mRNA-LNP Vaccines Encoding for Dengue Optimized prM/ENV Proteins Induce Protective Immunity without ADE

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ABSTRACT

Dengue virus (DENV) is the most common mosquito-borne virus in the world, causing nearly 400 million infections annually. Despite this prevalence there are no approved vaccine for DENV naïve individuals or therapeutics. DENV consists of four distinct serotypes, DENV 1-4, that share 60-70% homology. Development of a safe and efficacious pan-Dengue vaccine has been complicated by the potential of antibody-dependent enhancement, in which cross-reactive non-neutralizing antibodies can enhance infection and pathology. We previously demonstrated that a mRNA-LNP that encodes for the DENV-1 prM and Envelope proteins generates homotypic neutralizing immune responses and protects against a lethal challenge. The DENV-1 vaccine avoided ADE by 3-point mutations, G106R, L107D, and F108A, to the fusion loop (FL), one of the dominant ADE epitopes. Using a similar design methodology, here we present mRNA-LNP chimeric prM-E vaccines for DENV 2, 3, and 4. These vaccines maintained the ∆FL modifications, but encode a chimeric E protein with Japanese Encephalitis Virus' (JEV) stem/transmembrane (st/tm) to improve VLP expression and stability. All three vaccines elicited neutralizing titers of serotype specific antibodies in immunocompetent C57/BL6 mice and are protected against a lethal homotypic DENV challenge in susceptible mice. Mutation of the FL epitope lowered ADE in the sera from vaccinated mice. We formulated monoclonal vaccines against all DENV serotypes into a single tetravalent vaccine and evaluated for immunogenicity and protection in mice. The tetravalent vaccine elicited a mixed response with antiviral CD4 and CD8 T cells against all four DENV serotypes and neutralizing humoral responses against DENV 3 and DENV 4. Tetravalent vaccine recipient mice were protected against a lethal DENV 3 or DENV 4 challenge. Cumulatively, we have produced monovalent DENV mRNA-LNP vaccines against all four DENV serotypes that generate protective immunity and lower the potential for ADE.

46 **INTRODUCTION**

47 Dengue virus (DENV) is the most common vector-borne virus to infect humans, infecting an
48 estimated 100-400 million people annually.[1] DENV's ~11kb positive sense ssRNA genome
49 encodes a single polypeptide that contains three structural proteins and seven nonstructural
50 proteins.[1–3] The structural proteins premembrane (prM) and envelope (E) comprise the virion and
51 are critical to the antigenic landscape, while the capsid (C) binds and stabilizes the viral genome.[4]
52 The primary DENV vector are *Aedes* genus mosquitos, predominately *Aedes aegypti*, and to a
53 lesser degree *Aedes albopictus.*[1] Approximately 3.9 billion people throughout Central/South
54 America, Asia, and various Pacific islands currently live in the range of DENV's vectors, posing
55 an enormous public health threat.[1,5] DENV is a member of the family *Flaviviridae*, where it is most
56 closely related to Zika virus (ZIKV) and more distantly related to West Nile virus (WNV), Yellow
57 Fever virus (YFV), and Japanese Encephalitis virus (JEV).[6] DENV consists of 4 distinct
58 serotypes, DENV 1, 2, 3, and 4, which can cause similar disease outcomes.[7] While these
59 serotypes share 60-70% sequence homology, they are mostly antigenically unique, with some
60 cross-reactive epitopes.[1]
61
62 The majority of DENV infections are asymptomatic, but the most common symptomatic disease
63 outcome is Dengue Fever (DF).[8] DF consists of severe widespread pain as well as high fevers
64 (>103°F/40°C), rashes, and nausea.[1,2] DF symptoms typically resolve 1-2 weeks after onset. In
65 rare cases, DF can progress into more severe Dengue Hemorrhagic Fever (DHF) and Dengue
66 Shock Syndrome (DSS)[2], with the additional symptoms of vascular leakage, resulting in bleeding
67 in mucosal membranes, urine, and stool.[1,8,9] Without medical intervention, untreated DHF/DSS
68 can be fatal via hypovolemic shock and organ failure. Currently there are no approved
69 therapeutics and no widely approved vaccines for DENV, with the current standard of care of
70 DHF/DSS patients to be hospitalization with IV fluids and pain management.[2,8]
71
72 Typically, primary infection of any DENV serotype results in long term protection against the
73 infecting serotype, but not the other three serotypes.[10–12] Despite this long-lived protection,
74 neutralizing antibodies comprise a minority of the total polyclonal response. The majority of the
75 antibodies in the polyclonal response target cross-reactive and poorly/non-neutralizing epitopes
76 on immature prM or the fusion loop (FL) within domain II of the E protein.[11,12] These antibodies
77 can drive antibody dependent enhancement (ADE) in heterotypic secondary infections, in which
78 case the infecting virus is bound by non-neutralizing antibodies from the primary infection, leading
79 to FcγR-mediated phagocytosis. Once the opsonized-DENV enters endosome of the FcγR+ cell,

DENV proteins undergo conformational changes that reduce efficacy of bound antibodies and induce viral fusion in the acidified endosome, subsequently infecting the cell.[13–16] Because ADE is significantly faster than normal receptor mediated endocytosis, ADE mediated infection dramatically increases the infection rate and is known to be associated with more severe disease outcomes such as DHF, DSS, and death.[1]

Because of the potential of ADE, the epitopes within a DENV vaccine must be selected carefully. Antibodies specific to tertiary and quaternary epitopes, which are only found on intact viral particles, are necessary for neutralizing antibody responses.[12,17] To prevent ADE, a Dengue vaccine must elicit a balanced, neutralizing response to all serotypes targeting neutralizing epitopes and not ADE-enhancing epitopes. The first widely approved DENV vaccine, Dengvaxia, was a tetravalent live attenuated vaccine (LAV) based off the YFV-17D vaccine.[18] While Dengvaxia was protective in seropositive individuals, it failed to drive a long lasting and protective immune response to all DENV serotypes in DENV-naïve individuals.[19,20] Because of this, seronegative children who received Dengvaxia were at a higher risk of severe disease or death during a primary infection due to ADE.[18,21] Because of this increased risk of severe disease, all future DENV vaccine candidates must be designed to mitigate ADE, while maintaining relevant neutralizing epitopes.

The RNA vaccine platform has proven to be safe and efficacious with billions of doses delivered worldwide during the COVID-19 pandemic. For flaviviruses, the RNA platform represents a major advantage over the leading LAV platforms. All leading DENV LAV's include an unmodified FL to support viral replication. With the mRNA-LNP platform we can engineer mutations into the coding RNA at the FL in order to direct the host immune response away from ADE-driving epitopes. Unlike other viruses, such as SARS-CoV-2 and the spike protein, where expression of a single structural protein is adequate to elicit a neutralizing humoral response, recombinant DENV E (rE) likely lacks the most important neutralizing epitopes. rE subunit vaccines drive inadequate immunogenicity in preclinical settings likely due to structural issues and a lack of quaternary epitopes.[22–24] Upon translation of the prM/ENV polyprotein from ribosomes in the host ER, host proteases cleave the prM-E and these viral proteins are folded and anchored in the ER membrane. Appropriate folding and trafficking of the viral proteins is aided by a signal peptide at the N-terminus of the polyprotein. Expression of prM and ENV results in the de novo formation of a non-replicative viral like particle (VLP). VLPs preserve the majority of highly neutralizing

113 quaternary epitopes, which direct the host immune response towards neutralizing B and T cell
114 epitopes.[25–27].
115
116 We first developed a novel mRNA-LNP DENV 1 vaccine that achieves high levels of *in vitro* VLP
117 expression. *In vivo*, the vaccine elicited high levels of protective and non-cross-reactive antibodies
118 in an immunocompetent C57BL/6 mouse model, and was 100% protective in
119 immunocompromised AG129 mice.[28] Here, we have developed three novel monovalent prM-E
120 mRNA-LNP vaccines for DENV 2, 3, and 4 based on our original DENV 1 vaccine. Each
121 monovalent vaccine encodes prM and modified E of a given serotype. These monovalent
122 vaccines showed strong neutralizing antibody responses in WT C57/BL6 mice, while eliminating
123 or reducing ADE. All monovalent vaccines protected mice from a lethal homotypic challenge. We
124 then combined the DENV 1, 2, 3, and 4 monovalent mRNA-LNPs into a tetravalent vaccine.
125 Tetravalent vaccines drove a mixed response against the four DENV serotypes with protective
126 immunity against DENV 3 and DENV 4. These data indicate a significant step towards an effective
127 and safe tetravalent DENV vaccine.

129 **RESULTS**

130 **Design of DENV 1, 2, 3, and 4 prM/E constructs and *in vitro* expression**

131 Similar to our previously published DENV 1 approach, we designed nucleotide sequences for
132 dengue serotypes 2, 3, and 4 containing prM and E chimeric proteins from DENV 2 (strain
133 Thailand 16684/84), DENV 3 (strain Sri Lanka D3/H/IMISSA/SRI/2000/1269), and DENV 4 (strain
134 Dominica/ 814669/1981).[29] Similar to previous observations, we found that robust expression and
135 VLP secretion of the prM/ENV antigens of DENV2, 3, and 4 required optimization of the
136 stem/transmembrane domain of ENV.[25,30,31] The native stem and transmembrane domains of
137 each E protein was replaced with the sequence from JEV.[32] Lastly, variants of the DENV 2, 3,
138 and 4 sequences were modified to include 3 mutations (G106R, L107D, and F108A) in the fusion
139 loop to ablate the epitope, which has been shown to reduce the generation of poorly neutralizing
140 but cross-reactive antibodies associated with ADE.[29,31] Directly upstream of the prM and E
141 sequence, we included the sequence for the tissue plasminogen activator (tPa) signal peptide
142 which we have shown previously to increase VLP secretion with our DENV 1 vaccine.[29] All
143 sequences contain a 5' UTR and 3' UTRs that have been used in various mRNA vaccines.[26,29,33–35]
144 The 3'UTR is from human hemoglobin subunit alpha 1 mRNA (HBA1).[29,36]

145 All nucleotide sequences were *in vitro* transcribed via a T7 RNA polymerase promoter site directly
146 upstream from the 5' UTR. To camouflage our vaccine RNA from host innate RNA sensors, we
147 include the modified ribonucleotide N1M-Ψ Uridine in lieu of standard Uridine.[37–39] *In vitro*
148 transcription produces double stranded RNA artifacts that can activate intracellular innate immune
149 sensors that are known to reduce translation of exogenous mRNA. Double-stranded RNA
150 contamination was removed from IVT-synthesized RNA thought a cellulose purification step.[40]
151 Finally, a 5' cap-1 structure and 3' poly(A) tails were added enzymatically to yield RNA that mimics
152 host mRNA (Fig 1A). These mRNAs were encapsulated into lipid nanoparticles using either
153 Genvoy (DENV 2) or SM102 (DENV 3/4) ionizable lipids. To confirm VLP expression *in vitro*,
154 HEK293T cells were transfected with vaccine RNA and supernatants were harvested 48 hours
155 post transfection. Purified VLP's were then imaged with a transmission electron microscope, and
156 particles of approximately 30nm were detected with all constructs (Fig. 1B). Thus, deletion of the
157 fusion loop did not inhibit VLP formation and secretion, similar to our previous results with DENV
158 1 ΔFL prM/E mRNA vaccines. To evaluate the *in vivo* humoral response, 20µg of the mRNA-LNP
159 vaccines encoding for either the WT or ΔFL constructs were evaluated in immunocompetent
160 C57BL/6 mice in a two-dose prime-boost manner (Fig 1C). Mice received the boost dose 28 days
161 post prime, and animals were sacrificed 28 days post boost.

162 **DENV 2 prM/E Vaccine adaptive immune response**

163 C57BL/6 mice were vaccinated as described above. We included a group of mice that received a
164 mRNA-LNP encoding GFP as a negative control. We included a positive control cohort of mice
165 that were infected with 1E5 FFUs of DENV2 (strain New Guinea C, NGC) to compare the humoral
166 response of the vaccine to that of a natural infection. Total DENV specific IgG from heat
167 inactivated vaccinated mouse sera was quantified via endpoint dilution ELISA against purified
168 DENV 2. All ΔFL vaccinated mice and four out of five WT vaccinated mice seroconverted and had
169 significantly higher DENV 2 specific IgG levels than GFP vaccinated mice (Fig. 2A). DENV 2 WT
170 vaccinated animals had an average endpoint dilution of ~1/33,000 and DENV 2 ΔFL vaccinated
171 animals had an average endpoint dilution of ~1/55,000 compared to the GFP control of ~1/3,400
172 (*P* values = .0065 and .0013 respectively). DENV specific IgG titers from both vaccine variants
173 were not significantly different compared to DENV 2 infected animals (*P* values = .5887 and .9680
174 respectively). These data indicate the mRNA vaccines elicit a humoral response of similar
175 magnitude to a natural infection.

176 The neutralizing antibody titer was determined by Focus Reduction Neutralization Test (FRNT)
177 against DENV 2 NGC (Fig 2B). The serum concentration at which 50% viral neutralization occurs
178 was reported as EC50 for individual mice (Fig 2C). Despite significant DENV 2 specific IgG the
179 WT vaccine failed to drive a significant neutralizing response, with a mean EC50 of ~1/100 and a
180 maximum EC50 of ~1/626. This, compared to the mean GFP EC50 of ~1/60 was insignificant (*P*
181 value = .82). However, the ΔFL vaccine had a mean EC50 of ~1/780 with a maximum EC50 of
182 ~1/7,700 compared to GFP vaccinated control (*P* value = .0068). Neutralization titers were
183 equivalent between the ΔFL vaccinated mice and the naturally infected mice, again indicating a
184 humoral response of similar magnitude.

185 In a natural DENV infection, cross-reactive heterotypic antibodies comprise the majority of the
186 polyclonal response, which can cause ADE.[12] We characterized the ADE capacity of both variants
187 of our DENV 2 vaccines. We quantified ADE by performing serial dilutions of our DENV 2 vaccine
188 and opsonizing those dilutions with a constant MOI of DENV-1 virus. We then infected FcγR+
189 K562 cells with the sera opsonized DENV-1. The frequency of infected cells was determined via
190 flow cytometry using intracellular staining with 4G2, a pan-DENV monoclonal antibody specific to
191 the FL. Sera from WT DENV 2 vaccinated mice elicited a ~1.7 fold higher infection rate compared
192 to virus only controls, indicating a modest enhancement. Peak infection with the ΔFL DENV 2
193 vaccine was ~4.2 fold lower than virus only infection, indicating a lack of enhancement (Fig. 2D).
194 ADE of the individual mice are plotted as area under the curve. The sera from WT vaccinated

195 caused significantly more ADE compared to sera from the ΔFL vaccinated mice (Fig 2E, p-value
196 = 0.002)

197 To understand the protective capacity of the DENV 2 vaccines, a separate study was conducted
198 with susceptible mice which lack the which lack the type I IFN receptor (IFNAR$^{-/-}$). In the same
199 vaccine administration schedule, IFNAR$^{-/-}$ mice were given 10 µg's of the DENV2 ΔFL or the mock
200 GFP vaccine. Vaccinated IFNAR$^{-/-}$ mice were challenged with 1x10^4 FFU of DENV 2 strain
201 D2S20. All DENV 2 vaccinated mice survived the challenge, while all GFP vaccinated animals
202 died by day 7 (*P* value = .0003) (Fig. 2F). Minor weight loss was observed with DENV-2 vaccinated
203 animals, with a ~5% mean weight loss occurring at day 5 (Fig 2G). By day 7, the mean weights
204 returned to day 0 levels, and by day 9 all animals had fully returned to day 0 weight. These data
205 demonstrate that the DV2 prM/ENV mRNA-LNP vaccines elicit protective immune responses.

206 **DENV 3 prM/E Vaccine adaptive immune response**

207 *In vivo* immunogenicity assays of the DENV 3 mRNA-LNP vaccine were conducted as previously
208 described (Fig 1C). Both WT and ΔFL variants of the DENV 3 vaccine were used at 20µg doses
209 in a prime-boost schedule. Animals were sacrificed 56 days post prime, and sera was collected
210 for humoral analysis. To quantify the DENV 3-specific IgG response, heat inactivated sera from
211 vaccinated animals was quantified via endpoint dilution ELISA coated with purified DENV 3. All
212 vaccinated animals seroconverted and had statistically significant DENV 3 specific IgG's
213 compared to a naïve control. The mean endpoint dilution of both WT and ΔFL variants were
214 1/213,484 and 1/205,966 respectively which was significantly elevated compared to the endpoint
215 of naïve control sera at 1/1,268 (*P* values = <0.0001) (Fig. 3A). To quantify the neutralizing
216 antibody response, heat-inactivated sera was analyzed via homotypic FRNT. Compared to the
217 naïve cohort (EC50= 1/26), both WT and ΔFL cohorts had significantly elevated homotypic
218 neutralizing antibody titers, with the average EC50 of ~1/337 for the WT cohort and ~1/269 for
219 the ΔFL cohort (*P* value = <.0001 and .0002 respectively) (Fig. 3B/C).

220 DENV 3 vaccinated sera was also analyzed via ADE assays against heterotypic DENV2 (Fig
221 3D/E). Sera from WT vaccinated animals showed a modestly enhanced peak infection of (~1.6%
222 compared to the ~1.45% DENV-2 only infection) while the ΔFL vaccinated sera had a peak
223 infection of .7%. These data indicate that antibodies from the DENV 3 ΔFL vaccine do not drive
224 heterotypic enhancement with DENV 2.

225 To investigate the protective capacity of the DENV-3 vaccines, IFNAR$^{-/-}$ animals were vaccinated
226 in a prime-boost manner with 10µg of the WT or ΔFL variants, or mock vaccinated controls. Mice

227 were challenged with 1x10^7 FFU DENV 3 C0360/94. Both vaccine variants were significantly
228 protective from a lethal DENV-3 challenge compared to a mock vaccinated control. While both
229 variants were not 100% protective, the WT vaccine yielded a 90% survival rate (*P* value = .0029)
230 and the ΔFL vaccine yielding a 77% survival rate (*P* value = .0204) (Fig. 3F). The vaccinated
231 animals also had significantly lower weight loss compared to mock vaccinated animals (Fig 3G).
232 Both DENV-3 vaccines elicit neutralizing, serotype specific, and protective humoral immunity.

233 **DENV 4 prM/E Vaccine adaptive immune response**

234 In vivo evaluation of the DENV-4 vaccines followed an identical vaccination scheme used with
235 the DENV 2 and DENV 3 vaccines. C57BL/6 mice were vaccinated with 20µg of the WT or ΔFL
236 variant of the mRNA-LNP vaccine in a prime-boost manner. 56 days post prime animals were
237 sacrificed, and sera was collected for analysis. To quantify the total DENV 4 specific IgG response
238 to vaccination, heat inactivated sera was analyzed via endpoint dilution ELISA against purified
239 DENV 4. All ΔFL DENV 4 vaccinated animals and 3/5 WT DENV4 vaccinated animals
240 seroconverted (Fig. 4A). Mean endpoint dilutions for the WT and ΔFL variants were significant
241 compared to naïve control at ~1/47,785 and ~1/101,728 respectively (Fig 4B, *P* value = .0042 and
242 0005 respectively).

243 Sera neutralization was also analyzed via FRNT with a homotypic DENV-4 strain. Despite the
244 ΔFL vaccine yielding ~2X higher DENV specific IgG, the ΔFL had a slightly lower average EC50
245 compared to the WT variant (Fig. 4B/C). The WT variant yielded a mean EC50 of ~307, while the
246 ΔFL variant had a mean of ~1/240. Despite the marginally lower mean, the peak EC50 for the
247 ΔFL variant was ~1/780, while the peak EC50 for the WT variant was ~1/477. The EC50 for both
248 variants' EC50's was significantly elevated compared to naïve control, indicating both vaccine
249 variants induced neutralizing humoral responses (*P* value = .001 and .006 respectively).

250 Antibody cross-enhancement was evaluated via ADE assay, using DENV 3 virus. Both WT and
251 ΔFL showed increased enhancement compared to naïve sera. Notably, the WT vaccine elicited
252 higher, but statistically insignificant, mean enhancement compared to the ΔFL variant, indicating
253 the ΔFL mutations had a modest effect at reducing cross reactive antibodies (Fig 4D). When
254 looking at the area under the curve of individual mice (Fig 4E), both vaccine groups had
255 considerable heterogeneity in their cross-reactive enhancement response.

256 To investigate the protective capacity of the DENV 4 vaccines, IFNAR$^{-/-}$ animals were vaccinated
257 in a prime-boost manner with 10µg of the WT or ΔFL variants, or mock vaccinated controls. Mice
258 were challenged with 5x10^6 FFU DENV 4 TVP376. Both vaccine variants completely protected

259 against a DENV-4 challenge. Mock vaccinated control mice had 75% lethality, P value < 0.001
260 (Fig. 4F). The vaccinated animals also had significantly lower weight loss compared to mock
261 vaccinated animals (Fig 4G). Both DENV-3 vaccines elicit neutralizing, serotype specific, and
262 protective humoral immunity.

263 **Tetravalent Vaccination Adaptive Immune Response**

264 As we have developed effective monovalent vaccines for all DENV serotypes, we now
265 characterized the vaccines in a tetravalent setting. Five C57BL/6 mice were injected with an anti-
266 IFNAR1 mAb 24 hours prior to vaccination. Previously we and others have found immune
267 responses to mRNA vaccines are elevated in the absence of IFN signaling.[29,41,42] Using the ΔFL
268 variants of all vaccines, animals were vaccinated intramuscularly with 3μg of DENV 1 and 13μg
269 of DENV 2, 3, and 4. We previously published that 3μg of our DENV 1 vaccine was sufficient to
270 elicit a neutralizing humoral response, and 13μg was chosen for DENV 2-4 to balance dosage
271 with a minimum effective dose. Animals were vaccinated on an identical prime-boost timeline as
272 previously described. Four weeks post boost, animals were sacrificed, and sera was collected for
273 humoral analysis.

274 To quantify the gross anti-DENV IgG, we performed endpoint dilution ELISA's with heat
275 inactivated sera against all DENV serotypes (Fig. 5A-D). The only significant IgG response was
276 against DENV 4 (*P* value = .0165), with a near significant response against DENV 3 (*P* value =
277 .0628). There were no detectable anti-DENV 1 or DENV 2 IgG above background levels. We also
278 quantified the neutralizing titer against all four serotypes. We found significant neutralization
279 against DENV 3 and 4. Against DENV 3, the tetravalent vaccine elicited a mean EC50 of ~1/165,
280 with a peak EC50 of ~1/402 (Fig. 5G) (*P* value = .01, compared to naïve controls). Against DENV
281 4, we found a mean EC50 of ~1/207 and peak EC50 of ~1/302 (Fig. 5H) (*P* value = .026). We
282 found no neutralization against DENV 1 or 2 (Fig. 5E/F), in agreement with the lack of antiviral
283 IgG titers. Interestingly, despite reducing the dose of the DENV 3 and 4 vaccines by 35% (13μg
284 vs 20μg), we saw nearly identical mean EC50 values to the monovalent study (Fig. 5I).

285

286 The previous tetravalent study revealed our vaccines were able to drive neutralizing immune
287 responses to DENV serotypes 3 and 4 at similar magnitudes found in a monovalent setting. To
288 characterize the protective capacity of the tetravalent vaccines, we vaccinated IFNAR$^{-/-}$ mice with
289 all four ΔFL vaccines and then split mice into four groups. Each group was challenged with a lethal
290 dose of an individual serotype. We vaccinated 18 animals with 10μg of the DENV 1 vaccine and

291 20µg of the DENV 2, 3, and 4 vaccines in a 4-week prime boost manner (Fig. 6A). 4 weeks post
292 boost, animals were divided into 3 groups of 6 and challenged with lethal doses of DENV 1, 2, or
293 4 serotype.

294 We found the tetravalent vaccine to be 100% protective against lethal DENV 1, 2, and 4
295 challenges (Fig. 6B-G). All mock vaccinated animals died, with 100% mortality occurring at day
296 18 with DENV 1, day 5 with DENV 2, and day 17 with DENV 4 (Fig. 6 B, D, E). Modest weight
297 loss was observed in the vaccinated, DENV 4 challenged group, with a peak mean weight losses
298 of ~9% occurring 4 days post infection, with nearly full recovery by day 9 (Fig. 6G). Mean weight
299 loss was <2% in vaccinated, DENV 1 and DENV 2 challenged groups (Fig. 6C, E).

300

302 **DISCUSSION**

303 While there has been significant innovation and development, there is still no widely approved
304 Dengue vaccine that is safe and broadly efficacious against all four Dengue serotypes. DENV
305 vaccine design requires a balance between preserving neutralizing epitopes while minimizing
306 epitopes that drive ADE. The majority of long-lasting humoral immunity is granted by the presence
307 of circulating tertiary and quaternary epitope specific antibodies that are only naturally found on
308 DENV virions.[13,15,43] This epitope preservation is a major strength of DENV LAV's, especially
309 considering the generally poor immunogenicity from DENV subunit vaccines.[22–24] However,
310 because LAV's are replicative they must also maintain the FL epitope and thus must include
311 epitopes that are known to drive ADE. With DENV, the immunodominant humoral response is
312 weighted heavily towards epitopes on the FL and immature prM.[12,14–16] If LAV-derived humoral
313 immunity wains to sub-neutralizing levels, vaccine mediated ADE will be possible. With our
314 vaccine design, we are able to preserve the neutralizing epitopes found on LAV's while also
315 reducing the risk of ADE. Here, along with our previous publication, we have developed nucleotide
316 modified mRNA-LNP vaccine designs for each DENV serotype that drives a protective immune
317 response. In a monovalent setting, these vaccines drive a neutralizing humoral response that is
318 protective against lethal challenges.

319 While the neutralizing humoral response is critical from an efficacy standpoint, it cannot be the
320 only benchmark in which future DENV vaccines are evaluated. The cornerstone of any future
321 DENV vaccine must rest on a deliberate effort to eliminate ADE to make the safest possible
322 vaccine. Emphasizing the safety of any future DENV vaccine is critical in order to regain public
323 confidence given the mistrust generated from Dengvaxia's initial rollout.[18,21,44] Crucially, by
324 ablating the FL epitope, we are able to eliminate or reduce ADE *in vitro*. When administered as a
325 tetravalent vaccine, we achieve significant humoral responses against 2/4 DENV serotypes. With
326 DENV 2 and DENV 3 vaccinated animals, no ADE was identified with ΔFL variants, and a
327 reduction in ADE was observed with DENV 4. When comparing the AUC's of the groups, only the
328 WT vs. Naïve areas were significantly different (Fig. 4D). 3/6 ΔFL vaccinated animals had little to
329 no observable ADE, in comparison to 4/6 WT vaccinated animals having a >5% enhancement
330 compared to naïve. While the FL is a significant driver of ADE, other epitopes are known to drive
331 ADE such as immature prM. Elimination of the immature prM epitopes could be achieved by
332 further modification of the coding sequence, including replacement of the native furin cleavage
333 site with a more efficient sequence to drive VLP maturation and hide the pr epitope.[45]

334

335

336 Any Dengue vaccine must elicit a balanced, neutralizing response against all four serotypes.
337 When our monovalent vaccine was administered into a tetravalent formulation, we achieved
338 neutralizing, protective immune responses against DENV 3 and 4, but failed to drive protective
339 immunity against DENV 1 and DENV 2. Notably the monovalent dose required for effective DENV
340 2, 3, and 4 vaccine-driven immunity was significantly higher than the DV1 vaccine. The prM/ENV
341 Dengue vaccine against DENV 1 yielded robust immunity at low doses of 3µg RNA in a
342 prime/boost immunization strategy. To achieve similar vaccine-driven immunity, we had to
343 increase the dose to 20µg for DENV 2, 3, and 4 monovalent vaccines. While effective, 20ug
344 remains a large dose, thus challenging to incorporate into a tetravalent vaccine formulation that
345 achieves balanced immunity. Because these vaccines were delivered in the same injection, it
346 is likely that DENV 3 and 4 dominated the initial priming of the humoral immune response and/or
347 cellular translation. However, when used in tetravalent setting with 10µg's of DENV 1 ΔFL
348 and 20µg's each of DENV 2, 3, and 4 ΔFL vaccinees in an IFNAR$^{-/-}$ mouse model, we
349 achieved 100% protection against lethal DENV 1, 2, and 4 challenges. These findings
350 demonstrate that further optimization is required to reduce the required dosage of monovalent
351 vaccines prior to tetravalent formulation.
352

353 Ultimately, the "ideal" DENV vaccine strategy has yet to be fully identified by any group. Many
354 studies have shown the need to maintain strongly neutralizing epitopes to drive a durable and
355 protective humoral response. Other studies have shown the risk ADE poses, as well as potential
356 strategies to mitigate it. However, as the interaction between DENV and the adaptive immune
357 system is continued to be mapped, the engineering gap to actually create an effective and safe
358 DENV vaccine is not necessarily clear. Here, we have taken an important design leap towards
359 establishing a pan-DENV vaccine by synthesizing the first mRNA-LNP vaccines that drives
360 potently neutralizing antibody responses against all DENV serotypes. We have successfully
361 generated monovalent mRNA vaccines that induce protective immunity against all four DENV
362 serotypes. Further we have demonstrated that deletion of the fusion loop epitope can dampen
363 antibody dependent enhancement, thereby increasing vaccine safety. These preliminary data
364 establish the feasibility of the mRNA vaccine encoding for prM/ENV as a viable vaccination
365 strategy that supports the further development and optimization of this platform.

366

367

368 **MATERIALS AND METHODS**

369 **Ethics Statement**

370 All mice were stored in accordance with the Animal Welfare Act and all efforts were made to
371 minimize suffering. C57BL/6J mice were purchased from Jackson Laboratory and housed in the
372 pathogen-free Biomedical Resources Laboratory at the University of Illinois College of Medicine.
373 IFNAR$^{-/-}$ mice were bred in the animal facilities at St. Louis University or the University of Kentucky.
374 The vaccination and viral challenge protocols were approved by the Institutional Animal Care and
375 Use Committee (IACUC) at the University of Illinois Chicago, St. Louis University, or the University
376 of Kentucky.

377 **Viruses and Cells**

378 DENV serotype 1 16007 and DENV 2 serotype New Guinea C (NGC) were provided by Michael
379 Diamond at Washinton University in St. Louis, MO. DENV 3 serotype H87 and DENV 4 serotype
380 UIS 497 were obtained through BEI resources (VR-80 and NR-49724 respectively) NIAID, NIH,
381 as part of the WRCEVA program. All DENV serotypes were cultured in C6/36 cells at 28C and
382 titer determined by focus forming assay (FFA). Several detection monoclonal antibodies used in
383 FFA's, including 9.F.10 (Santa Cruz Biotech, cat: SC-70959) and 4G2 (BEI resources, NR-
384 50327). All DENV experiments were conducted under BSL-2 (BSL-2) conditions at either the
385 University of Illinois Chicago (UIC) College of Medicine, St. Louis University, or the University of
386 Kentucky. Vero E6 (CRL 1586) and K562 (CCL-243) cells were obtained from American Type
387 Culture Collection (ATCC) and maintained per provided guidelines. HEK 293T cells were provided
388 by Donna MacDuff at UIC.

389 **mRNA synthesis and LNP encapsulation**

390 Constructs were generated encoding for the tPA signal sequence upstream of the native DV2,
391 DV3, or DV4 prM/ENV coding regions. Strains selected were: DENV2 (strain Thailand 16684/84),
392 DENV 3 (strain Sri Lanka D3/H/IMISSA/SRI/2000/1269), and DENV 4 (strain Dominica/
393 814669/1981). The stem/transmembrane domains of envelope proteins were replaced with the
394 JEV stem/transmembrane domain, and the fusion loop epitope was mutated with the three amino
395 acid mutations (G106R, L107D, and F108A). The sequence of constructs is provided in
396 Supplemental Document. Source DNA plasmids encoding the vaccine mRNA were linearized,
397 and the T7 promoter 5' of the vaccine sequence was used with T7 *in vitro* transcription (IVT) kits

398 from ThermoFisher/Invitrogen (AM1334 or A57622). All vaccine RNA's were synthesized with N1
399 Methylpseudouridine (cat: N-1081) from Trilink Biotech. The 5' cap-1 structures (cat: SCCS2250)
400 and 3' polyA tails (cat: C-PAP5104H) were enzymatically added to all vaccine RNA's from
401 Cellscript. DENV-2 mRNA's used in monovalent humoral analyses were diluted in Formulation
402 Buffer (Precision Nanosystems, cat: 1002775) were encapsulated into LNP's using PNI
403 nanosystems NanoAssemblr using the Genvoy-ILM LNP (Precision Nanosystems, cat: 1002763)
404 formulations. All other vaccines were encapsulated into LNP's using the PNI Ignite using SM102
405 ionizable lipids (Cayman chemical cat: 35425). All mRNA's were encapsulated with a 3:1
406 aqueous:ethanolic ratio at a 12mL/min flow rate. mRNA-LNP's were concentrated using Amicon
407 Ultra Centrifugal Filter 10kDa MWCO (Milipore Sigma, cat: UFC9019). Encapsulation efficiency
408 of these mRNA-LNP's were quantified by RiboGreen assay (Invitrogen cat: R11490).

409 **Mouse experiments**

410 12 week old C57BL/6 mice were purchased from Jackson Laboratory and housed in the
411 pathogen-free Biomedical Resources Laboratory (BRL) at UIC College of Medicine. 8-16-week
412 old IFNAR$^{-/-}$ mice were vaccinated intramuscularly with 50µL of mRNA-LNP diluted in PBS. All
413 mouse sera was heat inactivated at 56C for 30 minutes before any analysis. WT mice often
414 present little to no symptoms from a DENV challenge so it is common to conduct lethal challenge
415 studies with the AG129 mouse.

416 ***In vitro* transfection and electron microscopy**

417 HEK 293T cells were transfected at ~70% confluence with vaccine mRNA using the Mirus TransIT
418 RNA transfection kit (cat: 2225). Supernatant was collected 24 hours post-transfection and
419 dialyzed overnight at 4C in circulating PBS with a 20,000 molecular-weight-cutoff dialysis
420 cartridge (ThermoFisher cat: 6603). Dialyzed sample was provided to UIC electron microscopy
421 core for imaging using the following parameters. 10-15µl of sample was loaded drop-wise onto a
422 300-mesh, Formvar/Carbon-coated copper EM grid with excess removed by filter paper via
423 capillary action. One drop 2% Uranyl acetate solution was deposited onto EM grid with excess
424 removed by filter paper via capillary action. Once grid was allowed to dry further, sample was
425 examined via transmission electron microscopy using JEOL JEM-1400F transmission electron
426 microscope, operating at 80 kV. Digital micrographs were acquired using an AMT BioSprint 12M-
427 B CCD Camera and AMT software (Version 701).

428 **Endpoint Dilution ELISA**

429 Viral stocks of all DENV serotypes were grown at 28C in C6/36 cells for 5-7 days. Supernatant
430 from infected cells was centrifuged at 3,200XG for 10 minutes at 4C to remove cellular debris,
431 then further centrifuged at 141,000XG for 2 hours at 4C with a 20% sucrose cushion. Remaining
432 viral pellets were resuspended in PBS then used to coat ELISA plates. Plates were coated with
433 ~1E3 FFU's of purified virus diluted in coating buffer (0.1 M sodium carbonate, 0.1 sodium
434 bicarbonate, 0.02% sodium azide, at pH 9.6) overnight at 4C. Plates were blocked with 2% BSA,
435 .025% Sodium Azide in PBS/T for 1 hour at 37C. Serial dilutions of vaccinated or control mouse
436 sera were added to coated plates and incubated overnight at 4C. Plates were stained with goat
437 anti-mouse IgG-HRP (Invitrogen cat: A16072) for 1 hour at room temperature. ELISA plates were
438 developed briefly using 3,3′,5,5′-tetramethylbenzidine (TMB) (ThermoFisher cat: 34029) and the
439 reaction was stopped using 2N H2SO4. Absorbance was measured using a BioTek ELISA
440 microplate reader at 450nm. Endpoint dilution was calculated by the concentration of sera with
441 signal 2X background level.

442 **Serum neutralization assay**

443 Focus Reduction Neutralization Tests (FRNT) were performed as described previously.[29,36] Serial
444 dilutions of vaccinated mouse sera was opsonized with ~70 FFU's of a single DENV serotype for
445 1 hour at 37C. Opsonized virus was added to ~70% confluent monolayer of VeroE6 cells in a 96
446 well plate in 2% FBS DMEM. After 1 hour of infection, plates were overlaid with 1% w/v
447 methylcellulose in 2%FBS MEM. Plates were incubated at 37C for 48 (DENV 1, 2, and 3) or 65
448 hours (DENV 4), then fixed with 4% paraformaldehyde (PFA). Plates were stained with a
449 combination of 9.F.10 (500ng/mL), 4G2, and 1-A1D2 in PermWash Buffer (.1% saponin and BSA
450 in PBS). Plates were washed and stained with goat anti-mouse IgG-HRP secondary antibody in
451 PermWash buffer, then resolved with TrueBlue peroxidase (KPL). Resolved plates were imaged
452 on an ImmunoSpot ELISpot plate scanner (Cellular Technology Limited) and foci were counted
453 with Viridot running on RStudio 3.4.1.[46]

454

455 **ADE Assay**

456 Serial dilutions of vaccinated or control mouse sera were opsonized with 1 MOI of a single
457 heterotypic DENV serotype (DENV 1 16007, DENV 2 New Guinea C, or DENV 3 H87) for 1 hour
458 at 37C. Opsonized virus was mixed with FcγR+ K562 cells in a 96 well V-bottomed plate. 16 hours
459 post infection, cells were fixed with 4% PFA and stained with a 4G2 chimeric rabbit mAb

460 (Invitrogen cat: MA5-47848) overnight at 4C. Plates were washed and stained with an anti-rabbit
461 fluorescent secondary antibody (Invitrogen cat: A-11008) and analyzed via flow-cytometry.

462

463 **Data analysis**

464 Figures and statistical analysis were predominately conducted on GraphPad Prism. Statistical
465 significance was was determined by unpaired one-way ANOVA's for multiple comparisons in sera
466 analysis. Students T-Test's were used in ADE assays, and log-rank tests were used for survival
467 curve comparisons. DENV foci were counted with Viridot: An automated virus plaque
468 (immunofocus) counter for the measurement of serological neutralizing responses with
469 application to dengue virus running on RStudio 3.4.1. Flow data was analyzed with BD
470 Biosciences FlowJo. Infographic's in figures were designed using BioRender.

471 **ACKNOWLEDGEMENTS**

472 The following reagent was obtained through BEI Resources, NIAID, NIH: Monoclonal Anti-
473 Flavivirus Group Antigen, Clone D1-4G2-4-15 (produced in vitro), NR-50327.

474

475

476 **FIGURE CAPTIONS**

477 **Figure 1: DENV prM/E vaccine design, viral protein expression, and *in vivo* vaccination**
478 **scheme.** (A) DENV genome and schematic showing previously published and current vaccine
479 design. Four separate mRNA's were engineered to encode prM and E proteins from a single
480 DENV serotype. (B) TEM images of extracellular VLP's. HEK 293T cells were transfected with
481 each vaccine mRNA and supernatant was harvested. Dialyzed supernatants were imaged via
482 TEM. (C) Vaccine schedule used for all *in vivo* analysis. C57BL/6 were vaccinated on a two-dose
483 prime-boost schedule intramuscularly.

484 **Figure 2: DENV 2 prM/E ΔFL vaccine induces neutralizing, non-enhancing antibody**
485 **responses.** (A-F) C57BL/6 mice were vaccinated with the WT or ΔFL DENV 2 vaccine or a GFP
486 control (N=5/group). Another group of mice (N=3) were infected with 1E5 FFU's of DENV 2 NGC.
487 (A) Serial dilutions of sera was analyzed for binding of purified DV2 via ELISA assay. The endpoint
488 dilution was determined for each animal. (B) Focus reduction neutralization tests (FRNT) were
489 conducted with serial dilutions of sera against DENV 2. Each point represents the mean of the
490 experimental group +/- the standard error of the mean (SEM) (C) EC50's from neutralization
491 curves of individual vaccinated mice are reported. (D) Serial dilutions of vaccinated animal sera
492 were analyzed for enhancement of heterologous DENV 1 16007. (E) The area under the
493 enhancement curves for each individual mouse is also reported. F-G) IFNAR$^{-/-}$ were vaccinated
494 with 10µg of the ΔFL DENV 2 vaccine or a GFP encoding mRNA-LNP. Animals were challenged
495 with1x10^4 FFU of DENV 2. Survival curve (F) and weight loss (G) was reported. *, $P < 0.05$; **,
496 $P < 0.01$; ***, $P < 0.001$.

497 **Figure 3: DENV 3 prM/E vaccines induces neutralizing, non-enhancing antibody**
498 **responses.** Immunocompetent C56BL/6 mice (N=6) were vaccinated prime-boost with either
499 20µg of the WT or ΔFL DENV 3 vaccine. (A) Serial dilutions of heat inactivated vaccinated mouse
500 sera was applied to an ELISA plate coated with purified DENV 3 to quantify total antiviral IgG.
501 The endpoint dilution of each mouse is plotted. (B) Focus reduction neutralization tests (FRNT)
502 were conducted with serial dilutions of vaccinated heat inactivated animal sera against DENV 3
503 H87. (C) EC50's from neutralization curves of individual vaccinated mice is reported. (D) Serial
504 dilutions of vaccinated animal sera were analyzed for heterotypic enhancing antibodies with
505 DENV 2. (E) The area under the enhancement curves for each individual mouse is plotted. (F-G)
506 IFNAR1$^{-/-}$ mice (N=9-11) were vaccinated with 10µg of either the WT or ΔFL DENV 3 vaccine, or
507 a PBS control. Animals were challenged with 1x10^7 FFU of DENV 3. Survival curve (F) and
508 weight loss (G) was reported. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

509 **Figure 4: DENV 4 prM/E vaccines induces neutralizing, non-enhancing antibody**
510 **responses.** C57BL/6 mice (N=6/group) were vaccinated with either 20µg of the WT or ∆FL DENV
511 4 vaccine. (A) Serial dilutions were analyzed for total anti-DENV 4 IgG via ELISA assay and
512 endpoint dilution of individual mice determined. (B) FRNT were conducted with serial dilutions of
513 vaccinated sera against DENV 4. (C) EC50's from neutralization curves of individual vaccinated
514 mice is reported. (D) Sera were analyzed for heterotypic enhancing antibodies with DENV 3. (E)
515 Area under the curve of individual mice are reported. (F-G) IFNAR1$^{-/-}$ mice (N=8-10/group) were
516 vaccinated with 10µg of either the WT or ∆FL DENV 4 vaccine, or a PBS control. Animals were
517 challenged with 5x10^6 FFU of DENV 4. Survival curve (F) and weight loss (G) was reported. *,
518 P < 0.05; **, P < 0.01; ***, P < 0.001.

519 **Figure 5: Tetravalent vaccine elicits neutralizing antibody responses against DENV 3 and**
520 **4**. Five C57BL/6 mice were treated with 1mg of an anti-IFNAR1 antibody 24 hours pre-
521 vaccination. Mice were injected intramuscularly with combination of the four monovalent ∆FL
522 vaccines; 3µg of DENV 1 and 13µg of DENV 2, 3, and 4 in a 4-week prime boost manner. 4
523 weeks post boost, animals were sacrificed and sera was collected. (A-D) Heat inactivated sera
524 was analyzed via ELISA plates coated with a sucrose purified DENV serotype and endpoint
525 dilution is reported. (E-H) Sera was serially diluted and analyzed via FRNT with one of four DENV
526 serotypes. EC50's from neutralization curves are reported. (I) Mean EC50 from experimental
527 groups from monovalent (Fig.'s 2-4) and tetravalent vaccinated mice plotted on a radar graph. *,
528 P < 0.05; **, P < 0.01.

529 **Figure 6: Tetravalent vaccine protects against lethal challenge**

530 IFNAR-/- mice were injected intramuscularly with ΔFL mRNA-LNP vaccines of each variant in a
531 prime-boost vaccination schedule separated by 4 weeks between the doses. For each dose mice
532 received 10ug DENV1ΔFL and 20ug each of DENV2 ΔFL, DENV3 ΔFL, DENV4 ΔFL mRNA-LNP
533 vaccines. B-C) Six vaccinated mice and 8 naïve IFNAR$^{-/-}$ mice were challenged with 5x10^6 FFU
534 of DENV 1 West Pac. D-E) Six vaccinated mice and three naïve IFNAR$^{-/-}$ mice were challenged
535 with 1x10^4 FFU of DENV2 D2S20. F-G) Six vaccinated mice and five naïve IFNAR$^{-/-}$ were
536 challenged with 5x10^6 FFU DENV TVP376. All mice were tracked for survival (B, D, F) and
537 weight loss (C, E, G).

538

539

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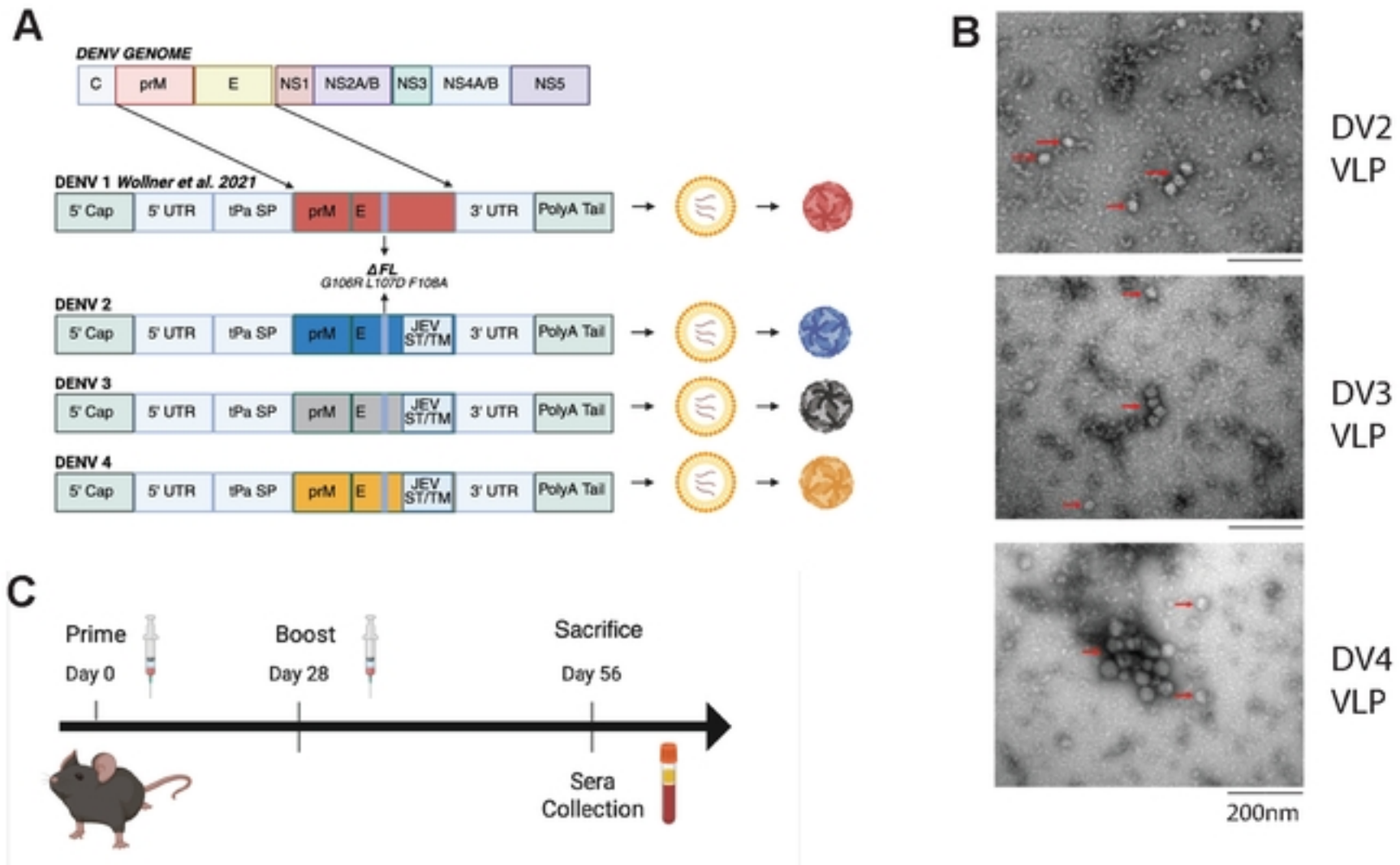
A
DENV GENOME
C
prM
E
NS1
NS2A/B
NS3
NS4A/B
NS5
DENV 1 Wollner et al. 2021
5' Cap
5' UTR
tPa SP
prM
E
3' UTR
PolyA Tail
ΔFL
G106R L107D F108A
DENV 2
JEV ST/TM
DENV 3
DENV 4
B
DV2 VLP
DV3 VLP
DV4 VLP
200nm
C
Prime
Day 0
Boost
Day 28
Sacrifice
Day 56
Sera Collection

Figure 1

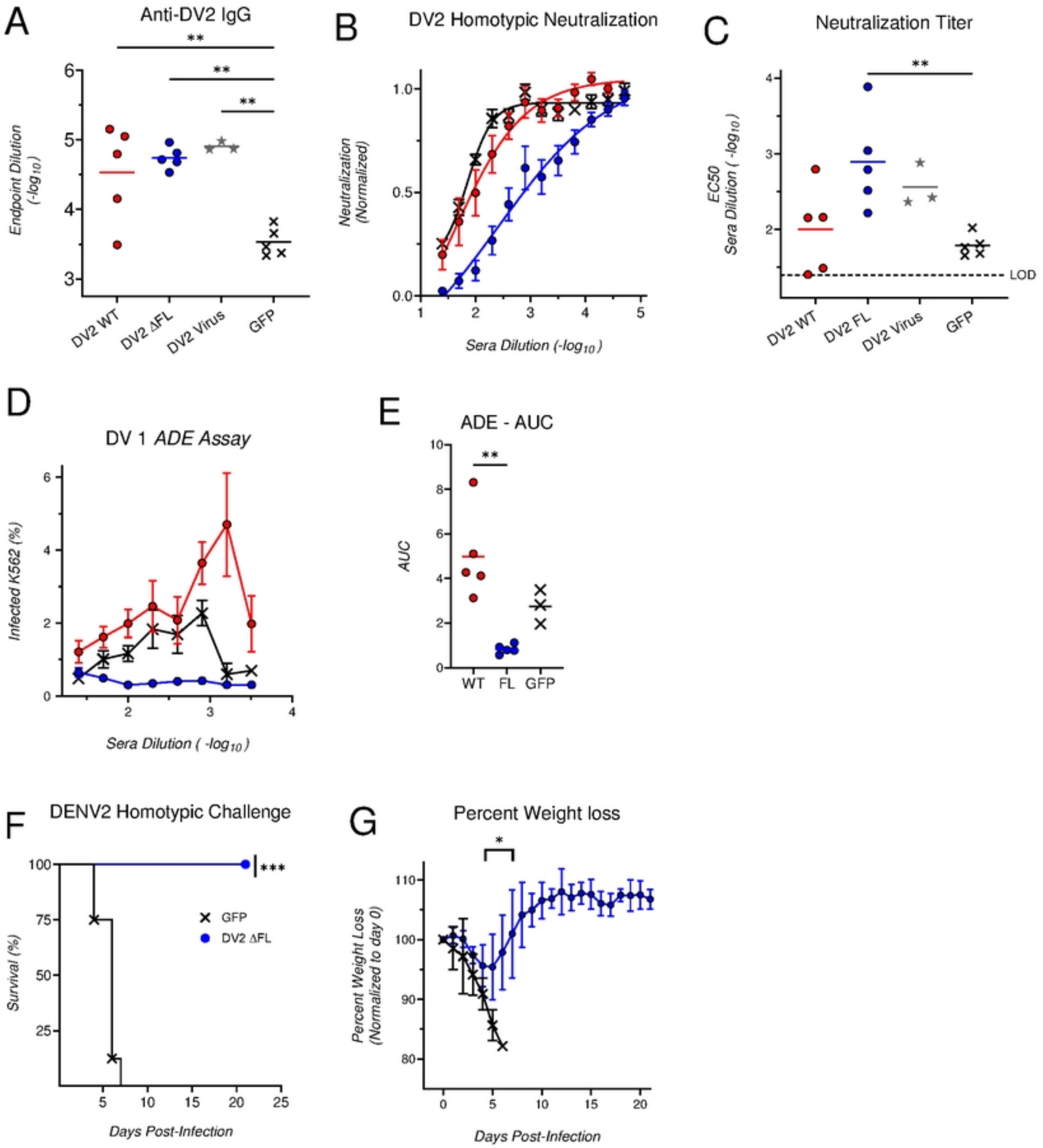
A
Anti-DV2 IgG
Endpoint Dilution (-log10)
DV2 WT
DV2 ΔFL
DV2 Virus
GFP
B
DV2 Homotypic Neutralization
Neutralization (Normalized)
Sera Dilution (-log10)
C
Neutralization Titer
EC50 Sera Dilution (-log10)
LOD
D
DV 1 ADE Assay
Infected K562 (%)
Sera Dilution (-log10)
E
ADE - AUC
AUC
WT
FL
GFP
F
DENV2 Homotypic Challenge
Survival (%)
GFP
DV2 ΔFL
Days Post-Infection
G
Percent Weight loss
Percent Weight Loss (Normalized to day 0)
Days Post-Infection

Figure 2

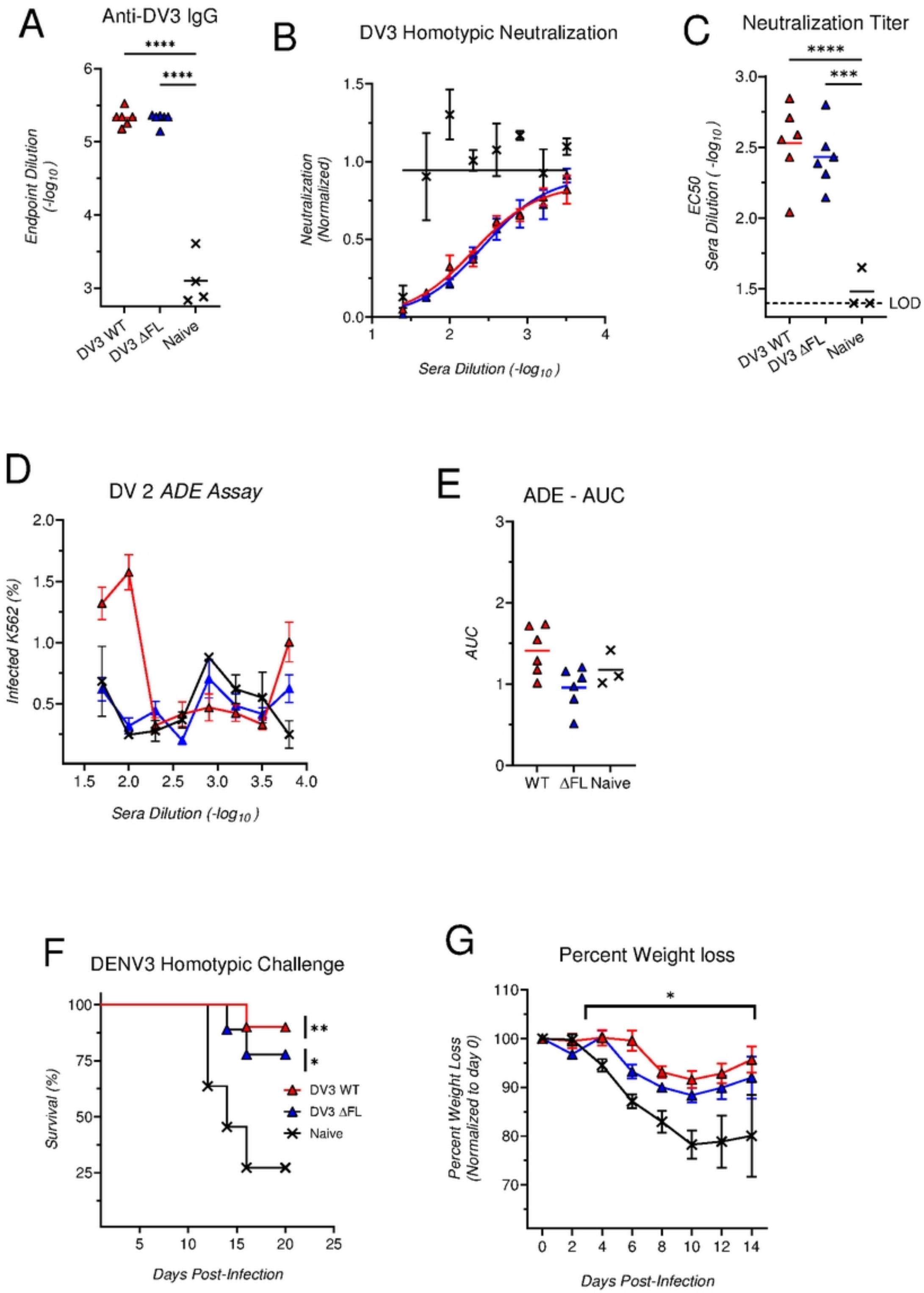
A
Anti-DV3 IgG
****
****
Endpoint Dilution (-log10)
DV3 WT
DV3 ΔFL
Naive
B
DV3 Homotypic Neutralization
Neutralization (Normalized)
Sera Dilution (-log10)
C
Neutralization Titer
****
***
EC50 Sera Dilution (-log10)
LOD
DV3 WT
DV3 ΔFL
Naive
D
DV 2 ADE Assay
Infected K562 (%)
Sera Dilution (-log10)
E
ADE - AUC
AUC
WT
ΔFL
Naive
F
DENV3 Homotypic Challenge
Survival (%)
**
*
DV3 WT
DV3 ΔFL
Naive
Days Post-Infection
G
Percent Weight loss
*
Percent Weight Loss (Normalized to day 0)
Days Post-Infection

Figure 3

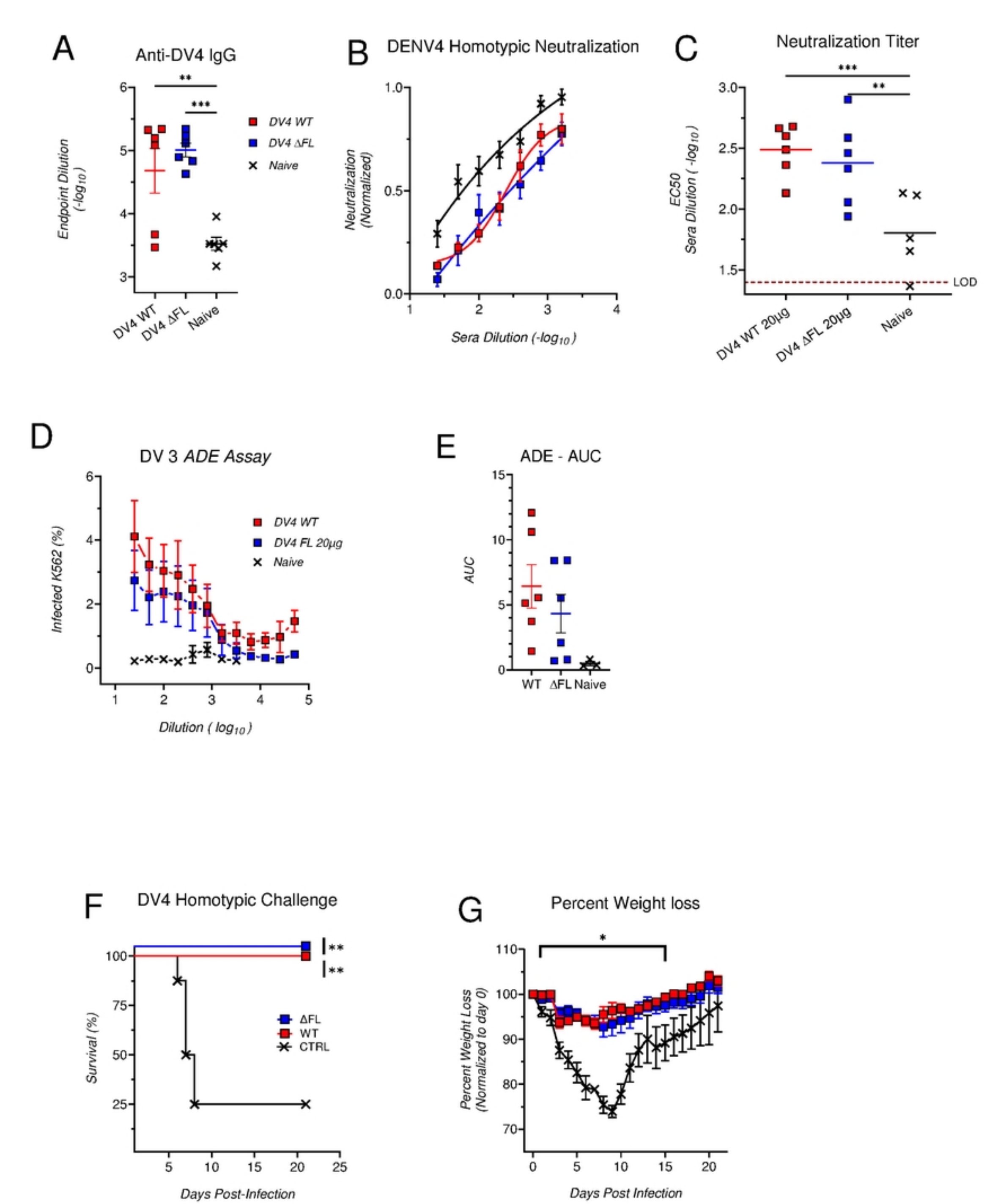
A
Anti-DV4 IgG
Endpoint Dilution (-log10)
DV4 WT
DV4 ΔFL
Naive
B
DENV4 Homotypic Neutralization
Neutralization (Normalized)
Sera Dilution (-log10)
C
Neutralization Titer
EC50 Sera Dilution (-log10)
LOD
DV4 WT 20µg
DV4 ΔFL 20µg
Naive
D
DV 3 ADE Assay
Infected K562 (%)
DV4 WT
DV4 FL 20µg
Naive
Dilution (log10)
E
ADE - AUC
AUC
WT
ΔFL
Naive
F
DV4 Homotypic Challenge
Survival (%)
ΔFL
WT
CTRL
Days Post-Infection
G
Percent Weight loss
Percent Weight Loss (Normalized to day 0)
Days Post Infection

Figure 4

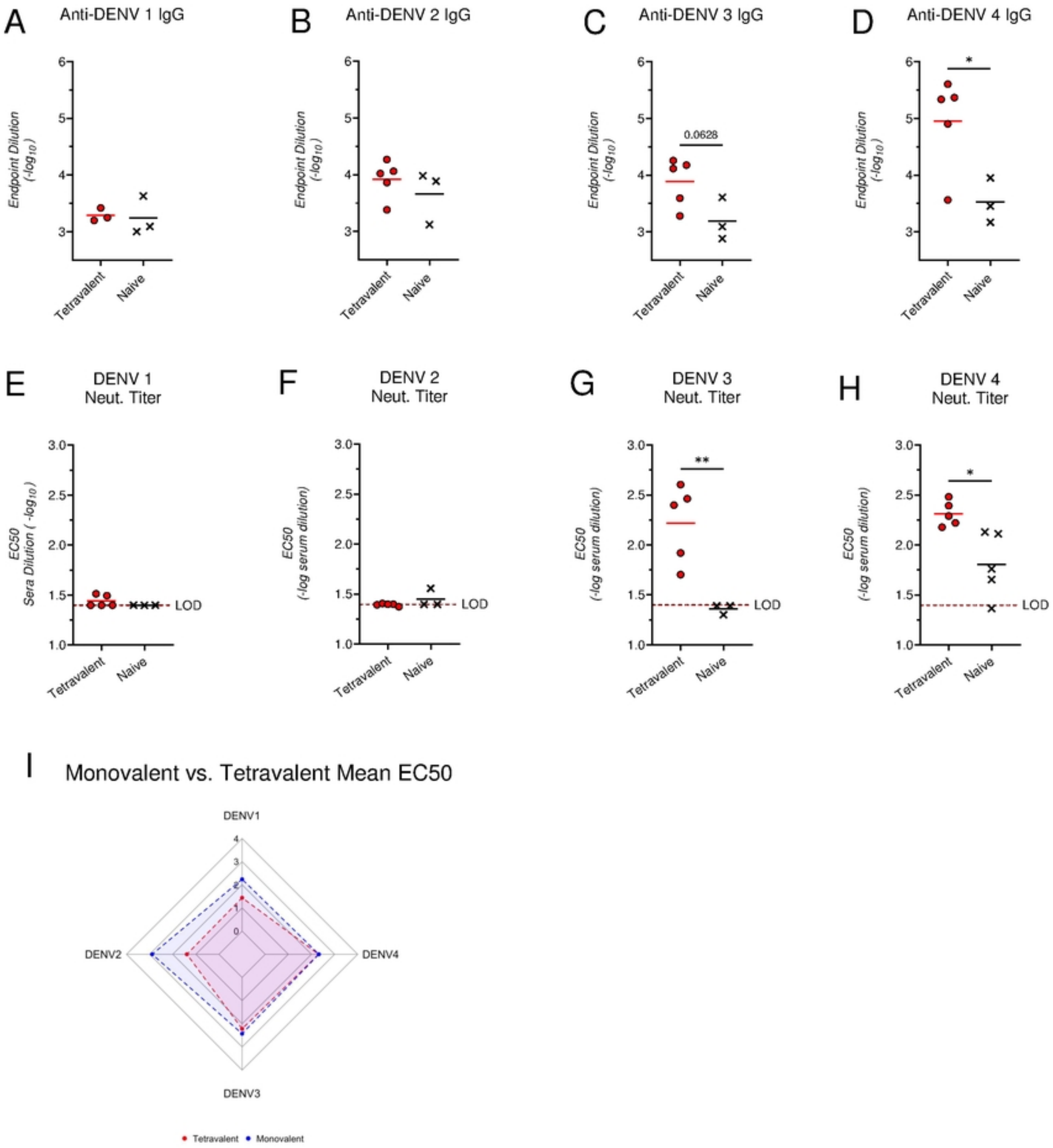
A
Anti-DENV 1 IgG
Endpoint Dilution (-log10)
Tetravalent
Naive
B
Anti-DENV 2 IgG
C
Anti-DENV 3 IgG
0.0628
D
Anti-DENV 4 IgG
*
E
DENV 1 Neut. Titer
EC50 Sera Dilution (-log10)
LOD
F
DENV 2 Neut. Titer
EC50 (-log serum dilution)
G
DENV 3 Neut. Titer
**
H
DENV 4 Neut. Titer
I
Monovalent vs. Tetravalent Mean EC50
DENV1
DENV2
DENV3
DENV4
Tetravalent
Monovalent

Figure 5

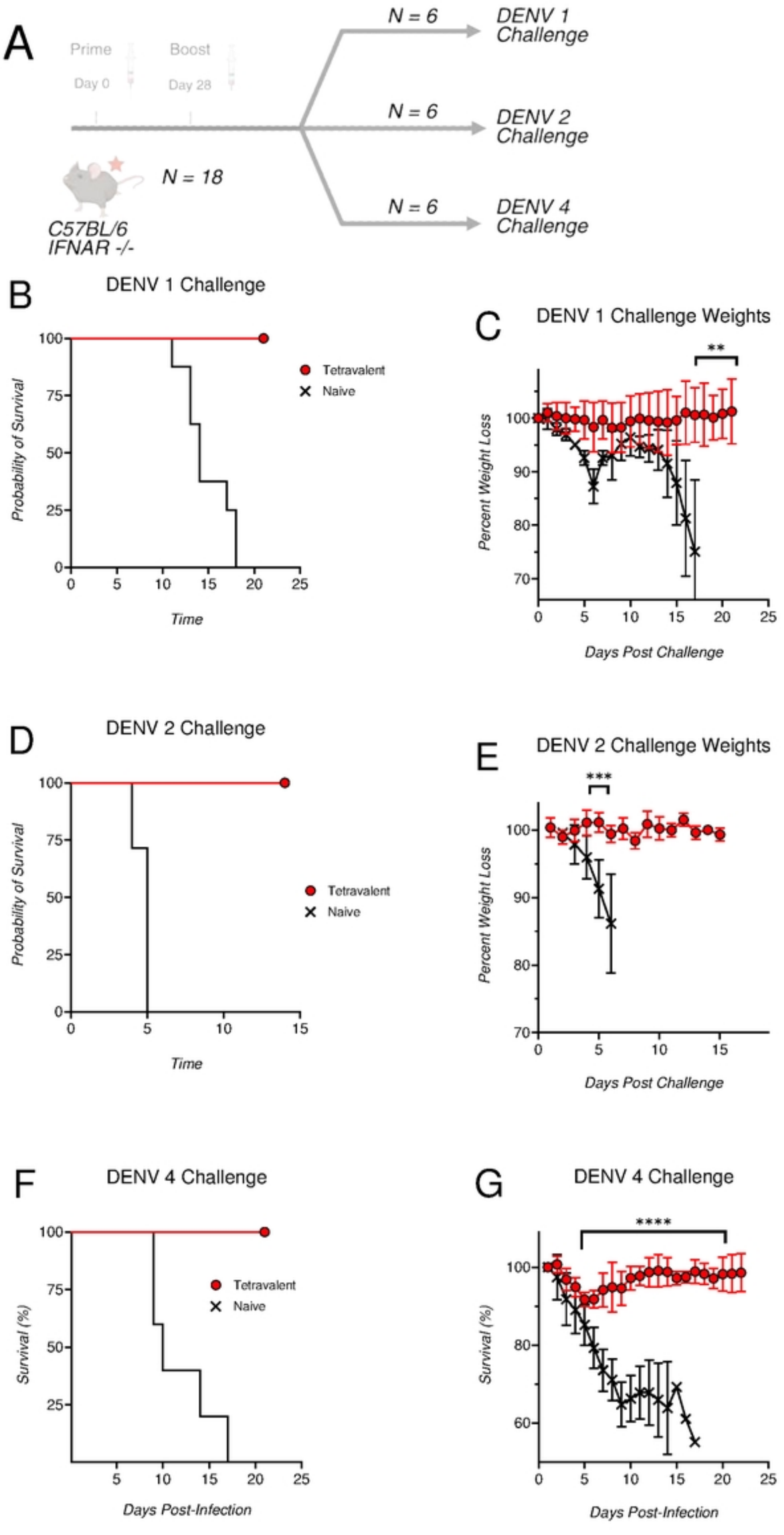
A
Prime
Day 0
Boost
Day 28
N = 18
C57BL/6
IFNAR -/-
N = 6
DENV 1
Challenge
N = 6
DENV 2
Challenge
N = 6
DENV 4
Challenge
B
DENV 1 Challenge
Probability of Survival
Time
Tetravalent
Naive
C
DENV 1 Challenge Weights
**
Percent Weight Loss
Days Post Challenge
D
DENV 2 Challenge
Probability of Survival
Time
Tetravalent
Naive
E
DENV 2 Challenge Weights
***
Percent Weight Loss
Days Post Challenge
F
DENV 4 Challenge
Survival (%)
Days Post-Infection
Tetravalent
Naive
G
DENV 4 Challenge
****
Survival (%)
Days Post-Infection

Figure 6