

1 **Title: Efficacy and breadth of adjuvanted SARS-CoV-2 receptor-binding domain nanoparticle**
2 **vaccine in macaques**

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45 **ABSTRACT**

46 Emergence of novel variants of the severe acute respiratory syndrome coronavirus-2 (SARS-
47 CoV-2) underscores the need for next-generation vaccines able to elicit broad and durable
48 immunity. Here we report the evaluation of a ferritin nanoparticle vaccine displaying the
49 receptor-binding domain of the SARS-CoV-2 spike protein (RFN) adjuvanted with Army
50 Liposomal Formulation QS-21 (ALFQ). RFN vaccination of macaques using a two-dose regimen
51 resulted in robust, predominantly Th1 CD4+ T cell responses and reciprocal peak mean
52 neutralizing antibody titers of 14,000-21,000. Rapid control of viral replication was achieved in
53 the upper and lower airways of animals after high-dose SARS-CoV-2 respiratory challenge, with
54 undetectable replication within four days in 7 of 8 animals receiving 50 µg RFN. Cross-
55 neutralization activity against SARS-CoV-2 variant B.1.351 decreased only ~2-fold relative to
56 USA-WA1. In addition, neutralizing, effector antibody and cellular responses targeted the
57 heterotypic SARS-CoV-1, highlighting the broad immunogenicity of RFN-ALFQ for SARS-like
58 betacoronavirus vaccine development.

59

60 **Keywords:** SARS-CoV-2, vaccine, receptor-binding domain

61

62 **Significance Statement**

63 The emergence of SARS-CoV-2 variants of concern (VOC) that reduce the efficacy of current
64 COVID-19 vaccines is a major threat to pandemic control. We evaluate a SARS-CoV-2 Spike
65 receptor-binding domain ferritin nanoparticle protein vaccine (RFN) in a nonhuman primate
66 challenge model that addresses the need for a next-generation, efficacious vaccine with increased
67 pan-SARS breadth of coverage. RFN, adjuvanted with a liposomal-QS21 formulation (ALFQ),

68 elicits humoral and cellular immune responses exceeding those of current vaccines in terms of
69 breadth and potency and protects against high-dose respiratory tract challenge. Neutralization
70 activity against the B.1.351 VOC within two-fold of wild-type virus and against SARS-CoV-1
71 indicate exceptional breadth. Our results support consideration of RFN for SARS-like
72 betacoronavirus vaccine development.

73 INTRODUCTION

74 The coronavirus infectious disease 2019 (COVID-19) pandemic, precipitated by severe acute
75 respiratory syndrome coronavirus-2 (SARS-CoV-2), continues to threaten global public health
76 and economies. Threats of future outbreaks also loom, as evidenced by three emergent SARS-
77 like diseases caused by zoonotic betacoronaviruses in the last two decades. While several
78 emergency use authorized (EUA) vaccines currently in use are expected to curb both disease and
79 transmission of SARS-CoV-2 (1-6), the emergence of circulating variants of concern (VOC) less
80 sensitive to vaccine-elicited immunity has raised concerns for sustained vaccine efficacy (7).
81 Logistic challenges of vaccine production, distribution, storage and access for these vaccines will
82 need to be resolved equitably to achieve resolution to the pandemic (8, 9). The rapid and
83 unparalleled spread of SARS-CoV-2 has driven an urgent need to deploy scalable vaccine
84 platforms to combat the ongoing pandemic and mitigate future outbreaks.

85
86 Current vaccines primarily focus the immune response to the spike glycoprotein (S) on the virion
87 surface as it mediates host cell viral fusion and entry. The receptor-binding domain (RBD) of S
88 engages the primary host cell receptor, Angiotensin-converting enzyme 2 (ACE2), for both
89 SARS-CoV-2 and SARS-CoV-1, making RBD a promising domain for vaccine elicited immune
90 focus (10-12). Moreover, many of the potently neutralizing monoclonal antibodies isolated
91 against SARS-CoV-2 target the RBD (13, 14). Vaccination of nonhuman primates with RBD-
92 encoding RNA or DNA protects against respiratory tract challenge, indicating that immune
93 responses to the RBD can prevent viral replication (15, 16). RBD vaccination also elicits cross-
94 reactive responses to circulating SARS-CoV-2 VOC in both animals and humans (17, 18), with
95 decrements against the more difficult to neutralize B.1.351 variant similar to that seen with S

96 immunogens (19). The breadth of RBD immunogenicity is further supported by the ability of
97 RBD-specific monoclonal antibodies isolated from SARS-CoV-1 convalescent individuals to
98 cross-neutralize SARS-CoV-2 (20, 21). These findings suggest potential for RBD-based
99 vaccines being efficacious against SARS-CoV-2 variants and other coronavirus species.
100
101 Approaches to improve immunogenicity of S or RBD protein vaccines include optimizing
102 antigen presentation and co-formulating with adjuvants to enhance the protective immunity. One
103 common approach to enhance the elicitation of adaptive immune responses is the multimeric
104 presentation of antigen, for example, on the surface of nanoparticles or virus-like particles (22).
105 Presenting RBD in ordered, multivalent arrays on the surface of self-assembling protein
106 nanoparticles is immunogenic and efficacious in animals (23-28), with improved
107 immunogenicity relative to monomeric soluble RBD and cross-reactive responses to variants (17,
108 24, 26). However, it is unknown whether RBD nanoparticle vaccines are able to protect against
109 infection in primates, which have become a standard model for benchmarking performance of
110 vaccine candidates. Liposomal adjuvants incorporating QS-21, such as that used in the
111 efficacious varicella zoster vaccine, SHINGRIX[®], may augment protective immunity to SARS-
112 CoV-2 vaccines. Such adjuvants have previously demonstrated superior humoral and cellular
113 immunogenicity relative to conventional adjuvants (29, 30).
114
115 Here, we evaluate the use of a ferritin nanoparticle vaccine presenting the SARS-CoV-2 RBD
116 (RFN) adjuvanted with the Army Liposomal Formulation QS-21 (ALFQ) (31). Both ferritin
117 nanoparticles and ALFQ have been evaluated for vaccination against multiple pathogens in
118 humans in phase 1 clinical trials (32-34). We demonstrate in a nonhuman primate model that

119 immunization with RFN induces robust and broad antibody and T cell responses, as well as
120 protection against viral replication and lung pathology following high-dose respiratory tract
121 challenge.

122

123 **RESULTS**

124 **Vaccine and animal study design**

125 A SARS-CoV-2 RBD ferritin nanoparticle vaccine (RFN) was designed as a ferritin-fusion
126 recombinant protein that self-assembles into a 24-mer nanoparticle displaying a multivalent,
127 ordered array of RBD on its surface. Briefly, the RBD protein sequence (residues 331-527)
128 derived from the Wuhan-Hu-1 genome sequence (GenBank accession number: MN908947.3)
129 was covalently linked to the C-terminal region of the *Helicobacter pylori* ferritin molecule.
130 Twenty-three rhesus macaques were immunized with either 50 or 5 µg of RFN, or sham-
131 immunized with PBS (n=7/8 per group), at study weeks 0 and 4 (Fig. 1A). RFN was adjuvanted
132 with ALFQ, which contains synthetic monophosphoryl 3-deacyl lipid A and QS-21. Animals
133 were challenged 4 weeks after the last immunization via combined intratracheal (IT, 1.0 mL) and
134 intranasal (IN, 0.5 mL per nostril) inoculation of a 10⁶ TCID₅₀ dose of SARS-CoV-2 virus
135 (USA-WA1/2020). Animals were followed for 7 (n=12) or 14 days (n=11) following challenge
136 for immunologic, virologic, and pathologic assessments.

137

138 **Humoral responses to vaccination**

139 Multiple vaccine-matched humoral immune responses were measured longitudinally in serum
140 following vaccination. First, binding antibody responses to the SARS-CoV-2 prefusion stabilized
141 S protein (S-2P) (35) were assessed by MSD. Immunization with either 5 or 50 µg of RFN

142 elicited S-specific IgG two weeks following the prime (21,896 and 79,109 AU/mL, respectively)
143 (Fig. 1B). These responses increased two weeks following the second immunization (420,249
144 and 293,509 AU/mL). Boosting was greater with the 5 μ g dose, achieving a 19-fold increase
145 relative to post-prime versus \sim 3.7-fold with 50 μ g. Responses continued to marginally increase
146 four weeks following the second immunization as well as two weeks post-challenge.
147 Unvaccinated control animals mounted responses \sim 1,000-fold over baseline within two weeks
148 post challenge and these responses were \sim 65-fold lower than those in vaccinated animals after
149 challenge.

150
151 Given the importance of the RBD in mediating viral entry and the majority of neutralizing
152 antibody responses targeting this domain, RBD-specific humoral responses were also measured.
153 RFN induced binding antibodies four weeks following the second immunization, with no
154 significant difference between vaccine dose groups (Fig. 1C). Responses in vaccinated animals
155 were \sim 60,000-fold over the background in unvaccinated controls and comparable in magnitude
156 to those against the S protein, consistent with an RBD-focused response. To confirm these
157 findings, the on-rate association between serum antibodies and RBD antigen was measured by
158 biolayer interferometry and longitudinal responses tracked with S-binding and pseudovirus
159 neutralizing responses (Fig. S1). Again, vaccine dose groups did not differ. Functional activity of
160 serum antibodies to inhibit ACE2 binding to the RBD antigen was also measured and present,
161 with high magnitude responses elicited by RFN at both the 5 and 50 μ g doses (Fig. 1D).

162
163 Neutralizing antibody responses against SARS-CoV-2 using a pseudovirus neutralization assay
164 followed a similar pattern as the S-specific binding responses (Fig. 1E). Peak ID₅₀ titers of

165 14,540 and 21,298 were observed two weeks following the boost for the 5 and 50 μg RFN doses,
166 respectively. Neutralizing responses increased markedly between the prime and boost, rising 48-
167 and 32-fold between study weeks two and six. Among the 50 μg RFN vaccinated animals
168 followed two weeks post-challenge, neutralizing responses declined six weeks post-boost by
169 approximately one log relative to peak values, indicating neutralizing responses may decay more
170 quickly than binding antibodies.

171
172 Neutralizing responses were also evaluated using an authentic SARS-CoV-2 virus neutralization
173 assay (USA-WA1 isolate). Robust neutralizing titers were detected in all RFN vaccinated
174 animals (Fig. 1F). Median EC_{50}^{-1} titers were $\sim 3,800$ for both dose groups, though slightly more
175 variable with 5 μg dosing. This result paralleled responses assessed by a pseudovirus assay (Fig.
176 1E,G). Since serum from convalescent COVID-19 human cases is frequently used as a
177 benchmarking reference for vaccine immunogenicity in clinical and pre-clinical studies, we
178 compared RFN-vaccinated macaque pseudovirus neutralizing titers to those of 41 convalescent
179 individuals 4-8 weeks post-COVID infection. Responses in the 50 μg group were on average 13-
180 fold higher than the convalescent individuals, indicating that RFN elicited higher antibody titers
181 than observed in the first months following human infection. Summarizing, RFN vaccination
182 generated strong RBD-specific binding antibodies with potent neutralization activity which block
183 the interaction between the RBD region of SARS-CoV-2 S and the host ACE2 receptor.

184
185 Non-neutralizing antibody effector functions associated with vaccine-mediated protection against
186 other viruses may also be important for SARS-CoV-2 (36, 37). Strong IgG-mediated cellular
187 opsonization responses were observed following the second immunization, while IgM and IgA

188 were more modest (Fig. S2A-C). Serum antibody-dependent phagocytosis mediated by either
189 monocytes (ADCP) or neutrophils (ADNP) as well as complement deposition (ADCD)
190 responses were also robust in both vaccinated groups and consistently peaked at week six (Fig.
191 S2D-F). A similar pattern was seen for antibody-dependent trogocytosis (38) (Fig. S2G).
192 Overall, 5 μ g RFN achieved equal Fc-mediated effector functions compared to 50 μ g, though
193 ADCD responses trended \sim 1.25-fold greater with the higher dose.

194

195 **Virus-specific T cell responses**

196 SARS-CoV-2-specific T cell immunity is associated with reduced disease severity and can
197 influence antibody responses (39, 40). We assessed S-specific T cells in PBMCs by *in vitro*
198 peptide stimulation and intracellular cytokine staining using a 19-color multiparameter flow
199 cytometry panel for detailed functional characterization of T cell responses from RFN
200 vaccination. A vigorous, dose-dependent Th1 (TNF, IL-2, IFN- γ) CD4⁺ T cell response was
201 observed in all RFN vaccinated animals 4 weeks after the second vaccination, ranging from 0.4-
202 5.2% of memory cells (Fig. 2A). These S-specific Th1 cells were polyfunctional in quality, a
203 property associated with control of other pathogens (41), as a large proportion simultaneously
204 expressed multiple Th1 cytokines (Fig. S3A). Th2 responses were low or undetectable (Fig. 2B),
205 with median Th1/Th2 ratios of \sim 20 among 50 μ g-vaccinated animals with evidence of a Th2
206 response (Fig. S3B). CD8⁺ T cell responses were observed in about half of the animals and were
207 more prominent in recipients of 50 μ g than 5 μ g RFN (Fig. S3C). Response magnitude was
208 \sim 0.1-0.4% of memory CD8⁺ T cells.

209

210 Additional CD4⁺ T cell functions important for the development of antibody responses were
211 evaluated. S-specific CD4⁺ T cell IL-21 responses, a surrogate marker of peripheral T follicular
212 helper cell activity, were observed in the majority of animals vaccinated with 50 µg and in half
213 of the animals vaccinated with 5 µg RFN (Fig. 2C). The average frequency in responders was
214 0.15%. The CD4⁺ T cell activation marker, CD40L, which promotes B cell antibody isotype
215 switching, was highly expressed by S-specific cells (Fig. 2D). Responses ranged from ~1-7%
216 after 50 µg RFN and were observed in all eight animals, while response rates and magnitude
217 were slightly reduced with the 5 µg dose (~0.7-2% in six of seven animals). Overall, these data
218 show that adjuvanted RFN induced robust Th1-polarized polyfunctional CD4⁺ T cells favorable
219 for viral clearance and with critical B-cell help capability.

220

221 **SARS-CoV-2 challenge efficacy**

222 To assess the protective efficacy of RFN vaccination, animals were challenged with high-dose
223 (10^6 TCID₅₀) SARS-CoV-2 USA-WA1 administered via the simultaneous IN/IT routes four
224 weeks following the second immunization. The presence of viral RNA was assessed in both the
225 upper (NP swabs and saliva) and lower (BAL) respiratory tract. Measurements were made of
226 both total RNA and subgenomic E mRNA (sgmRNA), considered a more specific indicator of
227 viral replication (42, 43). Unvaccinated control animals all showed evidence of a robust
228 infection, with mean levels of sgmRNA in the BAL of $\sim 10^6$ copies/mL, and in the NP swabs of
229 $\sim 10^7$ copies/mL at day 2 post-challenge (Fig. 3). Moreover, viral replication was sustained at
230 $>10^4$ copies/mL sgmRNA for 7 days in the upper airways. In RFN vaccinated animals, the
231 magnitude and duration of viral replication was markedly reduced. In the 50 µg group, day 1
232 sgmRNA was reduced by 1 and 2 logs in the BAL and NP swabs, respectively. Rapid clearance

233 was observed by day 2 in five of eight animals in the upper airways and four of eight in the lower
234 airways. Both airways were void of replicating virus in all but one animal by day 4. Viral
235 control was also apparent after 5 μ g RFN vaccination, though with slightly more breakthrough
236 replication early after challenge. The majority of animals had no detectable sgmRNA by day 4 in
237 both the upper and lower airways.

238

239 Viral replication was detected in saliva in all control animals on day 1 and persisted in five
240 animals through day 4 (Fig. 3C). Values were lower than those in BAL or NP swabs and tapered
241 to undetectable levels more rapidly. Overall fewer vaccinated animals contained sgmRNA in
242 their saliva and replicating virus was detected in only one animal from each vaccine dose group
243 starting on day 2. The kinetics of SARS-CoV-2 total RNA, which is more likely to reflect
244 material from the viral inoculum, paralleled results described above for sgmRNA in BAL, NP
245 swabs and saliva (Fig. S4).

246

247 **Respiratory tract pathology and antigen expression**

248 Vaccine efficacy was also assessed by histopathologic analysis of lung tissue from 3-5 macaques
249 from each group necropsied at day 7 post-challenge. By this point, all unvaccinated animals had
250 developed histopathologic evidence of multifocal, mild to moderate interstitial pneumonia (Fig.
251 4A). The pneumonia was characterized by type II pneumocyte hyperplasia, alveolar edema,
252 alveolar inflammatory and necrotic debris, thickening of alveolar septae, increased numbers of
253 pulmonary macrophages (including multinucleated giant cells), and vasculitis of small- to
254 medium- caliber blood vessels. The middle and caudal lung lobes were most severely affected in
255 all four unvaccinated animals. Histologic evidence of interstitial pneumonia was not observed in

256 animals from any of the vaccinated groups (Fig. 4B,C). However, in each of the vaccine groups,
257 there was minimal to mild mononuclear to mixed cellular infiltrates centered on small-to-
258 medium caliber blood vessels. Immunohistochemistry demonstrated SARS-CoV-2 viral antigen
259 in small numbers of alveolar pneumocytes and pulmonary macrophages in at least one lung
260 section of every unvaccinated animal (Fig. 4D). No viral antigen was detected in the lungs of
261 any of the animals in any of the vaccine groups (Fig. 4E,F). Overall, pathological findings were
262 significantly reduced by vaccination (Fig. 4G). No significant histopathologic differences were
263 observed between vaccinated and unvaccinated animals at day 14, consistent with transient
264 SARS-CoV-2 pathology in this model. Mild perivascular infiltrates occasionally remained in
265 some animals from all groups. In sum, vaccination with 5 or 50 μ g RFN prevented moderate
266 disease and viral protein expression in the lungs.

267

268 **Cross-reactive immunity to emergent SARS-CoV-2 variants and SARS-CoV-1**

269 Given concerns about circulating SARS-CoV-2 viral variants' increased resistance to currently
270 available vaccines, we assessed RFN vaccinated macaque serum for neutralizing antibody
271 responses against two variants of concern, B.1.1.7 and B.1.351. In an authentic virus
272 neutralization assay, reciprocal neutralization ID₅₀ GMT titers against B.1.1.7 were 73,983 two
273 weeks following the second 50 μ g dose (Fig. 5A). This translated to ~3.8-fold greater titers than
274 those against the wild-type, vaccine-matched USA-WA1 strain. Titers against the two strains
275 were similar when measured by the pseudovirus neutralization assay (Fig. 5B). These trends
276 were observed regardless of vaccine dose, though responses were slightly lower with 5 μ g RFN.
277 GMT neutralization titers against B.1.351 decreased approximately 2-fold to 8,070 and 9,876 in
278 the 50 μ g dose group in the authentic virus and pseudovirus assays respectively, indicating only a

279 minor diminution in potency compared to USA-WA1 (Fig. 5A-C). Thus, RFN vaccination
280 elicited broadly reactive neutralizing antibody responses with potent activity against two
281 important variants. Serum binding to the variant forms of SARS-CoV-2 was also assessed by
282 biolayer interferometry (Fig. S6). In both vaccine groups no changes in binding were observed to
283 B.1.1.7 (N501Y mutation), while responses to B.1.351 (K417N, E484K, N501Y mutations)
284 trended ~15% lower in both vaccine groups.

285

286 In addition to SARS-CoV-2 variants of concern, another open question in the field is the ability
287 of existing SARS-CoV-2 vaccine platforms to protect against future SARS-like coronavirus
288 outbreaks. Cross-protective vaccine-elicited immunity against SARS-CoV-1 may be a useful
289 metric to address this question. We measured IgG antibody responses able to bind SARS-CoV-1
290 RBD by biolayer interferometry in macaque serum at week 2 following the second vaccination.
291 All RFN vaccinated animals developed cross-reactive binding antibodies to SARS-CoV-1 at
292 levels approximately half those to SARS-CoV-2 (Fig. 5D, Fig. S1). Binding responses were also
293 measured to a series of SARS-CoV-1 and SARS-CoV-2 antigens using a Luminex assay (Fig.
294 S5), with strong binding responses observed to the SARS-1 S1 subunit and RBD, but not against
295 the S2 subunit or N-terminal domain. SARS-CoV-1 RBD-specific binding antibody responses
296 were ~70% that of the SARS-CoV-2 response. The functional capacity of these cross-binding
297 antibodies to mediate effector activity was assessed in an ADCP assay using SARS-CoV-1
298 trimeric S antigen. SARS-CoV-1 ADCP responses were observed in plasma of all vaccinated
299 animals and were comparable between the dose groups (Fig. 5E).

300

301 Neutralizing titers against SARS-CoV-1 were measured using both authentic virus and
302 pseudovirus neutralization assays, with cross-neutralizing responses observed in most RFN
303 vaccinated animals (Fig. 5F,G). Significant authentic virus neutralization titers were elicited by
304 50 µg RFN two weeks following the second immunization. SARS-CoV-1 pseudovirus
305 neutralization activity was also observed in both the 50 and 5 µg groups, though background in a
306 subset of control animals limited interpretation of both assays.

307

308 To assess T cell immunity cross-reactivity to SARS-CoV-1, we evaluated whether the RFN
309 vaccine-elicited T cells could recognize SARS-CoV-1 S. PBMC stimulated with SARS-CoV-1 S
310 peptide pools were stained for intracellular cytokine expression to quantitate cross-reactive T
311 cells. Significant CD4⁺ T cell Th1 responses were observed following the 50 µg RFN
312 vaccination series, though they were ~5-fold lower in magnitude than those to SARS-CoV-2 S
313 (Fig. 5H). SARS-CoV-1 S-specific CD40L responses were comparable to the Th1 responses for
314 both dose groups (Fig. S7A). IL-21 and Th2 CD4⁺ T cell responses were minimal or negligible
315 (Fig. S7B,C). Significant cross-reactive CD8⁺ T cells were elicited and similar in magnitude to
316 SARS-CoV-2-specific responses (~0.1-0.3%) (Fig. 5I), suggesting that the CD8⁺ T cell RBD
317 epitope specificities elicited by RFN vaccination may be relatively conserved. Again, responses
318 trended greater with the higher vaccine dose. These data indicate that S-specific CD4⁺ and
319 CD8⁺ T cells generated by ALFQ-adjuvanted RFN were able to cross-react with sequence
320 divergent SARS-CoV-1.

321 **DISCUSSION**

322 New SARS-CoV-2 vaccines may be needed to address concerns regarding emerging virus
323 variants less sensitive to immunity elicited by current vaccines (1, 44-47). In this study, we
324 evaluated a candidate RFN vaccine adjuvanted with ALFQ in rhesus macaques and observed
325 robust and broad humoral and T cell responses and protection from high-dose respiratory tract
326 challenge. Binding, neutralizing, and effector antibody responses were elicited in all animals and
327 of comparable or greater magnitude to that observed in pre-clinical studies of EUA vaccines (15,
328 48, 49). Spike-specific CD4+ T cell responses exceeded 0.5% of memory cells and were
329 predominantly of the Th1 phenotype. Using a rigorous challenge model in which viral loads of
330 control animals exceeded that of published macaque studies, replicating virus was rapidly
331 cleared in the airways of vaccinated animals. Cross-reactive antibody responses were either
332 higher or similar against the B.1.1.7 VOC in authentic and pseudovirus neutralization assays,
333 while B.1.351 reactivity was diminished by only ~2-fold. Additionally, binding and functional
334 antibodies were also reactive to SARS-CoV-1, which is 36% amino acid sequence divergent
335 from SARS-CoV-2 in the RBD (50). Overall, these data indicate broad, potent and efficacious
336 immunity elicited by RFN-ALFQ.

337
338 This study provides strong evidence that RBD-directed vaccination in primates is able to protect
339 against SARS-CoV-2 infection and elicit neutralization breadth against variant B.1.351, which
340 has shown the greatest resistance to neutralization by vaccinee sera (19, 51, 52). While many
341 RBD-based immunogens have been shown to be immunogenic in small and large animal models
342 (24-27), limited studies assessed efficacy against viral challenge and neutralization activity
343 against VOC. A recent macaque study investigated the immunogenicity and protective efficacy

344 of a three-dose regimen of an RBD-ferritin nanoparticle-based vaccine (RBD scNP) and reported
345 efficacy upon challenge, with no sgRNA detected in the upper or lower airways of vaccinated
346 animals at day 2 post-challenge (53). Neutralizing antibody titers were observed for the B.1.1.7
347 variant but were not assessed for B.1.351. Here, RFN vaccination elicited similar B.1.1.7
348 neutralization as RBD scNP, but also neutralized B.1.351 with minimal reduction in potency
349 relative to USA-WA1. In another study, vaccination with RBD fused to the Fc domain of human
350 IgG1 reduced total viral RNA following challenge in cynomolgus macaques, though virus
351 replication was not assessed (54). Our findings demonstrate RBD-specific immunity elicited by a
352 condensed two-dose vaccine regimen is protective and, importantly, cross-neutralizes the more
353 resistant B.1.351 variant.

354

355 The immunogenicity and efficacy of ALFQ-adjuvanted RFN compares favorably to pre-clinical
356 macaque data reported for three COVID-19 vaccines authorized for emergency use. RFN
357 vaccination elicited peak mean SARS-CoV-2 pseudovirus neutralization reciprocal titers of
358 14,540 and 21,298 for the 5 and 50 μ g groups, respectively, exceeding those of the EUA
359 vaccines, which ranged from 408-1,862 (48, 49, 55). While neutralizing activity is unlikely to be
360 the sole determinant of vaccine-mediated protection, it has been predictive of efficacy in human
361 trials (1). Therefore, ~10-50-fold greater neutralizing titers by RFN relative to those elicited in
362 NHP studies by efficacious vaccines currently in clinical use strongly suggests that RFN would
363 be protective in humans. In addition, breadth against the B.1.351 VOC appears greater than that
364 of EUA vaccines in humans, as the modest ~2-fold reduction in B.1.351 neutralization activity
365 relative to wild-type virus reported here is less than the ~6-12-fold reduction in mRNA vaccinee
366 sera (19). The most advanced platform closest in design and composition to RFN is NVX-

367 CoV2373, a prefusion spike nanoparticle vaccine delivered with a saponin-based Matrix-M
368 adjuvant. NVX-CoV2373 elicited neutralizing antibody titers of 6,400-17,000 in macaques (56,
369 57), greater than those achieved by current EUA vaccines and less than or similar to those
370 elicited by RFN. T cell immunity was also more pronounced with RFN. S-specific Th1 CD4+ T
371 cells ranged from 0.5-5% following 50 µg RFN, compared to peak values of 0.1-0.2% in NHP
372 vaccinated with the EUA vaccines (48, 49, 55).

373

374 When considering vaccination strategies for clinical use in humans, cost and manufacturability
375 are important considerations. The comparison of low or high dose vaccine regimens presented
376 here demonstrated that immune responses did not significantly differ between the 5 and 50 µg
377 doses, although the 50 µg group did trend towards higher responses and a slightly earlier
378 resolution of viral load. The power to detect these differences may have been limited by small
379 sample sizes. It is likely that as doses decrease further protective efficacy will wane, and such
380 experiments may allow further elucidation of correlates of protection. This absence of a strong
381 dose titration effect suggests that vaccination with the lower dose may be possible for dose
382 sparing purposes, although clinical testing and assessment of response durability are required.

383

384 In addition to the RFN vaccine described here, we have also developed a similar ferritin
385 nanoparticle immunogen displaying the full prefusion stabilized SARS-CoV-2 spike
386 glycoprotein (SpFN) and reported its immunogenicity and efficacy in non-human primates (58).
387 Compared to two-dose SpFN regimens, RFN elicited binding and neutralizing antibody and T
388 cell responses of a similar magnitude, albeit with a trend towards slightly lower titers. Post-
389 challenge control of viral replication was also similar, though viral clearance after SpFN

390 vaccination trended faster from the BAL at day 2 post-challenge and from NP swabs by day 4.
391 The overall magnitude of these differences was small and suggests that both the RBD and S
392 proteins are similarly immunogenic and protective when complexed to ferritin nanoparticles and
393 administered with ALFQ adjuvant at these vaccine doses. However, S-based immunogens may
394 offer the advantage of broadening the specificity of the immune response to other domains and
395 subdomains of the spike protein, limiting potential for viral escape. These findings support
396 further clinical development of both products.

397

398 There exists a strong potential for future pandemics arising from zoonotic SARS-like
399 betacoronaviruses entering into humans. We report SARS-CoV-2 RFN vaccine-elicited
400 responses that cross-react with the S glycoprotein SARS-CoV-1, including binding antibody
401 titers within an order of magnitude of those to SARS-CoV-2. The observed cross-neutralizing
402 and binding reactivity to SARS-CoV-1 suggests that adjuvanted RFN may be a viable candidate
403 for vaccination against future betacoronavirus outbreaks. Work is ongoing to elucidate the
404 potential mechanisms of cross-protective responses in this study, including epitope mapping of
405 the antibody binding responses. Taken together, these findings support continued development of
406 RFN vaccines for managing COVID-19 and SARS-like betacoronavirus outbreaks.

407 **MATERIALS AND METHODS**

408 **Vaccine and adjuvant production**

409 *DNA plasmid construction and preparation*

410 The SARS-CoV-2 RBD-ferritin construct was derived from the Wuhan-Hu-1 strain genome
411 sequence (GenBank MN9089473) comprising residues 331 – 527. RBD was attached
412 to *Helicobacter pylori* ferritin using a GSGGGG linker followed by a short sequence
413 (ESQVRQQFSK) derived from bullfrog ferritin (59) and synthesized by GenScript, to include a
414 N-terminal hexa-histadine (his) tag for purification. Additional point mutations (Y453R, L518N,
415 L519K, H520S) were introduced in the RBD, using a modified QuikChange site-directed
416 mutagenesis protocol (Agilent Technologies, Santa Clara, CA) and designated as construct
417 RFN_131. The construct used a prolactin leader (PL) sequence (60). Plasmid DNA generated by
418 site-directed mutagenesis was prepared from *Eschericia coli* Stbl3 cells. Large-scale DNA
419 isolation was performed using either endo free Maxiprep, Megaprep or Gigaprep kits (Qiagen,
420 Hilden, Germany).

421 *Immunogen expression and purification*

422 SARS-CoV-2 RFN_131 immunogen (RFN) was expressed in 293Expi mammalian cell lines by
423 transient transfection using Turbo293 transfection reagent (Speed Biosystems, Gaithersburg,
424 MD). Expression cultures were grown in polycarbonate baffled shaker flasks at 34°C and 8%
425 CO₂ at 120 rpm. Cells were harvested five days post-transfection via centrifugation at 3,500 x g
426 for 30 min. Culture supernatants were filtered with a 0.22-µm filter and stored at 4°C prior to
427 purification. RFN was purified using Ni-NTA affinity chromatography. 1 mL Ni-NTA resin
428 (Thermo Scientific) was used to purify protein from 1 L of expression supernatant. Ni-NTA resin
429 was equilibrated with 5 column volumes (CV) of phosphate buffered saline (PBS) (pH 7.4)

430 followed by supernatant loading at room temperature. Unbound protein was removed by
431 washing with 200 CV of PBS, followed by 50 CV 10mM imidazole in PBS. Bound protein was
432 eluted with 220mM imidazole in PBS. Purification purity was assessed by SDS-PAGE. RFN was
433 concentrated in the presence of 5% glycerol and then further purified by size-exclusion
434 chromatography using a 16/60 Superdex-200 purification column. Endotoxin levels for ferritin
435 nanoparticle immunogens were evaluated (Endosafe nexgen-PTS, Charles River Laboratories)
436 and 5 % v/v glycerol was added prior to filter-sterilization with a 0.22- μ m filter, flash-freezing in
437 liquid nitrogen, and storage at -80°C. Ferritin nanoparticle formation was confirmed by negative-
438 stain electron microscopy and dynamic light scattering by determining the hydrodynamic
439 diameter at 25 °C using a Malvern Zetasizer Nano S (Malvern, Worcestershire, UK) equipped
440 with a 633-nm laser.

441 *Adjuvant preparation*

442 Army Liposomal Formulation with QS21 (ALFQ), formulation was prepared as previously
443 described (61, 62). ALFQ is a unilamellar liposome comprised of dimyristoyl
444 phosphatidylcholine (DMPC), dimyristoyl phosphatidylglycerol (DMPG), cholesterol (Chol),
445 and synthetic monophosphoryl lipid A (3D-PHAD[®]) (Avanti Polar Lipids, Alabaster, AL) and
446 QS-21 (Desert King, San Diego, CA). DMPC and cholesterol were dissolved in chloroform and
447 DMPG and 3D-PHAD[®] were dissolved in chloroform:methanol 9:1. Lipids were mixed in a
448 molar ratio of 9:1:12.2:0.114 (DMPC:DMPG:Chol:3D-PHAD[®]) and the solvent was removed by
449 rotary evaporation. Lipids were suspended in Sorenson's PBS, pH 6.2, microfluidized to form
450 small unilamellar vesicles and filtered. QS-21 was solubilized in Sorenson's PBS, pH 6.2, filtered
451 and added to the vesicles to form ALFQ. The final lipid ratio was 9:1:12.2:0.114:0.044
452 (DMPC:DMPG:Chol:3D-PHAD[®]:QS-21).

453 ***Immunogen formulation***

454 RFN was diluted in dPBS to 0.1 mg/mL or 0.01 mg/mL and mixed 1:1 with 2X ALFQ on a tilted
455 slow-speed roller at room temperature for 10 min, followed by incubation at 4°C for 50 min.
456 Reagents were equilibrated to room temperature before use and immunizations were performed
457 within 4 h of vaccine formulation. Each vaccine comprised a 1.0 mL solution of RFN formulated
458 with ALFQ. The 3D-PHAD[®] and QS-21 doses were 200 and 100 µg, respectively.

459 **Study design and procedures**

460 Twenty-three male and female specific-pathogen-free, research-naïve Chinese-origin rhesus
461 macaques (age 3 - 7 years) were distributed—on the basis of age, weight and sex—into 3 cohorts
462 of 7-8 animals (Table S1). Animals were vaccinated intramuscularly with either 50 or 5 µg of
463 RFN, formulated with ALFQ, and control group animals received 1 mL of PBS, in the
464 anterior proximal quadriceps muscle, on alternating sides with each dose in the series.
465 Immunizations were administered twice 4 weeks apart. Animals were challenged 4 weeks after
466 the second immunization with virus stock obtained through BEI Resources, NIAID, NIH: SARS-
467 Related Coronavirus 2, Isolate USA-WA1/2020, NR-53780 (Lot# 70038893). Virus was stored
468 at -80°C prior to use, thawed by hand and placed immediately on wet ice. Stock was diluted to
469 5×10^5 TCID₅₀/mL in PBS and vortexed gently for 5 sec prior to inoculation via combined
470 intratracheal and intranasal routes (1 mL each).

471 All procedures were carried out in accordance with institutional, local, state and national
472 guidelines and laws governing animal research included in the Animal Welfare Act. Animal
473 protocols and procedures were reviewed and approved by the Animal Care and Use Committee
474 of both the US Army Medical Research and Development Command (USAMRDC, protocol
475 11355007.03) Animal Care and Use Review Office as well as the Institutional Animal Care and

476 Use Committee of Bioqual, Inc. (protocol number 20-092), where nonhuman primates were
477 housed for the duration of the study. USAMRDC and Bioqual, Inc. are both accredited by the
478 Association for Assessment and Accreditation of Laboratory Animal Care and are in compliance
479 with the Animal Welfare Act and Public Health Service Policy on Humane Care and Use of
480 Laboratory Animals.

481 **Convalescent Plasma Samples**

482 A panel of 41 human convalescent-phase plasma samples were obtained from BEI Resources
483 Repository (N=30), StemExpress (East Norritin, PA) (N=7) and a Walter Reed Army Institute of
484 Research institutional review board-approved leukapheresis protocol (#1386H) (N=4) for which
485 written informed consent was provided by participants. Samples were collected from males
486 (N=20) and females (N=21) ranging in age from 31 to 71 years. Individuals donated plasma
487 specimens approximately four-to-eight weeks after laboratory-confirmed SARS-CoV-2 infection
488 and had histories of asymptomatic-to-mild-to-moderate clinical presentation.

489 **Antibody responses**

490 *Binding antibodies*

491 SARS-CoV-2-specific binding IgG antibodies and ACE2 inhibiting antibodies were measured
492 using MULTI-SPOT[®] 96-well plates, (Meso Scale Discovery (MSD), Rockville, MD). Multiplex
493 wells were coated with SARS-CoV-2 antigens, S and RBD, at a concentration of 200-400 ng/mL
494 and BSA, which served as a negative control. Four-plex MULTISPOT plates were blocked with
495 MSD Blocker A buffer for 1 h at room temperature (RT) while shaking at 700 rpm. Plates were
496 washed with buffer before the addition of reference standard and calibrator controls. Serum
497 samples were diluted at 1:1,000 - 1:100,000 in diluent buffer, then added to each of four wells.
498 Plates were incubated for 2 h at room temperature while shaking at 700 rpm, then washed. MSD

499 SULFO-TAGTM anti-IgG antibody was added to each well. Plates were incubated for 1 h at RT
500 with shaking at 700 rpm and washed, then MSD GOLDTM Read buffer B was added to each
501 well. Plates were read by the MESO SECTOR SQ 120 Reader. IgG concentration was calculated
502 using DISCOVERY WORKBENCH[®] MSD Software and reported as arbitrary units (AU)/mL.

503 For binding antibodies that block S or RBD binding to ACE2, antigen-coated plates were
504 blocked and washed as described above. Assay calibrator and samples were diluted at 1:25 -
505 1:1,000 in MSD Diluent buffer, then added to the wells. Plates were incubated for 1 h at room
506 temperature while shaking at 700 rpm. ACE2 protein conjugated with MSD SULFO-TAGTM was
507 added and plates were incubated for 1 h at room temperature while shaking at 700rpm and
508 washed and read as described above.

509 Binding antibody measurements by octet biolayer interferometry were made using
510 HIS1K biosensors hydrated in PBS prior to use, using an Octet FortéBio Red96 instrument
511 (Sartorius, Fremont CA). All assay steps were performed at 30°C with agitation set at 1,000 rpm.
512 Baseline equilibration of the HIS1K biosensors (Sartorius, Fremont, CA)) was carried out with
513 PBS for 15 sec, prior to SARS-CoV2 RBD molecules (30 µg/mL diluted in PBS) loading for 120
514 sec. Biosensors were dipped in assay buffer (15 sec in PBS), dipped in the serum samples (100-
515 fold dilution) for 180 sec, and binding response (nm) was recorded for 180 sec.

516 ***Virus neutralization***

517 *SARS-CoV-1 and SARS-CoV-2 pseudovirus neutralization*

518 The S expression plasmid sequence for SARS-CoV-2 was codon optimized and modified to
519 remove an 18 amino acid endoplasmic reticulum retention signal in the cytoplasmic tail to
520 improve S incorporation into pseudovirions (PSV). PSV were produced by co-transfection of
521 HEK293T/17 cells with a SARS-CoV-2 S plasmid (pcDNA3.4), derived from the Wuhan-Hu-1

522 genome sequence (GenBank accession number: MN908947.3) and an HIV-1 NL4-3 luciferase
523 reporter plasmid (pNL4-3.Luc.R-E-, NIH HIV Reagent Program, Catalog number 3418).
524 Infectivity and neutralization titers were determined using ACE2-expressing HEK293 target cells
525 (Integral Molecular, Philadelphia, PA) in a semi-automated assay format using robotic liquid
526 handling (Biomex NXp Beckman Coulter, Brea, CA). Virions pseudotyped with the vesicular
527 stomatitis virus (VSV) G protein were used as a non-specific control. Test sera were diluted 1:40
528 in growth medium and serially diluted; then 25 μ L/well was added to a white 96-well plate. An
529 equal volume of diluted PSV was added to each well and plates were incubated for 1 h at 37°C.
530 Target cells were added to each well (40,000 cells/well) and plates were incubated for an
531 additional 48 h. Relative light units (RLU) were measured with the EnVision Multimode Plate
532 Reader (Perkin Elmer, Waltham, MA) using the Bright-Glo Luciferase Assay System (Promega,
533 Madison, WI). Neutralization dose–response curves were fitted by nonlinear regression using the
534 LabKey Server. Final titers are reported as the reciprocal of the serum dilution necessary to
535 achieve 50% inhibition SARS-CoV-2 (ID₅₀, 50% inhibitory dose) or 90% inhibition for SARS-
536 CoV-1 (ID₉₀, 90% inhibitory dose). Assay equivalency was established by participation in the
537 SARS-CoV-2 Neutralizing Assay Concordance Survey run by the Virology Quality Assurance
538 Program and External Quality Assurance Program Oversight Laboratory at the Duke Human
539 Vaccine Institute.

540 *Authentic SARS-CoV-2 wild-type neutralization assay*

541 Authentic virus neutralization was measured using SARS-CoV-2 (2019-nCoV/USA_WA1/2020)
542 obtained from the Centers for Disease Control and Prevention and passaged once in Vero CCL81
543 cells (ATCC). Rhesus sera were serially diluted and incubated with 100 focus-forming units of
544 SARS-CoV-2 for 1 h at 37°C. Serum-virus mixtures were added to Vero E6 cells in 96-well

545 plates and incubated for 1 h at 37°C. Cells were overlaid with 1% (w/v) methylcellulose in
546 MEM. After 30 h, cells were fixed with 4% PFA in PBS for 20 min at room temperature then
547 washed and stained overnight at 4°C with 1 µg/mL of CR3022 antibody in PBS supplemented
548 with 0.1% saponin and 0.1% bovine serum albumin. Cells were subsequently stained with HRP-
549 conjugated goat anti-human IgG for 2 h at room temperature. SARS-CoV-2-infected cell foci
550 were visualized with TrueBlue peroxidase substrate (KPL) and quantified using ImmunoSpot
551 microanalyzer (Cellular Technologies). Neutralization curves were generated with Prism
552 software (GraphPad Prism 8.0).

553 *Authentic SARS-CoV-2 variant and SARS-CoV-1 neutralization assay*

554 The SARS-CoV-2 viruses USA-WA1/2020 (WA1), USA/CA_CDC_5574/2020 (B.1.1.7), and
555 hCoV-19/South Africa/KRISP-EC-K005321/2020 (B.1.351) were obtained from BEI Resources
556 (NIAID, NIH) and propagated for one passage using Vero-E6 cells. Virus infectious titer was
557 determined by an end-point dilution and cytopathic effect (CPE) assay on Vero-E6 cells. In brief,
558 serum samples were heat inactivated and subjected to successive 3-fold dilutions starting from
559 1:50. Triplicates of each dilution were incubated with SARS-CoV-2 at an MOI of 0.1 in EMEM
560 with 7.5% inactivated fetal calf serum (FCS) for 1 h at 37°C. Virus-antibody mixture was
561 transferred onto a monolayer of Vero-E6 cells grown overnight and incubated for ~70 h. CPE of
562 viral infection was visually scored for each well in a blinded fashion by two independent
563 observers. Results were reported as percentage of neutralization at a given sample dilution. A
564 SARS-CoV-1 authentic plaque reduction virus neutralization assay was performed similarly to
565 previously described (63) with the following modifications. The starting dilution of serum was
566 1:5 and ~100 PFU of virus was used for virus/serum incubation. The overlay used after virus

567 adsorption was DMEM containing 2% FBS and 20% methylcellulose. Plates were then
568 incubated for 5 days, and post crystal violet staining the washing step utilized water.
569 Plaques were graded as follow: ~50 plaques/50% MD (+); ~75 plaques/75% MD (++); ~100
570 plaques/100% MD (+++); ~25 plaques/25%. All negative control wells were solid monolayers.

571 ***Antibody-dependent neutrophil phagocytosis (ADNP)***

572 Biotinylated SARS-CoV-2 prefusion stabilized S trimer was incubated with yellow-green
573 streptavidin-fluorescent beads (Molecular Probes, Eugene, OR) for 2 h at 37°C. 10 µL of a 100-
574 fold dilution of protein-coated beads was incubated for 2 h at 37°C with 100 µL of 8,100-fold
575 diluted plasma samples before addition of effector cells (50,000 cells/well). Fresh human
576 peripheral blood mononuclear cells were used as effector cells after red blood cell lysis with
577 ACK lysing buffer (ThermoFisher Scientific, Waltham, MA). After 1 h incubation at 37°C, cells
578 were washed, surface stained, fixed with 4% formaldehyde solution (Tousimis, Rockville, MD)
579 and fluorescence was evaluated on an LSRII flow cytometer (BD Bioscience, San Jose, CA).
580 Antibodies used for flow cytometry included anti-human CD3 AF700 (clone UCHT1), anti-
581 human CD14 APC-Cy7 (clone MφP9) (BD Bioscience, San Jose, CA) and anti-human CD66b
582 Pacific Blue (clone G10F5) (Biolegend, San Diego, CA). Phagocytic score was calculated by
583 multiplying the percentage of bead-positive neutrophils (SSC high, CD3- CD14- CD66+) by the
584 geometric mean of the fluorescence intensity of bead-positive cells; and dividing by 10,000.

585 ***Antibody-dependent cellular phagocytosis (ADCP)***

586 ADCP was measured as previously described (64). Briefly, biotinylated SARS-CoV-1 or SARS-
587 CoV-2 prefusion-stabilized S trimer was incubated with red streptavidin-fluorescent beads
588 (Molecular Probes, Eugene, OR) for 2 h at 37°C. 10 µL of a 100-fold dilution of beads–protein
589 was incubated for 2 h at 37°C with 100 µL of 8,100-fold (SARS-CoV-2) or 900-fold (SARS-

590 CoV-1) diluted plasma samples before addition of THP-1 cells (20,000 cells per well; Millipore
591 Sigma, Burlington, MA). After 19 h incubation at 37°C, the cells were fixed with 2%
592 formaldehyde solution (Tousimis, Rockville MD) and fluorescence was evaluated on an LSRII
593 flow cytometer (BD Bioscience, San Jose, CA). The phagocytic score was calculated by
594 multiplying the percentage of bead-positive cells by the geometric mean of the fluorescence
595 intensity of bead-positive cells and dividing by 10,000.

596 ***Opsionization***

597 SARS-CoV-2 Spike-expressing expi293F cells were generated by transfection with linearized
598 plasmid (pcDNA3.1) encoding codon-optimized full-length SARS-CoV-2 Spike protein
599 matching the amino acid sequence of the IL-CDC-IL1/2020 isolate (GenBank ACC#
600 MN988713). Stable transfectants were single-cell sorted and selected to obtain a high-level
601 Spike surface expressing clone (293F-Spike-S2A). SARS-CoV-2 S expressing cells were
602 incubated with 200-fold diluted plasma samples for 30 min at 37°C. Cells were washed twice and
603 stained with anti-human IgG PE, anti-human IgM Alexa Fluor 647, and anti-human IgA FITC
604 (Southern Biotech, Birmingham, AL). Cells were then fixed with 4% formaldehyde solution and
605 fluorescence was evaluated on an LSRII flow cytometer (BD Bioscience, San Jose, CA).

606 ***Antibody-dependent complement deposition (ADCD)***

607 ADCD was adapted from methods described previously (65). Briefly, SARS-CoV-2 S
608 expressing expi293F cells were incubated with 10-fold diluted, heat-inactivated (56°C for 30
609 min) plasma samples for 30 min at 37°C. Cells were washed twice and resuspended in R10
610 media. During this time, lyophilized guinea pig complement (CL4051, Cedarlane, Burlington,
611 Canada) was reconstituted in 1 mL cold water and centrifuged for 5 min at 4°C to remove
612 aggregates. Cells were washed with PBS and resuspended in 200 µL of guinea pig complement,

613 which was prepared at a 1:50 dilution in Gelatin Veronal Buffer with Ca^{2+} and Mg^{2+} (IBB-300x,
614 Boston BioProducts, Ashland, MA). After incubation at 37°C for 20 min, cells were washed in
615 PBS 15mM EDTA (ThermoFisher Scientific, Waltham, MA) and stained with an anti-Guinea
616 Pig Complement C3 FITC (polyclonal, ThermoFisher Scientific, Waltham, MA). Cells were then
617 fixed with 4% formaldehyde solution and fluorescence was evaluated on a LSRII flow cytometer
618 (BD Bioscience, San Jose, CA).

619 *Trogocytosis*

620 Trogocytosis was measured using a previously described assay (38). Briefly, SARS-CoV-2
621 Spike expressing expi293F cells were stained with PKH26 (Sigma-Aldrich, St-Louis, MO). Cells
622 were then washed with and resuspended in R10 media. Cells were then incubated with 200-fold
623 diluted plasma samples for 30 min at 37°C. Effector peripheral blood mononuclear cells were
624 next added to the R10 media at an effector to target (E:T) cell ratio of 50:1 and then incubated
625 for 5 h at 37°C. After the incubation, cells were washed, stained with live/dead aqua fixable cell
626 stain (Life Technologies, Eugene, OR) and CD14 APC-Cy7 (clone M ϕ P9) for 15 min at room
627 temperature, washed again, and fixed with 4% formaldehyde (Tousimis, Rockville, MD) for 15
628 min at room temperature. Fluorescence was evaluated on a LSRII flow cytometer (BD
629 Bioscience, San Jose, CA). Trogocytosis was evaluated by measuring the PKH26 mean
630 fluorescence intensity of the live CD14+ cells.

631 **Antigen-specific T cell responses**

632 Cryopreserved peripheral blood mononuclear cells were thawed and rested for 6 h in R10 with
633 50 U/mL Benzonase Nuclease (Sigma-Aldrich, St. Louis, MO). They were then stimulated with
634 peptide pools for 12 h. Stimulations consisted of two pools of peptides spanning the Spike
635 protein of SARS-CoV-2 or SARS-CoV-1 (1 $\mu\text{g}/\text{mL}$, JPT, PM-WCPV-S and PM-CVHSA-S

636 respectively) in the presence of Brefeldin A (0.65 μ L/mL, GolgiPlugTM, BD Cytotfix/Cytoperm
637 Kit, Cat. 555028), co-stimulatory antibodies anti-CD28 (BD Biosciences Cat. 555725 1 μ g/mL)
638 and anti-CD49d (BD Biosciences Cat. 555501; 1 μ g/mL) and CD107a (H4A3, BD Biosciences
639 Cat. 561348, Lot 9143920 and 253441). Following stimulation, cells were stained serially with
640 LIVE/DEAD Fixable Blue Dead Cell Stain (ThermoFisher #L23105) and a cocktail of
641 fluorescent-labeled antibodies (BD Biosciences unless otherwise indicated) to cell surface
642 markers CD4-PE-Cy5.5 (S3.5, ThermoFisher #MHCD0418, Lot 2118390 and 2247858), CD8-
643 BV570 (RPA-T8, BioLegend #301038, Lot B281322), CD45RA BUV395 (5H9, #552888, Lot
644 154382 and 259854), CD28 BUV737 (CD28.2, #612815, Lot 0113886), CCR7-BV650
645 (GO43H7, # 353234, Lot B297645 and B316676) and HLA-DR-BV480 (G46-6, # 566113, Lot
646 0055314). Intracellular cytokine staining was performed following fixation and permeabilization
647 (BD Cytotfix/Cytoperm, BD Biosciences) with CD3-Cy7APC (SP34-2, #557757, Lot 6140803
648 and 121752), CD154-Cy7PE (24-31, BioLegend # 310842, Lot B264810 and B313191), IFN γ -
649 AF700 (B27, # 506516, Lot B187646 and B290145), TNF α -FITC (MAb11, # 554512, Lot
650 15360), IL-2-BV750 (MQ1-17H12, BioLegend #566361, Lot 0042313), IL-4 BB700 (MP4-
651 25D2, Lot 0133487 and 0308726), MIP-1b (D21-1351, # 550078, Lot 9298609), CD69-ECD
652 (TP1.55.3, Beckman Coulter # 6607110, Lot 7620070 and 7620076), IL-21-AF647 (3A3-N2.1, #
653 560493, Lot 9199272 and 225901), IL-13-BV421 (JES10-5A2, # 563580, Lot 9322765, 210147
654 and 169570) and IL-17a-BV605 (BL168, Biolegend #512326, B289357). Sample staining was
655 measured on a FACSymphonyTM A5 SORP (Becton Dickenson) and data was analyzed using
656 FlowJo v.9.9 software (Tree Star, Inc.). CD4⁺ and CD8⁺ T cell subsets were pre-gated on
657 memory markers prior to assessing cytokine expression as follows: single-positive or double-
658 negative for CD45RA and CD28. Boolean combinations of cells expressing one or more

659 cytokines were used to assess the total S-specific response of memory CD4⁺ or CD8⁺ T cells.
660 Responses from the two-peptide pools spanning SARS-CoV-2 S or SARS-CoV-1 S were
661 summed. Display of multicomponent distributions were performed with SPICE v6.0 (NIH,
662 Bethesda, MD).

663 **Total and subgenomic messenger (sgm) RNA quantification**

664 Real-time quantitative PCR was carried out for subgenomic messenger RNA (sgmRNA) and
665 viral load RNA quantification from NP swab, BAL fluid and saliva samples. Primers targeted the
666 envelope (E) gene of SARS-CoV-2 (Table S2). RNA was extracted from 200 μ L of specimen
667 using the EZ1 DSP Virus kit (Qiagen) on the EZ1 Advanced XL instrument (Qiagen). Briefly,
668 samples were lysed in 200 μ L of ATL buffer (Qiagen) and transferred to the Qiagen EZ1 for
669 extraction. Bacteriophage MS2 (ATCC, Manassas, VA) was added to the RNA carrier and used
670 as an extraction control to monitor efficiency of RNA extraction and amplification (66). Purified
671 RNA was eluted in 90 μ L elution buffer (AVE). The RT-qPCR amplification reactions were
672 performed in separate wells of a 96-well Fast plate for the 3 targets: sgmRNA, RNA viral load,
673 and MS2 RNA using 10 μ L of extracted material 0.72 μ M of primers, 0.2 μ M of probe and 1x
674 TaqPath™ 1-Step RT-qPCR (Life Technologies, Thermo Fisher Scientific, Inc.). Amplification
675 cycling conditions were: 2 min at 25°C, 15 min at 50°C, 2 min at 95°C and 45 cycles of 3 sec at
676 94°C and 30 sec at 55°C with fluorescence read at 55°C. An RNA transcript for the SARS-CoV-
677 2 E gene was used as a calibration standard. RNA copy values were extrapolated from the
678 standard curve and multiplied by 45 to obtain RNA copies/mL. A negative control (PBS) and
679 two positive controls, contrived using heat-inactivated SARS-CoV-2 (ATCC, VR-1986HK), at
680 10⁶ and 10³ copies/mL, were extracted and used to assess performance of both assays.

681 **Histopathology**

682 Formalin-fixed sections of lung tissue were evaluated by light microscopy and
683 immunohistochemistry. Lungs were perfused with 10% neutral-buffered formalin. Lung sections
684 were processed routinely into paraffin wax, then sectioned at 5 μ m, and resulting slides were
685 stained with hematoxylin and eosin. Immunohistochemistry (IHC) was performed using the
686 Dako Envision system (Dako Agilent Pathology Solutions, Carpinteria, CA, USA). Briefly, after
687 deparaffinization, peroxidase blocking, and antigen retrieval, sections were covered with a
688 mouse monoclonal anti-SARS-CoV nucleocapsid protein (#40143-MM05, Sino Biological,
689 Chesterbrook, PA, USA) at a dilution of 1:4,000 and incubated at room temperature for 45 min.
690 They were rinsed, and the peroxidase-labeled polymer (secondary antibody) was applied for 30
691 min. Slides were rinsed and a brown chromogenic substrate 3,3' Diaminobenzidine (DAB)
692 solution (Dako Agilent Pathology Solutions) was applied for 8 min. The substrate-chromogen
693 solution was rinsed off the slides, and slides were counterstained with hematoxylin and rinsed.
694 The sections were dehydrated, cleared with Xyless, and then cover slipped. Tissue section slides
695 were evaluated by a board-certified veterinary anatomic pathologist who was blinded to study
696 group allocations. Immunohistochemistry (IHC) was performed with Dako Envision. Three
697 tissue sections from each of the right and left lung lobes were used to evaluate the lung
698 pathology. The histopathology of each section was evaluated on a scale of 0-5: 0 - absent, 1 -
699 minimal (<10% of tissue section affected); 2 - mild (11-25% of tissue section affected); 3 -
700 moderate (26-50% of tissue section affected); 4 - marked (51-75% affected); 5- severe (>75% of
701 tissue section affected). Sections were evaluated for edema, hyaline membranes, cellular
702 infiltrates, alveolar histiocytes, type II pneumocyte hyperplasia, interstitial fibroplasia, BALT
703 hyperplasia, bronchiolar degeneration, megakaryocytes in capillaries, and thrombosis. The

704 scores for all six sections of each pathologic finding were combined to create the final score
705 (TIIPH score) for individual animals.

706 **Statistical analysis**

707 Primary immunogenicity outputs of binding and neutralizing antibody titers as well as T cell
708 responses were compared across vaccination groups using the Kruskal-Wallis test. Non-
709 parametric pairwise comparisons between groups were made using the post-hoc Dunn's test.
710 Statistical significance was preset at an alpha level of 0.05.

711 **Data Availability**

712 All data are available in the manuscript or the supplementary materials.

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726 adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC
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728

729 **Author Contributions**

730 H.A.D.K., M.G.J., S.V., N.L.M., K.M. and D.L.B. designed the study. I.E.N, A.A., C.M.C., C.S.,
731 K.K.P., H.H.H., R.E.C, P.V.T., W-H.C., R.S.S., A.H., E.J.M., C.E.P., W.C.C., M.C., A. Ahmed,
732 K.M.W., M.D., I.S., J.B.C., K.G.L, V.D., S.M., K.A., R.C., S.J.K. D.P.P., N.K., V.R.P., Y.H.,
733 L.L.J., G.D.G. performed immunologic and virologic assays. H.A.E, A.C., M.G.L. led the
734 clinical care of the animals. S.P.D., X.Z., E.K.D performed histopathology. K.M., M.G.J.,
735 P.V.T., W-H.C., R.S.S., A.H., E.J.M., C.E.P., W.C.C., and M.C. designed the immunogens.
736 M.R., G.R.M., and A. Anderson designed and provided the adjuvant. H.A.D.K., M.G.J., C.S.,
737 J.A.H., M.F.A., S.P.D., P.T.S., D.D.H., M.S.D., M.R., G.D.D., S.A.P., N.L.M., K.M. and D.L.B.
738 analyzed and interpreted the data. H.A.D.K., C.S., K.M. and D.L.B. wrote the paper with
739 assistance from all coauthors.

740

741 **Competing Interests**

742 M.G.J. and K.M. are named as inventors on International Patent Application No. WO/2021/21405
743 entitled “Vaccines against SARS-CoV-2 and other coronaviruses.” M.G.J. is named as an inventor
744 on International Patent Application No. WO/2018/081318 entitled “Prefusion Coronavirus Spike
745 Proteins and Their Use.” M.S.D. is a consultant for Inbios, Vir Biotechnology, NGM
746 Biopharmaceuticals and Carnival Corporation and on the Scientific Advisory Boards of Moderna
747 and Immunome. The Diamond laboratory has received funding support in sponsored research
748 agreements from Moderna, Vir Biotechnology and Emergent BioSolutions. The other authors
749 declare no competing interests.

750

751 **Materials & Correspondence**

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903
904

905 **Figure Legends**

906

907 **Figure 1. RFN vaccine-elicited binding and neutralizing antibody responses to SARS-CoV-**

908 **2.** Humoral immune responses were measured in vaccinated macaques. (A) Rhesus macaque
909 vaccination, challenge, and sampling schedule. Animals were immunized with either 50 or 5 μ g
910 RFN-ALFQ at weeks 0 and 4; control animals received PBS (N= 7-8 per group). 1×10^6 TCID₅₀
911 of SARS-CoV-2 was administered four weeks after the last vaccination. (B) Serum SARS-CoV-
912 2 S-specific IgG responses assessed by MSD every two weeks following vaccination. Data are
913 depicted as the arbitrary units (AU)/ml of IgG binding. Thick lines indicate geometric means
914 within each group and thin lines represent individual animals. Serum SARS-CoV-2 RBD-
915 specific IgG (C) and inhibition of angiotensin-converting enzyme 2 (ACE2) binding to the RBD
916 (D) four weeks after last vaccination were measured by MSD. (E) Serum SARS-CoV-2 S-
917 specific pseudovirus neutralization every two weeks following vaccination. Virus neutralization
918 reciprocal 50% inhibitory dilution (ID₅₀) is shown. Thick lines indicate geometric means within
919 each group and thin lines represent individual animals. (F) Authentic SARS-CoV-2 WA1 virus
920 neutralization at four weeks after last vaccination. (G) Pseudovirus neutralization activity four
921 weeks post-boost was compared to a panel of human convalescent sera (N=41 samples). Bars
922 indicate the geometric mean titer. Symbols represent individual animals and overlap with one
923 another for equal values where constrained. Gray arrows indicate the time of immunization,
924 maroon arrow indicates time of challenge. Significance was assessed using a Kruskal-Wallis test
925 followed by a Dunn's post-test.

926

927 **Figure 2. RFN vaccine elicited SARS-CoV-2 S-specific CD4+ T cell responses.**

928 T cell responses were assessed by SARS-CoV-2 S peptide pool stimulation and intracellular cytokine
929 staining of peripheral blood mononuclear cells collected four weeks after last vaccination. S-
930 specific memory CD4+ T cells expressing the indicated markers are shown as follows: (A) Th1
931 cytokines (IFN γ , TNF and IL-2); (B) Th2 cytokines (IL-4 and IL-13); (C) IL-21; (D) and
932 CD40L. Boolean combinations of cytokine-positive memory CD4+ T cells were summed.
933 Probable positive responses, defined as >3 times the group background at baseline, are depicted
934 as closed symbols. Positivity rates within each group are shown below each graph as a fraction.
935 Box plot horizontal lines indicate the mean; top and bottom reflect the minimum and maximum.
936 Significance was assessed using a Kruskal-Wallis test followed by a Dunn's post-test.

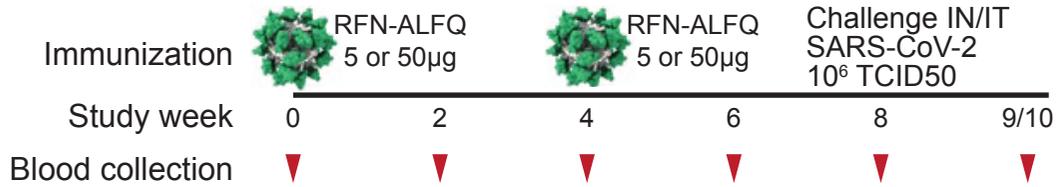
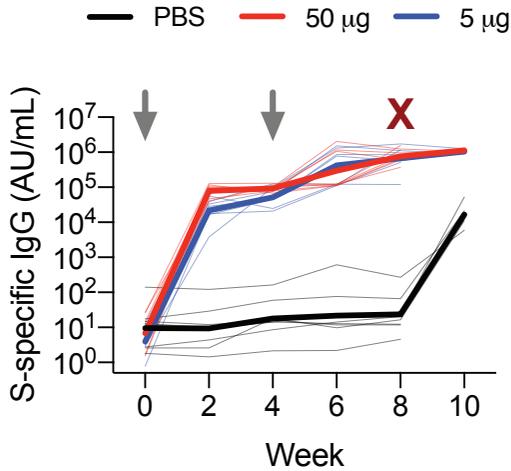
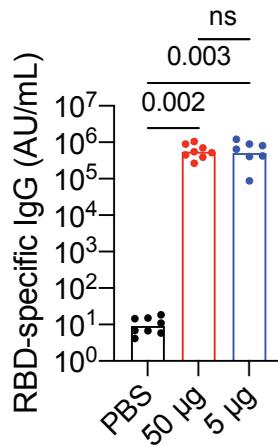
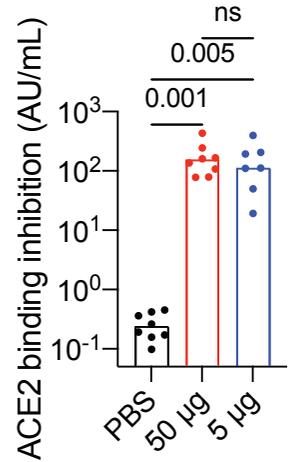
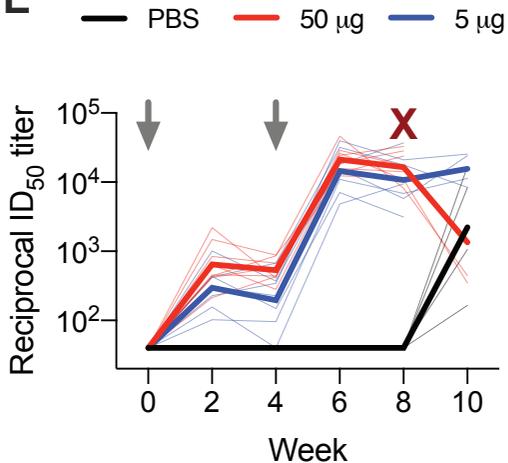
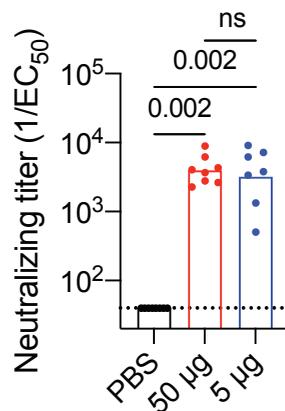
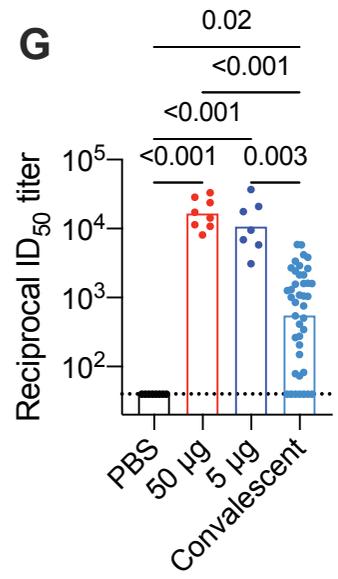
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938 **Figure 3. Viral replication in the lower and upper airways after RFN vaccination and**

939 **subsequent SARS-CoV-2 respiratory challenge.** Subgenomic E messenger RNA (sgmRNA)
940 copies per milliliter were measured following challenge. (A) Bronchoalveolar lavage fluid
941 (BAL), (B) nasopharyngeal (NP) swabs and (C) saliva of vaccinated and control animals for one
942 (N=7-8 per group) or two weeks (N=3-4 per group) following intranasal and intratracheal SARS-
943 CoV-2 (USA-WA1/2020) challenge of vaccinated and control animals. Specimens were
944 collected 1, 2, 4, 7, 10 and 14 days post-challenge. Dotted lines demarcate assay lower limit of
945 linear performance range (log₁₀ of 2.65 corresponding to 450 copies/mL); positive values below
946 this limit are plotted as 450 copies/ml. Open symbols represent animals with viral loads below
947 the limit of detection of the assay. Box plot horizontal lines indicate the mean; top and bottom
948 reflect the minimum and maximum. Significant differences between control and vaccinated
949 animals at day 2 post-challenge are indicated. Significance was assessed using a Kruskal-Wallis
950 test followed by a Dunn's post-test.

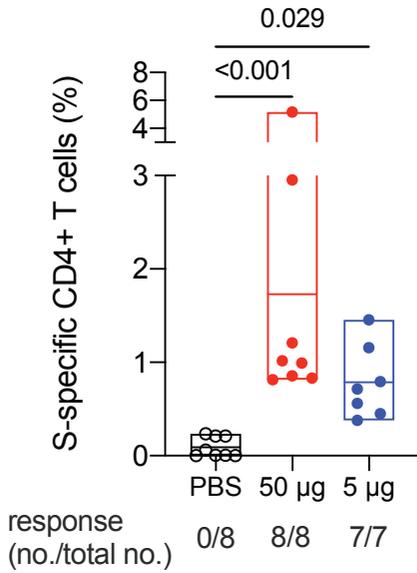
951
952 **Figure 4. Histopathology and virus detection in the lungs following SARS-CoV-2 challenge.**
953 Lung parenchymal tissue was assessed for pathology and viral antigen seven days post-
954 challenge. (A-C) Paraffin-embedded sections were stained with hematoxylin and eosin and
955 shown for animals that received PBS (A), 50 µg RFN (B) and 5 µg RFN (C). Inflammatory
956 debris (white star), type II pneumocyte hyperplasia (black arrow), edema (triangle) and vasculitis
957 of small- to medium-caliber blood vessels (white arrows) is indicated. Scale bars represent 50
958 µm. (D-F) SARS-CoV-2 nucleocapsid detected by immunohistochemistry in alveolar
959 pneumocytes, pulmonary macrophages and endothelial cells, appears as brown aggregates. Scale
960 bars represent 100 µm. Representative images are shown. (G) Each pathologic finding was
961 quantified for six lung sections and reported as a combined TIIPH score for all animals
962 necropsied at day seven post-challenge.

963
964 **Figure 5. Cross-reactive immune responses to SARS-CoV-2 variants and SARS-CoV-1.**
965 Serum and PBMC collected two weeks after the last vaccination was assessed for cross-
966 reactivity to variants of concern and SARS-CoV-1. (A) Authentic virus and (B) pseudovirus
967 neutralizing antibody responses to variants B.1.1.7 and B.1.351. Corresponding responses to
968 SARS-CoV-2 USA-WA1 authentic virus and Wuhan-1 pseudovirus are shown. Bars indicate the
969 geometric mean titer. (C) Reciprocal ID50 geometric mean titer (GMT) fold-change from wild-
970 type neutralization (WA1 or Wuhan-1) with statistical significance set at a p-value of < 0.05. (D)
971 Serum binding responses to SARS-CoV-1 RBD assessed by biolayer interferometry. (E)
972 Antibody-dependent cellular phagocytosis of SARS-CoV-1 spike trimer-coated fluorescent
973 beads. (F) Authentic SARS-CoV-1 (Urbani) neutralization titers (ID50). (G) SARS-CoV-1
974 (Urbani) pseudovirus neutralization (ID90). (H) SARS-CoV-1 (Urbani) S-specific memory
975 CD4⁺ Th1 and (I) CD8⁺ responses assessed by peptide pool stimulation and ICS (INF γ , IL-2
976 and TNF). Significance was assessed with a Kruskal-Wallis test followed by a Dunn's post-test.
977 Bars indicate the geometric mean titer.

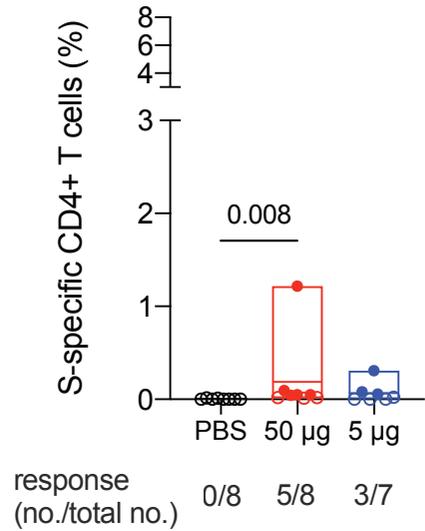
A**B****C****D****E****F****G**

A

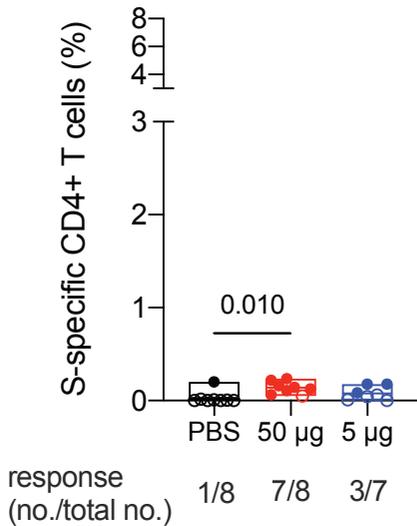
Th1 Responses

**B**

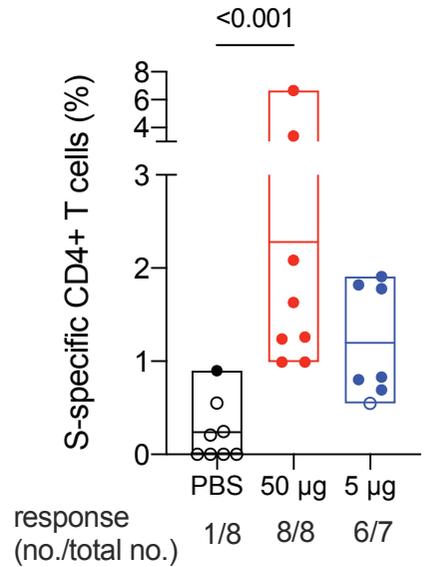
Th2 Responses

**C**

IL-21

**D**

CD40L



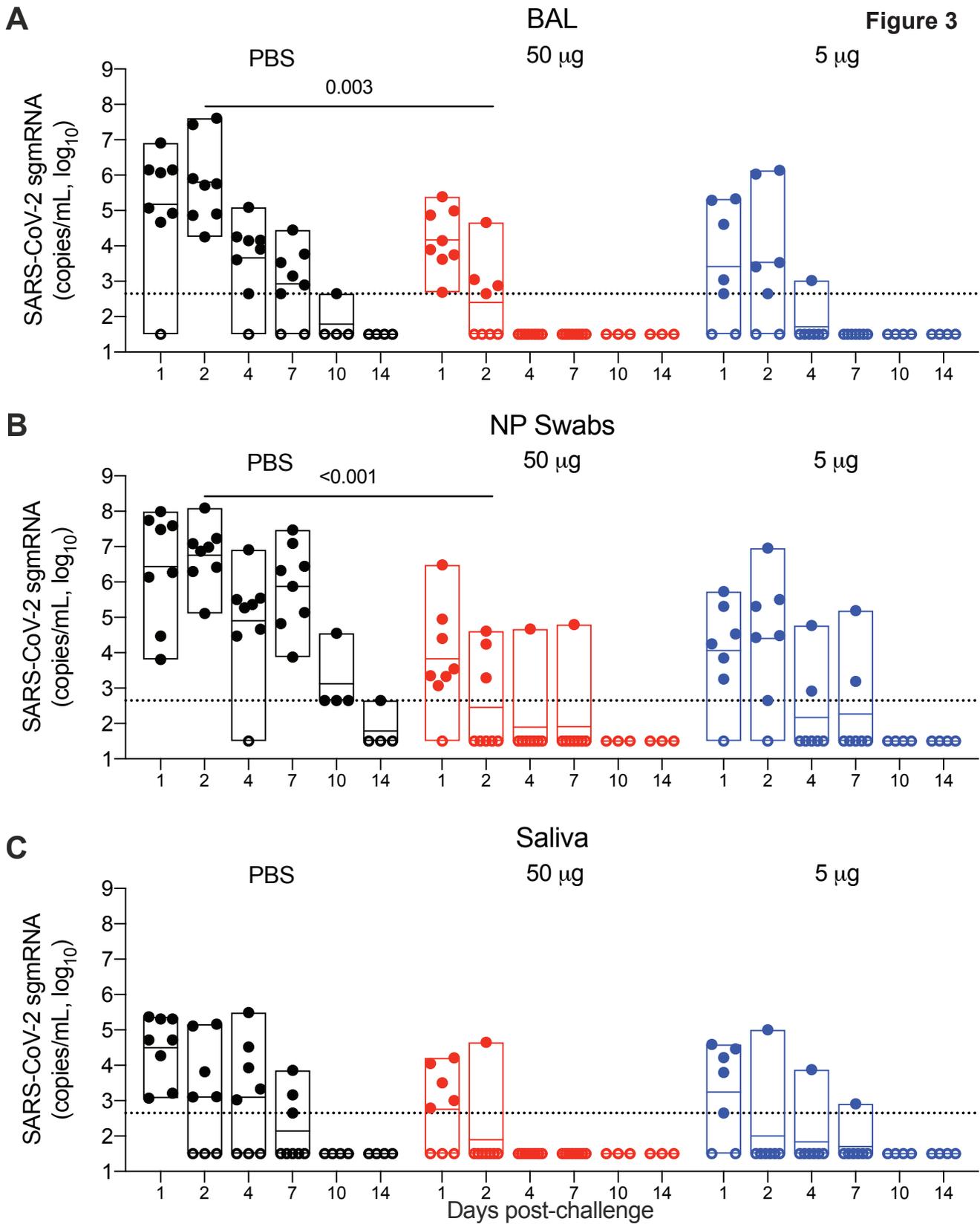
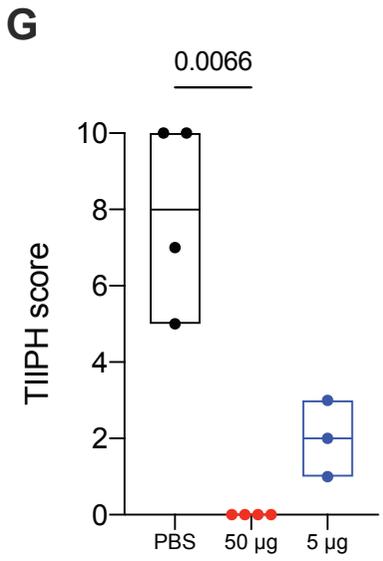
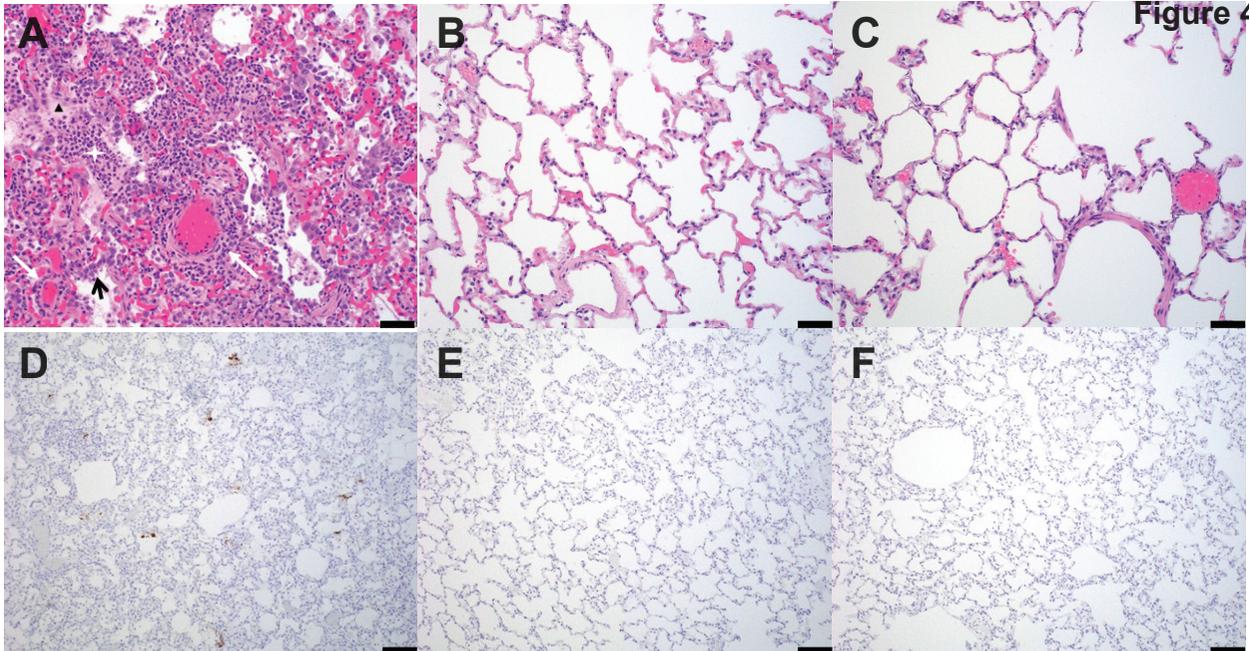
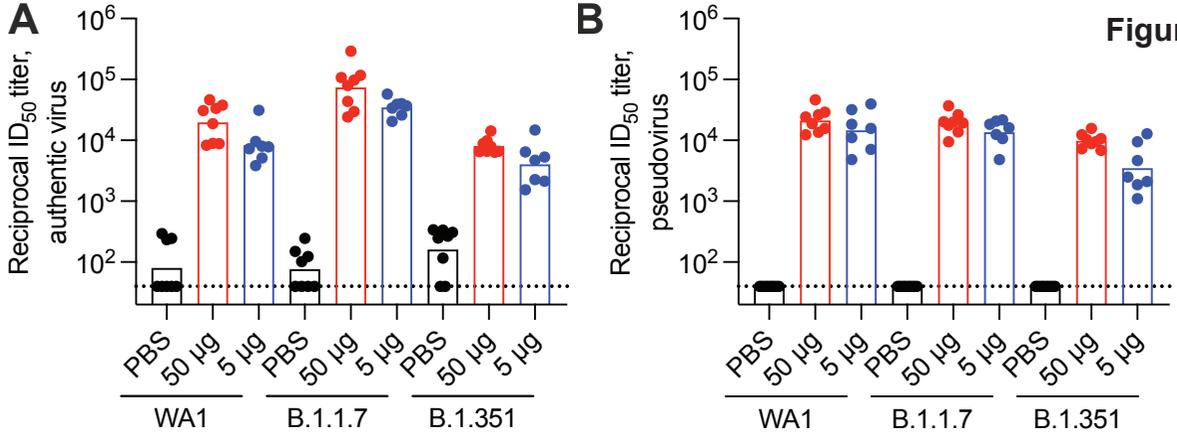


Figure 4





C

Immunization	Neutralization assay	ID50 GMT			ID50 GMT fold-change from WA1	
		WA1	B.1.1.7	B.1.351	B.1.1.7	B.1.351
50 µg	Authentic virus	19643	73983	8070	3.8	-2.2
5 µg	Authentic virus	8299	34637	4049	4.2*	-2.0
50 µg	Pseudovirus	21299	19070	9876	-1.1	-2.2**
5 µg	Pseudovirus	14540	13616	3480	-1.1	-4.2*

