue virus type 2 by locking E protein dimers. Science (80-) 349:88–
506 91.

507 21. Rouvinski A, Guardado-Calvo P, Barba-Spaeth G, Duquerroy S, Vaney MC, Kikuti CM,
508 Navarro Sanchez ME, Dejnirattisai W, Wongwiwat W, Haouz A, Girard-Blanc C, Petres S,
509 Shepard WE, Desprès P, Arenzana-Seisdedos F, Dussart P, Mongkolsapaya J, Screaton GR,
510 Rey FA. 2015. Recognition determinants of broadly neutralizing human antibodies
511 against dengue viruses. Nature 520:109–113.

512 22. Gallichotte EN, Baric TJ, Yount BL, Widman DG, Durbin A, Whitehead S, Baric RS, de Silva
513 AM. 2018. Human dengue virus serotype 2 neutralizing antibodies target two distinct
514 quaternary epitopes. PLoS Pathog 14:e1006934.

515 23. Smith SA, de Alwis AR, Kose N, Harris E, Ibarra KD, Kahle KM, Pfaf JM, Xiang X, Doranz BJ,
516 de Silva AM, Austin SK, Sukupolvi-Petty S, Diamond MS, Crowe JE. 2013. The potent and

517 broadly neutralizing human dengue virus-specific monoclonal antibody 1C19 reveals a
518 unique cross-reactive epitope on the bc loop of domain II of the envelope protein. MBio
519 4.

520 24. Cerofolini L, Giuntini S, Ravera E, Luchinat C, Berti F, Fragai M. 2019. Structural
521 characterization of a protein adsorbed on aluminum hydroxide adjuvant in vaccine
522 formulation. npj Vaccines 4.

523 25. Zhu DR, Rajesh AJ, Meganck RM, Young EF, Munt JE, Tse VL, Yount B, Conrad H, White L,
524 Henein S, DeSilva AM, Baric RS. 2023. Dengue virus 4/2 envelope domain chimeric virus
525 panel maps type-specific responses against dengue serotype 2. MBio 14.

526 26. Gallichotte EN, Widman DG, Yount BL, Wahala WM, Durbin A, Whitehead S, Sariol CA,
527 Crowe JE, De Silva AM, Barica RS. 2015. A new quaternary structure epitope on dengue
528 virus serotype 2 is the target of durable type-specific neutralizing antibodies. MBio 6.

529 27. Renner M, Flanagan A, Dejnirattisai W, Puttikhunt C, Kasinrerk W, Supasa P, Wongwiwat
530 W, Chawansuntati K, Duangchinda T, Cowper A, Midgley CM, Malasit P, Huiskonen JT,
531 Mongkolsapaya J, Screaton GR, Grimes JM. 2018. Characterization of a potent and highly
532 unusual minimally-enhancing antibody directed against dengue virus. Nat Immunol
533 19:1248.

534 28. Deng SQ, Yang X, Wei Y, Chen JT, Wang XJ, Peng HJ. 2020. A Review on Dengue Vaccine
535 Development. Vaccines 8.

536 29. Martinez DR, Yount B, Nivarthi U, Munt JE, Delacruz MJ, Whitehead SS, Durbin AP, de

537 Silva AM, Baric RS. 2020. Antigenic Variation of the Dengue Virus 2 Genotypes Impacts
538 the Neutralization Activity of Human Antibodies in Vaccinees. Cell Rep 33:108226.

539 30. Gallichotte EN, Baric TJ, Nivarthi U, Delacruz MJ, Graham R, Widman DG, Yount BL,
540 Durbin AP, Whitehead SS, de Silva AM, Baric RS. 2018. Genetic Variation between
541 Dengue Virus Type 4 Strains Impacts Human Antibody Binding and Neutralization. Cell
542 Rep 25:1214–1224.

543 31. Raut R, Corbett KS, Tennekoon RN, Premawansa S, Wijewickrama A, Premawansa G,
544 Mieczkowski P, Rückert C, Ebel GD, De Silva AD, De Silva AM. 2019. Dengue type 1 viruses
545 circulating in humans are highly infectious and poorly neutralized by human antibodies.
546 Proc Natl Acad Sci U S A 116:227–232.

547 32. Tse L V., Meganck RM, Dong S, Adams LE, White LJ, Mallory ML, Jadi R, de Silva AM, Baric
548 RS. 2022. Generation of Mature DENVs via Genetic Modification and Directed Evolution.
549 MBio 13.

550 33. Pierson TC, Diamond MS. 2015. A game of numbers: The stoichiometry of antibody-
551 mediated neutralization of flavivirus infection, p. 141–166. *In* Progress in Molecular
552 Biology and Translational Science.

553 34. Nanaware N, Banerjee A, Bagchi SM, Bagchi P, Mukherjee A. 2021. Dengue Virus
554 Infection: A Tale of Viral Exploitations and Host Responses. Viruses 13.

555 35. Wahala WMPB, Kraus AA, Haymore LB, Accavitti-Loper MA, de Silva AM. 2009. Dengue
556 virus neutralization by human immune sera: Role of envelope protein domain III-reactive

557 antibody. Virology 392:103–113.

558 36. Wahala WMPB, de Silva AM. 2011. The human antibody response to dengue virus
559 infection. Viruses 3:2374–2395.

560 37. Wahala WMPB, Donaldson EF, De Alwis R, Accavitti-Loper MA, Baric RS, De Silva AM.
561 2010. Natural Strain Variation and Antibody Neutralization of Dengue Serotype 3 Viruses.
562 PLOS Pathog 6:e1000821.

563 38. Sukupolvi-Petty S, Austin SK, Engle M, Brien JD, Dowd KA, Williams KL, Johnson S, Rico-
564 Hesse R, Harris E, Pierson TC, Fremont DH, Diamond MS. 2010. Structure and Function
565 Analysis of Therapeutic Monoclonal Antibodies against Dengue Virus Type 2. J Virol
566 84:9227–9239.

567 39. Munt JE, Henein S, Adams C, Young E, Hou YJ, Conrad H, Zhu D, Dong S, Kose N, Yount B,
568 Meganck RM, Tse LP V., Kuan G, Balmaseda A, Ricciardi MJ, Watkins DI, Crowe JE, Harris
569 E, DeSilva AM, Baric RS. 2023. Homotypic antibodies target novel E glycoprotein domains
570 after natural DENV 3 infection/vaccination. Cell Host Microbe 31:1850-1865.e5.

571 40. Young E, Carnahan RH, Andrade D V., Kose N, Nargi RS, Fritch EJ, Munt JE, Doyle MP,
572 White L, Baric TJ, Stoops M, DeSilva A, Tse L V., Martinez DR, Zhu D, Metz S, Wong MP,
573 Espinosa DA, Montoya M, Biering SB, Sukulpolvi-Petty S, Kuan G, Balmaseda A, Diamond
574 MS, Harris E, Crowe JE, Baric RS. 2020. Identification of Dengue Virus Serotype 3 Specific
575 Antigenic Sites Targeted by Neutralizing Human Antibodies. Cell Host Microbe 27:710-
576 724.e7.

577 41. Gallichotte EN, Henein S, Nivarthi U, Delacruz M, Scobey T, Bonaparte M, Moser J,
578 Munteanu A, Baric R, de Silva AM. 2022. Vaccine-induced antibodies to contemporary
579 strains of dengue virus type 4 show a mechanistic correlate of protective immunity. Cell
580 Rep 39.

581 42. Dahora L, Castillo IN, Medina FA, Vila F, Munoz-Jordan J, Segovia B, de Silva AM,
582 Premkumar L. 2023. Flavivirus Serologic Surveillance: Multiplex Sample-Sparing Assay for
583 Detecting Type-Specific Antibodies to Zika and Dengue Viruses. Prepr with LANCET 7 Apr
584 2023.

585 43. Katzelnick LC, Coello Escoto A, McElvany BD, Chávez C, Salje H, Luo W, Rodriguez-
586 Barraquer I, Jarman R, Durbin AP, Diehl SA, Smith DJ, Whitehead SS, Cummings DAT.
587 2018. Viridot: An automated virus plaque (immunofocus) counter for the measurement
588 of serological neutralizing responses with application to dengue virus. PLoS Negl Trop Dis
589 12:e0006862.

590 **Figure legends**

591 **Figure 1. Immunogenicity of recombinant DENV2 E protein vaccines: 2 dose study.** (**A**) DENV2
592 soluble E structure (PDB 1OAN) of the vaccine antigens: wild-type (WT) and SD*FL with
593 stabilizing amino acid mutations shown. (**B**) 6-week old female Balb/c mice were immunized
594 with 5µg of WT or SD*FL rE ±Alum (125ug) at week 0 (W0), and boosted with the same antigen
595 ±Alum at week 3 (W3).
596 IgG binding titer at W12 against (**C**) DENV2, (**D**) DENV2 rE monomer (M2P4), and (**E**) DENV2 rE
597 stabilized dimer with mutated FL (SD*FL) were measured by serially diluting the mouse sera,

598 and the resulting area under the curve (AUC) were reported. Due to limited samples availability,
599 the number of mice in SD*FL group in experiment (**D**) and (**E**) was reduced to n=4. (**F**) To
600 determine the ratio of dimer:monomer binding Ab, AUC value from dimer- and monomer-
601 binding was used. The horizontal line represents the mean titer of all mice within the group.
602 Statistical analysis was done using unpaired t-test (two-tailed, $p<0.05$). (**G**) DENV2 50%
603 neutralizing Ab titers at week 12. Limit of detection (LoD) for this assay was 1:10. The horizontal
604 line represents the geometric mean titer (GMT) of all mice within the group. Statistical analysis
605 was done on log-transformed values, followed by unpaired t-test (two-tailed, $p<0.05$). The color
606 and symbols match individual mice tested in different assays.

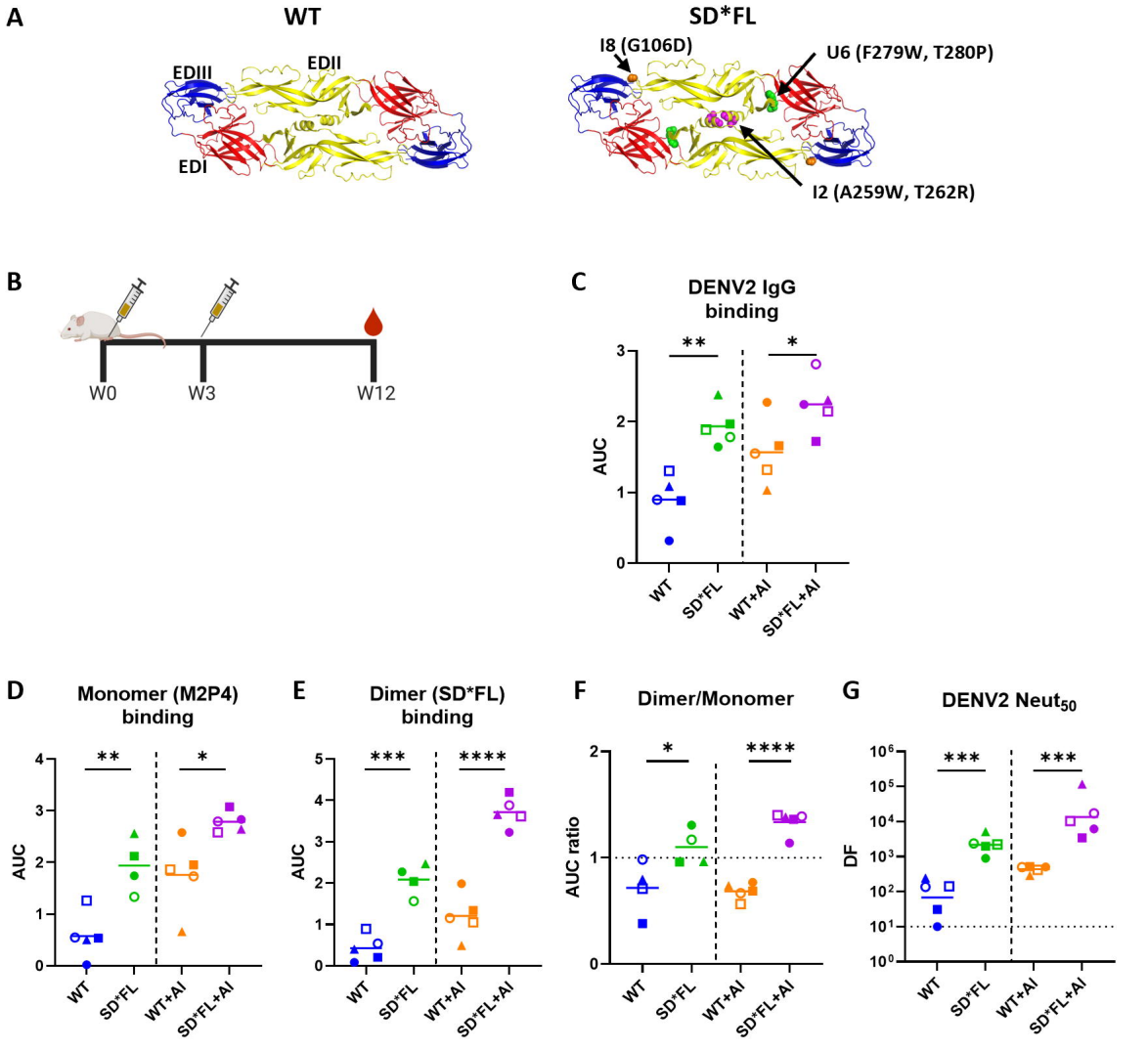
607 **Figure 2. Immunogenicity of recombinant DENV2 E protein vaccines: 3 dose study.** (**A**) 6-week
608 old female Balb/c mice were immunized with 5µg of WT or SD*FL rE following two different
609 vaccination schedules: 2 dose schedule- W0 and W3; 3 dose schedule- W0, W3 and W8. Serum
610 samples from each animal were collected pre- (W3, W8) and post-boost (W5, W10, W12). IgG
611 binding against (**B**) DENV2, (**C**) DENV2 rE monomer (M2P4), and (**D**) DENV2 rE dimer with
612 mutated fusion loop (SD*FL) were measured on W12 sera and the resulting AUC was
613 determined. (**E**) We took the AUC value of the monomer and dimer binding to quantify the
614 dimer:monomer Ab ratio. The horizontal line represents the mean titer of all mice within the
615 group. Statistical analysis was done using unpaired t-test (two-tailed, $p<0.05$). (**F**) DENV2 50%
616 neutralizing Ab titers at week 12. LoD for this assay was 1:10. The horizontal line represents the
617 GMT of all mice within the group. Statistical analysis was done on log-transformed values,
618 followed by unpaired t-test (two-tailed, $p<0.05$). The color and symbols match individual mice
619 tested in different assays.

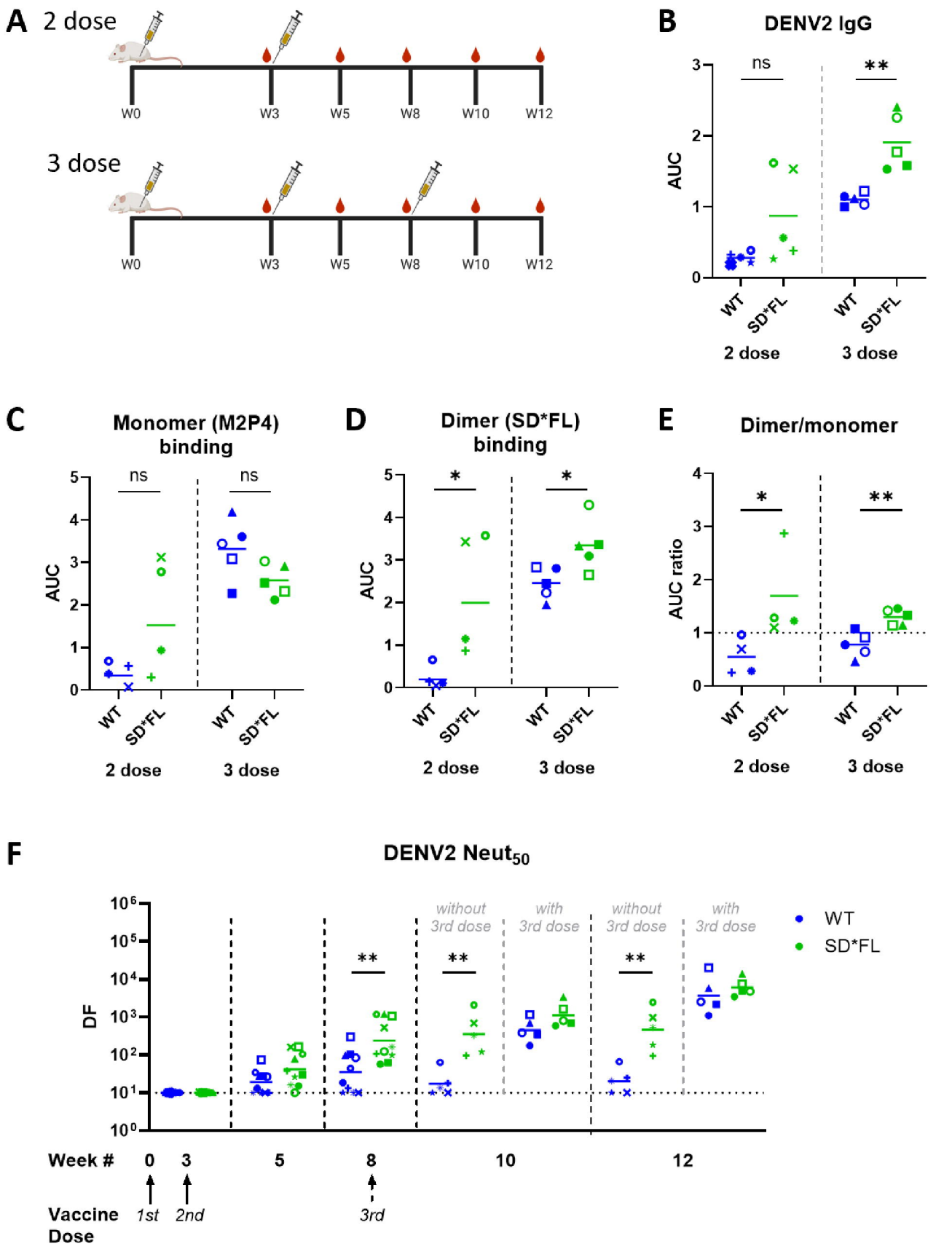
620 **Figure 3. Mapping DENV2 E-domains targeted by neutralizing Ab using DENV4/2 chimeric**
621 **viruses.** (**A**) DENV1-4 50% neutralizing Ab titers at week 12. (**B**) DENV4/2 chimera schematic
622 with DENV2 domain substitution highlighted in yellow (PBD 4CBF). The neutralizing IgG titer
623 elicited by WT and SD*FL rE vaccines are reported as the $Neut_{50}$ value against the parental
624 viruses (**A**) DENV2, DENV4 and against the various domains of DENV2 on a DENV4 backbone (**C**)
625 DENV4/2 EDI, (**D**) DENV4/2 EDII, (**E**) DENV4/2 EDIII. LoD for these assays is 1:60. The horizontal
626 line represents the GMT of all mice within the group. Statistical analysis was done on log-
627 transformed values, followed by unpaired t-test (two-tailed, $p<0.05$). The same mouse in
628 different experiments is represented with the same color and symbols.

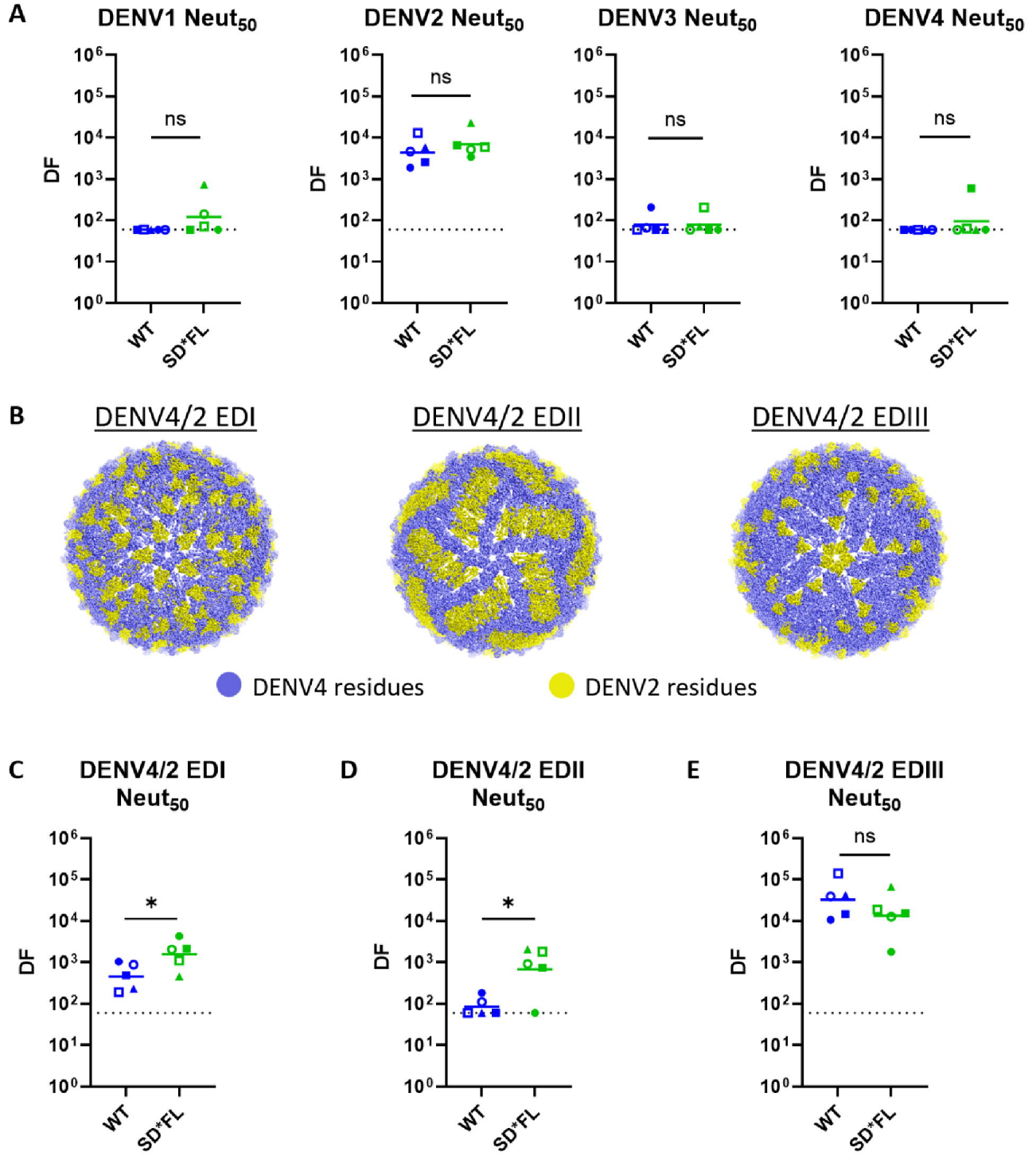
629 **Figure 4. Characterization of EDIII binding antibody.** (**A**) A schema for DENV2 EDIII depletion.
630 First, we conjugated Magne HaloTag beads with either EDIII or HaloTag. Next, we treated the
631 mouse sera with either EDIII- or HaloTag (control)-coated beads. Magnetic pulldown will
632 separate any antibodies that bind to the coated beads, leaving behind unbound antibodies
633 (non-simple EDIII or non-HaloTag antibodies) in the sera. Downstream depleted samples
634 analysis include (**B**) DENV2 EDIII IgG binding, (**C**) DENV2 stabilized dimer with intact FL (SD) IgG
635 binding, and (**D**) DENV2 neutralization, with LoD of DF120. (**E**) Percentage of NAb that target
636 other areas outside of simple EDIII region. Each data point is the average of each sample's
637 duplicates. (**B, C, E**) The horizontal line represents the mean of all mice within the group.
638 Statistical differences between Halo- vs EDIII-depleted samples (**B, C**) were determined by
639 paired t-test (one-tailed, $p<0.05$). Unpaired t-test (one-tailed, $p<0.05$) was used to determine
640 the statistical differences for (**E**). (**D**) The horizontal line represents the GMT of all mice within

641 the group. Statistical analysis was done on log-transformed values, followed by paired t-test

642 (one-tailed, $p<0.05$). The color and symbols match individual mice tested in different assays.


A
WT
EDIII
EDII
EDI
SD*FL
I8 (G106D)
U6 (F279W, T280P)
I2 (A259W, T262R)
B
W0
W3
W12
C
DENV2 IgG binding
AUC
WT
SD*FL
WT+AI
SD*FL+AI
D
Monomer (M2P4) binding
E
Dimer (SD*FL) binding
F
Dimer/Monomer
AUC ratio
G
DENV2 Neut50
DF


A
2 dose
W0
W3
W5
W8
W10
W12
3 dose
W0
W3
W5
W8
W10
W12
B
DENV2 IgG
ns
**
AUC
WT
SD*FL
WT
SD*FL
2 dose
3 dose
C
Monomer (M2P4) binding
ns
ns
AUC
WT
SD*FL
WT
SD*FL
2 dose
3 dose
D
Dimer (SD*FL) binding
*
*
AUC
WT
SD*FL
WT
SD*FL
2 dose
3 dose
E
Dimer/monomer
*
**
AUC ratio
WT
SD*FL
WT
SD*FL
2 dose
3 dose
F
DENV2 Neut50
without 3rd dose
with 3rd dose
without 3rd dose
with 3rd dose
**
**
**
DF
WT
SD*FL
Week #
0
3
5
8
10
12
Vaccine Dose
1st
2nd
3rd


A
DENV1 Neut50
DENV2 Neut50
DENV3 Neut50
DENV4 Neut50
DF
ns
WT
SD*FL
B
DENV4/2 EDI
DENV4/2 EDII
DENV4/2 EDIII
DENV4 residues
DENV2 residues
C
DENV4/2 EDI Neut50
D
DENV4/2 EDII Neut50
E
DENV4/2 EDIII Neut50
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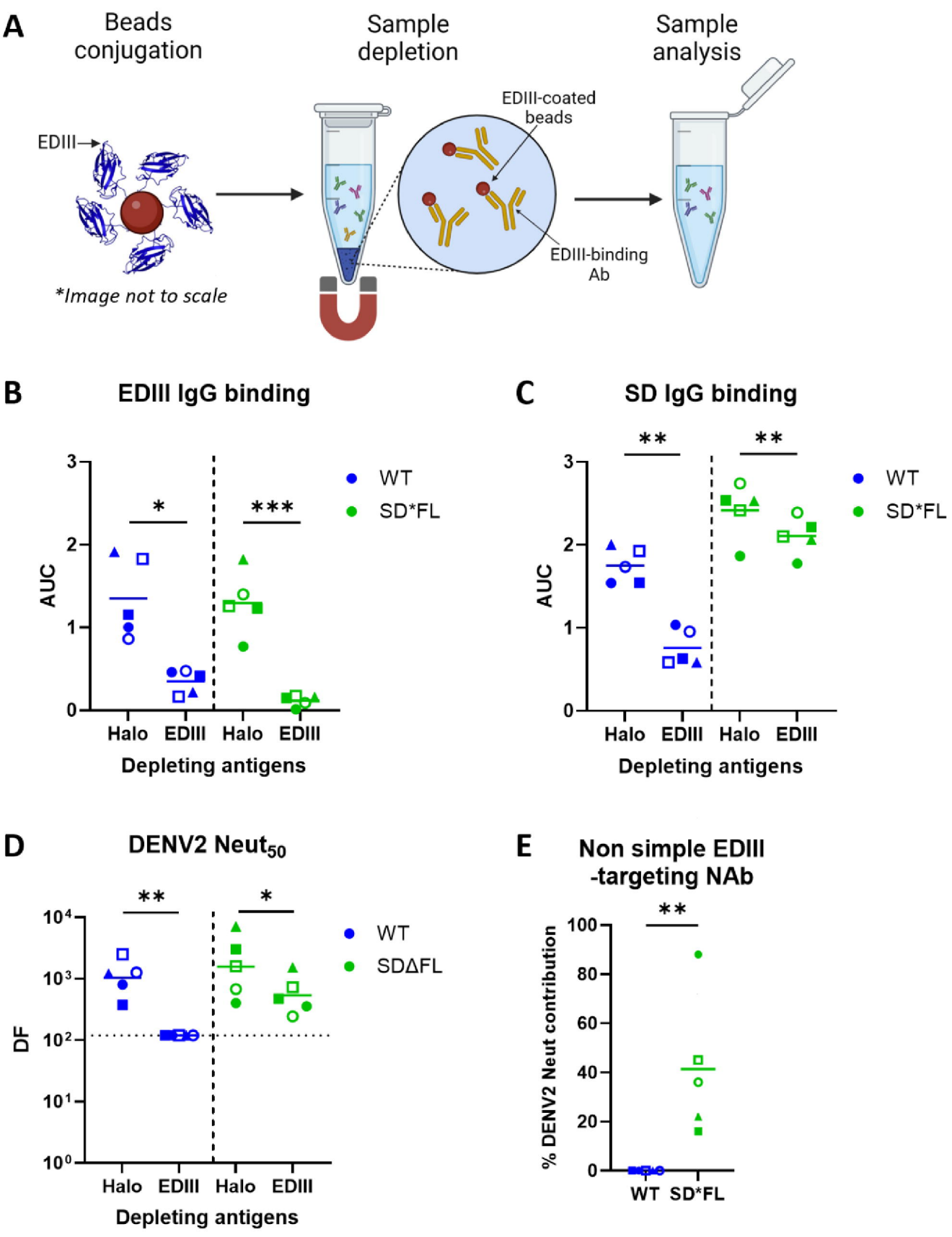
A
Beads conjugation
Sample depletion
Sample analysis
EDIII
*Image not to scale
EDIII-coated beads
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B
EDIII IgG binding
AUC
Halo
EDIII
Depleting antigens
WT
SD*FL
C
SD IgG binding
D
DENV2 Neut50
DF
SDΔFL
E
Non simple EDIII -targeting NAb
% DENV2 Neut contribution