

# 1        **Tetravalent SARS-CoV-2 S1 Subunit Protein Vaccination Elicits** 2        **Robust Humoral and Cellular Immune Responses in SIV-Infected** 3        **Rhesus Macaque Controllers**

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45           **Abstract**

46           The COVID-19 pandemic has highlighted the need for safe and effective vaccines to be  
47 rapidly developed and distributed worldwide, especially considering the emergence of new  
48 SARS-CoV-2 variants. Protein subunit vaccines have emerged as a promising approach due to  
49 their proven safety record and ability to elicit robust immune responses. In this study, we  
50 evaluated the immunogenicity and efficacy of an adjuvanted tetravalent S1 subunit protein  
51 COVID-19 vaccine candidate composed of the Wuhan, B.1.1.7 variant, B.1.351 variant, and P.1  
52 variant spike proteins in a nonhuman primate model with controlled SIVsab infection. The  
53 vaccine candidate induced both humoral and cellular immune responses, with T- and B cell  
54 responses mainly peaking post-boost immunization. The vaccine also elicited neutralizing and  
55 cross-reactive antibodies, ACE2 blocking antibodies, and T-cell responses, including spike  
56 specific CD4<sup>+</sup> T cells. Importantly, the vaccine candidate was able to generate Omicron variant  
57 spike binding and ACE2 blocking antibodies without specifically vaccinating with Omicron,  
58 suggesting potential broad protection against emerging variants. The tetravalent composition of  
59 the vaccine candidate has significant implications for COVID-19 vaccine development and  
60 implementation, providing broad antibody responses against numerous SARS-CoV-2 variants.  
61

## 62            **Introduction**

63  
64            The coronavirus disease 2019 (COVID-19) pandemic caused by the severe acute  
65 respiratory syndrome coronavirus 2 (SARS-CoV-2) has had an unprecedented impact on global  
66 health, economy, and society. The COVID-19 pandemic consisted of over 675 million cases,  
67 with 6.5 million deaths, and 13 billion COVID-19 vaccine doses administered across the human  
68 population, as of February 3<sup>rd</sup> 2023.<sup>1</sup> Although approved COVID-19 vaccines have been  
69 effective in reducing mortality and morbidity caused by SARS-CoV-2 infection, the emergence  
70 of new variants that are able to evade the immune response has raised concerns about their long-  
71 term efficacy. Furthermore, the uneven distribution of vaccines worldwide has resulted in many  
72 low to middle income countries being left without access to variant-specific vaccines that are  
73 better suited for the evolving SARS-CoV-2 variant landscape. This highlights the need for the  
74 development of vaccines that can provide broad protection against a range of SARS-CoV-2  
75 variants, as well as the importance of equitable distribution of vaccines to mitigate the risk of  
76 further virus evolution and spread.<sup>2-5</sup> Since its emergence in late 2019, SARS-CoV-2 has  
77 continuously evolved, at a higher-than-expected rate, giving rise to multiple variants with  
78 multiple genetic mutations and various phenotypic properties, including increased  
79 transmissibility, virulence, and immune escape.<sup>5,6</sup> The emergence of these variants has raised  
80 concerns about the efficacy of current vaccines and the potential for future outbreaks. Therefore,  
81 there is a critical need to develop effective vaccines that can provide broad and durable  
82 protection against SARS-CoV-2 and its variants. SARS-CoV-2 variants such as B.1.1.7 (Alpha),  
83 B.1.351 (Beta), and P.1 (Gamma) have exhibited substantial increases in immune escape from  
84 wildtype (WU) vaccine or infection induced immunity.<sup>7,8</sup>

85           The spike (S) protein of SARS-CoV-2 has been the main target of currently approved  
86 COVID-19 vaccines and of most COVID-19 vaccines in development.<sup>9</sup> S protein allows for  
87 virus binding and infection of susceptible cells through interaction with host receptor  
88 angiotensin-converting enzyme 2 (ACE2).<sup>10</sup> The S1 subunit of the S protein contains the receptor  
89 binding domain (RBD) that binds with ACE2, while the S2 subunit allows for cell fusion and  
90 viral entry.<sup>11,12</sup> It has been widely acknowledged that antibodies targeting the S protein,  
91 particularly those binding to the RBD, are able to block the binding of SARS-CoV-2 to the cell  
92 receptor and prevent infection of susceptible cells.<sup>13-17</sup> We have previously demonstrated the  
93 immunogenicity of S1 subunit targeting vaccines against various Beta-coronaviruses including  
94 SARS-CoV-1, SARS-CoV-2, and MERS.<sup>18-23</sup>

95           A focus for next-generation SARS-CoV-2 vaccine design is the investigation of novel  
96 vaccines which may be able to induce a broader immune response effective against multiple  
97 SARS-CoV-2 variants. A multivalent vaccine is a traditional approach used to increase antigen  
98 immunity coverage against multi-variant viruses such as SARS-CoV-2. We have previously  
99 demonstrated the immunogenicity of a trivalent protein subunit vaccine in BALB/c mice.<sup>22</sup> Here,  
100 we assessed our S1 protein subunit vaccine, at an increased valency to tetravalent, in an  
101 advanced animal model more closely related to humans. Nonhuman primates (NHPs) are  
102 commonly used as preclinical models to evaluate the safety and efficacy of vaccines and  
103 therapeutics for infectious diseases, including SARS-CoV-2.<sup>24-27</sup> We employed a rhesus  
104 macaque (RM) model of controlled simian immunodeficiency virus (SIV) infection to evaluate  
105 the immunogenicity of a tetravalent SARS-CoV-2 S1 protein subunit vaccine delivered with  
106 AddaVax adjuvant. Controlled SIV infection in RMs mimic a situation of chronic viral infection  
107 which can be encountered in humans, which may influence the development of immune

108 responses to vaccination. Indeed, some studies reported lower SARS-CoV-2 antibody responses  
109 for people living with HIV.<sup>28,29</sup> Several studies have demonstrated the utility of RMs as a  
110 preclinical model for SARS-CoV-2 vaccine development. For example, macaques have been  
111 used to evaluate the immunogenicity and the correlates of protection, as well as the protective  
112 efficacy of various vaccine platforms, including viral vector-based vaccines, mRNA vaccines,  
113 and protein subunit vaccines.<sup>26,27,30-34</sup> Moreover, the use of NHP models can provide critical  
114 insights into the mechanisms of vaccine-induced immunity, including the kinetics, specificity,  
115 and durability of the immune responses.

116         Here, we evaluated the immunogenicity of a tetravalent SARS-CoV-2 vaccine approach  
117 with S1 subunit protein vaccine targeting Wuhan S1, B.1.1.7 (Alpha), B.1.351 (Beta), and P.1  
118 (Gamma). We chose these variants because, at the time of the start of the study, they represented  
119 a diverse and relevant set of SARS-CoV-2 strains that were circulating in different regions of the  
120 world and had distinct mutations in the spike protein, which is the main target of neutralizing  
121 antibodies. We found that vaccination induced robust humoral and cellular immune responses  
122 which resulted in antibodies capable of blocking ACE2 binding to 15 different SARS-CoV-2  
123 variants, including multiple Omicron variants. Vaccination also induced antibodies that were  
124 able to block SARS-CoV-2 infection of susceptible cells by live wild-type (WU), Beta, and Delta  
125 variant viruses. We profiled the lymphocyte response to immunization for 2 months post initial  
126 prime vaccination through quantifying the number of T and B cells, investigating markers of T-  
127 cell activation, and memory subsets in peripheral blood mononuclear cells (PBMCs) and showed  
128 robust immune activation, primarily after boost immunization. We were also able to measure a  
129 spike-specific CD4<sup>+</sup> T-cell response in the PBMC's of RMs 42 days post-prime immunization,  
130 although, no CD8<sup>+</sup> T-cell response was found. Our study further demonstrates the

131 immunogenicity of protein subunit vaccines against SARS-CoV-2 targeting the S1 subunit of the  
132 spike protein while also contributing insights on approaches to further increase valency of  
133 currently approved COVID-19 vaccines.

134

## 135 **Results**

### 136 **Design and expression of recombinant proteins**

137 To produce recombinant proteins of SARS-CoV-2-S1 pAd/S1Wu, pAd/S1Alpha,  
138 pAd/S1Beta, and pAd/S1Gamma were generated by subcloning the codon-optimized SARS-  
139 CoV-2-S1 gene having C-tag into the shuttle vector, pAd (GenBank U62024) at *Sal* I and *Not* I  
140 sites (**Fig. 1A**). Variant-specific mutations for B.1.1.7 (Alpha), B.1.351 (Beta), and P.1 (Gamma)  
141 SARS-CoV-2 recombinant S1 proteins are outlined. To determine SARS-CoV-2-S1 expression  
142 from each plasmid, Expi293 cells were transfected with pAd/ S1WU, pAd/S1Alpha,  
143 pAd/S1Beta, and pAd/S1Gamma or pAd as a control. At 5 days after transfection, the  
144 supernatants of Expi293 cells were characterized by Western blot analysis. As shown in **Fig. 1B**,  
145 each S1 recombinant proteins were recognized by a polyclonal anti-spike of SARS-CoV-2  
146 Wuhan antibody at the expected glycosylated monomeric molecular weights of about 110 kDa  
147 under the denaturing reduced conditions, while no expression was detected in the mock-  
148 transfected cells (lane1). The purified rS1WU, rS1Apha, rS1Beta, and rS1Gamma proteins using  
149 C-tagXL affinity matrix were determined by silver staining (**Fig. 1C**).

150

### 151 **Binding antibody and cross-variant live virus neutralizing antibody response**

152 Prior to immunization, RMs were infected with a simian immunodeficiency virus (SIV) that  
153 naturally infects African green monkeys (SIVsab).<sup>35</sup> This virus is completely controlled in

154 RMs,<sup>36</sup> in spite of retaining the replicative abilities.<sup>37</sup> At the time of SARS-CoV-2 immunization,  
155 the RMs were controlling SIVsab for over a year. Upon prime and boost immunization, SIVsab  
156 viral loads remained undetectable suggesting no SIV activation upon vaccination. RMs were  
157 primed and boosted on week 3 with 60 µg total of rS1WU, rS1Apha, rS1Beta, and rS1Gamma,  
158 15 µg of each antigen, mixed with 300 µl of AddaVax<sup>TM</sup>, squalene-based oil in water nano-  
159 emulsion adjuvant (**Fig. 2A**). To assess the magnitude of the antibody response we first  
160 determined Wuhan IgG antibody endpoint titers (EPT) in the sera of vaccinated RMs with  
161 ELISA. Serum samples collected prior to immunization, week 3, week 7, and week 9-11 after  
162 immunization were serially diluted to determine SARS-CoV-2-S1-specific IgG titers against  
163 Wuhan S1 using ELISA (**Fig. 2B**). RMs had detectable anti-S1 binding antibody response prior  
164 to immunization (**Fig 2B**), however, no neutralizing antibody response was found (**Fig. 2C**). S1-  
165 specific IgG titers were statistically increased at week 7 and week 9-11 when compared to week  
166 0 (**Fig. 2B**,  $p < 0.05$ , Kruskal-Wallis test, followed by Dunn's multiple comparisons). To  
167 evaluate the functional quality of vaccine-generate antigen-specific antibodies, we used a  
168 microneutralization assay (NT<sub>90</sub>) to test the ability of sera from immunized RMs to neutralize the  
169 infectivity of SARS-CoV-2. Sera, collected from RMs on week 3 (prior to booster  
170 immunization) and week 7 (4 weeks post boost) after primary immunization were tested for the  
171 presence of SARS-CoV-2-specific neutralizing antibodies with live SARS-CoV-2 Wuhan, Beta,  
172 and Delta viruses (**Fig. 2C**). High levels of neutralizing antibodies were detected in sera at week  
173 3 and week 7 against Wuhan, Beta, and Delta SARS-CoV-2 variants (**Fig. 2C**) and showed a  
174 similar pattern with IgG endpoint titers in each RM (**Supplementary Fig. 2**). Furthermore, the  
175 geometric mean titers (GMT) of neutralizing antibodies at week 7 against the Wuhan, Beta, and  
176 Delta strain were increased with 6.4-, 5.4-, 3.2-fold compared at week 3, respectively, while

177 only neutralizing antibody response against live Wuhan SARS-CoV-2 at week 7 was  
178 significantly increased when compared to preimmunized sera (**Fig. 2C**,  $p < 0.05$ , Kruskal-Wallis  
179 test, followed by Dunn's multiple comparisons). Neutralization against highly immune-evasive  
180 Beta and Delta SARS-CoV-2 variants of concern (VOC) were found at slightly lower levels than  
181 Wuhan at both week 3 and week 7 (**Fig. 2C**). While Beta VOC S1 was included in the tetravalent  
182 immunization regimen, Delta VOC was not, highlighting the diverse response induced by  
183 tetravalent immunization in RMs.

184

### 185 **Potent ACE2 binding inhibition effective against 15 different SARS-COV-2 VOC's** 186 **spikes**

187 For further insight into the neutralizing capabilities of antibodies induced by vaccination  
188 we used the Meso Scale Discovery (MSD) V-PLEX SARS-CoV-2 (ACE2) Kit to measure the  
189 inhibition of binding between angiotensin converting enzyme-2 (ACE2) and trimeric spike  
190 protein of SARS CoV-2 variants. Initially, we used kit Panel 18 including Wuhan S and spikes  
191 from variants; Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), Delta (B.1.617, B.1.617.2), Zeta  
192 (P.2), Kappa (B.1.617.1), B.1.526.1, B.1.617, and B.1.617.3 (**Fig. 3**). Sera from vaccinated RMs  
193 were examined at week 7, due to that being the peak of measured IgG binding antibody response  
194 and compared to preimmunized sera (**Fig. 2A, Fig. 3**). Antibodies blocking ACE2 and trimeric S  
195 binding of all variants, by over 90% inhibition, were detected in all 1:10 diluted RM sera at  
196 Week 7 (**Fig. 3**). Week 7 sera ACE2 binding inhibition for RMs was significantly increased,  
197 when compared to preimmunized sera, for Wuhan, B.1.1.7, B.1.351, P.1, B.1.617.2, P.2,  
198 B.1.617.1, B.1.526.1, B.1.617, and B.1.617.3 Spike (**Fig. 3**,  $p < 0.05$ , Mann-Whitney Test).



199 To assess the neutralizing capabilities of RM vaccine induced antibodies against Omicron  
200 (BA.1) VOC, and Omicron sub-variants (BA.2, BA.3, BA.1+R346K, BA.1+L452R) we used  
201 MSD V-Plex SARS-CoV-2 ACE2 Kit Panel 25 (**Fig. 4**). Panel 25 includes SARS-CoV-2  
202 Wuhan, BA.1, BA.2, AY.4, BA.3, BA.1+R346K, BA.1+L452, B.1.1.7, B.1.351, and B.1.640.2  
203 trimeric spike. Sera from vaccinated RMs were examined at week 3, week 7, and week 9-11 post  
204 vaccination and compared to preimmunized sera at a 1:10 dilution (**Fig. 4A**) and 1:100 dilution  
205 (**Fig 4B**). Week 7 and Week 9-11 RM sera ACE2-binding inhibition were significantly  
206 increased when compared to preimmunized sera for Wuhan, AY.4 (Delta lineage), BA.1+L452R,  
207 B.1.1.7, B.1.351, and B.1.640.2 VOC spikes at 1:10 dilution (**Fig. 4A**,  $p < 0.05$ , Kruskal-Wallis  
208 test, followed by Dunn's multiple comparisons). Week 7 RM sera ACE2-binding inhibition were  
209 significantly increased when compared to preimmunized sera for BA.1 VOC spike at 1:10  
210 dilution (**Fig. 4A**  $p < 0.05$ , Kruskal-Wallis test, followed by Dunn's multiple  
211 comparisons). While not statistically significantly increased when compared to preimmunized  
212 RM sera; RMs demonstrated moderate ACE2-binding inhibition for BA.2, BA.3, and  
213 BA.1+R346K VOC spikes weeks 7 and 9-11 post immunization at 1:10 dilution (**Fig. 4A**,  $p >$   
214  $0.05$ , Kruskal-Wallis test, followed by Dunn's multiple comparisons). To further interrogate the  
215 vaccine-induced neutralizing capabilities of RMs, we further substantially diluted RM sera to  
216 1:100 (**Fig. 4B**). Week 7 RM 1:100 diluted sera ACE-2 binding inhibition was significantly  
217 increased when compared to preimmunized sera for Wuhan, AY.4, B.1.1.7, B.1.351, B.1.640.2  
218 VOC spikes (**Fig. 4B**,  $p < 0.05$ , Kruskal-Wallis test, followed by Dunn's multiple comparisons).  
219 At 1:100 dilution, RM sera did not have ACE-2 binding inhibition above preimmunized sera for  
220 BA.1, BA.2, BA.3, BA.1+R346K, BA.1+L452R VOC spikes (**Fig. 4B**). Results suggest the  
221 necessity of the booster immunization to induce potent and cross variant recognizing antibodies.

222 Results also suggest that vaccination induced antibodies that are able to potently recognize and  
223 block ACE2 binding of a wide range of SARS-CoV-2 variants spikes by week 7 post prime  
224 immunization.

225

## 226 **Longitudinal lymphocyte dynamics and cell-mediate immune response to vaccination** 227 **shows immune activation primarily observed after boost**

228 To investigate the kinetics and magnitude of immune responses induced by the tetravalent  
229 SARS-CoV-2 vaccine, we monitored the peripheral blood mononuclear cells (PBMCs) of  
230 vaccinated rhesus macaques over a 60-day period. PBMCs are a mixture of different immune  
231 cell types, including T cells and B cells, and are a useful tool for investigating the immune  
232 response to vaccination in vivo.

233 **Fig. 5** shows the dynamics of CD3<sup>+</sup> T-cells (**Fig. 5A**), CD4<sup>+</sup> T-cells (**Fig. 5B**), CD8<sup>+</sup> T-cells  
234 (**Fig. 5C**), and CD20<sup>+</sup> B cell (**Fig. 5D**) counts over 60 days. We observed increases in all T-cell  
235 subsets (CD3<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup>) and B cells (CD20<sup>+</sup>) after the prime and especially after the  
236 boost, demonstrating clear increases for all subsets, with the CD8<sup>+</sup> T cell count showing the  
237 greatest increase after boost immunization compared to the other cell types.

238 **Fig. 6** shows the fraction of activating and proliferating CD4<sup>+</sup> and CD8<sup>+</sup> T cells. We used the  
239 activation markers CD69 and HLDR and CD38, as previously described in the literature.<sup>38-40</sup> We  
240 also used Ki-67 as a marker for cell proliferation. CD69<sup>+</sup> CD4<sup>+</sup> T-cell induction was mainly  
241 observed in RM177 (**Fig. 6A**). Ki67<sup>+</sup> CD4<sup>+</sup> T cells showed moderate increases in percentage  
242 after boost vaccination (**Fig. 6B**). HLA-DR<sup>+</sup> CD38<sup>+</sup> CD4<sup>+</sup> T-cells showed activation post prime  
243 and boost with a return to near baseline by Day 40 (**Fig. 6C**). The fraction of CD69<sup>+</sup> CD8<sup>+</sup> T-  
244 cells increased in all RMs post prime and boost, with most starting to return to prevaccination

245 levels at day 60 (**Fig. 6D**). The induction of Ki-67<sup>+</sup> CD8<sup>+</sup> T-cells was primarily seen at day 40  
246 postimmunization (**Fig. 6E**), while HLA-DR<sup>+</sup> CD38<sup>+</sup> CD8<sup>+</sup> T-cell activation was mainly seen in  
247 RM175 and RM176 at different timepoints (**Fig. 6F**). However, the induction of HLA-DR<sup>+</sup>  
248 CD38<sup>+</sup> CD8<sup>+</sup> T cells was not as robust as that of CD69<sup>+</sup> CD8<sup>+</sup> T cells and Ki-67<sup>+</sup> CD8<sup>+</sup> T cells  
249 (**Fig. 6F, Fig. 6D, Fig. 6E**).

250 **Fig. 7** shows the changes in the distribution of T-cell memory subsets over time. We  
251 defined naïve, central memory (CM), and effector memory (EM) T cells using CD28<sup>+</sup> and  
252 CD95<sup>+</sup> markers. Naïve T cells are CD28<sup>+</sup> CD95<sup>neg</sup>, CM T-cells are CD28<sup>+</sup> CD95<sup>+</sup>, and EM T  
253 cells are CD28<sup>neg</sup> CD95<sup>+</sup>. We observed that both CD4<sup>+</sup> and CD8<sup>+</sup> central memory T cells (**Fig.**  
254 **7A & 7D**), along with naïve CD4<sup>+</sup> naïve CD8<sup>+</sup> T cells (**Fig. 7C & 7F**), decreased in abundance  
255 after prime and boost, while CD4<sup>+</sup> and CD8<sup>+</sup> effector memory T cells (**Fig. 7B & 7E**) increased  
256 in abundance after prime boost. This finding suggests that the tetravalent S1 protein vaccine  
257 induces a shift towards an effector memory phenotype and away from a central memory  
258 phenotype, which may be beneficial in generating a rapid and robust response to vaccination.

259 Intracellular cytokine staining was performed to evaluate the spike-specific T-cell responses  
260 in CD4<sup>+</sup> and CD8<sup>+</sup> T cells after stimulation with a spike peptide pool at day 0 and day 42  
261 postvaccination in PBMCs (**Fig. 8**). We tested for interferon-gamma (IFN- $\gamma$ ), interleukin-2 (IL-  
262 2), and tumor necrosis factor-alpha (TNF- $\alpha$ ) cytokine staining. Only RM212 induced an IFN- $\gamma$   
263 CD4<sup>+</sup> T-cell response, while no such response was observed in the other four RMs (**Fig. 8A**). In  
264 **Fig. 8B**, we observed an induction of IL-2 CD4<sup>+</sup> T-cell response in RM212 and to a lesser extent  
265 in RM101, but not in the other three RMs. **Fig. 8C** shows an induction of TNF $\alpha$  CD4<sup>+</sup> T-cell  
266 response in RM212, RM176 and, to a minimal extent, in RM101, RM175, and RM177. Notably,  
267 we were not able to detect a spike specific CD8<sup>+</sup> T-cell response at day 0 or day 42 post

268 vaccination (data not shown). RM212 mounted a robust CD4<sup>+</sup> T-cell response for all three  
269 cytokines at day 42. These results suggest that there is a variable induction of cytokine responses  
270 in CD4<sup>+</sup> T cells among different RMs at day 42 postvaccination.

271 Overall, the use of PBMC's allowed for the unique assessment of the dynamics of immune  
272 activation after vaccination. The results showed a clear increase in T-cell counts and activation  
273 after boost immunization, with the CD8<sup>+</sup> T-cell counts showing the greatest increase. The use of  
274 CD markers allowed for the differentiation of T-cell subsets and their activation status, with the  
275 CD8<sup>+</sup> T cells expressing either CD69 or Ki-67 CD8<sup>+</sup> T cells showing the most robust dynamics.  
276 Additionally, there was evidence of a functional spike-specific CD4<sup>+</sup> T-cell response in RMs at  
277 day 42 post vaccination, albeit in the context of no CD8<sup>+</sup> T-cell response. These findings  
278 highlight the potential of this vaccine candidate to induce a robust cellular immune response,  
279 which is critical for controlling viral infections.

280

## 281 **Discussion**

282 We evaluated the immunogenicity and efficacy of a tetravalent COVID-19 vaccine  
283 candidate based on the spike S1 protein of SARS-CoV-2 in an NHP model of controlled SIV  
284 infection. RMs infected with SIVsab from African green monkeys are able to control viral  
285 replication and disease progression through maintaining a healthy immune system, unlike HIV-1  
286 in humans.<sup>36</sup> The SIVsab-infected RMs in this study were elite controllers for about a year prior  
287 to SARS-CoV-2 immunization.

288 There were weaker band in western blot of the supernatant after a transient transfection  
289 with pAd/S1Alpha, pAd/S1Beta, and pAd/S1Gamma compared with pAd/S1WU (**Fig. 1B**),  
290 which might be explained by the usage of anti-spike of SARS-CoV-2 Wuhan as a primary

291 antibody. Indeed, no big differences were observed in yield pre or post C-tag purification of each  
292 recombinant proteins after transient transfection by sandwich ELISA with standard of each  
293 purified rS1 proteins (**Supplementary Fig.1**).

294 Our vaccine formulation induced high levels of binding antibodies against the Wuhan  
295 strain of SARS-CoV-2, as well as neutralizing antibodies against live B.1.351 (Beta), and  
296 B.1.617.2 (Delta) VOC (**Fig. 2**). The sera of vaccinated RMs exhibited potent ACE2-binding  
297 inhibition capabilities against a suite of SARS-CoV-2 VOC spikes including Omicron (BA.1)  
298 and Omicron subvariants (BA.2, BA.3, BA.1+R246K, and BA.1+L452R) (**Fig. 3 & Fig. 4**).  
299 These findings are consistent with previous studies demonstrating the immunogenicity and cross-  
300 reactivity of COVID-19 vaccines NHP models.<sup>26,27,30-33,41</sup>

301 Importantly, the vaccine candidate also induced cellular immune responses, including T  
302 cell responses, which have been shown to play a critical role in COVID-19 immunity and  
303 protection.<sup>42-49</sup> We investigated the cellular immune response to the tetravalent SARS-CoV-2  
304 vaccine in vaccinated RMs, using a range of markers to examine T-cell subsets and activation  
305 status. The results showed that all T-cell subsets and B cells increased after the prime and  
306 especially after the boost, with the CD8<sup>+</sup> T-cell count showing the greatest increase after boost  
307 immunization compared to other cell types (**Fig. 5**). We demonstrate that the tetravalent S1  
308 subunit protein COVID-19 vaccine candidate induces CD4<sup>+</sup> and CD8<sup>+</sup> T-cell activation, as  
309 indicated by increased expression of CD69, HLA-DR, CD38, and Ki-67 activation and  
310 proliferation markers on both T-cell subsets (**Fig. 6**). The distribution of T-cell memory subsets  
311 over time was also investigated, revealing a decrease in abundance of both CD4<sup>+</sup> and CD8<sup>+</sup>  
312 central memory T cells, along with CD4<sup>+</sup> and CD8<sup>+</sup> naive T cells after prime and boost (**Fig. 7**).  
313 In contrast, CD4<sup>+</sup> and CD8<sup>+</sup> effector memory T cells increased in abundance after prime boost,

314 indicating a shift towards an effector memory phenotype and away from a central memory  
315 phenotype induced by the tetravalent S1 protein vaccine (**Fig. 7**). Furthermore, intracellular  
316 cytokine staining was performed to evaluate the spike-specific responses of CD4<sup>+</sup> and CD8<sup>+</sup> T  
317 cells after stimulation with a spike peptide pool (**Fig. 8**). Cytokine staining for IFN- $\gamma$ , IL-2, and  
318 TNF- $\alpha$  was tested and a variable induction of cytokine responses by CD4<sup>+</sup> T cells among  
319 different RMs at day 42 postvaccination was observed (**Fig. 8**). However, no spike-specific  
320 response of the CD8<sup>+</sup> T cells was detected at day 0 or day 42. It is possible that the spike-specific  
321 CD8<sup>+</sup> T cells were present, but were not detected by the intracellular staining assay, as this assay  
322 may not be sensitive enough to detect low-frequency antigen-specific CD8<sup>+</sup> T cells. It is also  
323 possible that the undetectable spike-specific CD8<sup>+</sup> T-cell response at day 42 post-vaccination  
324 was related to the time-point used, which was too late after boost, such as the vaccine-specific T  
325 cells had already started to wane in abundance, as shown by Arunachalam et al.<sup>50</sup> Altogether, our  
326 study demonstrates that the tetravalent S1 protein vaccine candidate was able to induce a robust  
327 SARS-CoV-2-specific immune response in RMs, which is promising for future development and  
328 testing of COVID-19 vaccines in humans.

329         The results of our study have important implications for COVID-19 vaccine development  
330 and implementation in humans. The vaccine candidate induced not only humoral immune  
331 responses but also cellular immune responses, which have been shown to be important for long-  
332 term immunity.<sup>51</sup> The use of RMs as an animal model for studying vaccine efficacy has been  
333 widely accepted in the scientific community.<sup>25,26,34,52</sup> Here we have used RM controllers based  
334 on the rationale that SIV controllers have a nearly healthy immune system (able to control SIV  
335 replication).<sup>36</sup> We also wanted to assess whether the induction of T-cell activation at the effector  
336 sites would result in a burst of SIV replication. Such a boosting of SIV was reported to occur

337 after administration of vectorized vaccines.<sup>53</sup> The use of NHP models has been shown to be  
338 highly informative for predicting vaccine efficacy in humans.<sup>54,55</sup>

339 The results showed that the vaccine induced both humoral and cellular immune responses  
340 against SARS-CoV-2, including neutralizing antibodies, ACE2 blocking antibodies, and T-cell  
341 responses. Furthermore, the vaccine candidate was able to generate Omicron variant binding and  
342 ACE2 blocking antibodies without specifically vaccinating with Omicron, suggesting the  
343 potential for broad protection against emerging variants.<sup>56–60</sup> This is particularly significant given  
344 the emergence of highly diverged SARS-CoV-2 variants, such as Omicron, which have raised  
345 concerns about vaccine efficacy and the need for updated vaccines.<sup>56,58,59,61</sup> Another significant  
346 feature of the vaccine candidate is its tetravalent composition, which targets the spike proteins of  
347 four different SARS-CoV-2 variants. This approach has the potential to provide broad protection  
348 against multiple SARS-CoV-2 variants, as well as to minimize the risk of immune escape and  
349 emergence of new variants.

350 Protein subunit vaccines are known for their safety, ease of large-scale production, and  
351 distribution, and have been used in other successful vaccine campaigns, such as the hepatitis B  
352 vaccine.<sup>54,62–64</sup> This makes protein subunit vaccines an ideal candidate for worldwide vaccine  
353 equity, particularly for countries that may not have access to the more complex mRNA or viral  
354 vector vaccine platforms. Furthermore, the ability to store and transport protein subunit vaccines  
355 at a relatively low temperature (-20°C to 4°C), compared to the ultra-low temperature required  
356 for mRNA vaccines, makes their distribution and administration easier in resource-limited  
357 settings.<sup>65,66</sup> The protein subunit platform is also amenable to alternative routes of administration,  
358 such as intradermal delivery, which has been shown to increase immunogenicity in other vaccine  
359 studies.<sup>20,67–69</sup> In summary, the tetravalent S1 protein subunit vaccine represents a promising

360 vaccine candidate against SARS-CoV-2, particularly for populations that may not have access to  
361 other vaccine platforms and could potentially be further optimized to enhance its  
362 immunogenicity.

363         However, it should be noted that this study has limitations. The sample size was small  
364 and we did not perform a SARS-CoV-2 virus challenge in our vaccinated RMs to fully assess  
365 vaccine efficacy.<sup>27,50</sup> While our results show promising immune responses to the tetravalent  
366 SARS-CoV-2 vaccine in RMs, a virus challenge would have provided further insights into the  
367 effectiveness of the vaccine in preventing infection and disease. Additionally, our study did not  
368 evaluate the durability of the antibody response generated by the vaccine over a longer period.  
369 Studies have shown that antibody responses to SARS-CoV-2 vaccines may wane over time,  
370 which highlights the importance of evaluating the longevity of vaccine-induced immunity.<sup>70-75</sup>  
371 Finally, we did not assess mucosal immunity in our study, which is an important aspect of  
372 immune protection against respiratory viruses like SARS-CoV-2. Mucosal immunity may  
373 provide an additional layer of protection against infection and transmission, and future studies  
374 should investigate the mucosal immune response to the tetravalent SARS-CoV-2 vaccine.<sup>31,76-79</sup>

375         The tetravalent S1 subunit protein COVID-19 vaccine candidate evaluated in this study  
376 contained SARS-CoV-2 S1 antigens from the Wuhan strain, as well as the B.1.1.7 variant,  
377 B.1.351 variant, and P.1 variant. Our study demonstrates that this vaccine candidate can induce  
378 both humoral and cellular immune responses, as evidenced by increased cell counts in both T  
379 and B cells, and the production of neutralizing and cross-reactive antibodies, as well as ACE2  
380 blocking antibodies and T cell responses. It is important to note that the RMs used in this study  
381 were infected with SIVsab and controlled the infection for a year prior to immunization. The  
382 ability of these animals to control the SIVsab infection, without reactivation of virus upon



383 immunization, while mounting immune responses to the vaccine candidate, further demonstrates  
384 the potential of this vaccine candidate to provide robust protection against SARS-CoV-2, even in  
385 individuals with pre-existing conditions. Moreover, the tetravalent composition of the vaccine  
386 candidate has significant implications for COVID-19 vaccine development and implementation,  
387 with the potential to provide broad protection against multiple SARS-CoV-2 variants and to  
388 minimize the risk of immune escape and emergence of new variants.

389

## 390 **Materials and methods**

### 391 **Construction of recombinant protein expressing vectors**

392 The coding sequence for SARS-CoV-2-S1 amino acids 1 to 661 of full-length from  
393 BetaCoV/Wuhan/IPBCAMS-WH-05/2020 (GISAID accession id. EPI\_ISL\_403928) having C-  
394 terminal tag known as ‘C-tag’, composed of the four amino acids (aa), glutamic acid-proline-  
395 glutamic acid-alanine (E-P-E-A) flanked with *Sal* I & *Not* I was codon-optimized using the  
396 UpGene algorithm for optimal expression in mammalian cells (68) and synthesized (GenScript).  
397 The construct also contained a Kozak sequence (GCCACC) at the 5' end. For Alpha variant  
398 (B.1.1.7), SARS-CoV-2-S1 mutated Del69-70; Del144; N501Y; A570D; D614G was  
399 synthesized. Also, Beta variant (B.1.351) of SARS-CoV-2-S1 (Del144; K417N; E484K; N501Y;  
400 A570D; D614G) and Gamma variant (P.1) of SARS-CoV-2-S1 (L18F; T20N; P26S; D138Y;  
401 R190S; K417T; E484K; N501Y; H655Y) were synthesized based on above codon-optimized  
402 SARS-CoV-2-S1 Wuhan. pAd/S1WU, pAd/S1Alpha, pAd/S1Beta, and pAd/S1Gamma, were  
403 then created by subcloning the four variants of codon-optimized SARS-CoV-2-S1 inserts into the  
404 shuttle vector, pAdlox (GenBank U62024), at *Sal* I/*Not* I sites. The plasmid constructs were  
405 confirmed by DNA sequencing.

406

### 407 **Transient Production in Expi293 Cells**

408 pAd/S1WU, pAd/S1Alpha, pAd/S1Beta, and pAd/S1Gamma, were amplified, and  
409 purified using ZymoPURE II plasmid maxiprep kit (Zymo Research). For Expi293 cell  
410 transfection, we used ExpiFectamie™ 293 Transfection Kit (ThermoFisher) and followed the  
411 manufacturer's instructions. Cells were seeded  $3.0 \times 10^6$  cells/ml one day before transfection and  
412 grown to  $4.5\text{-}5.5 \times 10^6$  cells/ml. 1µg of DNA and ExpiFectamine mixtures per 1ml culture were  
413 combined and incubated for 15 min before adding into  $3.0 \times 10^6$  cells/ml culture. At 20 h post-  
414 transfection, enhancer mixture was added, and culture was shifted to 32°C. The supernatants  
415 were harvested 5 days post transfection and clarified by centrifugation to remove cells, filtration  
416 through 0.8 µm, 0.45 µm, and 0.22 µm filters and either subjected to further purification or  
417 stored at 4°C before purification.

418

### 419 **SDS-PAGE and western blot**

420 To evaluate the expression of S1 from the plasmids, Expi293 cells were transfected with  
421 pAd/S1WU, pAd/S1Alpha, pAd/S1Beta, and pAd/S1Gamma, respectively. At 5 days after  
422 transfection, 10 µl each supernatant of Expi293 cells was subjected to sodium dodecyl sulfate  
423 polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot as previously described.<sup>20</sup>  
424 Briefly, after the supernatants were boiled in Laemmli sample buffer containing 2% SDS with  
425 beta-mercaptoethanol (β-ME), the proteins were separated by Tris-Glycine SDS-PAGE gels and  
426 transferred to nitrocellulose membrane. After blocking for 1 hour at room temperature (RT) with  
427 5% non-fat milk in PBST, rabbit anti-SARS-CoV Wuhan spike polyclonal antibody (1:3000)  
428 (Sino Biological) was added and incubated overnight at 4°C as primary antibody, and

429 horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:10000) (Jackson  
430 immunoresearch) was added and incubated at RT for 2 hours as secondary antibody. After  
431 washing three times with PBST, the signals were visualized on an iBright FL 1500 Imager  
432 (ThermoFisher).

433

#### 434 **Purification of recombinant proteins**

435 The recombinant proteins named rS1WU, rS1Alpha, rS1Beta, and rS1Gamma were  
436 purified using a CaptureSelect™ C-tagXL Affinity Matrix prepacked column (ThermoFisher)  
437 and followed the manufacturer's guidelines. Briefly, The C-tagXL column was conditioned with  
438 10 column volumes (CV) of equilibrate/wash buffer (20 mM Tris, pH 7.4) before sample  
439 application. Supernatant was adjusted to 20 mM Tris with 200 mM Tris (pH 7.4) before being  
440 loaded onto a 5-mL prepacked column per the manufacturer's instructions at 5 ml/min rate. The  
441 column was then washed by alternating with 10 CV of equilibrate/wash buffer, 10 CV of strong  
442 wash buffer (20 mM Tris, 1 M NaCl, 0.05% Tween-20, pH 7.4), and 5 CV of equilibrate/wash  
443 buffer. The recombinant proteins were eluted from the column by using elution buffer (20 mM  
444 Tris, 2 M MgCl<sub>2</sub>, pH 7.4). The eluted solution was concentrated and desalted with preservative  
445 buffer (PBS) in an Amicon Ultra centrifugal filter devices with a 50,000 molecular weight cutoff  
446 (Millipore). The concentrations of the purified recombinant proteins were determined by the  
447 BCA protein assay kit (ThermoFisher) and separated by reducing SDS-PAGE and visualized by  
448 silver staining. The rest proteins were aliquoted and stored at -80°C until use.

449

#### 450 **ELISA**

451 Sera from all rhesus macaques were collected prior to immunization and on weeks 3 and  
452 7 after immunization. Sera was evaluated for SARS-CoV-2 S1-specific IgG using ELISA.  
453 ELISA plates were coated with 200 ng of recombinant SARS-CoV-2-S1 protein (Sino  
454 Biological) per well overnight at 4°C in carbonate coating buffer (pH 9.5) and then blocked with  
455 PBS-T and 2% bovine serum albumin (BSA) for one hour. Rhesus macaque sera was inactivated  
456 at 64°C for 40 minutes, then diluted in PBS-T with 1% BSA and incubated overnight. After the  
457 plates were washing, anti-monkey IgG-horseradish peroxidase (HRP) (1:50000, Sigma) were  
458 added to each well and incubated for one hour. The plates were washed three times, developed  
459 with 3,3',5,5'-tetramethylbenzidine, and the reaction was stopped with 1M H<sub>2</sub>SO<sub>4</sub>. Next,  
460 absorbance was determined at 450nm using a plate reader (Molecular Devices SPECTRAmax).

461

## 462 **Animals and Immunization**

463 At week 0, male RMs (n=5 animals per group) were bled and primed with 60 µg of  
464 tetravalent rS1 proteins of Wuhan, B.1.1.7 (Alpha), B.1.351 (Beta), and P.1 (Gamma) [15µg of  
465 each antigen]. Total volume of 300 µl of antigen was mixed with 300 µl of AddaVax adjuvant  
466 then administered to RMs (600 µl injection volume). RMs were bled on week 3 and received a  
467 homologous booster of 60 µg of tetravalent rS1 proteins. RMs were bled on weeks 7. RMs were  
468 also bled and serially euthanized after week 9 post-prime vaccination: on day 0 (RM177), 1  
469 (RM175), 6 (RM176), 8 (RM101), and 15 (RM175). PMBC's from RMs were collected and  
470 analyzed on Days -1, 3, 7, 10, 14, 21, 24, 28, 31, 35, 42, 49, and 64 days post prime  
471 immunization. RMs were maintained under specific pathogen-free conditions at the University of  
472 Pittsburgh, and all experiments were conducted in accordance with animal use guidelines and

473 protocols approved by the University of Pittsburgh's Institutional Animal Care and Use  
474 (IACUC) Committee.

475

#### 476 **SARS-CoV-2 microneutralization assay**

477 Neutralizing antibody (NT-Ab) titers against SARS-CoV-2 were defined according to the  
478 following protocol.<sup>80,81</sup> Briefly, 50 µl of sample from each mouse, starting from 1:10 in a  
479 twofold dilution, were added in two wells of a flat bottom tissue culture microtiter plate  
480 (COSTAR, Corning Incorporated, NY 14831, USA), mixed with an equal volume of 100  
481 TCID<sub>50</sub> of a SARS-CoV-2 Wuhan, Beta, or Delta strain isolated from symptomatic patients,  
482 previously titrated, and incubated at 33°C in 5% CO<sub>2</sub>. All dilutions were made in EMEM  
483 (Eagle's Minimum Essential Medium) with addition of 1% penicillin, streptomycin and  
484 glutamine and 5 γ/mL of trypsin. After 1 hour incubation at 33°C 5% CO<sub>2</sub>, 3×10<sup>4</sup> VERO E6 cells  
485 [VERO C1008 (Vero 76, clone E6, Vero E6); ATCC® CRL-1586™] were added to each well.  
486 After 72 hours of incubation at 33°C 5% CO<sub>2</sub> wells were stained with Gram's crystal violet  
487 solution (Merck KGaA, 64271 Damstadt, Germany) plus 5% formaldehyde 40% m/v (Carlo  
488 ErbaSpA, Arese (MI), Italy) for 30 min. Microtiter plates were then washed in running water.  
489 Wells were scored to evaluate the degree of cytopathic effect (CPE) compared to the virus  
490 control. Blue staining of wells indicated the presence of neutralizing antibodies. Neutralizing  
491 titer was the maximum dilution with the reduction of 90% of CPE. A positive titer was equal or  
492 greater than 1:10. The geometric mean titers (GMT) of NT<sub>90</sub> end point titer were calculated with  
493 4 as a negative shown <10. Sera from mice before vaccine administration were always included  
494 in microneutralization (NT) assay as a negative control.

495

496 **ACE2 Blocking Assay**

497 Antibodies blocking the binding of SARS-CoV-2 spike variants (Alpha (B.1.1.7), Beta  
498 (B.1.351), Gamma (P.1), Delta (B.1.617.2), Zeta (P.2), Kappa (B.1.617.1), New York  
499 (B.1.516.1), India (B.1.617 and B.1.617.3)) to ACE2 were detected with a V-PLEX SARS-CoV-  
500 2 Panel 18 (ACE2) Kit (Meso Scale Discovery (MSD) according to the manufacturer's  
501 instructions. Antibodies blocking the binding of SARS-CoV-2 spike including Wuhan and spikes  
502 from immune evasive variants; BA.1, BA.2, AY.4 (Delta lineage), BA.3, BA.1+R346K  
503 mutation, BA.1+L452R mutation, B.1.1.7 (Alpha), B.1.351 (Beta), and B.1.1640.2 to ACE2  
504 were detected with a V-PLEX SARS-CoV-2 Panel 25 (ACE2) Kit (Meso Scale Discovery  
505 (MSD) according to the manufacturer's instructions. Serum samples were diluted (1:10 and  
506 1:100). The assay plate was blocked for 30 min and washed. Serum samples were diluted (1:10  
507 for P18; 1:10 & 1:100 for P25) and 25 µl were transferred to each well. The plate was then  
508 incubated at room temperature for 60 min with shaking at 700 rpm, followed by the addition of  
509 SULFO-TAG conjugated ACE2, and continued incubation with shaking for 60 min. The plate  
510 was washed, 150 µl MSD GOLD Read Buffer B was added to each well, and the plate was read  
511 using the QuickPlex SQ 120 Imager. Electrochemiluminescent values (ECL) were generated for  
512 each sample. Results were calculated as % inhibition compared to the negative control for the  
513 ACE2 inhibition assay, and % inhibition is calculated as follows: % neutralization =  $100 \times (1 -$   
514 (sample signal/negative control signal).

515

516 **Flow Cytometry**

517 Absolute counts of immune cells in whole blood and immunophenotyping of circulating  
518 immune cells were determined by flow cytometry. First, 50 µl of whole blood were added to a

519 TruCount tube (BD Biosciences) containing an antibody mix, allowing to precisely quantify  
520 CD45<sup>+</sup> cell counts in blood, as well as CD4<sup>+</sup> and CD8<sup>+</sup> T cells, and CD20<sup>+</sup> B cells. Whole  
521 peripheral blood was stained with fluorescently-labeled antibodies (all purchased from BD  
522 Bioscience, San Jose, CA, USA, unless noted otherwise): CD3 (clone SP34-2, V450), CD4  
523 (clone L200, APC), CD8 (clone RPA-T8, PE-CF594), CD28 (clone CD28.2, PE-Cy7), CD38  
524 (clone AT-1, FITC) (Stemcell), CD45 (clone D058-1283, PerCP), CD69 (clone FN50, APC-H7),  
525 CD95 (clone DX2, FITC), HLA-DR (clone L243, PE-Cy7), Ki-67 (clone P56, PE). For  
526 intracellular staining, cells were fixed and permeabilized with 1X BD Fix/Perm, before being  
527 stained for Ki-67. Flow cytometry acquisitions were performed on an LSRFortessa flow  
528 cytometer (BD Biosciences), and flow data were analyzed using FlowJo® v10.8.0 (TreeStar,  
529 Ashland, OR, USA).

530

### 531 **Spike-Specific Intracellular Staining**

532 Antigen-specific T-cell responses in the PBMC's of RMs immunized as described above  
533 were analyzed after immunization by flow cytometry, adhering to the recently published  
534 guidelines.<sup>21,82</sup> PBMCs collected prior to immunization and on Day 42 post prime immunization  
535 were stimulated with PepTivator SARS-CoV-2-S1 (a pool of S1 MHC class I- and MHC class  
536 II- restricted peptides) overnight in the presence of protein transport inhibitors (Golgi Stop) for  
537 the last 4 hours. Unstimulated cells were used as negative controls. Phorbol myristate acetate  
538 (PMA) and ionomycin stimulated cells served as positive controls. Cell were washed with FACS  
539 buffer (PBS, 2 % FCS), incubated with Fc Block (BD Biosciences, 553142) for 5 min at 4°C,  
540 and stained with surface marker antibody (Ab) stain for 20 min at 4°C. Surface Abs were used as  
541 follows: CD3-V450 (SP34-2, V450, BD Biosciences), CD4-APC (L200, APC, BD Biosciences),

542 and CD8ab-PE-CF594 (RPA-T8, PE-CF594, BD Biosciences). For dead cell exclusion, cells  
543 were stained with Zombie NIR Fixable Viability dye (BioLegend) for 10 min at 4°C and washed  
544 in FACS buffer. Intracellular cytokine staining (ICS) was performed on surface Ab-stained cells  
545 by first fixing and permeabilizing cells using the FoxP3 Transcription Factor Staining Buffer kit  
546 (eBioscience, 00-5523-00) following manufacturer's instructions. Intracellular staining with  
547 IFN $\gamma$ -FITC (4S.B3, FITC, BD Biosciences), IL2-PE (MQ1-17H12, PE, BD Biosciences), and  
548 TNFa-AF700 (Mab11, AF700, BD Biosciences). Samples were run on an Aurora (Cytex) flow  
549 cytometer and flow data were analyzed using FlowJo® v10.8.0 (TreeStar, Ashland, OR, USA).

550

### 551 **Statistical Analysis**

552 Statistical analyses were performed using GraphPad Prism v9 (San Diego, CA).  
553 Significant differences are indicated by \*  $p < 0.05$ . Comparisons with non-significant differences  
554 are not indicated.

555

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566

## 567 **Disclosure**

568 The authors declare that they have competing interests in relation to the research  
569 presented in this manuscript. AG, EK, and MSK are co-founders of GAPHAS  
570 PHARMACEUTICAL INC., a private startup company that may potentially benefit from the  
571 findings of this research. AG, EK, and MSK have equity in GAPHAS PHARMACEUTICAL  
572 INC. However, the authors have taken measures to ensure that the research is conducted  
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577

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772

## 773 **FIGURE LEGENDS**

### 774 **Figure 1. Construction and expression of tetravalent recombinant SARS-CoV-2-S1**

775 **proteins. A.** A shuttle vector carrying the codon-optimized four variants of SARS-CoV-2-S1  
776 gene encoding N-terminal 1-661 with c-tag (EPEA) was designated as shown in the diagram.

777 Amino acid changes in the SARS-CoV-2-S1 region of in this study is shown. ITR: inverted  
778 terminal repeat; RBD: receptor binding domain. **B.** Detection of the SARS-CoV-2-S1 proteins by

779 western blot with the supernatant of Expi293 cells transfected with pAd/S1WU (lane2),

780 pAd/S1Alpha (lane3), pAd/S1Beta (lane4), and pAd/S1Gamma (lane5), respectively, using

781 rabbit anti spike of SARS-CoV Wuhan polyclonal antibody. As a negative control, mock-

782 transfected cells were treated the same (lane 1). **C.** Purified proteins, rS1WU (lane1), rS1Alpha

783 (lane2), rS1Beta (lane3), and rS1Gamma (lane4), isolated by c-tag affinity purification were

784 separated by SDS-PAGE and visualized by silver staining. Molecular weight marker (MW  
785 marker) is indicated on the left.

786

787 **Figure 2. Antigen-specific antibody responses in rhesus macaques immunized with**  
788 **tetravalent SARS-CoV-2 rS1 protein subunit vaccine. A.** Schedule of immunization and  
789 blood sampling for IgG end point titration. Rhesus macaques (N=5) were immunized with 60µg  
790 of tetravalent rS1 proteins of Wuhan, B.1.1.7 (Alpha), B.1.351 (Beta), and P.1 (Gamma) [15µg  
791 of each antigen] mixed with AddaVax adjuvant then administered to RMs arm at week 0 and 3.  
792 Syringes indicated the timing of immunization and the red drops denote times at which blood  
793 was drawn. The red crosses showed euthanized times of each RM. **B.** Sera were diluted and  
794 SARS-CoV-2-S1-specific antibodies were quantified by ELISA to determine the IgG endpoint  
795 titer. The IgG titers at each time points were showed in each RM. The bars represent geometric  
796 mean with geometric SD. **C.** Neutralizing antibodies in serum of mice prior to immunization,  
797 along with week 3 and week 7 post immunization were measured using a microneutralization  
798 assay (NT<sub>90</sub>) with SARS-CoV-2 Wuhan, Beta, and Delta. Serum titers that resulted in 90%  
799 reduction in cytopathic effect compared to the virus control were reported. Horizontal lines  
800 represent geometric mean titers. Groups were compared by Kruskal-Wallis test at each time  
801 point, followed by Dunn's multiple comparisons. Significant differences are indicated by \*p <  
802 0.05. N = 5 rhesus macaques per group for each experiment.

803 **Figure 3. Percent ACE2 binding inhibition of neutralizing antibodies against SARS-CoV-2**  
804 **variants.** Antibodies in sera (diluted 1:10) capable of neutralizing the interaction between  
805 SARS-CoV-2 Wuhan, Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2), Zeta  
806 (P.2), Kappa (B.1.617.1), New York (B.1.516.1), India (B.1.617 and B.1.617.3) variants spike

807 and ACE2 were examined in all animals preimmunization and Week 7 post prime immunization  
808 with V-PLEX SARS-CoV-2 Panel 18. Groups were compared by Kruskal-Wallis test at each  
809 time point, to preimmunized sera control, followed by Dunn's multiple comparisons. Significant  
810 differences are indicated by \* $p < 0.05$ . N = 5 rhesus macaques per group for each experiment.

811

812 **Figure 4. Percent ACE2 binding inhibition of neutralizing antibodies against Omicron**  
813 **SARS-CoV-2 variants.** Antibodies in sera, diluted **A.** 1:10 and **B.** 1:100 capable of blocking the  
814 binding of SARS-CoV-2 spike including Wuhan and spikes from immune evasive variants;  
815 BA.1, BA.2, AY.4 (Delta lineage), BA.3, BA.1+R346K mutation, BA.1+L452R mutation,  
816 B.1.1.7 (Alpha), B.1.351 (Beta), and B.1.1640.2 to ACE2 were detected with a V-PLEX SARS-  
817 CoV-2 Panel 25. Groups were compared by Kruskal-Wallis test at each time point, to  
818 preimmunized sera control, followed by Dunn's multiple comparisons. Significant differences  
819 are indicated by \* $p < 0.05$ . N = 5 rhesus macaques per group for each experiment.

820 **Figure 5. CD3, CD4, CD8, and CD20 cell counts post immunization and boost.** Absolute  
821 counts of immune cells in whole blood and immunophenotyping of circulating immune cells  
822 were determined by flow cytometry. 50  $\mu$ l of whole blood were added to a TruCount tube (BD  
823 Biosciences) containing an antibody mix, allowing to precisely quantify **A.** CD45<sup>+</sup> cells, **B.**  
824 CD4<sup>+</sup>, **C.** CD8<sup>+</sup> T cells, and **D.** CD20<sup>+</sup> B cells in blood per  $\mu$ l. PMBC's from RMs were collected  
825 and analyzed on Days -1, 3, 7, 10, 14, 21, 24, 28, 31, 35, 42, 49, and 64 days post prime  
826 immunization. Individual results for each RM are depicted.

827

828 **Figure 6. CD4 CD8 T cell activation post immunization and boost.** Whole peripheral blood  
829 was stained with fluorescently labeled antibodies for CD4<sup>+</sup>, CD8<sup>+</sup>, CD69<sup>+</sup>, Ki-67<sup>+</sup>, and HLA-

830 DR<sup>+</sup> to investigate CD4 and CD8 activation induced by vaccination with flow cytometry. **A.**  
831 Frequencies of CD4<sup>+</sup> CD69<sup>+</sup> T cells, **B.** Frequencies of CD4<sup>+</sup> Ki-67<sup>+</sup> T cells, **C.** Frequencies of  
832 CD4<sup>+</sup> HLA-DR<sup>+</sup> CD38<sup>+</sup> T cells, **D.** Frequencies of CD8<sup>+</sup> CD69<sup>+</sup> T cells, **E.** Frequencies of CD8<sup>+</sup>  
833 Ki-67<sup>+</sup> T cells, and **F.** Frequencies of CD8<sup>+</sup> HLA-DR<sup>+</sup> CD38<sup>+</sup> T cells. PMBC's from RMs were  
834 collected and analyzed on Days -1, 3, 7, 10, 14, 21, 24, 28, 31, 35, 42, 49, and 64 days post  
835 prime immunization. Individual results for each RM are depicted.

836

837 **Figure 7. T cell memory subset dynamics and induction post immunization and boost.**

838 Whole peripheral blood was stained with fluorescently labeled antibodies for CD4<sup>+</sup>, CD8<sup>+</sup>,  
839 CD28<sup>+</sup> and CD95<sup>+</sup>. Memory subsets were defined naive, central memory (CM), and effector  
840 memory (EM) T cells using CD28<sup>+</sup> and CD95<sup>+</sup> markers. Naive T cells are CD28<sup>+</sup>CD95<sup>-</sup>, CM T  
841 cells are CD28<sup>+</sup>CD95<sup>+</sup>, and EM T cells are CD28<sup>-</sup>CD95<sup>+</sup>. **A.** Frequencies of CD4<sup>+</sup> CM T cells,  
842 **B.** Frequencies of CD4<sup>+</sup> EM T cells, **C.** Frequencies of CD4<sup>+</sup> Naive T cells, **D.** Frequencies of  
843 CD8<sup>+</sup> CM T cells, **E.** Frequencies of CD8<sup>+</sup> EM T cells, and **F.** Frequencies of CD8<sup>+</sup> Naïve T  
844 cells. PMBC's from RMs were collected and analyzed on Days -1, 3, 7, 10, 14, 21, 24, 28, 31,  
845 35, 42, 49, and 64 days post prime immunization. Individual results for each RM are depicted.  
846

847 **Figure 8. Spike-specific CD4+ T cell responses at Day 0 and Day 42 post immunization in**

848 **PBMC's.** PBMC's collected prior to immunization and on Day 42 post prime immunization  
849 were stimulated with PepTivator SARS-CoV-2-S1 (a pool of S1 MHC class I- and MHC class  
850 II- restricted peptides), followed by intracellular staining (ICS) and flow cytometry to identify  
851 SARS-CoV-2 S1 specific T cells. **(A)** Frequencies of SARS-CoV-2 S1 CD4<sup>+</sup> IFN- $\gamma$ <sup>+</sup> T cells.  
852 Individual results for each RM are depicted. **(B)** Frequencies of SARS-CoV-2 S1 CD4<sup>+</sup> IL-2<sup>+</sup>

853 T cells. Individual results for each RM are depicted. (C) Frequencies of SARS-CoV-2 S1  
854 CD4<sup>+</sup> TNF $\alpha$  T cells. Individual results for each RM are depicted. Day 0 PBMC responses are  
855 indicated by solid circle. Day 42 PBMC responses are indicated by solid triangle.

856

857 **Supplementary Figure 1. Yield pre and post C-tag purification of each recombinant**

858 **proteins after transient transfection.** To evaluate the expression of rS1WU, rS1Alpha,

859 rS1Beta, and rS1Gamma recombinant proteins, ELISA plates were coated with chimeric

860 MAb 40150-D003 (1:750, Sino Biological) overnight at 4°C. **A.** The supernatants of Expi293<sup>TM</sup>

861 cells transfected with pAd/S1WU, pAd/S1Alpha, pAd/S1Beta, and pAd/S1Gammawas,

862 respectively, diluted 1:40 or **B.** purified each protein by a CaptureSelect<sup>TM</sup> C-tagXL Affinity

863 Matrix prepacked column diluted 1:1000 in PBS-T with 1% BSA and along with each purified

864 rS1 proteins for a standard curve were incubated overnight at 4°C. After the plates were

865 washed, chimeric MAb 40150-D001 HRP conjugated secondary antibody (1:10000, Sino

866 Biological) was added to each well. After the development with reagent, the reaction was

867 determined using an ELISA reader (Molecular Devices SPECTRAmax) in same as described in

868 materials and methods.

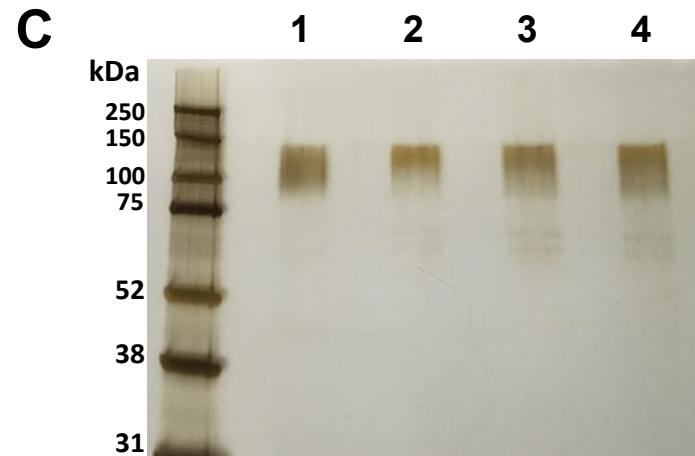
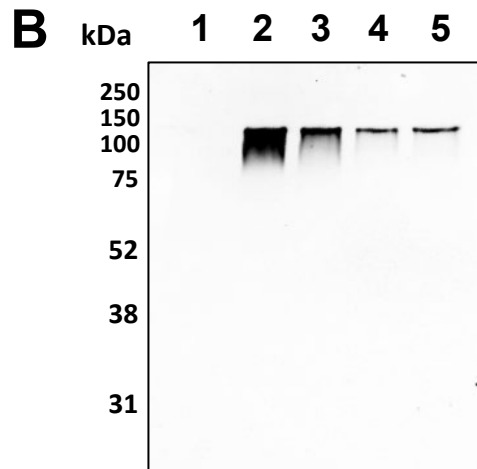
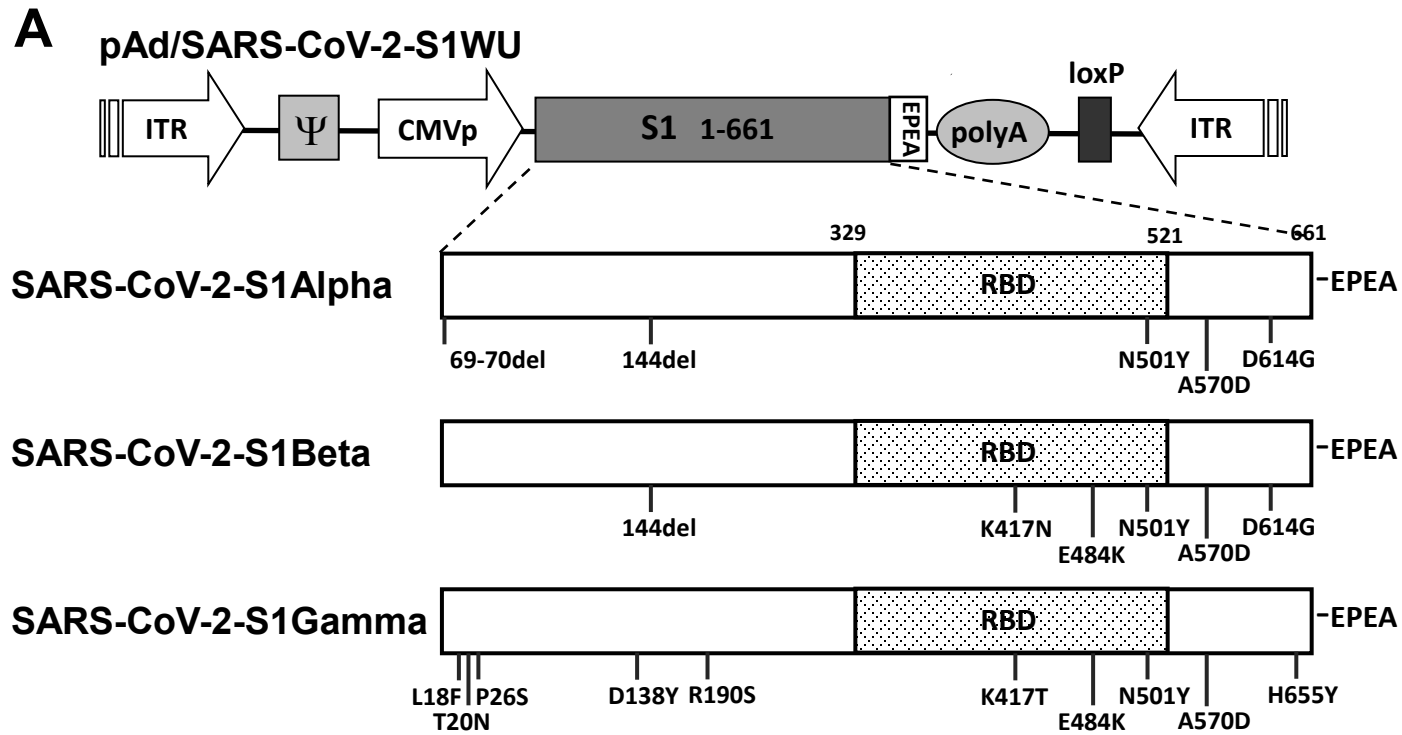
869

870 **Supplementary Figure 2.** Neutralizing antibodies at week 0, 3, and 7 using a

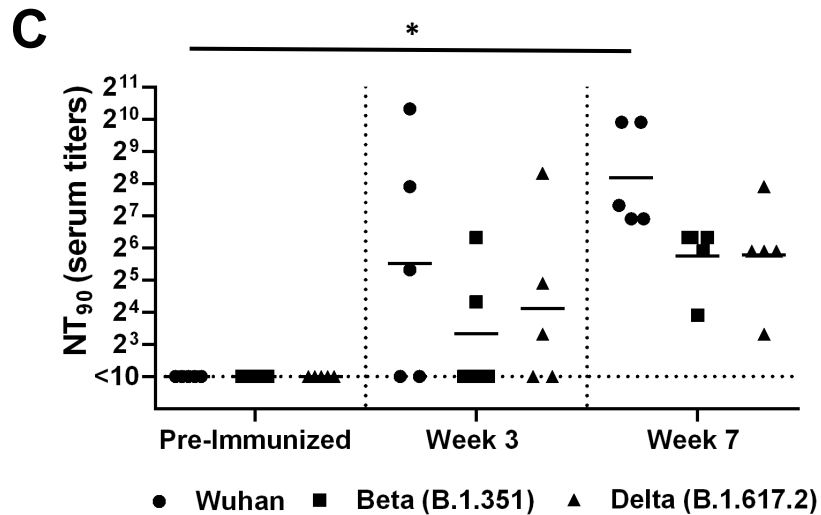
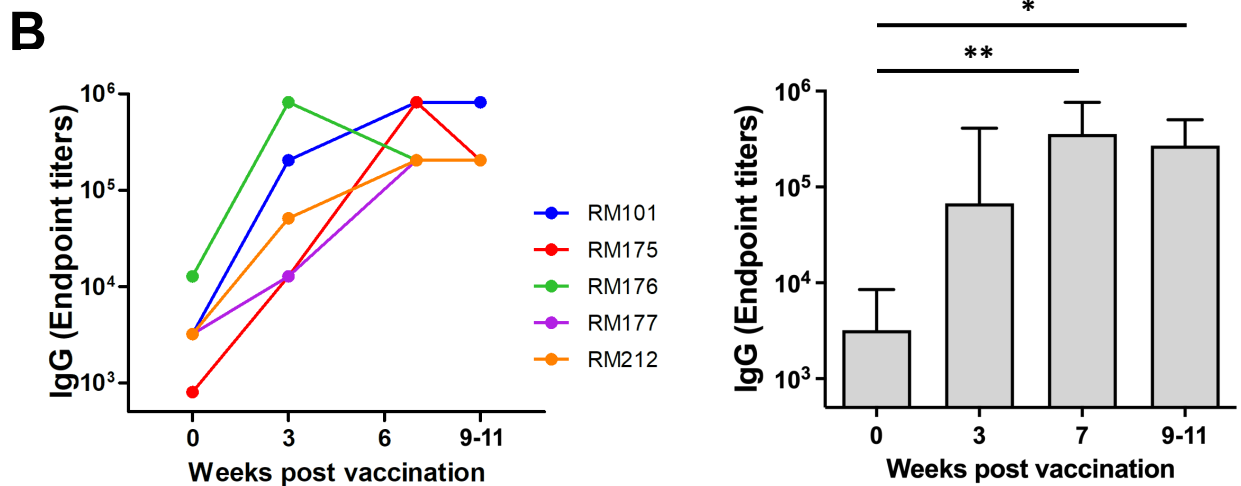
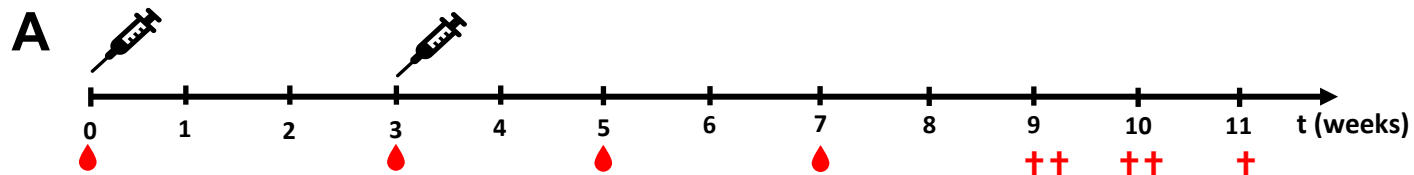
871 microneutralization assay (NT<sub>90</sub>) were showed in each RM with SARS-CoV-2 Wuhan, Beta, and

872 Delta variants.

873



**Figure 1**



**Figure 2**



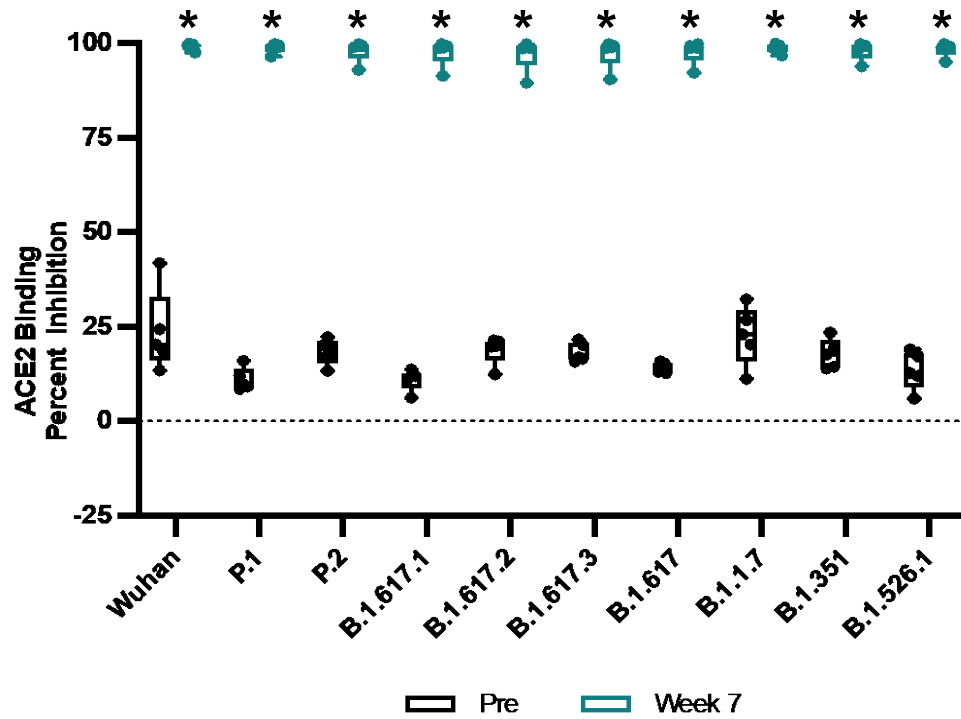
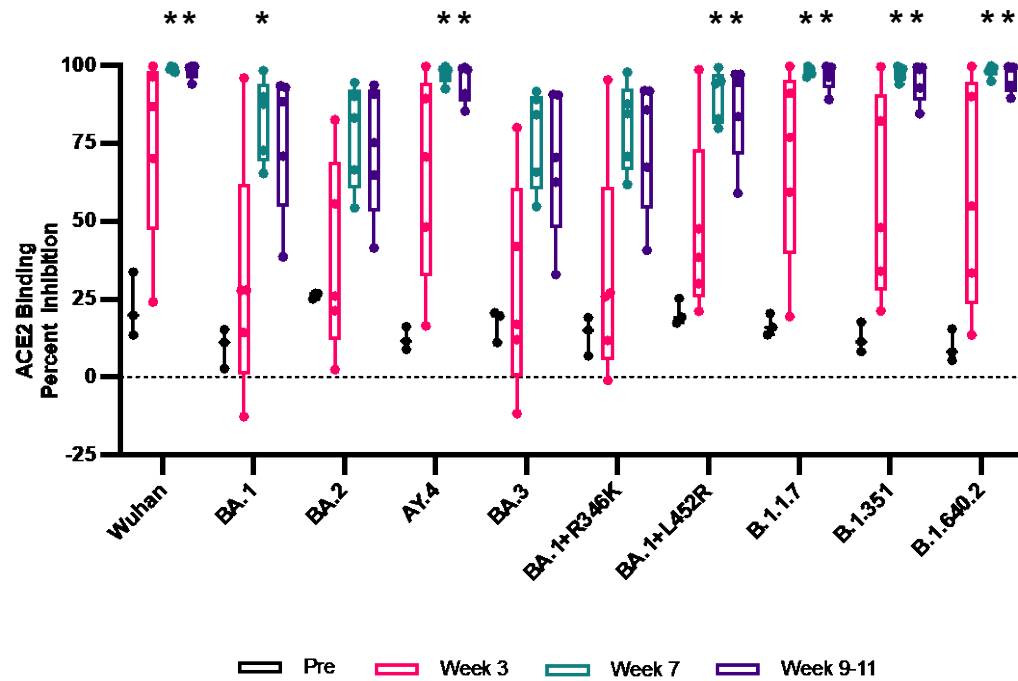
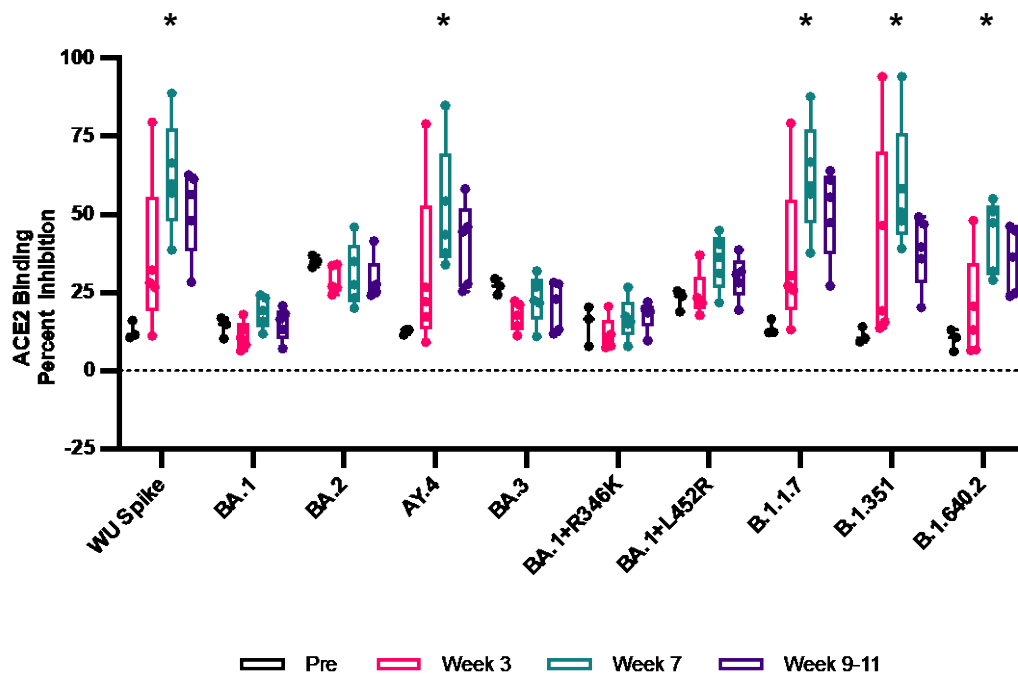


Figure 3

**A****B****Figure 4**

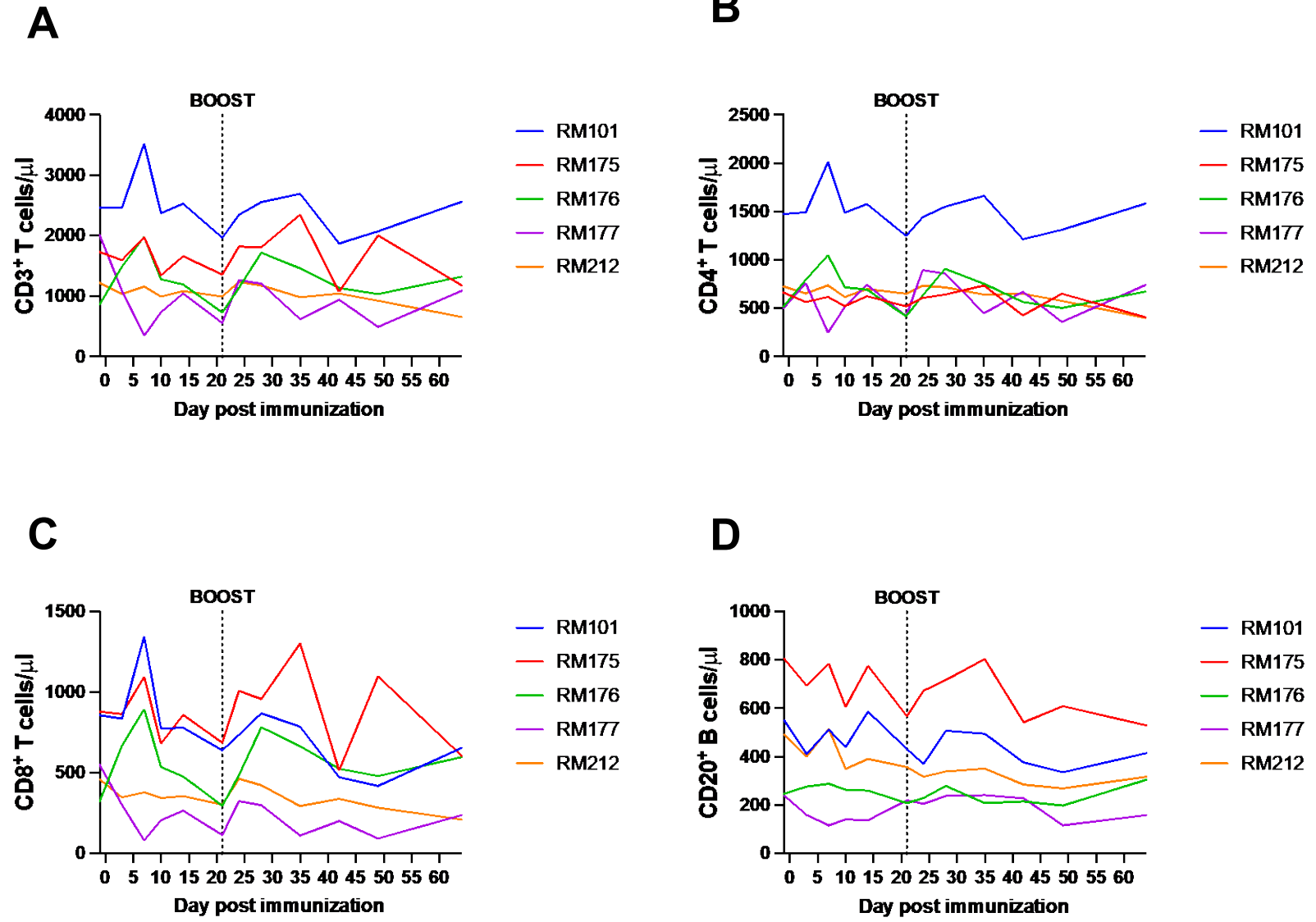


Figure 5

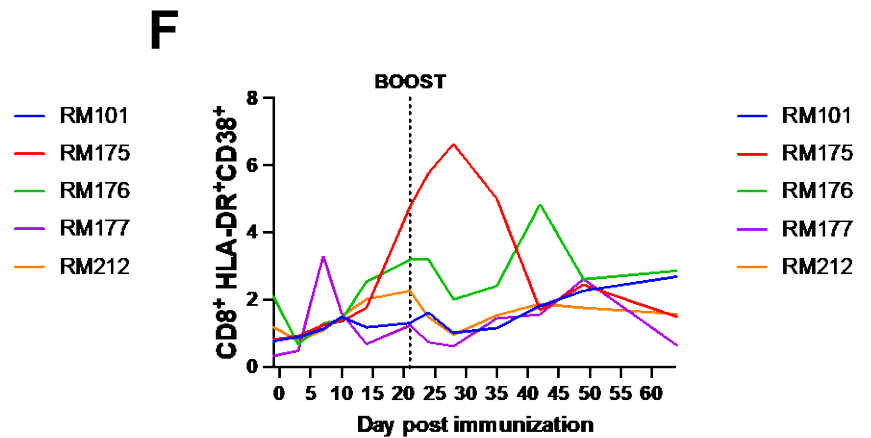
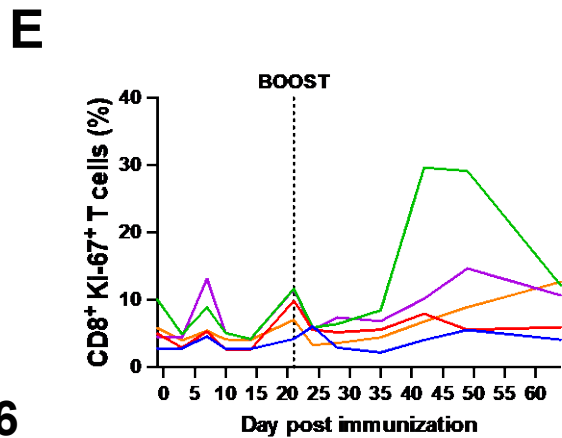
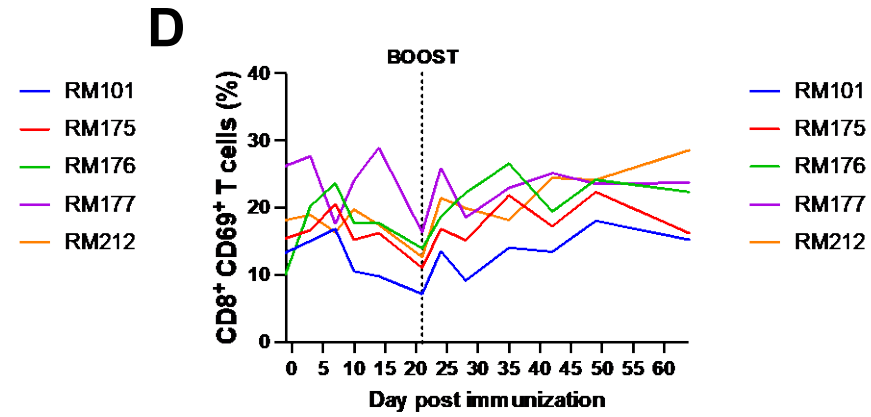
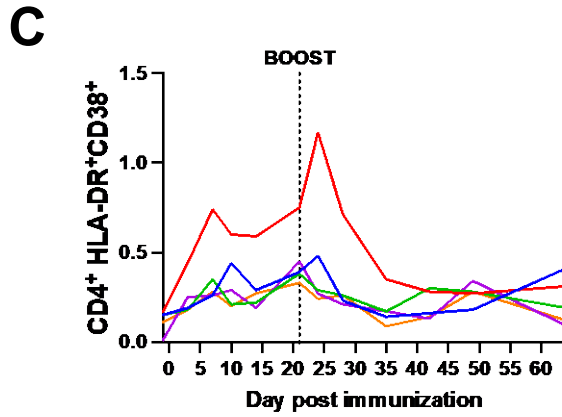
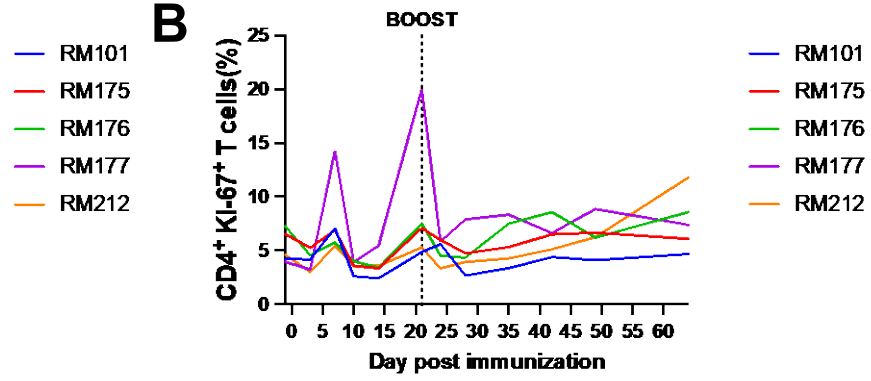
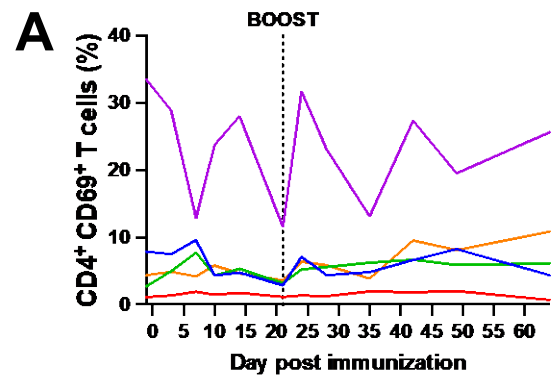


Figure 6

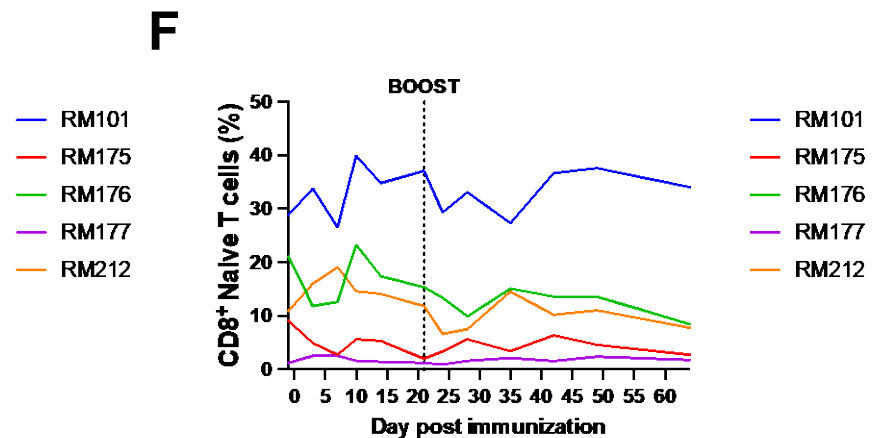
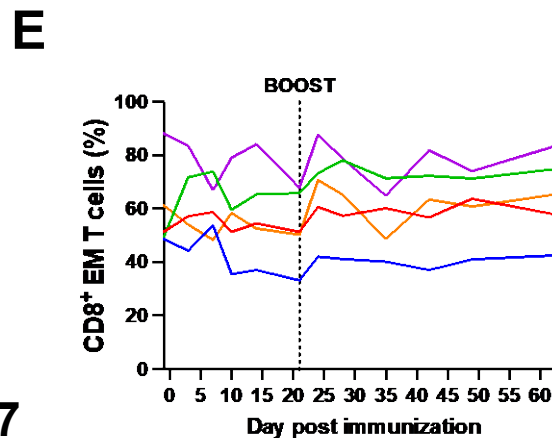
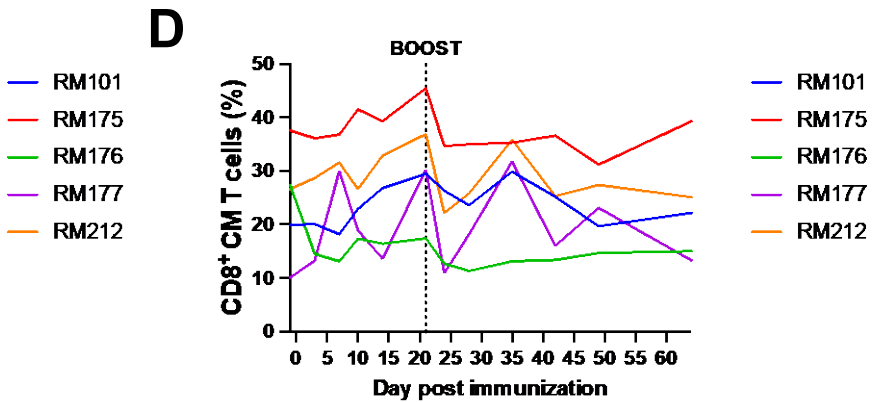
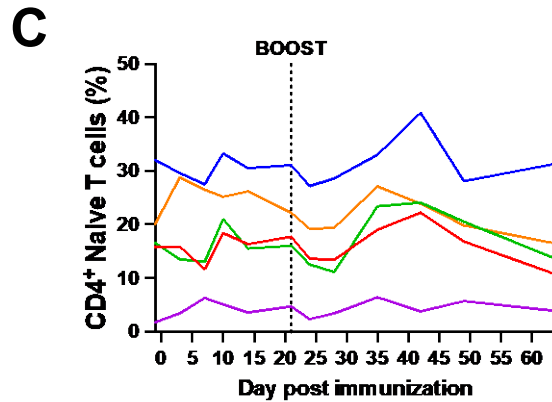
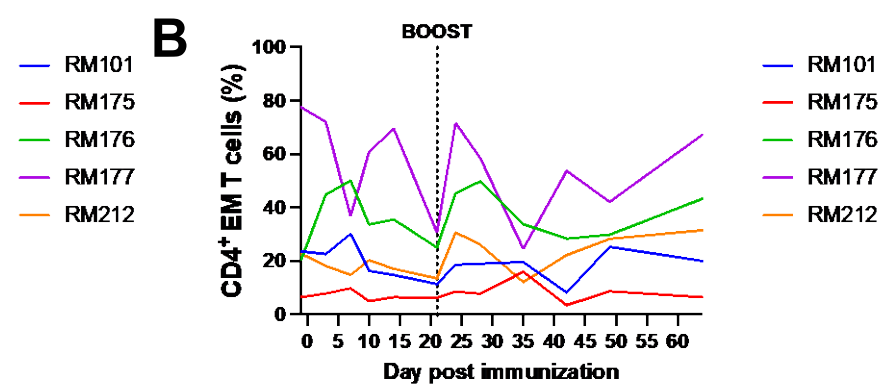
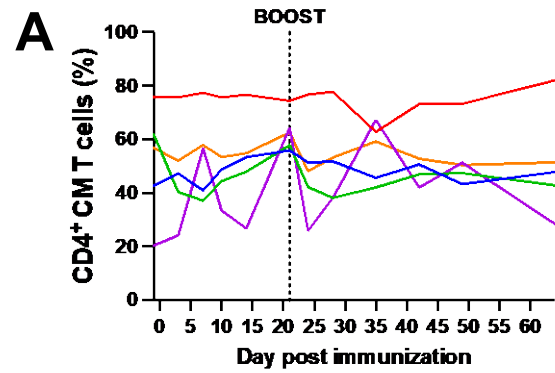
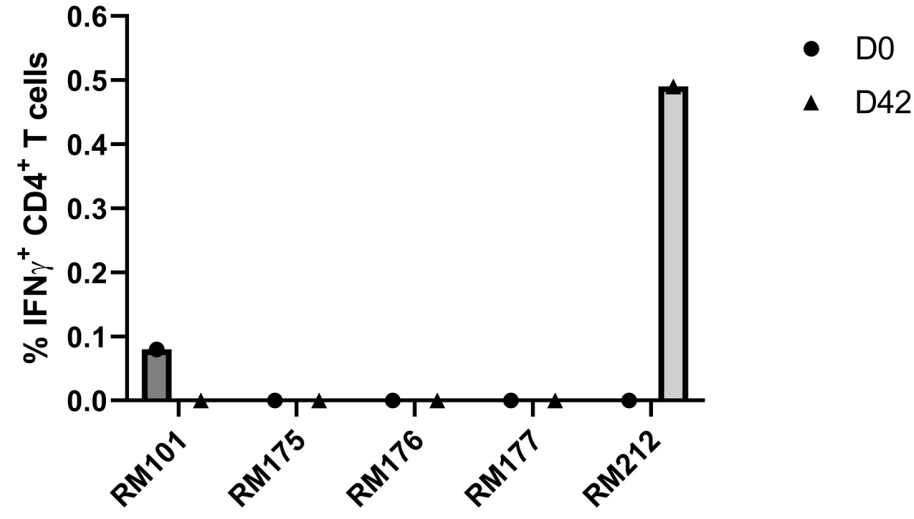
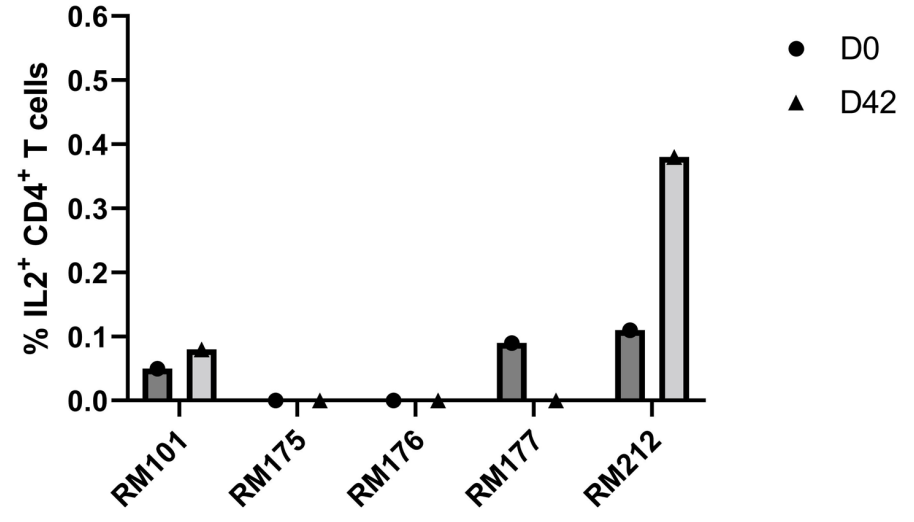
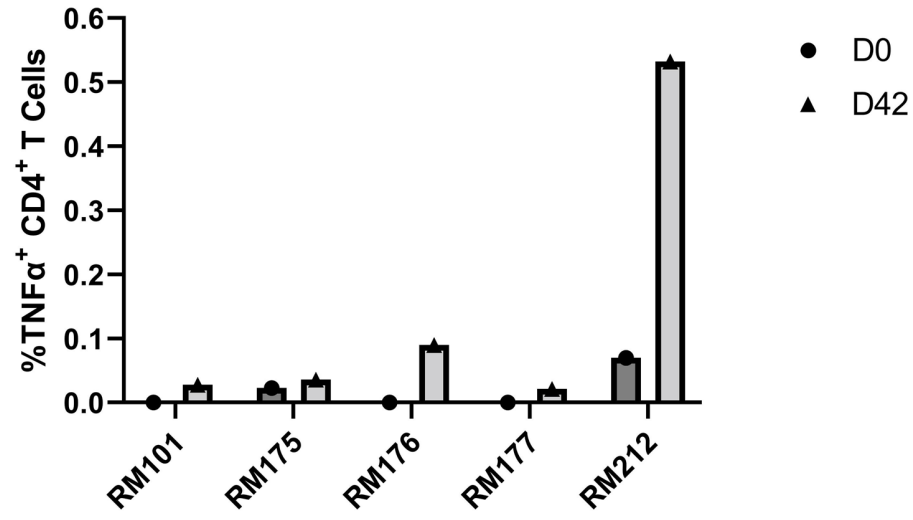
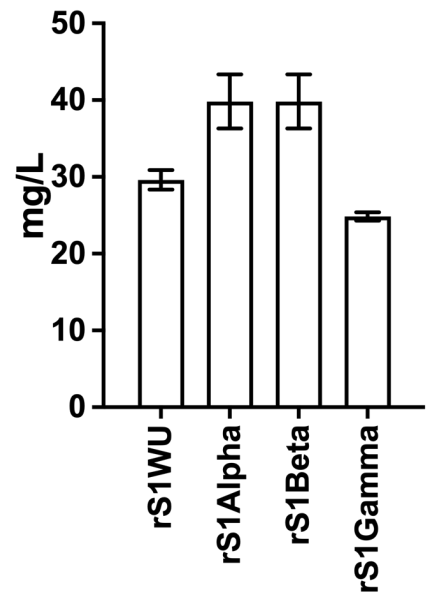


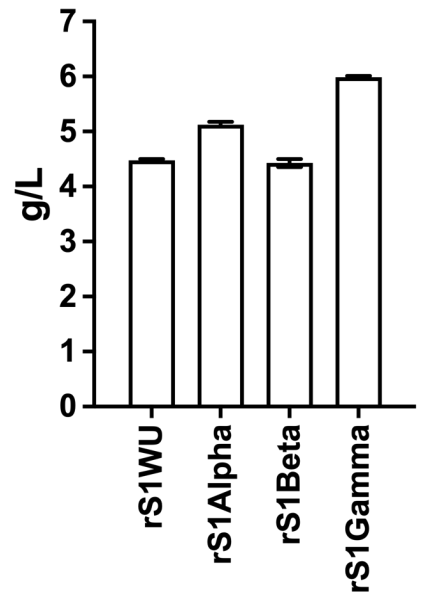
Figure 7

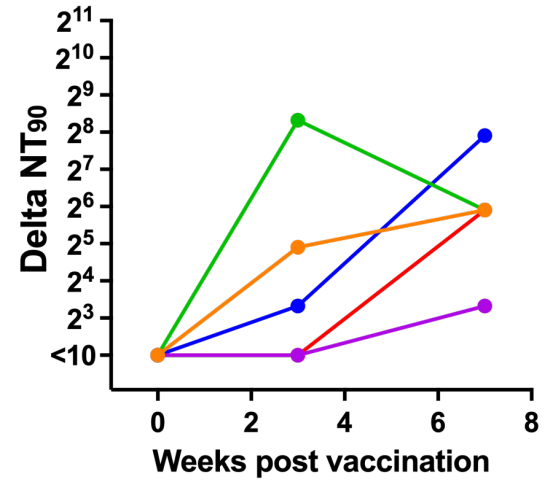
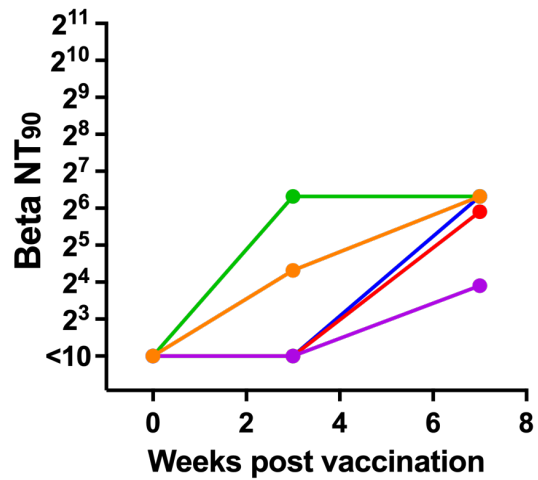
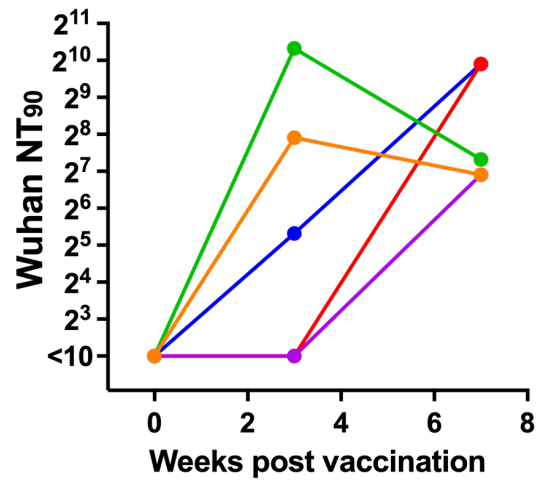
**A****B****C****Figure 8**

**A**



**B**





- RM101
- RM175
- RM176
- RM177
- RM212

Supplementary Figure 2