

1 Prime-boost vaccination regimens with INO-4800 and INO-4802 augment and broaden immune
2 responses against SARS-CoV-2 in nonhuman primates

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15

16 **SUMMARY**

17

18 The enhanced transmissibility and immune evasion associated with emerging SARS-CoV-2
19 variants demands the development of next-generation vaccines capable of inducing superior
20 protection amid a shifting pandemic landscape. Since a portion of the global population harbors
21 some level of immunity from vaccines based on the original Wuhan-Hu-1 SARS-CoV-2 sequence
22 or natural infection, an important question going forward is whether this immunity can be boosted
23 by next-generation vaccines that target emerging variants while simultaneously maintaining long-
24 term protection against existing strains. Here, we evaluated the immunogenicity of INO-4800, our
25 synthetic DNA vaccine candidate for COVID-19 currently in clinical evaluation, and INO-4802,
26 a next-generation DNA vaccine designed to broadly target emerging SARS-CoV-2 variants, as
27 booster vaccines in nonhuman primates. Rhesus macaques primed over one year prior with the
28 first-generation INO-4800 vaccine were boosted with either INO-4800 or INO-4802 in
29 homologous or heterologous prime-boost regimens. Both boosting schedules led to an expansion
30 of antibody responses which were characterized by improved neutralizing and ACE2 blocking
31 activity across wild-type SARS-CoV-2 as well as multiple variants of concern. These data
32 illustrate the durability of immunity following vaccination with INO-4800 and additionally support
33 the use of either INO-4800 or INO-4802 in prime-boost regimens.

34

35 INTRODUCTION

36

37 SARS-CoV-2 is a beta-coronavirus belonging to the same family as severe acute
38 respiratory coronavirus (SARS-CoV) and Middle East Respiratory Syndrome coronavirus
39 (MERS-CoV), which share similar structural features including the spike glycoprotein which has
40 been the primary target of vaccine development for each of these viruses [1]. Although the rollout
41 of the EUA vaccines has been underway for several months, global distribution of these vaccines
42 has fallen along entrenched socioeconomic lines, leaving many low- and middle-income countries
43 with inadequate supply [2]. For successful global coverage, many more vaccines will be needed.
44 The rapid expansion of SARS-CoV-2 variants of concern (VOC) has corresponded with a
45 reduction in neutralizing antibody activity in convalescent and vaccinated individuals, suggesting
46 that emerging mutations observed in some lineages are associated with immune escape [3-7].
47 Alarmingly, the Beta (B.1.351) variant has demonstrated a reduced sensitivity to neutralizing sera
48 from convalescent and immunized individuals [7]. Recently, it has been observed that vaccine
49 effectiveness (either BNT162b2 or ChAdOx1 nCoV-19) was notably lower against the now
50 dominant Delta (B.1.617.2) variant, compared to the Alpha (B.1.1.7) variant [8]. The combination
51 of viral escape mechanisms and waning immunity suggest that heterologous prime-boost strategies
52 may be needed to provide sufficient coverage against novel variants [9].

53 Synthetic DNA vaccines offer multiple advantages over other vaccine platforms including
54 shortened clinical development timetables for vaccines against emerging infectious diseases,
55 ability to scale up manufacture, and long-term temperature stability that facilitates rapid and
56 efficient deployment in resource-limited settings [10-12]. We have previously described the design
57 of a synthetic DNA vaccine encoding the wild-type (Wuhan-Hu-1) Spike protein, INO-4800,

58 which is currently in clinical evaluation [10]. In preclinical studies we have shown INO-4800
59 vaccination induces antigen-specific T cell responses and functional antibodies that neutralize
60 SARS-CoV-2 [10, 13, 14]. In a non-human primate (NHP) challenge model, INO-4800
61 vaccination was associated with reduced viral loads and protection against respiratory tract disease
62 [13, 14]. Phase 1 and 2 clinical trials of INO-4800 demonstrated a favorable safety and tolerability
63 profile and immunogenicity [15, 16].

64 In response to the increasing number of SARS-CoV-2 VOCs demonstrating evasion of
65 vaccine- or infection-induced humoral immunity, we have designed INO-4802, a next-generation
66 DNA vaccine expressing a pan-Spike immunogen which has been shown to raise immunity across
67 SARS-CoV-2 VOCs in mice [17] and confers broad protection in hamsters following intranasal
68 challenge with multiple VOCs [17].

69 Prime-boost regimens are widely used in the development of vaccines against a variety of
70 infectious diseases [18, 19], including DNA and viral-vector based approaches [20, 21]. DNA
71 vaccines have particular advantages in the prime-boost setting where they have been shown to
72 enhance both humoral and cellular responses without inducing anti-vector immunity [22]. In the
73 boost setting DNA vaccines were found to be superior to the adenovirus platform in expanding
74 responses to simian immunodeficiency virus (SIV) antigens in rhesus macaques [23]. In the clinic,
75 the DNA platform is not limited by the same dose-dependent reactogenicity observed following
76 administration of lipid nanoparticles carrying mRNA vaccines [24], which may be an important
77 consideration in booster acceptance.

78
79 In the current study, we investigated the durability and memory recall of antigen-specific SARS-
80 CoV-2 responses in a cohort of non-human primates that were initially primed with the first-

81 generation SARS-CoV-2 vaccine INO-4800. One year following the primary immunization series
82 with INO-4800, the animals were boosted with either INO-4800 or INO-4802 in homologous or
83 heterologous prime-boost regimens, respectively. Boosting with either INO-4800 or INO-4802 led
84 to the induction of potent neutralizing antibody responses across multiple SARS-CoV-2 VOCs
85 that correlated with ACE2 blocking activity. These data highlight the capability of DNA vaccines
86 to boost SARS-CoV-2 immunity.

87

88 **RESULTS**

89

90 ***Durability of SARS-CoV-2-specific humoral response following immunization with INO-4800***

91 Initial studies investigated the durability of immune responses in non-human primates (NHPs)
92 primed with INO-4800. NHPs were immunized at week 0 and 4 with either a 1 mg or 2 mg dose
93 of INO-4800, and blood was collected over the course of one year (**Figure 1A**). It should be noted
94 that, for Figure 1 and Supplemental Figure 1, the NHPs were initially treated on staggered
95 schedules, and therefore the data from the prime immunization portion of the study will show
96 collected data points for NHP IDs #7544, 7545, 7546, 7548, 7550 terminating at Week 35 and for
97 others, IDs #7514, 7520, 7523, 7524, terminating at Week 52. To measure levels of binding
98 antibodies in the sera we used an enzyme-linked immunosorbent assay (ELISA). Peak antibody
99 titers were observed at week 6 with a geometric mean endpoint titer of 258,032, two weeks
100 following the second immunization (**Figure 1B**). Detectable levels of binding antibodies persisted
101 in the serum for the duration of the study, and at the final timepoint prior to boosting, the 1 mg
102 dose group had geometric mean endpoint titers of 11143 for the S1+S2 ECD. The 2 mg dose
103 group had geometric mean endpoint titers of 4525 for the S1+S2 ECD. Similar trends were also

104 observed in the levels of binding antibodies against the SARS-CoV-2 S1, SARS-CoV-2 S2 and
105 RBD proteins (**Supplemental Figure 1**).

106

107 Functional antibody responses were measured in a pseudovirus neutralization assay against the
108 SARS-CoV-2 wild-type, Alpha (B.1.1.7), Beta (B.1.351) and Gamma (P.1) variants of concern
109 (VOCs) which were in circulation during this time period. Immunization with INO-4800 resulted
110 in the induction of neutralizing antibodies that were increased over baseline for all VOCs (**Figure**
111 **1C-F**). SARS-CoV-2 VOC neutralizing antibody responses were durable and remained elevated
112 over baseline at the last collected timepoint, with the 1 mg dose group having a geometric mean
113 titer (GMT) of 301 for the wild-type variant, 349 for B.1.1.7, 158 for B.1.351, and 317 for P.1. In
114 Figure 1E, the authors note that NHP #7545 showed reduced neutralizing activity at Week 14
115 which was attributed to sampling error during plating. The 2 mg dose group had a GMT of 174.6
116 for the wild-type variant, 58.2 for B.1.1.7, 100.3 for B.1.351, and 164.2 for P.1. Together, these
117 data illustrate that the primary INO-4800 vaccination schedule induced SARS-CoV-2 specific
118 antibodies harboring neutralizing activity that were maintained over the period of 35 – 52 weeks.

119

120 *Humoral responses following delivery of either INO-4800 or INO-4802*

121 We evaluated INO-4800 and INO-4802 as booster vaccines. The same rhesus macaques that were
122 initially primed with INO-4800 were randomized into two groups and boosted with either INO-
123 4800, homologous to the original vaccine, or INO-4802, an updated pan-SARS-CoV-2 Spike
124 immunogen in a heterologous boost regimen. Rhesus macaques #7544, 7545, 7546, 7548, 7550
125 were boosted 43 weeks after the initial vaccination while NHPs #7514, 7520, 7523, 7524, were
126 boosted at 64 weeks after the initial vaccination (**Figure 2A**).

127

128 The homologous boost with INO-4800 resulted in the induction of antibody titers at two weeks
129 post-boost that were increased over pre-boost levels (**Figure 2B**). Increases in binding antibody
130 levels showed similar patterns against the wild-type, B.1.351, P.1 and Delta (B.1.617.2) Spike
131 proteins, with GMTs of 87, 43, 43 and 342, respectively, pre-boost and 3077, 2338, 2077 and
132 21044, respectively, post-boost. Likewise, heterologous boost with INO-4802 also led to increased
133 binding antibodies against all variants tested with GMTs of 150, 187, 290 and 44, respectively pre-
134 boost and 6285, 6285, 6285 and 6285, respectively, two weeks post-boost for the wild-type,
135 B.1.351, P.1 and B.1.617.2 variants (**Figure 2B**).

136

137 Neutralizing activity against the wild-type, B.1.351, P.1 and B.1.617.2 variants was assessed by a
138 pseudovirus neutralization assay, which revealed increased neutralizing antibody responses
139 against all SARS-CoV-2 variants in animals boosted with either INO-4800 or INO-4802 (**Figure**
140 **2C**). The GMTs at Week 2 for the NHPs after the homologous INO-4800 boost were 2286.2,
141 1199.3, 1596.1 and 785.6 against the wild-type, B.1.351, P.1 and B.1.617.2 pseudoviruses,
142 respectively. The GMTs at Week 2 for the NHPs after the heterologous INO-4802 boost were
143 3712, 1452.1, 4389.6 and 1434.8 against the wild-type, B.1.351, P.1 and B.1.617.2 respectively.
144 As an additional readout of functional antibody responses, we measured ACE2/SARS-CoV-2
145 spike interaction blocking activity of serum antibodies using a Meso Scale Discovery (MSD)
146 assay, by quantifying the level of inhibition of ACE2 binding to a panel of variant SARS-CoV-2
147 Spike proteins. In line with the pseudovirus neutralization data, all animals showed an increase in
148 the level of functional anti-SARS-CoV-2 antibodies in their serum following the boost
149 immunization (**Figure 2D**). We observed positive correlations between pseudovirus neutralization

150 and inhibition of the ACE2/SARS-CoV-2 spike interaction (**Supplemental Figure 2A**),
151 supporting the overall functional antibody responses observed in animals receiving either booster
152 vaccine.

153
154 We next evaluated T follicular helper cells (T_{fh}) cells, an important cell type in the generation of
155 high-affinity antibodies [25-27]. The frequency of circulating T_{fh} cells positively correlated with
156 ACE2 blocking activity at week 2 in animals boosted with INO-4800 and INO-4802
157 (**Supplemental Figure 2B**), supporting the generation of functional antibody responses following
158 a boost with SARS-CoV-2 DNA vaccines. Together, these data show an augmentation of humoral
159 responses following a boost with either INO-4800 or INO-4802 in the context of existing SARS-
160 CoV-2 immunity, possibly increasing the breadth of immune response against multiple VOCs.

161

162 **DISCUSSION**

163 Emerging SARS-CoV-2 VOCs and waning immunity will likely lead to recurrent waves of
164 COVID-19 disease [28]. The frequency and severity of future outbreaks will depend on several
165 complex factors that will likely unfold differently across the world [29]. The duration of immunity
166 following SARS-CoV-2 infection and vaccination will be a key determinant of future SARS-CoV-
167 2 transmission dynamics [29]. Accumulating evidence suggests that durable immune responses
168 are maintained in COVID-19 convalescent individuals and vaccinees [30-33]. However, the
169 emergence of SARS-CoV-2 variants capable of evading humoral immune responses [5, 34]
170 highlights the potential need to update vaccines to mitigate the severity of future SARS-CoV-2
171 outbreaks. More broadly protective vaccines that can be administered as boosters may be critical
172 in maintaining levels of protection against new outbreak waves with antigenically divergent

173 SARS-CoV-2 lineages. Recent studies have shown that the mutations associated with emerging
174 VOCs have a negative impact on neutralizing antibody responses and on the efficacy of SARS-
175 CoV-2 vaccines[3-7, 35]. Data show that the adenovirus-vectored ChAdOx1 nCoV-19 vaccine
176 and nanoparticle-based NVX-CoV2373 vaccine have lower efficacy against the B.1.351 variant
177 compared to the overall vaccine efficacy [36, 37].

178

179 The development of next-generation vaccines is one approach to broadening immune coverage
180 against emerging SARS-CoV-2 variants, and the immunogenicity of booster vaccines that are
181 heterologous from previous vaccinations is will play an important role in informing global
182 immunization strategies. It was found that individuals who received a primary immunization series
183 of mRNA-1273 and subsequently received a booster shot of Moderna's updated mRNA vaccine
184 encoding the B.1.351 Spike protein, mRNA-1273.351, showed increases in antibody neutralization
185 titers against the B.1.351 and P.1 variants that were superior to those in individuals who received
186 a booster shot of mRNA-1273 [38]. Other studies have assessed the safety and immunogenicity of
187 heterologous prime-boost regimens involving different vaccine platforms. A recent study
188 involving individuals who originally received one of the three EUA vaccines (mRNA-1273,
189 Ad26.COV2.S, or BNT162b2) and were subsequently boosted with a homologous or heterologous
190 vaccine suggests that both boosting schedules increase protective efficacy against symptomatic
191 SARS-CoV-2 infection [39]. In a separate study, favorable increases in humoral responses were
192 observed in individuals previously vaccinated with the ChAdOx1 nCoV-19 vaccine and
193 subsequently boosted with BNT162b2 [40, 41]. Likewise, a heterologous boost of mRNA-1273
194 also resulted in an increase in humoral responses in individuals primed with ChAdOx1 nCoV-19
195 [42]. However, homologous boosting strategies have also shown promise in enhancing humoral

196 responses against SARS-CoV-2 VOCs. A third dose of BNT162b2, for instance, was reported to
197 increase neutralizing activity against the Delta variant over 5-fold in 18-55-year-olds and over 11-
198 fold in 65-85-year-olds (Pfizer Second Quarter 2021 Earnings Report). We have observed in Phase
199 1 trial participants that a third dose of INO-4800, our DNA vaccine candidate for COVID-19,
200 results in higher levels of cellular and humoral immune responses without increased levels of
201 adverse events [43].

202

203 We recently described the design, immunogenicity, and efficacy of INO-4802, a synthetic DNA
204 vaccine expressing a pan-Spike immunogen aimed at inducing broad immunity across SARS-
205 CoV-2 VOCs [17]. In a hamster challenge model, INO-4802 conferred protection following
206 intranasal challenge with either the Wuhan-Hu-1, B.1.1.7, B.1.351, P.1, or B.1.617.2 (Delta)
207 SARS-CoV-2 variants. Additionally, INO-4802 showed promise as a heterologous booster vaccine
208 by enhancing humoral responses against VOCs in hamsters previously immunized with INO-4800.
209 Here, we addressed the immunogenicity of INO-4800 and INO-4802 as booster regimens in rhesus
210 macaques previously immunized with INO-4800 using clinically relevant dosing parameters.

211 Rhesus macaques receiving booster immunizations of either INO-4800 or INO-4802 showed a
212 robust induction of humoral responses, supporting the use of either vaccine in a prime/boost
213 regimen. Importantly, boosting of INO-4800-primed animals with INO-4800 or INO-4802
214 resulted in neutralizing antibody responses that were magnitudes greater compared to pre-boost
215 levels. Both treatment groups induced humoral responses capable of neutralizing wild-type and
216 several VOC pseudoviruses, suggesting broad protection among SARS-CoV-2 variants.
217 Pseudovirus neutralizing activity against the Beta and Gamma variants trended higher in animals
218 boosted with the heterologous INO-4802 vaccine compared to those receiving INO-4800,

219 indicating potential for an enhanced level of protection against some emerging SARS-CoV-2
220 variants following boosting with the next-generation pan-SARS-CoV-2 vaccine.

221

222 Neutralizing antibody responses correlated with inhibition of ACE2 binding activity, further
223 supporting the functional antibody responses following either a homologous or heterologous boost
224 with synthetic DNA vaccine constructs. Levels of antibodies binding variant Spike proteins were
225 also increased following the boost immunization in both treatment groups. Together, these data
226 point to broad functional humoral responses following a boost with both the original INO-4800
227 and INO-4802 pan-SARS-CoV-2 DNA vaccines. The rapid boost in neutralizing antibody
228 responses can likely be attributed to the maintenance of a memory B cell pool following the
229 priming immunization. Similar increases in humoral responses are observed in COVID-19
230 convalescent individuals who later received SARS-CoV-2 mRNA vaccines [44]. Longitudinal
231 analyses have also found that SARS-CoV-2-reactive memory B cell clones are stably maintained
232 in convalescent COVID-19 patients for several months following infection [30, 45]. Memory B
233 cell responses persist despite the natural decline of SARS-CoV-2-specific IgG binding titers,
234 suggestive of high-quality and durable memory B cell responses [46].

235

236 Neutralizing antibody responses are predictive of immune protection against symptomatic SARS-
237 CoV-2 infection [47], and as such, neutralizing antibodies are an important readout in the
238 evaluation of SARS-CoV-2 vaccines [48-50]. Owing to the critical role of T follicular helper (Tfh)
239 cells in providing help to maturing B cells in germinal centers, Tfh responses serve as a
240 mechanistic indicator of neutralizing antibody responses in infection and vaccination, including
241 for EUA SARS-CoV-2 vaccines [25-27, 51-53]. We observed a positive correlation between the

242 frequency of circulating Tfh cells and functional antibody responses, further affirming the
243 immunogenicity of SARS-CoV-2 DNA vaccine boosters in animals with existing vaccine-induced
244 immunity.

245
246 Overall, the development of safe and effective booster vaccines will be critical in maintaining
247 control of SARS-CoV-2 in the long term. Ideal treatment regimens should seek to expand immune
248 coverage to emerging variants while maintaining immune responses to existing SARS-CoV-2
249 variants. Current focus has shifted to evaluating the cross-immunogenicity of booster vaccines
250 against wild-type SARS-CoV-2 antigens and other VOCs and re-designing vaccines to investigate
251 this important question. In this study, we report that the next-generation pan-SARS-CoV-2 vaccine
252 INO-4802 boosts immune responses in animals primed with the wild-type-matched SARS-CoV-2
253 DNA vaccine INO-4800. These data support the immunogenicity and boosting capability of INO-
254 4800 and INO-4802 in nonhuman primates, which may have broader application in the clinical
255 setting.

256

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262

263 **Author Contributions**

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273

274 **Declaration of Interests:**

275 **A.P., E.L.R., E.P., E.N.G., M.P., D.F., F.I.Z, A.K.,** declare no competing interests. **J.N.W., B.S.,**
276 **K.S., I.M., Z.E., A.D., D.E., A.G., V.M.A., J.J.K., L.M.H., S.J.R., T.R.F.S., K.E.B.** are
277 employees of Inovio Pharmaceuticals and as such receive salary and benefits, including ownership
278 of stock and stock options, from the company. **D.B.W.** discloses the following paid associations
279 with commercial partners: GeneOne (Consultant), Geneos (Advisory Board), Astrazeneca
280 (Advisory Board, Speaker), Inovio (BOD, SRA, Stock), Pfizer (Speaker), Merck (Speaker), Sanofi
281 (Advisory Board), BBI (Advisory Board).

282

283 **Methods**

284 ***Constructs***

285 The plasmid designs for INO-4800 and INO-4802 have been previously described [10, 17]. For INO-
286 4802, a SynCon® consensus sequence for the SARS-CoV-2 spike harboring focused RBD
287 mutations and 2P mutation was codon-optimized using Inovio's proprietary optimization
288 algorithm. The final sequence was subcloned into the pGX0001 vector (BamHI/XhoI) and
289 synthesized (Genscript, Piscataway, NJ).

290

291 *Animal Immunizations, sample collection*

292 All rhesus macaque experiments were approved by the Institutional Animal Care and Use
293 Committee at Bioqual (Rockville, Maryland), an Association for Assessment and Accreditation of
294 Laboratory Animal Care (AAALAC) International accredited facility. Nine Chinese rhesus
295 macaques, five males and four females roughly 4 years of age (weights ranging from 4.48kg-
296 8.50kg) were randomized prior to immunization and received one or two injections at 1mg per
297 dose of INO-4800, at weeks 0 and 4 by intradermal electroporation (ID-EP) administration using
298 the CELLECTRA 2000® Adaptive Constant Current Electroporation Device with a 3P array
299 (Inovio Pharmaceuticals). Approximately one year post prime immunization, the study animals
300 were randomized and received a boost immunization at 1mg per dose of INO-4800 or INO-4802
301 by ID-EP. At the indicated time-points, blood was collected to analyse blood chemistry and to
302 isolate peripheral blood mononuclear cells (PBMC) and serum.

303

304 *Peripheral blood mononuclear cell isolation and IFN- γ Enzyme-linked immunospot (ELISpot)*

305 Blood was collected from each study animal into sodium citrate cell preparation tubes (CPT, BD
306 Biosciences). The tubes were centrifuged to separate plasma and lymphocytes, according to the

307 manufacturer's protocol. Samples from the prime immunization were transported by same-day
308 shipment on cold-packs from Bioqual to The Wistar Institute, and boost samples were shipped
309 overnight to Inovio Pharmaceuticals for PBMC isolation. PBMCs were washed, and residual red
310 blood cells were removed using ammonium-chloride-potassium (ACK) lysis buffer. Cells were
311 counted using a ViCell counter (Beckman Coulter) and resuspended in RPMI 1640 (Corning),
312 supplemented with 10% fetal bovine serum (Seradigm), and 1% penicillin/streptomycin (Gibco).
313 Fresh cells were then plated for IFN γ ELISpot assay to detect cellular responses.

314

315 Monkey IFN- γ ELISpotPro plates (Mabtech, Sweden, Cat#3421M-2APW-10) were prepared
316 according to the manufacturer's protocol. Freshly isolated PBMCs were added to each well at
317 200,000 cells per well in the presence of either 1) SARS-CoV-2-specific peptide pools, 2) R10
318 with DMSO (negative control), or 3) anti-CD3 positive control (Mabtech, 1:1000 dilution), in
319 triplicate. Plates were incubated overnight at 37°C, 5% CO $_2$, then after a minimum incubation of
320 18 hours, plates were developed according to the manufacturer's protocol. Spots were imaged
321 using a CTL Immunospot plate reader and antigen-specific responses determined by subtracting
322 the R10-DMSO negative control wells from the wells stimulated with peptide pools.

323

324 *Antigen Binding ELISA*

325 Serum collected at each time point was evaluated for binding titers as previously described [10].
326 For prime immunization samples, ninety-six well immunosorbent plates (NUNC) were coated with
327 1 μ g/mL recombinant SARS-CoV-2 S1+S2 ECD protein (Sino Biological 40589-V08B1), S1
328 protein (Sino Biological 40591-V08H), S2 protein (Sino Biological 40590-V08B), or receptor-

329 binding domain (RBD) protein (Sino Biological 40595-V05H) in PBS overnight at 4°C. For boost
330 samples, ELISA half-area plates were also coated with 1 µg/mL recombinant spike wild-type spike
331 protein, B.1.351, P.1 and B.1.617.2 full length spike variant proteins (Acro Biosystems #SPN-
332 C52H8, #SPN-C52Hc, #SPN-C52Hg and #SPN-C52He respectively). Secondary antibodies
333 included IgG (Bethyl #A140-202P) at 1:50,000. Plates were washed three times with PBS + 0.05%
334 Tween20 (PBS-T) and blocked with 3% FBS in PBS-T for 2 hours at room temperature (RT).
335 Sera from vaccinated macaques were serially diluted in PBS-T + 1% FBS, added to the washed
336 ELISA plates, and then incubated for 2 hours at RT. Plates were then washed and incubated with
337 an anti-monkey IgG conjugated to horseradish peroxidase (Bethyl A140-202P) 1 hour at RT.
338 Within 30 minutes of development, plates were read at 450nm using a Biotek Synergy2 plate
339 reader.

340

341 ***Meso Scale Discovery ACE2 Inhibition Assay***

342 Meso Scale Discovery (MSD) V-PLEX SARS-CoV-2 ACE2 Neutralization Kit, Panels 5 and 14
343 were used to evaluate sera collected from immunized study animals according to the
344 manufacturer's instructions with the MSD Sector S 600 instrument. Briefly, MSD plates
345 containing SARS-CoV-2 Spike proteins (wildtype, B.1.1.7, B.1.351, P.1 and B.1.617.2) were
346 blocked, washed, and incubated with sera from vaccinated animals at a 1:27 dilution. Plates were
347 then washed and incubated with SULFO-TAG ACE2 and developed according to the
348 manufacturer's protocol. Functional antibody activity was measured as percent inhibition of
349 binding of SULFO-TAG ACE2 to Spike protein.

350

351 ***Pseudovirus Neutralization Assay***

352 SARS-CoV-2 pseudovirus stocks encoding for the WT, B.1.1.7, P.1, B.1.351 or B.1.617.2 Spike
353 protein were produced as previously described (Smith et al Nature 2020, Andrade et al bioRxiv
354 2021). To assess the extent of which neutralizing antibodies are present in the sera, CHO cells
355 stably expressing ACE2 (ACE2-CHOs – Creative Biolabs) were used as target cells at 10,000
356 cells/well. Sera was heat inactivated and serially diluted prior to incubation with the different
357 SARS-CoV-2 variant pseudoviruses. After a 90-minute incubation, sera-pseudovirus mixture was
358 added to ACE2-CHOs, then 72 hours later, cells were lysed using Bright-Glo™ Luciferase Assay
359 (Promega) and RLU was measured using an automated luminometer. Neutralization titers (ID₅₀)
360 were calculated using GraphPad Prism 8 and defined as the reciprocal serum dilution that is
361 reduced by 50% compared to the signal in the infected control wells.

362

363 **Flow Cytometry**

364 Thawed, cryopreserved PBMCs were assessed to determine the frequency of circulating T
365 follicular helper (Tfh) using a panel which included the following antibodies: CD3 (BD
366 Biosciences; clone SP34-2), CD4 (BD Biosciences; clone L200), CXCR5 (eBioscience; clone
367 MU5UBEE), and PD-1 (BioLegend; clone EH12.2H7). Tfh cells were identified as
368 CD3+/CD4+/CXCR5+/PD-1+. Samples were acquired on a BD Celesta flow cytometer and
369 analysed using FlowJo software version 10.7 (Treestar Inc.).

370

371 **Figure titles and legends**

372 **Figure 1. Study design and durability of humoral immune responses in rhesus macaques**
373 **primed with INO-4800. A)** Schematic depicting the prime immunization schedule and sample
374 collection timepoints. Note: The longitudinal collection for the NHPs in the 1mg dose group ended
375 at Week 35 and for 2mg dose group at Week 52. **B)** Longitudinal serum IgG binding titers in rhesus
376 macaques vaccinated with 1 or 2 mg INO-4800 at weeks 0 and 4. Antibody titers in the sera were
377 measured against the wildtype SARS-CoV-2 Spike protein antigen. **C-F)** Longitudinal serum
378 pseudovirus neutralizing activity in rhesus macaques between weeks XX. Neutralizing activity
379 (ID₅₀) was measured against the wildtype (Wuhan) SARS-CoV-2 (C), B.1.1.7 (D), B.1.351 (E) and
380 P.1 (F) pseudoviruses.

381

382 **Figure 2. Humoral immune responses following homologous or heterologous boost in INO-**
383 **4800-primed rhesus macaques.** Antibody responses were measured in animals boosted with 1
384 mg of either the homologous INO-4800 (purple symbols) or heterologous INO-4802 (blue
385 symbols) vaccines on the day of the boost (week 0) and at weeks 2 and 4 post-boost. **A)** Schematic
386 of the boost schedule showing the vaccine groups with the respective animal IDs. **B)** Serum IgG
387 binding titers in rhesus macaques boosted with INO-4800 or INO-4802. Binding titers were
388 measured against the wildtype (top left) B.1.351 (top right), P.1 (lower left), and B.1.617.2 (lower
389 right) Spike proteins. **C)** Serum pseudovirus neutralizing activity in rhesus macaques boosted with
390 INO-4800 or INO-4802. Neutralizing activity was measured against the wildtype (top left) B.1.351
391 (top right), P.1 (lower left), and B.1.617.2 (lower right) pseudoviruses. **D)** ACE2 blocking activity
392 in the serum collected from rhesus macaques boosted with INO-4800 or INO-4802. Inhibition of

393 ACE2 binding was measured against the wildtype (top left) B.1.351 (top right), P.1 (lower left),
394 and B.1.617.2 (lower right) Spike proteins.

395

396 **Figure S1. Humoral Responses in INO-4800 vaccinated rhesus macaques.** IgG binding was
397 measured in sera from INO-4800 vaccinated rhesus macaques to SARS-CoV-2 RBD (A), S1 (B)
398 and S2 (C) protein antigens.

399

400 **Figure S2. Functional antibody responses following homologous or heterologous boost in**
401 **INO-4800-primed rhesus macaques.** A) Spearman correlation of ACE2 blocking activity and
402 neutralizing activity among animals boosted with either INO-4800 or INO-4802. Correlations
403 relating to functional antibody responses against the wildtype (left) B.1.351 SARS-CoV-2 (center),
404 and B.1.617.2 (right) variants at weeks 2 and 4 post-boost are shown. B) Spearman correlation of
405 the frequency of circulating T follicular helper cells with ACE-2 binding inhibition at week 2 post-
406 boost.

407

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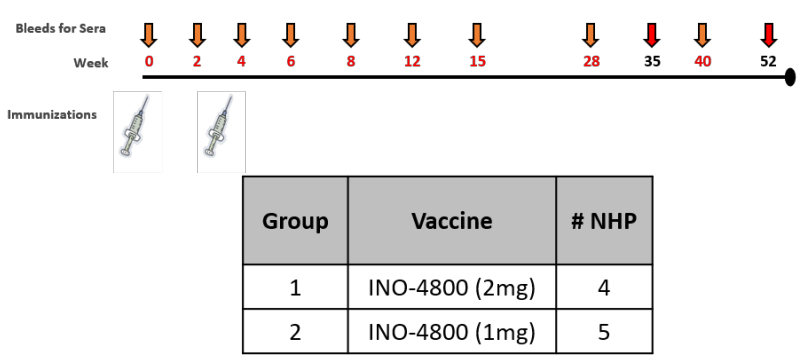
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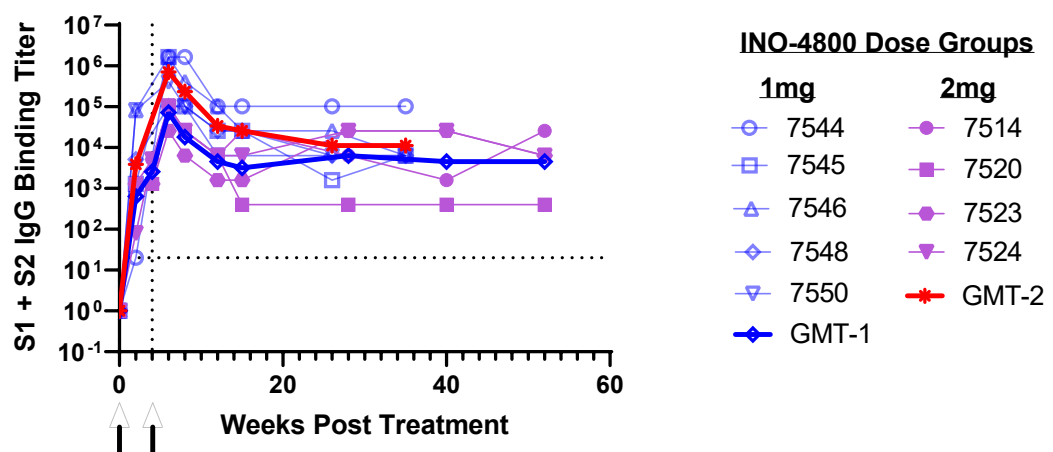
Figure 1

Prime

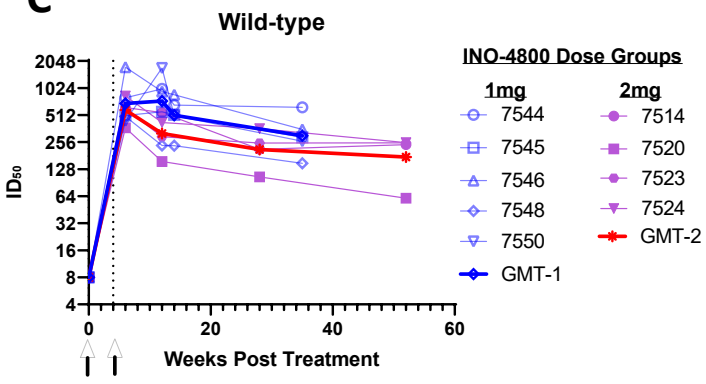
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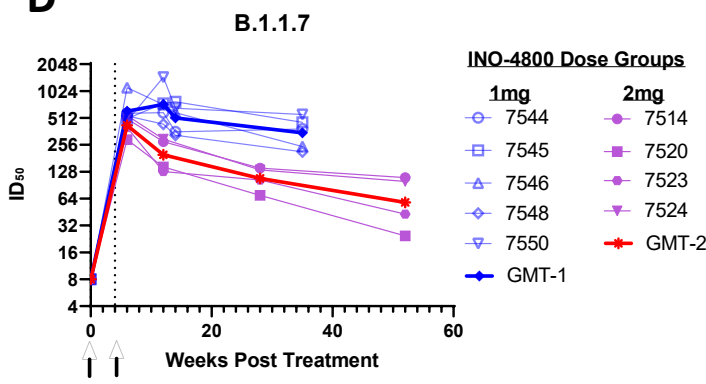
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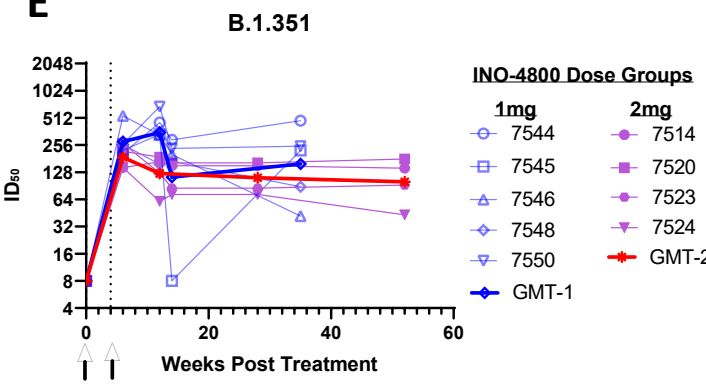
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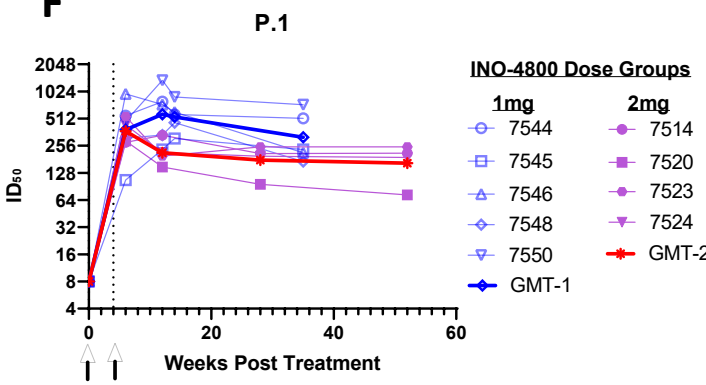
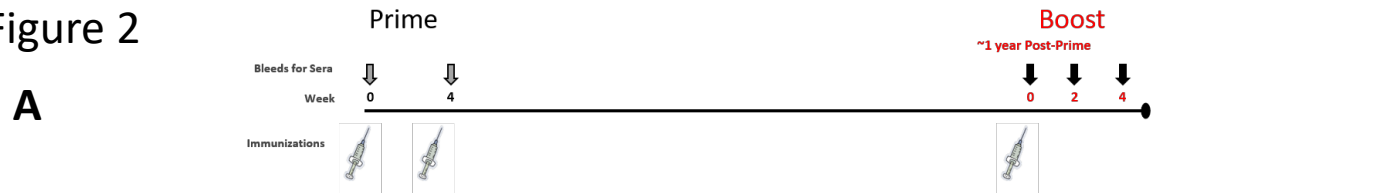
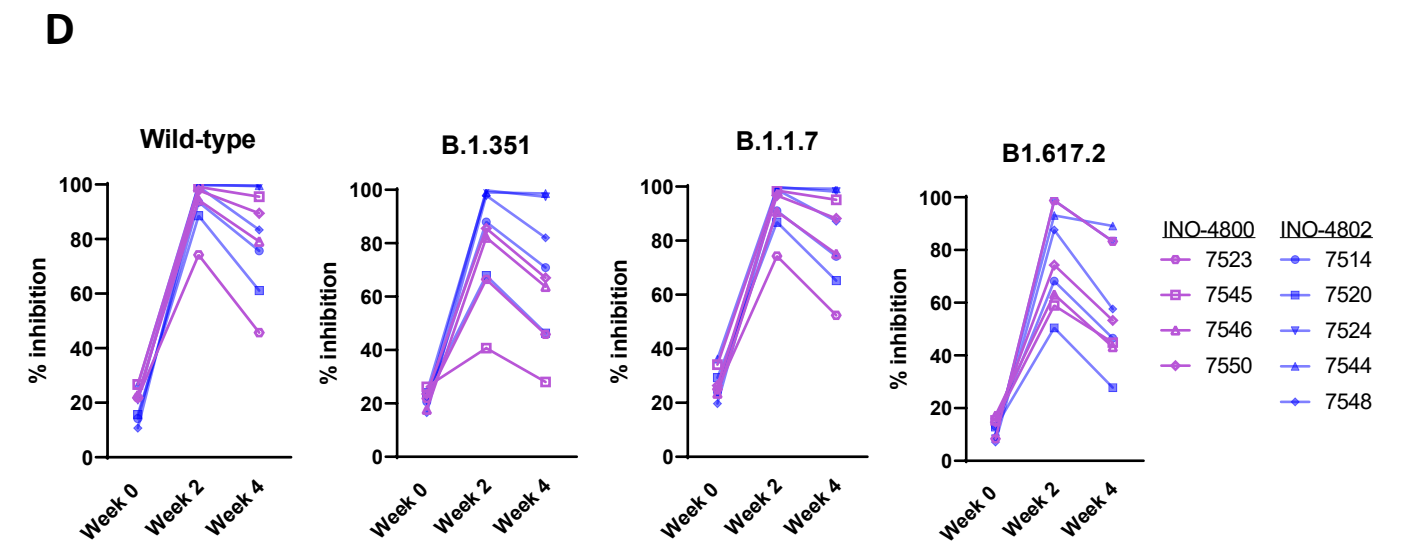
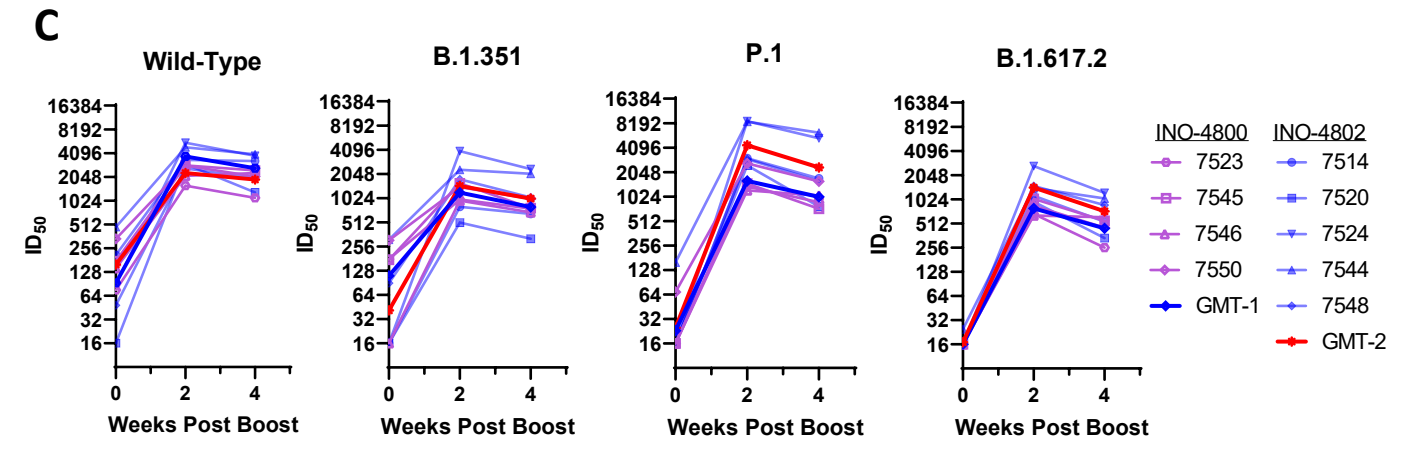
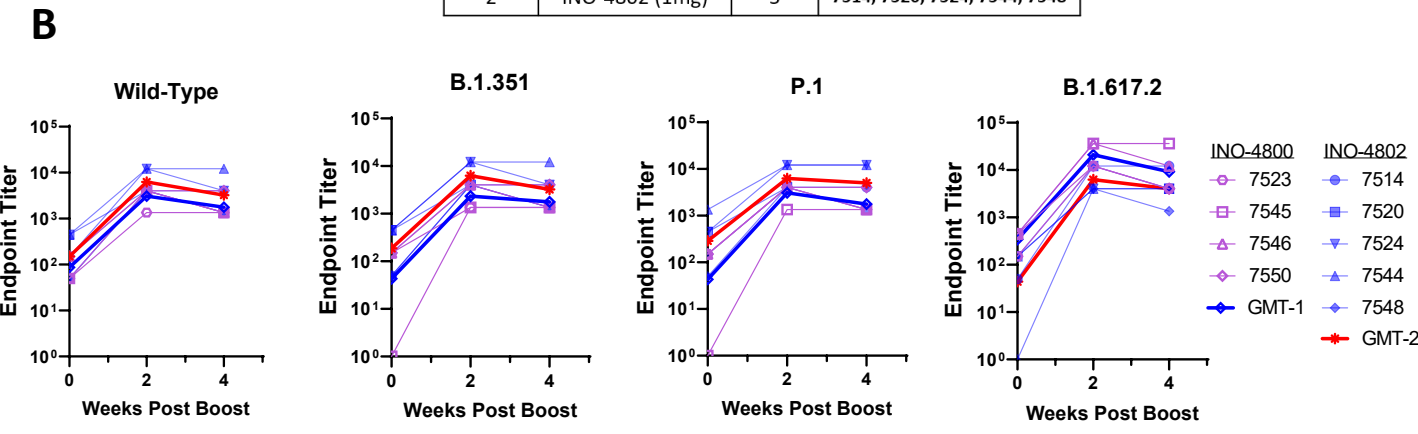


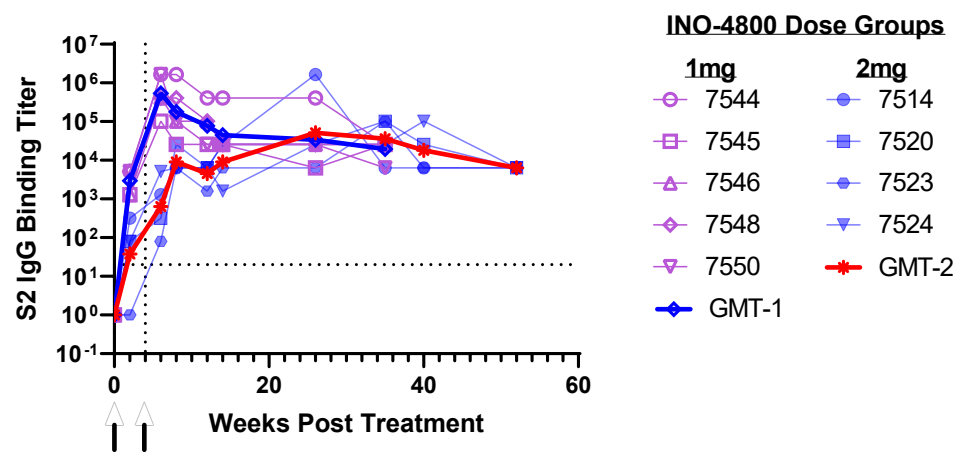
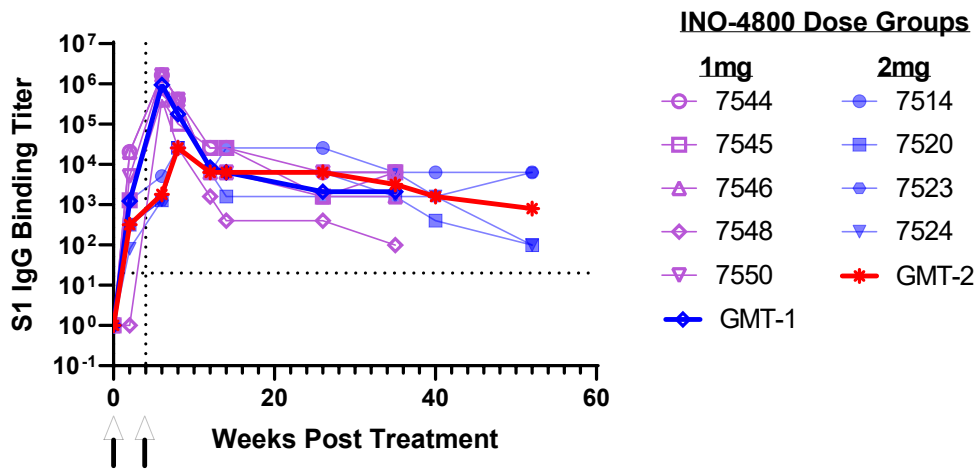
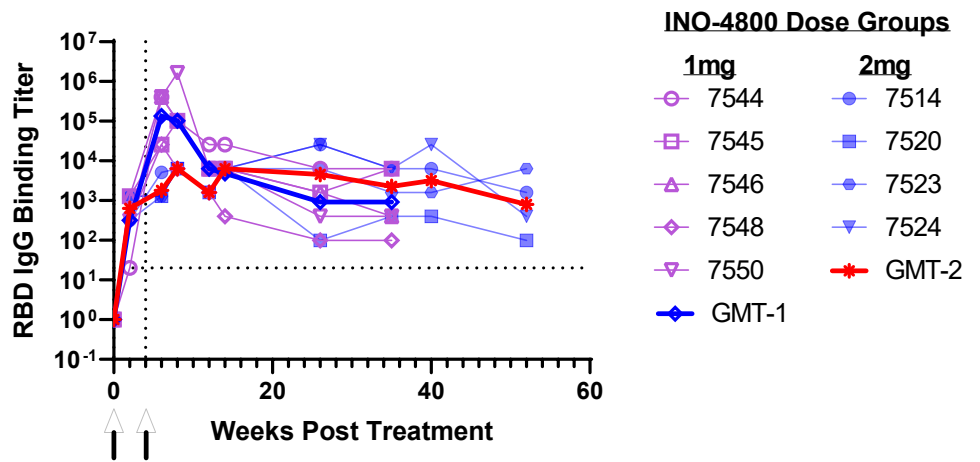
Figure 2



Randomized Animals from Prime			
Group	Vaccine	# NHP	Animal IDs
1	INO-4800 (1mg)	4	7523, 7545, 7546, 7550
2	INO-4802 (1mg)	5	7514, 7520, 7524, 7544, 7548

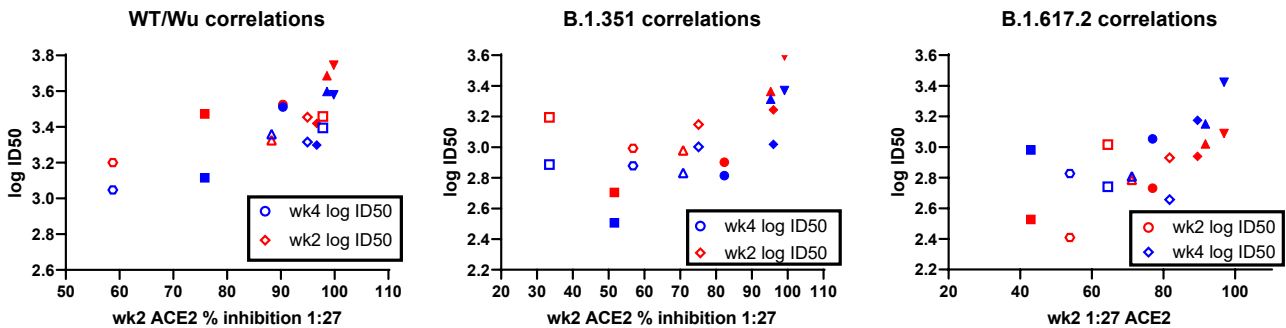


Supplemental Figure 1



Supplemental Figure 2

A



Spike Variant	Spearman r value (Week 2)	Spearman p value (Week 2)
WT	0.667	0.059
B.1.351	0.617	0.086
B.1.617.2	0.717	0.037

Spike Variant	Spearman r value (Week 4)	Spearman p value (Week 4)
WT	0.783	0.017
B.1.351	0.683	0.050
B.1.617.2	0.833	0.008

B

