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

Authors

Enzo LaMontia-Hankin[1], Clayton J Wollner[1], E Taylor Stone[2], Angélica Peña Rosado[2], Jill Pflugheber[2], Ashley Zalla[2], Sebastian Nieto[1], Amelia K Pinto[2], James D Brien[2], Justin M Richner[1]

Affiliations

1, Department of Microbiology and Immunology, University of Illinois College of Medicine, Chicago, Illinois, USA

2, Department of Microbiology, Immunology & Molecular Genetics, University of Kentucky, Lexington, Kentucky, USA.

ABSTRACT

Dengue virus (DENV) is the most common mosquito-borne virus in the world, causing nearly 400 million infections annually, with the number of cases predict to increase over time. Despite this prevalence there are no widely 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 disease. We previously demonstrated that a mRNA-LNP encoding for DENV-1 prM and Envelope proteins generates homotypic neutralizing immune responses and protects against a lethal challenge. The DENV-1 vaccine avoided ADE by eliminating the fusion loop (FL), one of the dominant ADE epitopes. Using a similar design methodology, here we present mRNA-LNP prM-E vaccines for DENV 2, 3, and 4. These vaccines maintained the ΔFL modifications, but encode a chimeric E protein to improve VLP expression and stability. All three vaccines elicited neutralizing titers of serotype specific antibodies mice and protected against a lethal homotypic DENV challenge. Mutation of the FL lowered ADE in vaccinated mouse sera. We formulated monotypic vaccines into a tetravalent vaccine and evaluated for immunogenicity and protection in mice. The tetravalent vaccine elicited neutralizing humoral responses against all DENV serotypes and protected against lethal challenges. Cumulatively, our monovalent DENV mRNA-LNP vaccines against all DENV serotypes generate protective immunity and lower the potential for ADE, leading to the development of a first-generation ADE-altered tetravalent DENV vaccine.

INTRODUCTION

Dengue virus (DENV) is the most common vector-borne virus to infect humans, infecting an estimated 100-400 million people annually.[1] DENV is a member of the family *Flaviviridae*, where it is most closely related to Zika virus (ZIKV) and more distantly related to West Nile virus (WNV), Yellow Fever virus (YFV), and Japanese Encephalitis virus (JEV).[6] DENV consists of 4 distinct serotypes, DENV 1, 2, 3, and 4, which can cause similar disease outcomes.[7] While these serotypes share 60-70% sequence homology, they are mostly antigenically unique, with some cross-reactive epitopes.[1] DENV's ~11kb positive sense ssRNA genome encodes a single polypeptide that contains three structural proteins and seven nonstructural proteins.[1–3] The structural proteins premembrane (prM) and envelope (E) comprise the virion and are critical to the antigenic landscape, while the capsid (C) binds and stabilizes the viral genome.[4] The primary DENV vector are *Aedes* genus mosquitos, predominately *Aedes aegypti*, and to a lesser degree *Aedes albopictus.*[1] Approximately 3.9 billion people throughout Central/South America, Asia, and various Pacific islands currently live in the range of DENV's vectors, posing an enormous public health threat.[1,5] DENV is a member of the family *Flaviviridae*, where it is most closely related to Zika virus (ZIKV) and more distantly related to West Nile virus (WNV), Yellow Fever virus (YFV), and Japanese Encephalitis virus (JEV).[6] DENV consists of 4 distinct serotypes, DENV 1, 2, 3, and 4, which can cause similar disease outcomes.[7] While these serotypes share 60-70% sequence homology, they are mostly antigenically unique, with some cross-reactive epitopes.[1]

The majority of DENV infections are asymptomatic, but the most common symptomatic disease outcome is Dengue Fever (DF).[8] DF consists of severe widespread pain as well as high fevers (>103°F/40°C), rashes, and nausea.[1,2] DF symptoms typically resolve 1-2 weeks after onset. In rare cases, DF can progress into more severe Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS)[2], with the additional symptoms of vascular leakage, resulting in bleeding in mucosal membranes, urine, and stool.[1,8,9] Without medical intervention, untreated DHF/DSS can be fatal via hypovolemic shock and organ failure. Currently there are no approved therapeutics and no widely approved vaccines for DENV, with the current standard of care of DHF/DSS patients to be hospitalization with IV fluids and pain management.[2,8]

Typically, primary infection of any DENV serotype results in long term protection against the infecting serotype, but not the other three serotypes.[10–12] Despite this long-lived protection, neutralizing antibodies comprise a minority of the total polyclonal response. The majority of the antibodies in the polyclonal response target cross-reactive and poorly/non-neutralizing epitopes

77 on immature prM or the fusion loop (FL) within domain II of the E protein.[11,12] These antibodies
78 can drive antibody dependent enhancement (ADE) in heterotypic secondary infections, in which
79 case the infecting virus is bound by non-neutralizing antibodies from the primary infection, leading
80 to FcγR-mediated phagocytosis. Once the opsonized-DENV enters the endosome of the FcγR+
81 cell, DENV E proteins undergo conformational changes that reduce efficacy of bound antibodies
82 and induce viral fusion in the acidified endosome, subsequently infecting the cell.[13–16] Because
83 ADE is significantly associated with more severe DENV disease outcomes, it is critical to evaluate
84 any DENV vaccine formulations' capacity to generate enhancing antibodies.[14]
85
86 Because of the potential of ADE, the antibody epitopes presented by within a DENV vaccine must
87 be selected carefully. Antibodies specific to tertiary and quaternary epitopes, which are only found
88 on intact viral particles, are necessary for neutralizing antibody responses.[12,17] To prevent ADE, a
89 DENV vaccine must elicit a balanced, neutralizing response to all serotypes targeting neutralizing
90 epitopes and not ADE-enhancing epitopes. The first widely approved DENV vaccine, Dengvaxia,
91 was a tetravalent live attenuated vaccine (LAV) based off the YFV-17D vaccine.[18] While
92 Dengvaxia was protective in seropositive individuals, it failed to drive a long lasting and protective
93 immune response to all DENV serotypes in DENV-naïve individuals.[19,20] Because of this,
94 seronegative children who received Dengvaxia were at an increased risk of severe disease or
95 death during a primary infection due to ADE.[18,21] Because of this increased risk of severe disease,
96 all future DENV vaccine candidates must be designed to mitigate ADE, while maintaining relevant
97 neutralizing epitopes.
98
99 The mRNA vaccine platform has proven to be safe and efficacious with billions of doses delivered
100 worldwide during the COVID-19 pandemic. For flaviviruses, the RNA platform represents a major
101 advantage over the leading LAV platforms. All leading DENV LAV's include an unmodified FL to
102 support viral replication. With the mRNA-LNP platform we can engineer mutations into the coding
103 RNA at the FL in order to direct the host immune response away from ADE-driving epitopes.
104 Unlike other viruses, such as SARS-CoV-2 and the spike protein, where expression of a single
105 structural protein is adequate to elicit a neutralizing humoral response, recombinant DENV E (rE)
106 likely lacks the most important neutralizing epitopes. rE subunit vaccines drive inadequate
107 immunogenicity in preclinical settings likely due to structural issues and a lack of quaternary
108 epitopes.[22–24] Upon translation of the prM/ENV polyprotein from ribosomes in the host ER, host
109 proteases cleave the prM-E and these viral proteins are folded and anchored in the ER
110 membrane. Appropriate folding and trafficking of the viral proteins is aided by a signal peptide at

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

128 **RESULTS**

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

130 Similar to our previously published DENV 1 approach, we designed nucleotide sequences for
131 dengue serotypes 2, 3, and 4 containing prM and E chimeric proteins from DENV 2 (strain
132 Thailand 16684/84), DENV 3 (strain Sri Lanka D3/H/IMISSA/SRI/2000/1269), and DENV 4 (strain
133 Dominica/ 814669/1981).[28] Similar to previous observations, we found that robust expression and
134 VLP secretion of the prM/ENV antigens of DENV2, 3, and 4 required optimization of the
135 stem/transmembrane domain of ENV.[25,29,30] The native stem and transmembrane domains of
136 each E protein was replaced with the sequence from JEV.[31] Lastly, variants of the DENV 2, 3, and
137 4 sequences were modified to include 3 mutations (G106R, L107D, and F108A) in the fusion loop
138 to ablate the epitope, which has been shown to reduce the generation of poorly neutralizing but
139 cross-reactive antibodies associated with ADE.[28,30] Directly upstream of the prM and E sequence,
140 we included the sequence for the tissue plasminogen activator (tPa) signal peptide which we have
141 shown previously to increase VLP secretion with our DENV 1 vaccine.[28] All sequences contain a
142 5' UTR and 3' UTRs that have been used in various mRNA vaccines.[26,28,32–34]

143 All nucleotide sequences were *in vitro* transcribed via a T7 RNA polymerase promoter site directly
144 upstream from the 5' UTR. To camouflage our vaccine RNA from host innate RNA sensors, we
145 include the modified ribonucleotide N1M-Ψ Uridine in lieu of standard Uridine.[35–37] *In vitro*
146 transcription produces double stranded RNA artifacts that can activate intracellular innate immune
147 sensors that are known to reduce translation of exogenous mRNA. Double-stranded RNA
148 contamination was removed from IVT-synthesized RNA thought a cellulose purification step.[38]
149 Finally, a 5' cap-1 structure and 3' poly(A) tails were added enzymatically to yield RNA that mimics
150 host mRNA (Fig 1A). These mRNAs were encapsulated into lipid nanoparticles using either
151 Genvoy (DENV 2) or SM-102 (DENV 3/4) ionizable lipids. To confirm VLP expression *in vitro*,
152 HEK293T cells were transfected with vaccine RNA and supernatants were harvested 48 hours
153 post transfection. Purified VLP's were then imaged with a transmission electron microscope, and
154 particles of approximately 30nm were detected with all constructs (Fig. 1B). While DENV virions
155 are ~50nm, it has been reported similar antigenicity between DENV VLP's and virions.[25] Thus,
156 deletion of the fusion loop did not inhibit VLP formation and secretion, similar to our previous
157 results with DENV 1 ΔFL prM/E mRNA vaccines. To evaluate the *in vivo* humoral response, 20μg
158 of the mRNA-LNP vaccines were evaluated identically as described above (Fig 1C). Mice received
159 the boost dose 28 days post prime, and animals were sacrificed 28 days post boost.

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

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

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

183 In a natural DENV infection, cross-reactive heterotypic antibodies comprise the majority of the
184 polyclonal response, which can cause ADE.[12] We characterized the ADE capacity of both variants
185 of our DENV 2 vaccines. We quantified ADE by performing serial dilutions of our DENV 2 vaccine
186 and opsonizing those dilutions with a constant MOI of DENV-1 virus. While sera is collected 28
187 days post boost, which is not enough time for neutralizing antibodies to wain for ADE to occur, we
188 do serial dilutions of the sera to simulate waning humoral immunity. 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. While infection with DENV 2 NGC elicits a humoral response, similar
210 infection with DENV 3 H87 failed to drive a detectable humoral response in WT mice, so no viral
211 infection comparator control was included. Animals were sacrificed 56 days post prime, and sera
212 was collected for humoral analysis. To quantify the DENV 3-specific IgG response, heat
213 inactivated sera from vaccinated animals was quantified via endpoint dilution ELISA coated with
214 purified DENV 3. All vaccinated animals seroconverted and had statistically significant DENV 3
215 specific IgG's compared to a naïve control. The mean endpoint dilution of both WT and ΔFL
216 variants were 1/213,484 and 1/205,966 respectively which was significantly elevated compared
217 to the endpoint of naïve control sera at 1/1,268 (*P* values = <0.0001) (Fig. 3A). To quantify the
218 neutralizing antibody response, heat-inactivated sera was analyzed via homotypic FRNT.
219 Compared to the naïve cohort (EC50= 1/26), both WT and ΔFL cohorts had significantly elevated
220 homotypic neutralizing antibody titers, with the average EC50 of ~1/337 for the WT cohort and
221 ~1/269 for the ΔFL cohort (*P* value = <.0001 and .0002 respectively) (Fig. 3B/C).

222 DENV 3 vaccinated sera was also analyzed via ADE assays against heterotypic DENV 2 (Fig
223 3D/E). Sera from WT vaccinated animals showed a modestly enhanced peak infection of (~1.6%
224 compared to the ~1.45% DENV-2 only infection) while the ΔFL vaccinated sera had a peak

225 infection of .7%. These data indicate that antibodies from the DENV 3 ΔFL vaccine do not drive
226 heterotypic enhancement with DENV 2.

227 To investigate the protective capacity of the DENV-3 vaccines, IFNAR$^{-/-}$ animals were vaccinated
228 in a prime-boost manner with 10µg of the WT or ΔFL variants, or mock vaccinated controls. Mice
229 were challenged with 1x10^{7} FFU DENV 3 C0360/94. Both vaccine variants were significantly
230 protective from a lethal DENV 3 challenge compared to a mock vaccinated control. While both
231 variants were not 100% protective, the WT vaccine yielded a 90% survival rate (*P* value = .0029)
232 and the ΔFL vaccine yielding a 77% survival rate (*P* value = .0204) (Fig. 3F). The vaccinated
233 animals also had significantly lower weight loss compared to mock vaccinated animals (Fig 3G).
234 Both DENV 3 vaccines elicit neutralizing, serotype specific, and protective humoral immunity.

235 **DENV 4 prM/E Vaccine Adaptive immune response**

236 In vivo evaluation of the DENV-4 vaccines followed an identical vaccination scheme used with the
237 DENV 2 and DENV 3 vaccines (Fig 1A). C57BL/6 mice were vaccinated with 20µg of the WT or
238 ΔFL variant of the mRNA-LNP vaccine in a prime-boost manner. Similar to DENV 3 H87, DENV
239 4 UIS 497 did not elicit a humoral response in immunocompetent mice and thus was omitted from
240 this analysis. 56 days post prime animals were sacrificed, and sera was collected for analysis. To
241 quantify the total DENV 4 specific IgG response to vaccination, heat inactivated sera was
242 analyzed via endpoint dilution ELISA against purified DENV 4. All ΔFL DENV 4 vaccinated animals
243 and 3/5 WT DENV4 vaccinated animals seroconverted (Fig. 4A). Mean endpoint dilutions for the
244 WT and ΔFL variants were significant compared to naïve control at ~1/47,785 and ~1/101,728
245 respectively (Fig 4B, *P* value = .0042 and 0005 respectively).

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

253 Antibody cross-enhancement was evaluated via ADE assay, using DENV 3 virus. Both WT and
254 ΔFL showed increased enhancement compared to naïve sera. Notably, the WT vaccine elicited
255 higher, but statistically insignificant, mean enhancement compared to the ΔFL variant, indicating
256 the ΔFL mutations had a modest effect at reducing cross reactive antibodies (Fig 4D). When

257 looking at the area under the curve of individual mice (Fig 4E), both vaccine groups had
258 considerable heterogeneity in their cross-reactive enhancement response.

259 To investigate the protective capacity of the DENV 4 vaccines, IFNAR$^{-/-}$ animals were vaccinated
260 in a prime-boost manner with 10µg of the WT or ΔFL variants, or mock vaccinated controls. Mice
261 were challenged with 5x10^{6} FFU DENV 4 TVP376. Both vaccine variants completely protected
262 against a DENV-4 challenge. Mock vaccinated control mice had 75% lethality, P value < 0.001
263 (Fig. 4F). The vaccinated animals also had significantly lower weight loss compared to mock
264 vaccinated animals (Fig 4G). Both DENV-3 vaccines elicit neutralizing, serotype specific, and
265 protective humoral immunity.

266 **Tetravalent Vaccination Adaptive Immune Response**

267 As we have developed effective monovalent mRNA-LNP vaccines for all DENV serotypes, we
268 now sought to characterize the vaccines in a tetravalent delivery setting. To characterize the
269 protective capacity of the tetravalent vaccine, we vaccinated 18 IFNAR$^{-/-}$ mice with all four ΔFL
270 vaccine variants with 10µg's of the DENV 1 vaccine and 20µg's each of the DENV 2, 3, and 4
271 vaccines. Animals were vaccinated on an identical prime-boost timeline as previously described
272 (Fig. 5A). Four weeks post boost, animals were bled to characterize pre-challenge humoral
273 responses. The 18 mice were then divided into three groups of 6 and subsequently challenged
274 with lethal doses of either DENV 1, 2, or 4. DENV 3 was omitted from challenge studies due to
275 limitations with DENV 3 lethal challenge stocks.

276 To quantify the anti-DENV IgG, we performed endpoint dilution ELISA's with heat inactivated pre-
277 challenge sera against all DENV serotypes (Fig. 5C-F). We found significant IgG responses
278 against all DENV serotypes as well as significant neutralizing humoral responses to all DENV
279 serotypes (Fig. 5 H-K). In comparison to the monovalent humoral responses, EC50's were
280 significantly higher against DENV 1, 3, and 4 in the tetravalent formulation (Fig. 5 L, Table: 1).
281 Despite the increase in neutralizing titer, the type specific IgG was significantly lower against
282 DENV 3 4, with near significance against DENV 1 (P = ~.08) (Fig. 5 G, Table: 1).

283 Vaccinated animals were challenged with either 5x10^{6} DENV 1 West Pac, 1x10^{4} FFU's of DENV
284 2 D2S20, or 5x10^{6} FFU DENV 4 TVP376. We found the tetravalent vaccine to be 100% protective
285 against lethal DENV 1, 2, and 4 challenges (Fig. 6 A-C). All mock vaccinated animals died, with
286 100% mortality occurring at day 18 with DENV 1, day 5 with DENV 2, and day 17 with DENV 4
287 (Fig. 6 A-C). Modest weight loss was observed in the vaccinated, DENV 4 challenged group, with
288 a peak mean weight losses of ~9% occurring 4 days post infection, with nearly full recovery by

289 day 9 (Fig. 6 F). Weight loss was negligible in vaccinated, DENV 1 and DENV 2 challenged groups
290 (Fig. 6 D, E).

291

292

293 **DISCUSSION**

294 While there has been significant innovation and development, there is still no widely approved
295 Dengue vaccine that is safe and broadly efficacious against all four dengue serotypes. DENV
296 vaccine design requires a balance between preserving neutralizing epitopes while minimizing
297 epitopes that drive ADE. The majority of long-lasting humoral immunity is granted by the presence
298 of circulating tertiary and quaternary epitope specific antibodies that are only naturally found on
299 DENV virions.[13,15,39] This epitope preservation is a major strength of DENV LAV's, especially
300 considering the generally poor immunogenicity from DENV subunit vaccines.[22–24] However,
301 because LAV's are replicative they must also maintain the FL epitope and thus must include
302 epitopes that are known to drive ADE. With DENV, the immunodominant humoral response is
303 weighted heavily towards epitopes on the FL and immature prM.[12,14–16] If LAV-derived humoral
304 immunity wains to sub-neutralizing levels, vaccine mediated ADE will be possible. With our
305 vaccine design, we are able to preserve the neutralizing epitopes found on LAV's while also
306 reducing the risk of ADE. Here, along with our previous publication, we have developed nucleotide
307 modified mRNA-LNP vaccine designs for each DENV serotype that drives a protective immune
308 response. In both monovalent and tetravalent settings, our prM-E vaccines drive significant
309 humoral responses to all serotypes, with the ability to protect against lethal challenges.

310

311 While the neutralizing humoral response is critical from an efficacy standpoint, it cannot be the
312 only benchmark in which future DENV vaccines are evaluated. The cornerstone of any future
313 DENV vaccine must rest on a deliberate effort to eliminate ADE to make the safest possible
314 vaccine. Emphasizing the safety of any future DENV vaccine is critical in order to regain public
315 confidence given the mistrust generated from Dengvaxia's initial rollout.[18,21,40] Crucially, by
316 ablating the FL epitope, we are able to eliminate or reduce ADE *in vitro*. With DENV 2 and DENV
317 3 vaccinated animals, no ADE was identified with ΔFL variants, and a reduction in ADE was
318 observed with DENV 4. When comparing the AUC's of these groups, only the WT vs. Naïve areas
319 were significantly different (Fig. 4D). 3/6 ΔFL vaccinated animals had little to no observable ADE,
320 in comparison to 4/6 WT vaccinated animals having a >5% enhancement compared to naïve.
321 While the FL is a significant driver of ADE, other epitopes are known to drive ADE such as
322 immature prM. Elimination of the immature prM epitopes could be achieved by further modification
323 of the coding sequence, including replacement of the native furin cleavage site with a more
324 efficient sequence to drive VLP maturation and hide the immature prM epitope.[41]

325

326 Any dengue vaccine must elicit a balanced, neutralizing response against all four serotypes.
327 Notably the monovalent dose required for effective DENV 2, 3, and 4 vaccine-driven immunity
328 was significantly higher than the DV1 vaccine. The prM/ENV Dengue vaccine against DENV 1
329 yielded robust immunity at low doses of 3μg RNA in a prime/boost immunization strategy in a
330 previous publication from our group.[28] To achieve similar vaccine-driven immunity, we had to
331 increase the dose to 20μg for DENV 2, 3, and 4 monovalent vaccines. Despite the high dosage,
332 when administered as a tetravalent vaccine, we achieve significant humoral responses against
333 all DENV serotypes. In previous studies, we found that 10μg of the ΔFL DENV 1 vaccine elicited
334 ~1 log higher neutralizing antibody titers in IFNAR/IFNGR double knock-out mice (AG129)
335 compared to immunocompetent C57BL/6 mice.[28] Here, we have also found a similar phenotype
336 in our tetravalent vaccine between WT and IFNAR KO C57BL/6 mice, with statistically significant
337 increases in EC50 against DENV 1, 3, and 4, compared to the monovalent setting (Table 1).

338

339 Interestingly, we observed lower serotype specific IgG titers against DENV 1, 3, and 4 (Table 1),
340 while an increase in the neutralization titers, when comparing tetravalent vaccination in the
341 immunodeficient IFNAR$^{-/-}$ mice compared to the monovalent vaccines in the immunocompetent
342 C57BL/6. These data are surprising given the increased antigenic load and the prevalence for
343 cross-reactive poorly neutralizing antibodies in the tetravalent setting as observed in historical
344 unbalanced immune responses from LAV tetravalent vaccines.[20,42] With our mRNA-LNP
345 approach, we achieve balanced immunity against all four serotypes counter to previous LAV
346 tetravalent vaccines which drive a dominant response to a single serotype. It is possible that the
347 VLP's encoded by the mRNA-LNP vaccine are driving a higher quality humoral response by
348 increasing the population of the rare potently neutralizing antibodies that often target tertiary and
349 quaternary epitopes. While our tetravalent vaccine encoded 20μg each of DENV 2, 3, and 4
350 vaccines, we conducted the monovalent survival studies with 10μg of each vaccine, all of which
351 were significantly protective compared to mock groups. While we were unable to conduct a
352 survival study with tetravalent vaccinated animals against DENV 3, we showed significant DENV
353 3 specific IgG and neutralization form in these animals, with significantly higher EC50's compared
354 to monovalent vaccinated animals. We also showed significant protection against a lethal DENV
355 3 challenge with a 10μg monovalent vaccine, which is half the dose used in the tetravalent
356 formulation.
357

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

372

373

374

375 **MATERIALS AND METHODS**

376 **Viruses and Cells**

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

388 **mRNA synthesis and LNP encapsulation**

389 Constructs were generated encoding for the tPA signal sequence upstream of the native DENV2,
390 DENV3, or DENV4 prM/ENV coding regions. Strains selected were: DENV2 (strain Thailand
391 16684/84), DENV 3 (strain Sri Lanka D3/H/IMISSA/SRI/2000/1269), and DENV 4 (strain
392 Dominica/ 814669/1981). The stem/transmembrane domains of envelope proteins were replaced
393 with the JEV stem/transmembrane domain, and the fusion loop epitope was mutated with the
394 three amino acid mutations (G106R, L107D, and F108A). The sequence of constructs is provided
395 in Supplemental Material. Source DNA plasmids encoding the vaccine mRNA were linearized,
396 and the T7 promoter 5' of the vaccine sequence was used with T7 *in vitro* transcription (IVT) kits
397 from ThermoFisher/Invitrogen (AM1334 or A57622). All vaccine RNA's were synthesized with N1
398 Methylpseudouridine (TriLink Biotech, cat: N-1081). The 5' cap-1 structures (CellSCript, cat:
399 SCCS2250) and 3' polyA tails (CellScript, cat: C-PAP5104H) were enzymatically added to all
400 vaccine RNA's. DENV 2 mRNA's used only in the immunocompetent monovalent humoral
401 analyses were diluted in Formulation Buffer (Precision Nanosystems, cat: 1002775) were
402 encapsulated into LNP's using PNI Nanosystems NanoAssemblr using the Genvoy-ILM LNP
403 (Precision Nanosystems, cat: 1002763). All other vaccines were encapsulated into LNP's using
404 the PNI Ignite with SM102 ionizable lipids (Cayman chemical cat: 35425). All mRNA's were
405 encapsulated with a 3:1 aqueous:ethanolic ratio at a 12mL/min flow rate. mRNA-LNP's were
406 concentrated using Amicon Ultra Centrifugal Filter 10kDa MWCO (Milipore Sigma, cat: UFC9019).

407 Encapsulation efficiency of these mRNA-LNP's were quantified by RiboGreen assay (Invitrogen
408 cat: R11490).

409 **Mouse experiments**

410 mRNA-LNP vaccines were diluted with PBS to contain their given mRNA dosage (10µg, 20µg,
411 etc) per 50µL and administered intramuscularly in all *in vivo* experiments. 12 week old C57BL/6
412 mice were purchased from Jackson Laboratory and housed in the pathogen-free Biomedical
413 Resources Laboratory (BRL) at UIC College of Medicine. 8-16-week old C57BL/6 or IFNAR$^{-/-}$ mice
414 were vaccinated intramuscularly in a four-week prime boost manner.

415

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. All mouse sera was heat inactivated at 56C for
436 30 minutes before any analysis. Serial dilutions of vaccinated or control mouse sera were added
437 to coated plates and incubated overnight at 4C. Plates were stained with goat anti-mouse IgG-

438 HRP (Invitrogen cat: A16072) for 1 hour at room temperature. ELISA plates were developed briefly
439 using 3,3′,5,5′-tetramethylbenzidine (TMB) (ThermoFisher cat: 34029) and the reaction was
440 stopped using 2N H2SO4. Absorbance was measured using a BioTek ELISA microplate reader
441 at 450nm. Endpoint dilution was calculated by the concentration of sera with signal 2X
442 background level.

443 **Serum neutralization assay**

444 Focus Reduction Neutralization Tests (FRNT) were performed as described previously.[28,43] Serial
445 dilutions of vaccinated mouse sera was opsonized with ~70 FFU's of a single DENV serotype for
446 1 hour at 37C. Opsonized virus was added to ~70% confluent monolayer of VeroE6 cells in a 96
447 well plate in 2% FBS DMEM. After 1 hour of infection, plates were overlaid with 1% w/v
448 methylcellulose in 2%FBS MEM. Plates were incubated at 37C for 48 (DENV 1, 2, and 3) or 65
449 hours (DENV 4), then fixed with 4% paraformaldehyde (PFA). Plates were stained with a
450 combination of 9.F.10 (500ng/mL), 4G2, and 1-A1D2 in PermWash Buffer (.1% saponin and BSA
451 in PBS). Plates were washed and stained with goat anti-mouse IgG-HRP secondary antibody in
452 PermWash buffer, then resolved with TrueBlue peroxidase (KPL). Resolved plates were imaged
453 on an ImmunoSpot ELISpot plate scanner (Cellular Technology Limited) and foci were counted
454 with Viridot running on RStudio 3.4.1.[44]

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. This work was
474 funded by NIH R01 AI150672 to Justin Richner, 5P20GM148326-02 to Amelia Pinto, and
475 3U01CA260541-02S2 to James Brien.

476

477

478

479

480

481 **FIGURE CAPTIONS**

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

489 **Figure 2: DENV 2 prM/E ΔFL vaccine induces neutralizing, non-enhancing antibody**
490 **responses.** (A-F) C57BL/6 mice were vaccinated with the WT or ΔFL DENV 2 vaccine or a GFP
491 control (N=5/group). Another group of mice (N=3) were infected with 1E5 FFU's of DENV 2 NGC.
492 (A) Serial dilutions of sera was analyzed for binding of purified DV2 via ELISA assay. The endpoint
493 dilution was determined for each animal. (B) Focus reduction neutralization tests (FRNT) were
494 conducted with serial dilutions of sera against DENV 2. Each point represents the mean of the
495 experimental group +/- the standard error of the mean (SEM) (C) EC50's from neutralization
496 curves of individual vaccinated mice are reported. (D) Serial dilutions of vaccinated animal sera
497 were analyzed for enhancement of heterologous DENV 1 16007. (E) The area under the
498 enhancement curves for each individual mouse is also reported. F-G) IFNAR$^{-/-}$ were vaccinated
499 with 10μg of the ΔFL DENV 2 vaccine or a GFP encoding mRNA-LNP. Animals were challenged
500 with1x10^4 FFU of DENV 2. Survival curve (F) and weight loss (G) was reported. *, $P < 0.05$; **,
501 $P < 0.01$; ***, $P < 0.001$.

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

512 control. Animals were challenged with 1x10^7 FFU of DENV 3. Survival curve (F) and weight loss
513 (G) was reported. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.

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

524 **Figure 5: Tetravalent vaccine elicits pan-serotype neutralizing antibody responses.** IFNAR-
525 /- mice were injected intramuscularly with ΔFL mRNA-LNP vaccines of each variant in a prime-
526 boost vaccination schedule separated by 4 weeks between the doses. For each dose mice
527 received 10ug DENV1ΔFL and 20ug each of DENV2 ΔFL, DENV3 ΔFL, DENV4 ΔFL mRNA-LNP
528 vaccines. (C-F) Heat inactivated sera was analyzed via ELISA plates coated with a sucrose
529 purified DENV serotype and endpoint dilution is reported. (H-K, B) Sera was serially diluted and
530 analyzed via FRNT with one of four DENV serotypes. EC50's from neutralization curves are
531 reported. (G, L) Mean EC50's from experimental groups from monovalent (Fig.'s 2-4) and
532 tetravalent vaccinated mice plotted on a radar graph. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****,
533 P < 0.0001.

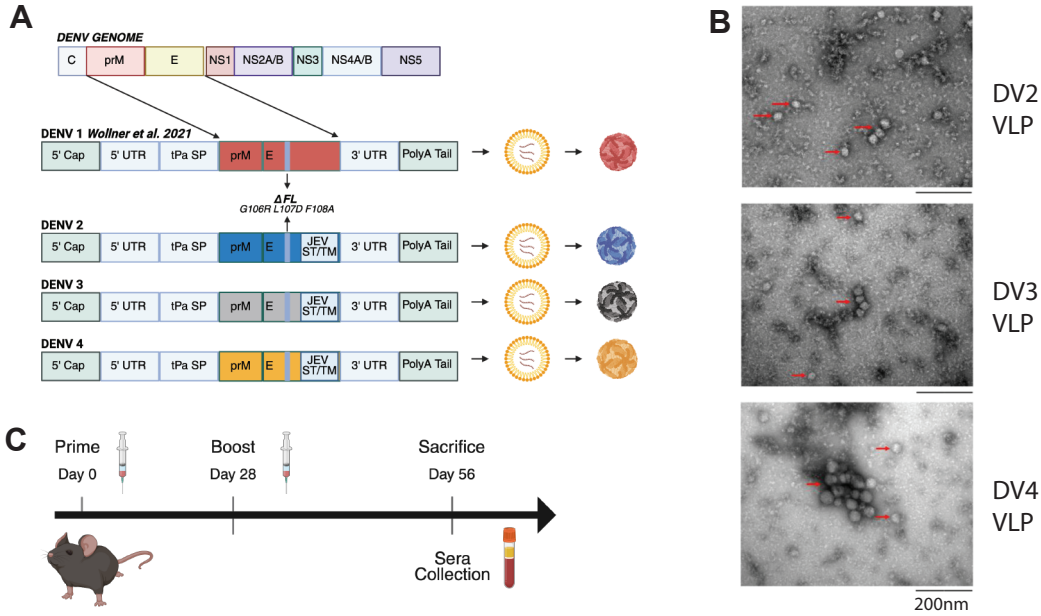
534 **Figure 6: Tetravalent vaccine protects against lethal challenge**. (A,D) Six vaccinated mice
535 and 8 naïve IFNAR$^{-/-}$ mice were challenged with 5x10^6 FFU of DENV 1 West Pac. (B,E) Six
536 vaccinated mice and three naïve IFNAR$^{-/-}$ mice were challenged with 1x10^4 FFU of DENV 2
537 D2S20. (C, F) Six vaccinated mice and five naïve IFNAR$^{-/-}$ were challenged with 5x10^6 FFU
538 DENV 4 TVP376. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.

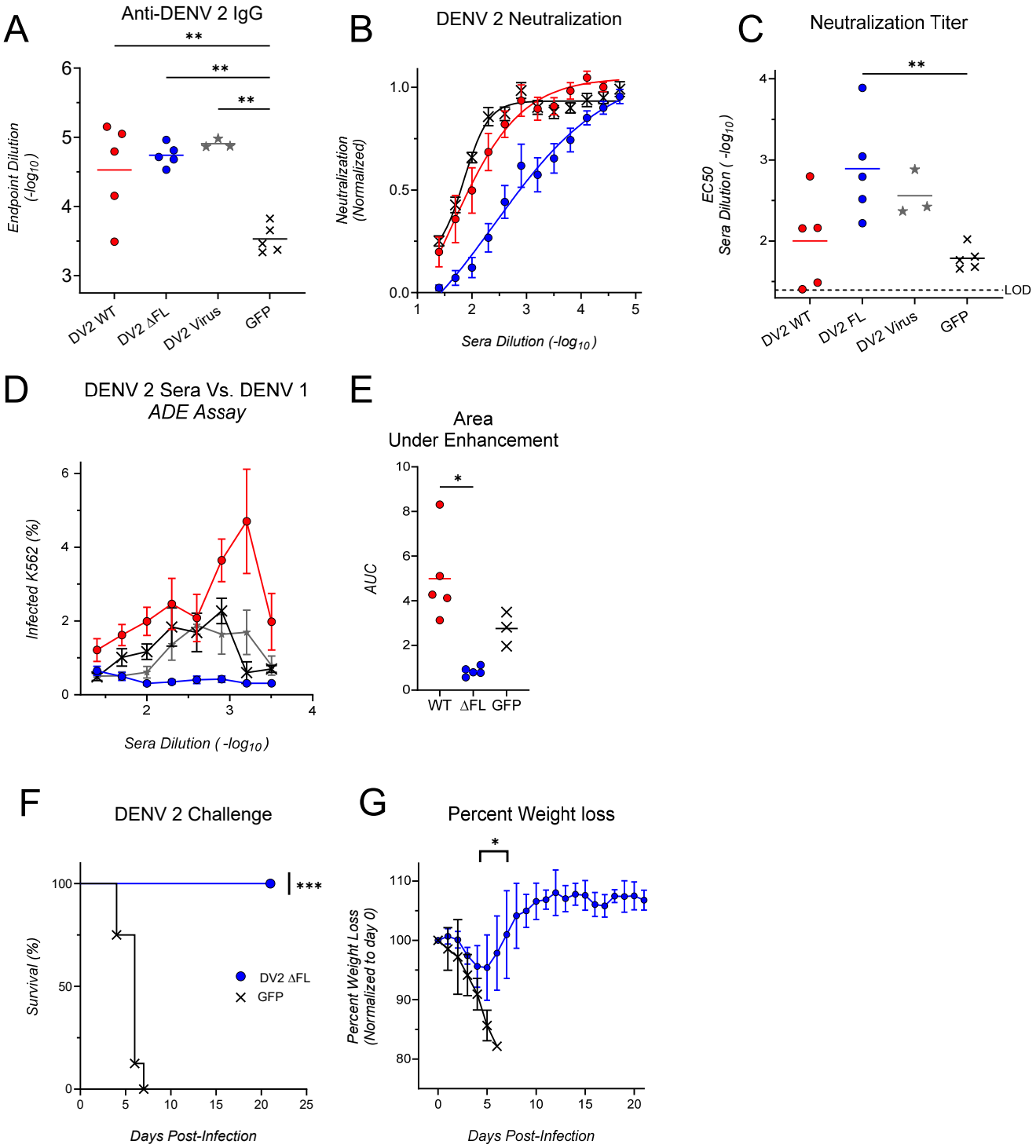
539 **Table 1**: Comparison of total DENV specific IgG and neutralization between monovalent
540 vaccinated and tetravalent vaccinated groups. All mice received the reported vaccine dose,
541 however mouse background differs between monovalent (C57BL/6) and tetravalent (C57BL/6
542 IFNAR$^{-/-}$).

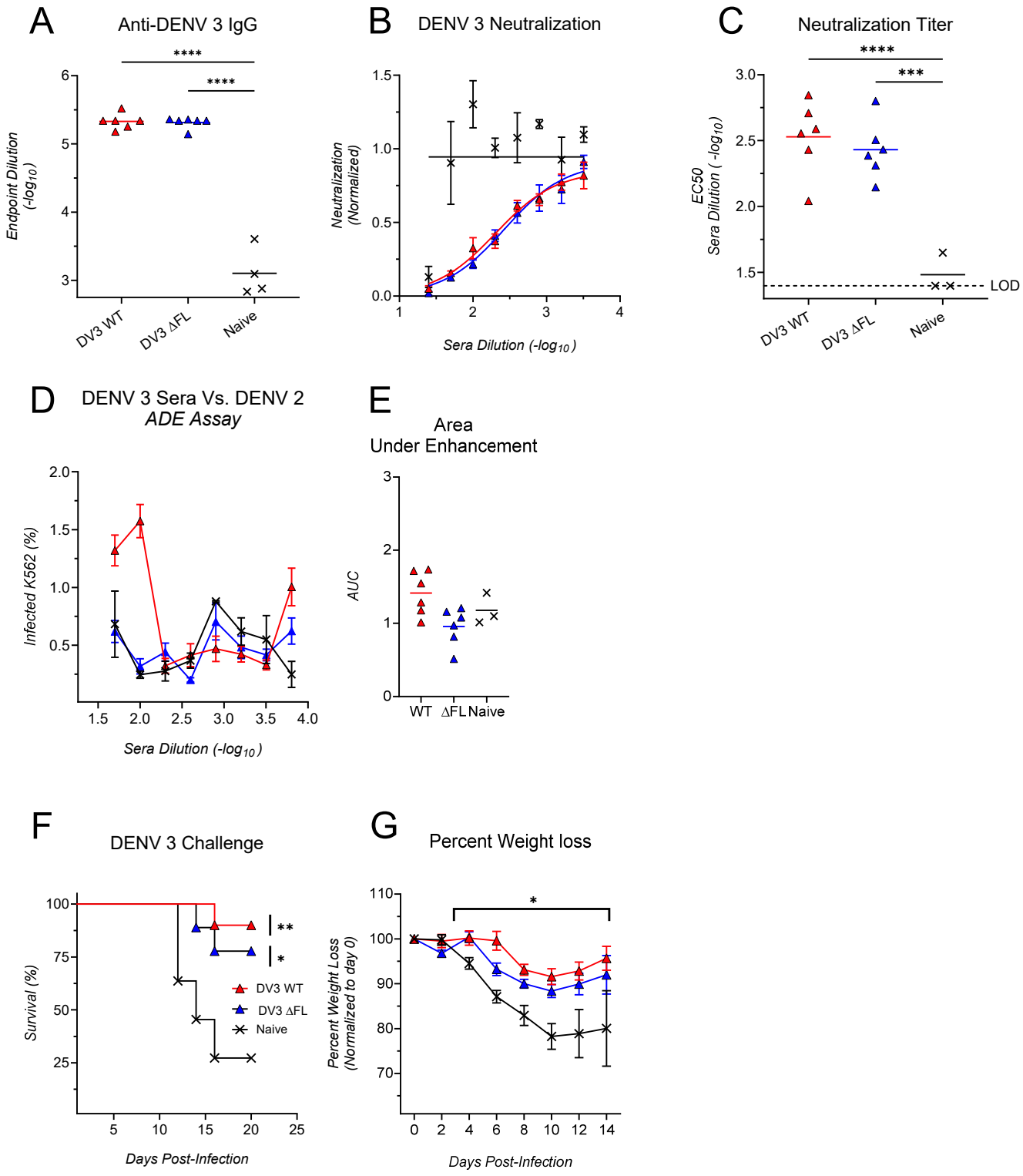
543

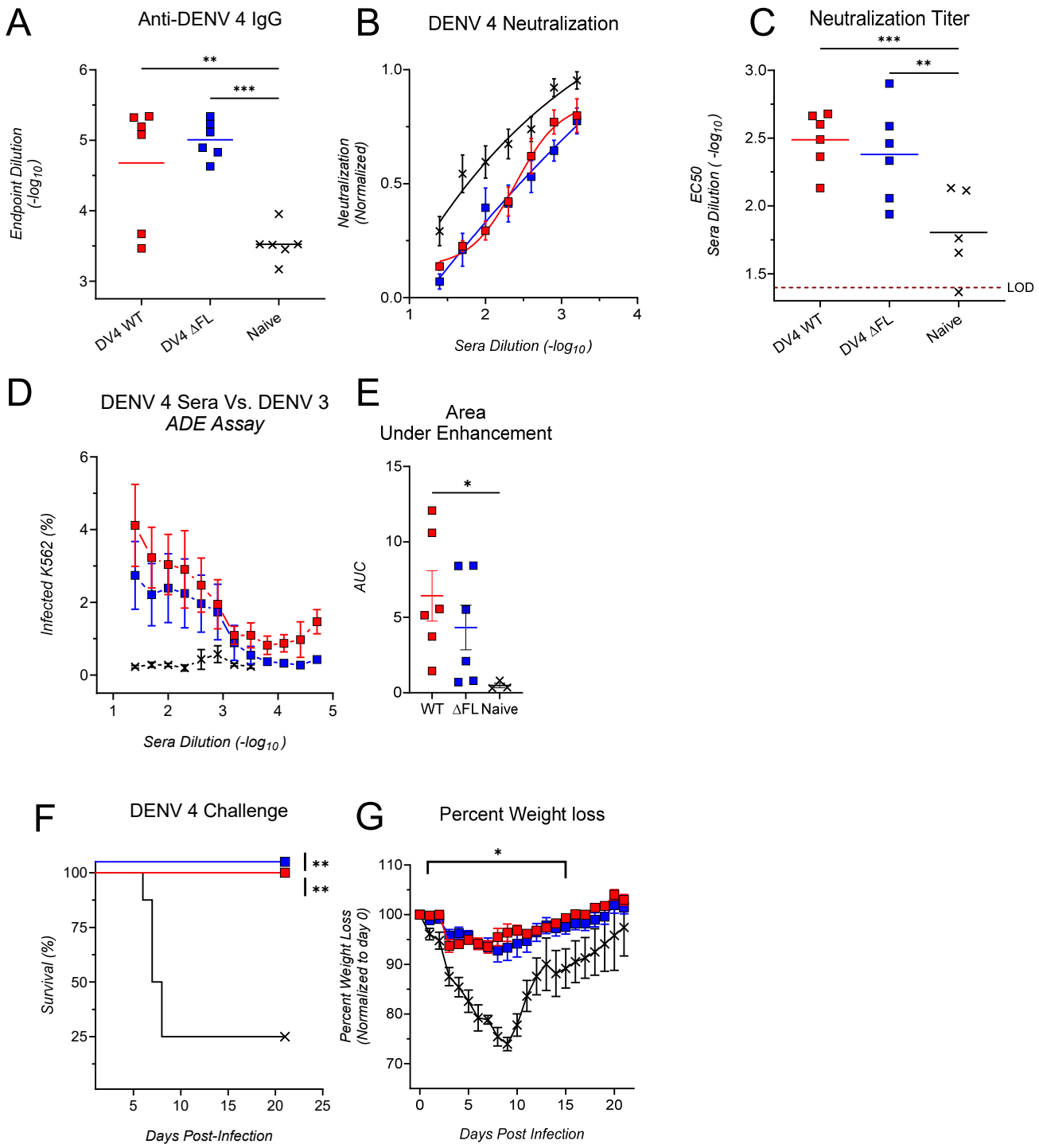
1. Guzman, M. G. & Harris, E. Dengue. *The Lancet* **385**, 453–465 (2015).
2. Khan, M. B. *et al.* Dengue overview: An updated systemic review. *J. Infect. Public Health* **16**, 1625–1642 (2023).
4. Sinha, S. *et al.* Dengue virus pathogenesis and host molecular machineries. *J. Biomed. Sci.* **31**, 43 (2024).
5. Messina, J. P. *et al.* The current and future global distribution and population at risk of dengue. *Nat. Microbiol.* **4**, 1508–1515 (2019).
6. *Fields Virology*. (Wolters Kluwer/Lippincott Williams & Wilkins Health, Philadelphia, PA, 2013).
7. Gould, E. & Solomon, T. Pathogenic flaviviruses. *The Lancet* **371**, 500–509 (2008).
8. Muller, D. A., Depelsenaire, A. C. I. & Young, P. R. Clinical and Laboratory Diagnosis of Dengue Virus Infection. *J. Infect. Dis.* **215**, S89–S95 (2017).
9. van Leur, S. W., Heunis, T., Munnur, D. & Sanyal, S. Pathogenesis and virulence of flavivirus infections. *Virulence* **12**, 2814–2838.
10. Beltramello, M. *et al.* The Human Immune Response to Dengue Virus Is Dominated by Highly Cross-Reactive Antibodies Endowed with Neutralizing and Enhancing Activity. *Cell Host Microbe* **8**, 10.1016/j.chom.2010.08.007 (2010).
11. Lai, C.-Y. *et al.* Antibodies to Envelope Glycoprotein of Dengue Virus during the Natural Course of Infection Are Predominantly Cross-Reactive and Recognize Epitopes Containing Highly Conserved Residues at the Fusion Loop of Domain II. *J. Virol.* **82**, 6631–6643 (2008).
12. Rey, F. A., Stiasny, K., Vaney, M., Dellarole, M. & Heinz, F. X. The bright and the dark side of human antibody responses to flaviviruses: lessons for vaccine design. *EMBO Rep.* **19**, 206–224 (2018).
14. Katzelnick, L. C. *et al.* Antibody-dependent enhancement of severe dengue disease in humans. *Science* **358**, 929–932 (2017).
15. Tsai, W.-Y., Lin, H.-E. & Wang, W.-K. Complexity of Human Antibody Response to Dengue Virus: Implication for Vaccine Development. *Front. Microbiol.* **8**, 1372 (2017).
16. Dejnirattisai, W. *et al.* Enhancing cross-reactive anti-prM dominates the human antibody response in dengue infection. *Science* **328**, 10.1126/science.1185181 (2010).
17. de Alwis, R. *et al.* Identification of human neutralizing antibodies that bind to complex epitopes on dengue virions. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 7439–7444 (2012).
18. Thomas, S. J. & Yoon, I.-K. A review of Dengvaxia®: development to deployment. *Hum. Vaccines Immunother.* **15**, 2295–2314 (2019).
19. Dayan, G. H. *et al.* Assessment of the long-term efficacy of a dengue vaccine against symptomatic, virologically-confirmed dengue disease by baseline dengue serostatus. *Vaccine* **38**, 3531–3536 (2020).
20. Henein, S. *et al.* Dissecting Antibodies Induced by a Chimeric Yellow Fever–Dengue, Live-Attenuated, Tetravalent Dengue Vaccine (CYD-TDV) in Naive and Dengue-Exposed Individuals. *J. Infect. Dis.* **215**, 351–358 (2017).
21. Halstead, S. B. Dengvaxia sensitizes seronegatives to vaccine enhanced disease regardless of age. *Vaccine* **35**, 6355–6358 (2017).
22. Kudlacek, S. T. & Metz, S. W. Focused dengue vaccine development: outwitting nature's design. *Pathog. Dis.* **77**, ftz003 (2019).
23. de Silva, A. M. & Harris, E. Which Dengue Vaccine Approach Is the Most Promising, and Should We Be Concerned about Enhanced Disease after Vaccination? *Cold Spring Harb. Perspect. Biol.* **10**, a029371 (2018).
24. Manoff, S. B. *et al.* Preclinical and clinical development of a dengue recombinant subunit vaccine. *Vaccine* **33**, 7126–7134 (2015).

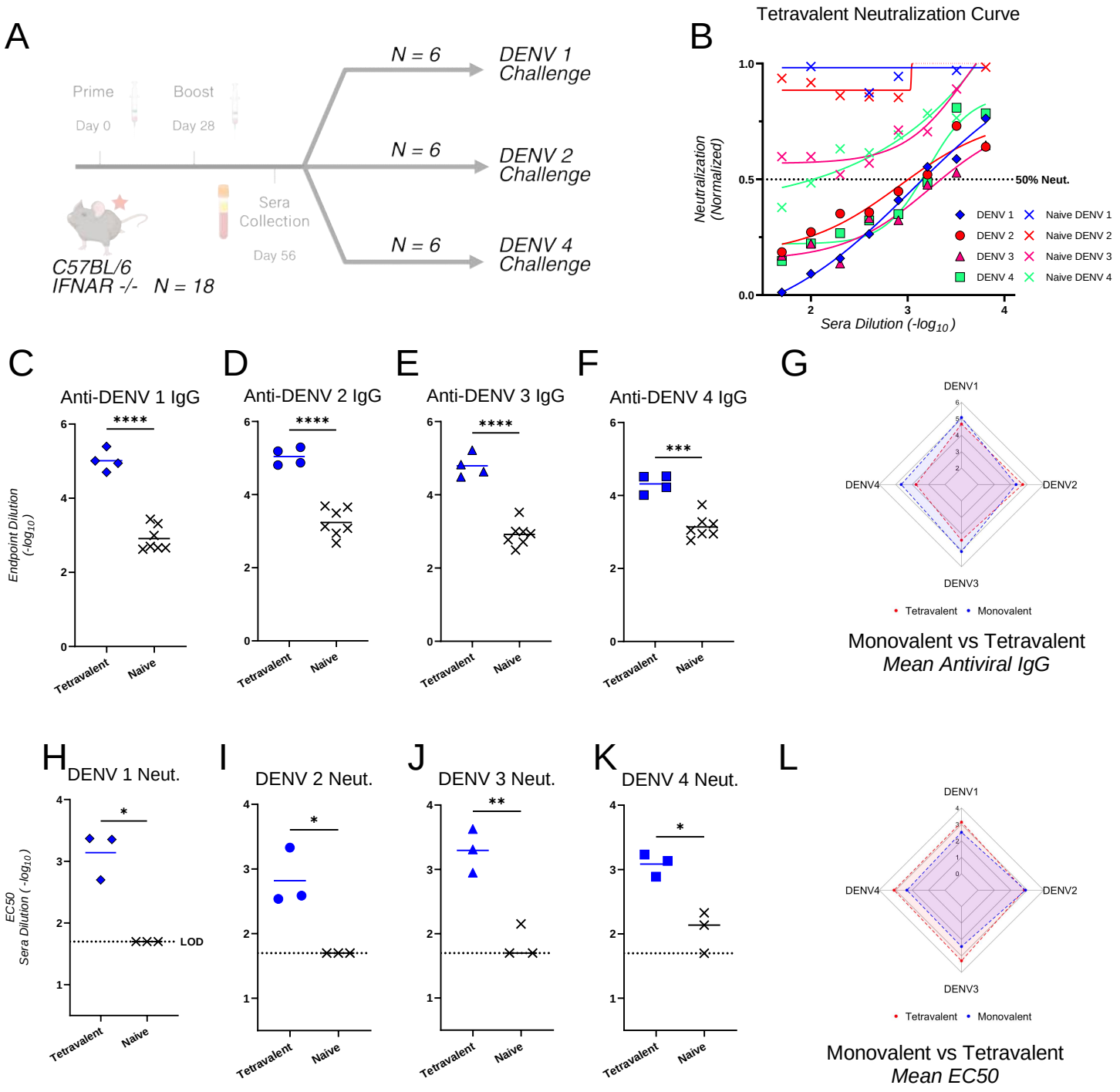
593 25. Metz, S. W. *et al.* Dengue virus-like particles mimic the antigenic properties of the
594 infectious dengue virus envelope. *Virol. J.* **15**, 60 (2018).
595 26. Palur, V. R. *et al.* An Integrative Approach to Rational Engineering of Dengue Virus-Like
596 Particles. 2025.03.11.642607 Preprint at https://doi.org/10.1101/2025.03.11.642607 (2025).
597 27. Shen, W.-F. *et al.* Epitope resurfacing on dengue virus-like particle vaccine preparation
598 to induce broad neutralizing antibody. *eLife* **7**, e38970 (2018).
599 28. Wollner, C. J. *et al.* A Dengue Virus Serotype 1 mRNA-LNP Vaccine Elicits Protective
600 Immune Responses. *J. Virol.* **95**, e02482-20 (2021).
601 29. Chang, G.-J. J. *et al.* Enhancing biosynthesis and secretion of premembrane and
602 envelope proteins by the chimeric plasmid of dengue virus type 2 and Japanese encephalitis
603 virus. *Virology* **306**, 170–180 (2003).
604 30. Urakami, A. *et al.* An Envelope-Modified Tetravalent Dengue Virus-Like-Particle Vaccine
605 Has Implications for Flavivirus Vaccine Design. *J. Virol.* **91**, e01181-17 (2017).
606 31. Purdy, D. E. & Chang, G.-J. J. Secretion of noninfectious dengue virus-like particles and
607 identification of amino acids in the stem region involved in intracellular retention of envelope
608 protein. *Virology* **333**, 239–250 (2005).
609 32. Tu, J. J. *et al.* An HTLV-1 envelope mRNA vaccine is immunogenic and protective in
610 New Zealand rabbits. *J. Virol.* **98**, e0162323 (2024).
611 33. Dangi, T. *et al.* Pre-existing immunity modulates responses to mRNA boosters. *Cell Rep.*
612 **42**, 112167 (2023).
613 34. Richner, J. M. *et al.* Vaccine Mediated Protection Against Zika Virus-Induced Congenital
614 Disease. *Cell* **170**, 273-283.e12 (2017).
615 35. Andries, O. *et al.* N(1)-methylpseudouridine-incorporated mRNA outperforms
616 pseudouridine-incorporated mRNA by providing enhanced protein expression and reduced
617 immunogenicity in mammalian cell lines and mice. *J. Control. Release Off. J. Control. Release*
618 *Soc.* **217**, 337–344 (2015).
619 36. Karikó, K., Buckstein, M., Ni, H. & Weissman, D. Suppression of RNA Recognition by
620 Toll-like Receptors: The Impact of Nucleoside Modification and the Evolutionary Origin of RNA.
621 *Immunity* **23**, 165–175 (2005).
622 37. Karikó, K. *et al.* Incorporation of Pseudouridine Into mRNA Yields Superior
623 Nonimmunogenic Vector With Increased Translational Capacity and Biological Stability. *Mol.*
624 *Ther. J. Am. Soc. Gene Ther.* **16**, 1833–1840 (2008).
625 38. Baiersdörfer, M. *et al.* A Facile Method for the Removal of dsRNA Contaminant from
626 In Vitro-Transcribed mRNA. *Mol. Ther. Nucleic Acids* **15**, 26–35 (2019).
627 39. Flipse, J. & Smit, J. M. The Complexity of a Dengue Vaccine: A Review of the Human
628 Antibody Response. *PLoS Negl. Trop. Dis.* **9**, e0003749 (2015).
629 40. Mabale, M. A. A. *et al.* Implications of information heard about Dengvaxia on Filipinos'
630 perception on vaccination. *Vaccine* **42**, 1673–1681 (2024).
631 41. Shen, W.-F. *et al.* Epitope resurfacing on dengue virus-like particle vaccine preparation
632 to induce broad neutralizing antibody. *eLife* **7**, e38970 (2018).
633 42. Hadinegoro, S. R. *et al.* Efficacy and Long-Term Safety of a Dengue Vaccine in Regions
634 of Endemic Disease. *N. Engl. J. Med.* **373**, 1195–1206 (2015).
635 43. Richner, J. M. *et al.* Modified mRNA Vaccines Protect against Zika Virus Infection. *Cell*
636 **169**, 176 (2017).
637 44. Katzelnick, L. C. *et al.* Viridot: An automated virus plaque (immunofocus) counter for the
638 measurement of serological neutralizing responses with application to dengue virus. *PLoS Negl.*
639 *Trop. Dis.* **12**, e0006862 (2018).
640

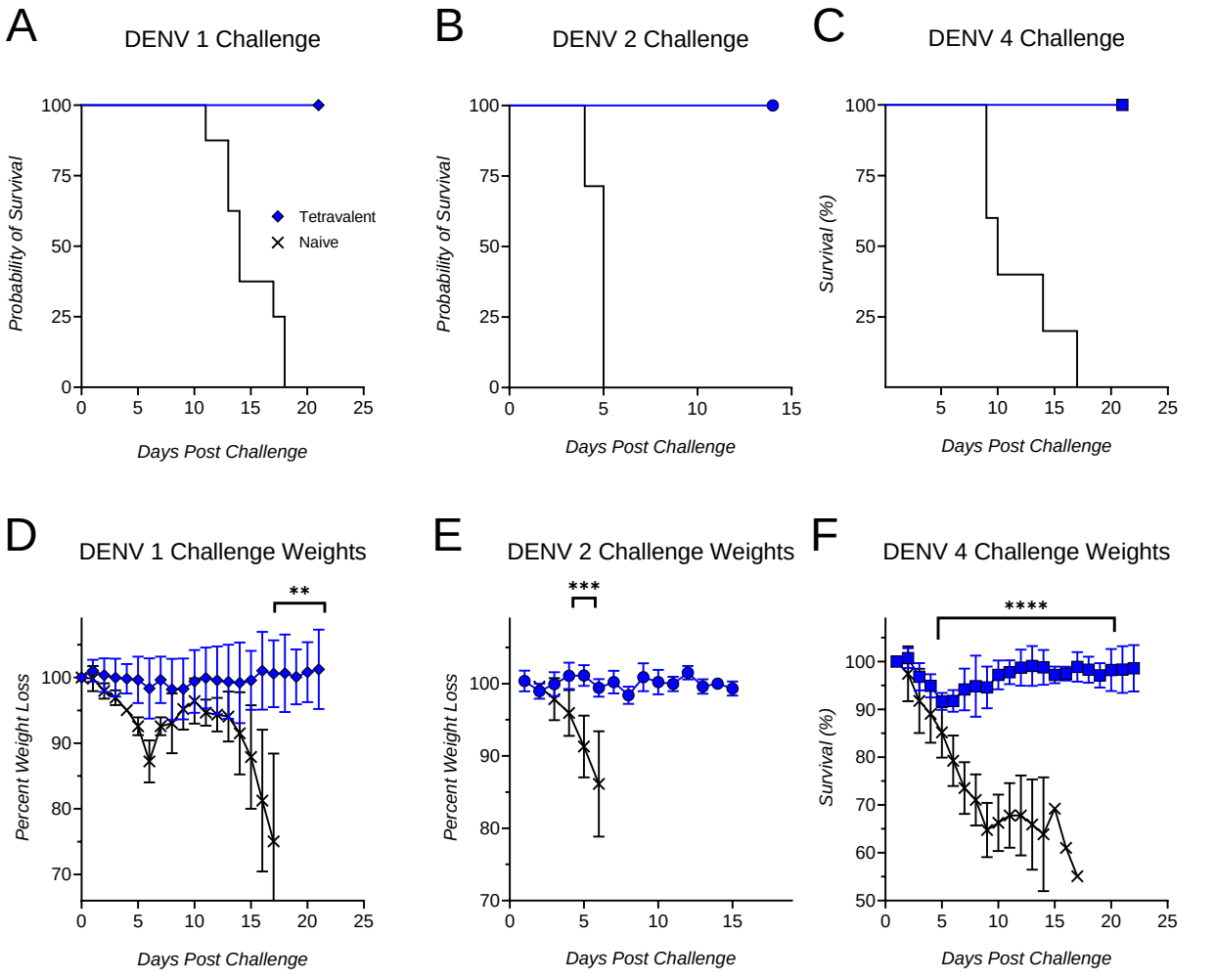

A
DENV GENOME
C
prM
E
NS1
NS2A/B
NS3
NS4A/B
NS5
DENV 1 Wollner et al. 2021
5' Cap
5' UTR
tPa SP
prM
E
3' UTR
PolyA Tail
ΔFL
G106R L107D F108A
DENV 2
JEV ST/TM
DENV 3
DENV 4
B
DV2 VLP
DV3 VLP
DV4 VLP
200nm
C
Prime
Day 0
Boost
Day 28
Sacrifice
Day 56
Sera Collection


A
Anti-DENV 2 IgG
Endpoint Dilution (-log10)
DV2 WT
DV2 ΔFL
DV2 Virus
GFP
B
DENV 2 Neutralization
Neutralization (Normalized)
Sera Dilution (-log10)
C
Neutralization Titer
EC50 Sera Dilution (-log10)
LOD
DV2 WT
DV2 FL
DV2 Virus
GFP
D
DENV 2 Sera Vs. DENV 1
ADE Assay
Infected K562 (%)
Sera Dilution (-log10)
E
Area
Under Enhancement
AUC
WT
ΔFL
GFP
F
DENV 2 Challenge
Survival (%)
DV2 ΔFL
GFP
Days Post-Infection
G
Percent Weight loss
Percent Weight Loss (Normalized to day 0)
Days Post-Infection


A
Anti-DENV 3 IgG
****
****
Endpoint Dilution (-log10)
DV3 WT
DV3 ΔFL
Naive
B
DENV 3 Neutralization
Neutralization (Normalized)
Sera Dilution (-log10)
C
Neutralization Titer
****
***
EC50 Sera Dilution (-log10)
LOD
DV3 WT
DV3 ΔFL
Naive
D
DENV 3 Sera Vs. DENV 2
ADE Assay
Infected K562 (%)
Sera Dilution (-log10)
E
Area
Under Enhancement
AUC
WT
ΔFL
Naive
F
DENV 3 Challenge
Survival (%)
**
*
DV3 WT
DV3 ΔFL
Naive
Days Post-Infection
G
Percent Weight loss
*
Percent Weight Loss (Normalized to day 0)
Days Post-Infection


A
Anti-DENV 4 IgG
Endpoint Dilution (-log10)
DV4 WT
DV4 ΔFL
Naive
B
DENV 4 Neutralization
Neutralization (Normalized)
Sera Dilution (-log10)
C
Neutralization Titer
EC50 Sera Dilution (-log10)
LOD
D
DENV 4 Sera Vs. DENV 3
ADE Assay
Infected K562 (%)
Sera Dilution (-log10)
E
Area Under Enhancement
AUC
WT
ΔFL
Naive
F
DENV 4 Challenge
Survival (%)
Days Post-Infection
G
Percent Weight loss
Percent Weight Loss (Normalized to day 0)
Days Post Infection


A
Prime
Day 0
Boost
Day 28
Sera Collection
Day 56
C57BL/6
IFNAR -/- N = 18
N = 6
DENV 1 Challenge
N = 6
DENV 2 Challenge
N = 6
DENV 4 Challenge
B
Tetravalent Neutralization Curve
Neutralization (Normalized)
Sera Dilution (-log10)
50% Neut.
DENV 1
DENV 2
DENV 3
DENV 4
Naive DENV 1
Naive DENV 2
Naive DENV 3
Naive DENV 4
C
Anti-DENV 1 IgG
D
Anti-DENV 2 IgG
E
Anti-DENV 3 IgG
F
Anti-DENV 4 IgG
Endpoint Dilution (-log10)
Tetravalent
Naive
G
DENV1
DENV2
DENV3
DENV4
Tetravalent
Monovalent
Monovalent vs Tetravalent
Mean Antiviral IgG
H
DENV 1 Neut.
I
DENV 2 Neut.
J
DENV 3 Neut.
K
DENV 4 Neut.
EC50 Sera Dilution (-log10)
LOD
L
Monovalent vs Tetravalent
Mean EC50


A
DENV 1 Challenge
Probability of Survival
Days Post Challenge
Tetravalent
Naive
B
DENV 2 Challenge
Probability of Survival
Days Post Challenge
C
DENV 4 Challenge
Survival (%)
Days Post Challenge
D
DENV 1 Challenge Weights
Percent Weight Loss
Days Post Challenge
**
E
DENV 2 Challenge Weights
Percent Weight Loss
Days Post Challenge
***
F
DENV 4 Challenge Weights
Survival (%)
Days Post Challenge
****

DENV-1 ΔFL Vaccine (10µg Dose)	DENV-1 Specific IgG	DENV-1 Neut. (EC50)
Monovalent	204,000	330
Tetravalent	102,000	1400
P-Value	NS 0.1692	* 0.05

DENV-2 ΔFL Vaccine (20µg Dose)	DENV-2 Specific IgG	DENV-2 Neut. (EC50)
Monovalent	55,000	780
Tetravalent	109,000	660
P-Value	NS 0.0814	NS 0.8536

DENV-3 ΔFL Vaccine (20µg Dose)	DENV-3 Specific IgG	DENV-3 Neut. (EC50)
Monovalent	206,000	270
Tetravalent	62,000	2000
P-Value	* 0.0421	* 0.0302

DENV-4 ΔFL Vaccine (20µg Dose)	DENV-4 Specific IgG	DENV-4 Neut. (EC50)
Monovalent	101,000	209
Tetravalent	21,000	1300
P-Value	** 0.0043	** 0.0054