

# 1 A trimeric NTD and RBD SARS-CoV-2 subunit vaccine induced protective 2 immunity in CAG-hACE2 transgenic mice and rhesus macaques

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25 **A trimeric NTD and RBD SARS-CoV-2 subunit vaccine induced protective  
26 immunity in CAG-hACE2 transgenic mice and rhesus macaques**  
27

28 **Abstract**

29 The coronavirus disease 2019 (COVID-19) pandemic caused by severe acute  
30 respiratory syndrome coronavirus 2 (SARS-CoV-2) has led to significant public health,  
31 economic and social problems. Development of effective vaccines is still a priority to  
32 contain the virus and end the global pandemic. In this study, we reported that ReCOV,  
33 a recombinant trimeric NTD and RBD two-component SARS-CoV-2 subunit vaccine  
34 adjuvanted with BFA03 (an AS03-like squalene adjuvant), induced high levels of  
35 neutralizing antibodies against SARS-CoV-2 and the circulating variants in mice,  
36 rabbits and rhesus macaques. Notably, two-dose immunizations of ReCOV provided  
37 complete protection against challenge with SARS-CoV-2 in hACE2 transgenic mice  
38 and rhesus macaques, without observable antibody-dependent enhancement of  
39 infection. These results support further clinical development of ReCOV and the vaccine  
40 is currently being evaluated in a phase I clinical trial in New Zealand (NCT04818801).

41

42 **Introduction**

43 Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection and the  
44 resulting disease, coronavirus disease 2019 (COVID-19), remains a global challenge  
45 [1]. In less than 2 years, since December 2019, COVID-19 has spread worldwide with  
46 millions infected and many innocent lives lost. The high infection rate, long incubation  
47 period, along with mild-to-moderate symptoms experienced by many, make COVID-

48 19 a troubling disease with wide spread negative impacts on health, social and  
49 economic issues. Global efforts to end the current pandemic hinge on necessary travel  
50 restrictions and precautions and, in the long run, require control by mass vaccinations,  
51 the most effective strategy proven to control infectious diseases [2,3].

52 The ongoing SARS-CoV-2 spread has propelled high-speed vaccine development. As  
53 of 20 October 2021, there are 21 vaccines now being rolled out in countries worldwide,  
54 10 COVID-19 vaccines being approved for vaccination among priority groups under  
55 an Emergency Use Authorization (EUA) [4]. Although the EUA of vaccines has  
56 brought hope to people under threat of the COVID-19 pandemic, the emergence of new  
57 variants of SARS CoV-2 has rendered the situation confusing [5,6]. A new wave of  
58 COVID-19 is engulfing many countries around the world primarily due to the  
59 increasingly prevalent and more transmissible new variants, which pose a serious threat  
60 to the success of vaccination [7,8]. Therefore, more efficacious vaccines that can  
61 stimulate potent protective immunity to prevent the transmission of SARS-CoV-2  
62 variants, is urgently needed.

63 Most of COVID-19 vaccines currently under development, including vaccines  
64 approved for EUA, use full-length spike (S) glycoprotein or the receptor-binding  
65 domain (RBD) of the S protein as target immunogen to trigger protective immune  
66 response, given that the coronavirus S protein is surface-exposed and mediates virus  
67 entry into host cells by interacting with angiotensin-converting enzyme 2 (ACE2), and  
68 is primary target for potent neutralizing antibodies [9-11]. More recently, we and others  
69 demonstrated that, in addition to RBD, a subset of antibodies targeting the N-terminal

70 domain (NTD) exhibit potent neutralizing activities against SARS-CoV-2 [12-14]. The  
71 inclusion of NTD in a COVID-19 vaccine would broaden the neutralizing epitopes and  
72 decrease the potential of viral escape of host immunity. Indeed, our proof-of-concept  
73 study demonstrated that the cocktails of antibodies containing NTD-directed as well as  
74 RBD-targeting NAbs act synergistically to confer protection against SARS-CoV-2,  
75 suggesting that NTD is a promising immunogenic partner of the SARS-CoV-2 RBD.

76 In fact, the combined immunogens of the NTD and RBD, elicited more robust  
77 neutralization activity compared with a single immunogen consisting of either the RBD  
78 or NTD [14]. In addition, accumulating evidence suggests multimerized antigens are  
79 better in engaging interactions with B cell receptors thereby facilitating generation of  
80 high-affinity antibodies compared to monomeric antigens [15]. Therefore, we assume  
81 that trimeric display of SARS-CoV-2 NTD and RBD protein as vaccine candidates may  
82 represent a promising strategy to induce potent neutralizing antibody responses to  
83 prevent SARS-CoV-2 spread.

84 In this study, we demonstrated that ReCOV, a recombinant trimeric NTD and RBD  
85 two-component SARS-CoV-2 subunit vaccine adjuvanted with BFA03(an AS03-like  
86 squalene adjuvant), induced high levels of neutralizing antibodies against SARS-CoV-2  
87 and the circulating variants in mice, rabbits and rhesus macaques. Notably, two-dose  
88 immunizations of ReCOV provided complete protection against challenge with SARS-  
89 CoV-2 in hACE2 humanized mice and rhesus macaques, without observable antibody-  
90 dependent enhancement of infection. These results support further clinical development  
91 of ReCOV and the vaccine is currently being evaluated in a phase I clinical trial in New

92 Zealand (NCT04818801).

93 **Material and Method**

94 **Ethics statement**

95 The protocol and procedures used in the studies with animals were reviewed and  
96 approved by the Laboratory Animal Welfare and Ethics Committee in Institute of  
97 Medical Biology, Chinese Academy of Medical Sciences, and Center of Laboratory  
98 Animal Sciences, Wuhan University (Wuhan, China), respectively.

99

100 **Construction, expression and purification of SARS-CoV-2 NTD-RBD-foldon**

101 To construct recombinant vector for expression of SARS-CoV-2 NTD-RBD-foldon in  
102 CHO-K1 cell, the fragment 1-541 of SARS-CoV-2 spike protein (strain Wuhan-1/2020)  
103 fused with foldon was codon-optimized and synthesized. The fragment 1-541 of spike  
104 protein and foldon were fused together with a GSGSG linker and inserted into the  
105 backbone vector pWX4.1(WuXi Biologics), yielding plasmid pWX4.1-Pr-7323-2. For  
106 expression of NTD-RBD-foldon in CHO cell, the NTD-RBD-foldon gene was PCR  
107 amplified and cloned separately into pWX039 and pWX040 vectors (WuXi Biologics),  
108 yielding expression plasmid pWX039-PR-Z-7323B and pWX040-PR-B-7323B. The  
109 NTD-RBD-foldon gene was validated using Sanger sequencing. CHO-K1 cells were  
110 transfected with recombinant plasmids harboring NTD-RBD-foldon DNA by  
111 electroporation. After transfection, the cells were transferred to preheated 10 ml CD  
112 CHO expression medium and cultivated in an incubator shaker (KUHNER) operated at  
113 37°C, 225 rpm, 6% CO<sub>2</sub>, and 75% relative humidity. After 15 days, the surviving cells  
114 were subjected to monoclonal screening. The high yield cell clones screened were  
115 grown and harvested cell suspension was purified by hydrophobic chromatography.  
116 Elution from hydrophobic chromatography was purified by anion exchange  
117 chromatography. Then eluted protein was loaded on to mixed-mode cation exchange  
118 resin, and eluted target protein was nanofiltrated and used for analysis experiments and  
119 animal immunization.

120

121 **SEC-MALS**

122 SEC-MALS was performed using an XBridge Protein BEH SEC, 450 Å column  
123 (Waters) combined with DAWN HELEOS- II /1790-H2 multi-angle light scattering  
124 (MALS) detector (Wyatt Technology). Purified protein was separated at 0.5ml/min in  
125 50mM PB and 300mM Arginine Hydrochloride (pH7.5). The molecular mass was  
126 determined across the protein elution peak.

127

128 **SPR**

129 The affinity of NTD-RBD-foldon binding Human ACE2 was measured by Biacore 8K  
130 (GE Healthcare) according to the manufacturer's instrument instructions. In brief, NTD-  
131 RBD-foldon was diluted with 10 mM NaAc (pH 5.5) to 6.0 $\mu$ g/mL, respectively, and  
132 injected into FC2 of the channel of the chip respectively to coupled with chips  
133 (v=10 $\mu$ L/min; t=30s). Finally, the chips were sealed with 1 M ethanolamine HCl for  
134 420s at a flow rate of 10  $\mu$  L/min. FC1 channel was activated and blocked as a  
135 reference channel. Human ACE2 was diluted with 1 $\times$ HBS-EP+ buffer to 9.38, 18.75,  
136 37.5, 75, 150 and 300 nM, respectively. The diluted samples flowed through the Fc1-  
137 Fc2 of the channel separately (note, The 75 nM sample was injected twice). The  
138 injection speed was 30  $\mu$ L/min, the sample binding time was 200 s, and the dissociation  
139 time was 300 s. After each binding and dissociation, the chip surface was regenerated  
140 with 10 mM glycine (pH 1.5) for 30 s, and the injection flow rate was 10  $\mu$ L/min.

141

142 **Vaccine formulation**

143 The purified recombinant proteins were mixed with BFA03 adjuvant (AS03-like water-  
144 in-oil emulsion). The formulations were prepared following protocol.

145

146 **Authentic SARS-CoV-2 neutralization assay**

147 The neutralizing activity of the vaccinated sera was tested in microneutralization (MN)  
148 assay based on cytopathic changes. Serum samples were inactivated at 56 °C for 30  
149 min before neutralization testing. The diluted samples were mixed with a virus  
150 suspension of 100 TCID50, followed by 2 h incubation in a 5% CO2 incubator. Vero

151 cells were then added to the serum-virus mixture, and the plates were incubated for 3–  
152 5 days in a 5% CO<sub>2</sub> incubator. The neutralization titer was calculated by the dilution  
153 number of 50% protective condition.

154

### 155 **Pseudotyped virus neutralization assay**

156 Vero cells were cultured in DMEM supplemented with 10% heat inactivated fetal  
157 bovine serum, 50 U/ml Penicillin–streptomycin solution at 37°C with 5% CO<sub>2</sub>.  
158 Inactivated serum samples were serially dilute and incubated with  $1.3 \times 10^4$  TCID<sub>50</sub>/ml  
159 SARS-CoV-2 pseudotyped virus for 1 h at 37 °C. Vero cells were added after 1 h and  
160 allowed to incubate for 24 h. Positive and negative control samples were prepared as  
161 same way. After infection, cells were lysed and RLU were measured using the  
162 Microplate Luminometer. Neutralization titers were calculated as the serum dilution at  
163 which RLU were reduced by 50% compared with RLU in virus control wells.

164

### 165 **Antigen-specific IgG, IgG1, IgG2a ELISA assay**

166 Blood samples were collected from the vaccinated animals. ELISA plates were coated  
167 with recombinant NTD-RBD protein in the coating buffer at 4 °C overnight. Following  
168 blocking and incubation with serial dilutions of sera, anti-mouse IgG, IgG1, IgG2a  
169 HRP-conjugated antibody were used as secondary Abs and incubated for 1 h at RT.  
170 The TMB was used as the substrate. After reaction stopping, plates were read at 450  
171 nm wavelength.

172

### 173 **Immunogenicity analysis of ReCOV in mice**

174 Two groups of female BALB/c mice (n=10) were intramuscularly administrated 4µg or  
175 8µg RECOV with BFA03 adjuvant in a two-dose regimen (D0/D21 interval), and two  
176 weeks after second dose, the levels of antigen specific IgG antibody, neutralizing  
177 antibody, cross-neutralization against the main prevalent variants and IgG2a/IgG1 ratio,  
178 were evaluated.

179

### 180 **Immunogenicity analysis of ReCOV in rabbits**

181 To explore the effect of immune enhancement of BFA03 adjuvant in rabbit , three  
182 groups of rabbit (n=6, male/female=1:1) were intramuscularly immunized with 0.5ml  
183 (1human dose , 1HD) BFA03 adjuvant alone, 40 $\mu$ g NR-foldon alone and 40 $\mu$ g NR-  
184 foldon adjuvanted with 0.5ml BFA03, respectively, at Day 0 and Day 21, and the levels  
185 of immune responses induced were evaluated two weeks after second immunization.  
186 To measure the effect of different dose of BFA03 adjuvant on immune response in  
187 rabbit. Three groups of rabbit (n=6, male/female=1:1) were intramuscularly immunized  
188 with 40 $\mu$ g NR-foldon adjuvanted with 0.5ml (1HD), 0.25 ml(1/2HD) and 0.125  
189 ml(1/4HD) BFA03, respectively, at Day 0 and Day 21, and the levels of immune  
190 responses induced were evaluated two weeks after second immunization. To determine  
191 the effect of antigen doses in rabbit. Two groups of rabbit (n=6, male/female=1:1) were  
192 intramuscularly immunized with 20 $\mu$ g, 40 $\mu$ g NR-foldon adjuvanted with 0.5ml (1HD)  
193 BFA03, respectively, at Day 0 and Day 21, and the levels of immune responses induced  
194 were evaluated two weeks after second immunization.

195

#### 196 **SARS-CoV-2 viral challenge study in CAG-hACE2 transgenic mice**

197 Experiments of CAG- hACE2 transgenic mice were performed at the Biosafety Level  
198 3 (BSL-3) in Center of Laboratory Animal Sciences, Wuhan University (Wuhan,  
199 China). 36 female CAG-hACE2 transgenic mice, 5-6 weeks old, were divided into 4  
200 groups, namely the negative control group(n=6), adjuvant control group(n=6), low-  
201 dose vaccine group (n=12) (4 $\mu$ g/dose) and high-dose vaccine group(n=12) (16 $\mu$ g/dose).  
202 The latter three groups of animals were intramuscularly immunized with 0.5ml BFA03,  
203 4 $\mu$ g RECOV+ 0.5ml BFA03 and 8 $\mu$ g RECOV+0.5ml BFA03, respectively, at Day 0  
204 and Day 21. Two weeks after the second immunization (D35), RECOV or BFA03  
205 adjuvant immunized CAG-hACE2 mice were challenged with  $2.5 \times 10^2$  PFU SARS-  
206 CoV-2 virus (Wuhan-1/2020 strain) intranasally (Figure4A). After challenge, clinical  
207 symptoms, including malaise, bristling, arched back, drowsiness, and body weight  
208 reduction were seen in mice received adjuvants only (the model group), and two-thirds  
209 mice of the adjuvant immunized group died four days after challenge, the remaining

210 mice were euthanized four or five days after challenge according to ethical principle.

211

## 212 **SARS-CoV-2 viral challenge study in rhesus macaques**

213 The study was performed in Biosafety Level 3 laboratory (BSL-3) in Institute of  
214 Medical Biology, Chinese Academy of Medical Sciences. Twelve rhesus macaques  
215 were randomly divided into two groups, with 6 animals in each group. The animals in  
216 the placebo group were intramuscularly injected with 0.5 ml BFA03; the animals in  
217 vaccine groups were intramuscularly injected with ReCOV vaccine (40  $\mu$ g) and 0.5 ml  
218 BFA03. Vaccines and placebos were injected intramuscularly into the right thigh of  
219 each rhesus monkey. All macaques were immunized with a two-dose regimen at days  
220 0 and 21. A challenge study was conducted 21 days after the second immunization by  
221 direct inoculation of SARS-CoV-2 virus of  $1 \times 10^5$  TCID50 through the intranasal and  
222 intratracheal route under anesthesia. The blood and tissue were taken and analyzed per  
223 protocol.

224

## 225 **Statistical analysis**

226 Statistical analyses were performed using GraphPad Prism 8.0 (GraphPad Software)  
227 and comparison between groups was performed using a two-tailed nonparametric  
228 Mann-Whitney U t test.

229

## 230 **Results**

### 231 **Design, expression and characterization of NTD-RBD-foldon in CHO cells**

232 Based on our previous proof-of-concept studies showing that the combination of NTD  
233 and RBD is superior to either RBD or NTD alone in eliciting stronger neutralizing  
234 activity [14], we chose the NTD-RBD domain of the S protein as the target antigen of  
235 RECOV to broaden the effective neutralizing antibody responses against SARS-CoV-2.

236 We fused the NTD-RBD domain with foldon (the natural trimerization domain of T4

237 fibritin) in an attempt to mimic the natural trimeric form of the S protein considering  
238 that multimeric display of the antigen can enhance the potency of the antibody response  
239 (Figure 1A, 1B) [15]. NTD-RBD-foldon was expressed in CHO cell and purified by  
240 three-step chromatography. The apparent molecular weight of NTD-RBD-foldon  
241 protein was determined by non-denaturing non-reducing electrophoresis (native PAGE)  
242 and denaturing non-reducing electrophoresis. The results showed that the NTD-RBD-  
243 foldon protein appeared as a single band in native PAGE with the apparent molecular  
244 weight of about 500 kDa due to glycosylation modification and electrophoresis  
245 conditions, while in denaturing non-reducing electrophoresis, the NTD-RBD-foldon  
246 protein appeared as three bands (80~90kDa, 160~180kDa and 250~ 270kDa, which  
247 corresponding to the monomer, dimer and trimer respectively) (Figure 1C).

248 The molecular weight of the NTD-RBD-foldon main peak analyzed by SEC-MALS is  
249 264.3kD, which is 3 times of the predicted monomer molecular weight, indicating that  
250 NTD-RBD-foldon is a trimer as designed. While the molecular weight of peak 2 was  
251 189.6kD, which corresponds to 2 copies of NTD-RBD-foldon and 1 copy of RBD  
252 molecule, assumed to be a degradation product (Figure 1D).

253 The Surface Plasmon Resonance (SPR) assay was performed to determine the binding  
254 affinity of CHO derived NTD-RBD-foldon and human ACE2. The results showed that  
255 the binding affinity between NTD-RBD-foldon and hACE2 was 28.7nM in the  
256 equilibrium dissociation constant (KD) (Figure 1E), which was at the comparable level  
257 as the affinity between RBD and hACE2. The results demonstrated that the NTD-RBD-  
258 foldon expressed in CHO cells is a trimer with the comparable affinity to hACE2 as its

259 monomer RBD.

260 **Immunogenicity of RECOV in BALB/c mice**

261 To assess the immunogenicity of RECOV, two groups of female BALB/c mice (n=10)  
262 were intramuscularly administrated 4 $\mu$ g or 8 $\mu$ g RECOV with BFA03 adjuvant in a two-  
263 dose regimen (D0/D21 interval), and two weeks after second dose, the levels of antigen  
264 specific IgG antibody, neutralizing antibody, cross-neutralization against the main  
265 prevalent variants and IgG2a/IgG1 ratio, were evaluated. The results showed that  
266 RECOV induced good humoral immunity in BALB/c mice. The seroconversion rate of  
267 antigen specific IgG antibody and neutralizing antibody in 4 $\mu$ g or 8 $\mu$ g groups are both  
268 100%. The antigen specific IgG antibody titers were  $13.0 \times 10^6$  and  $19.7 \times 10^6$  for 4 $\mu$ g  
269 and 8 $\mu$ g dose groups, respectively (Figure2A). The pseudovirus neutralizing antibody  
270 GMT titers is 16134 and 22626 for 4  $\mu$ g and 8 $\mu$ g groups, respectively, with 8 $\mu$ g group  
271 slightly higher than that of 4 $\mu$ g group, but no statistical significance was observed  
272 (Figure2C). The neutralization result in authentic virus system also showed that  
273 ReCOV induced high titer of neutralizing antibody responses, with GMT titers of 6208  
274 and 4096 for 4 $\mu$ g and 8 $\mu$ g groups, respectively (Figure2B).

275 The cross-neutralizing capacity of RECOV against the pseudoviruses of the main  
276 prevalent SARS-CoV-2 variants including the British mutant strain (B.1.1.7, Alpha),  
277 the South African mutant strain (B.1.351, Beta), the Brazil mutant strain (P1, Gamma),  
278 and the India mutant strain-2 (B.1.617.2, Delta) was explored in mice (Figure2C).  
279 Compared with the neutralizing activity against Wuhan-1 virus (GMT 16134 and 22626  
280 for 4 $\mu$ g and 8 $\mu$ g groups, respectively), the vaccinated sera of ReCOV were able to  
281 neutralize most of the above variants comparably, with neutralizing titers of 17923 and  
282 19722 against B.1.1.7 variant, 17082 and 23488 against B.1.351 variant, 47142 and  
283 69108 against P1 variant, and 4214 and 16833 against B.1.617.2 variant, respectively,  
284 except for the India mutant strain-2 (B.1.617.2, Delta), which was less sensitive to sera  
285 from the 4 $\mu$ g group as reported[16].

286 The antigen-specific cytokines induced by RECOV vaccination were determined by  
287 intracellular cytokine staining (ICS) assay. Overall, the cytokines including IL-2, IFN- $\gamma$ ,

288 IL-4 and IL-5 produced in CD4<sup>+</sup> T cells of the vaccinated mice was higher than that of  
289 the control group, with IL-2 significantly higher both in 4 $\mu$ g and 8 $\mu$ g groups, which  
290 implied a preferred CD4 T cell response (Figure2D). Moreover, in order to clarify the  
291 Th1 and Th2 type response induced by RECOV vaccination, IgG1 and IgG2a subtypes  
292 of Th-dependent antibodies in the sera of 2 weeks after the second immunization were  
293 measured, IgG2a/IgG1 ratio in serum after vaccine immunization is less than 1,  
294 indicated a biased Th1 response (Figure2E), the same as mRNA vaccine. No  
295 inflammation or other adverse effects were observed in the mice.

296 **Immunogenicity of RECOV adjuvanted with BFA03 in rabbit**

297 We investigated the immunogenicity of RECOV in rabbit (Figure 3). We first explored  
298 the effect of immune enhancement of BFA03 adjuvant in rabbit. Three groups of  
299 rabbit(n=6, male/female=1:1) were intramuscularly immunized with 0.5ml (1human  
300 dose, 1HD) BFA03 adjuvant alone, 40 $\mu$ g NR-foldon alone and 40 $\mu$ g NR-foldon  
301 adjuvanted with 0.5ml BFA03, respectively, at Day 0 and Day 21, and the levels of  
302 immune responses induced were evaluated two weeks after second immunization, the  
303 results demonstrated that NR-foldon adjuvanted with BFA03 induced a robust antigen  
304 specific IgG antibody response(Figure3A) that is 100-fold higher and a robust  
305 neutralizing antibody response (Figure3B) that is 300-fold higher compared to the  
306 response induced by NR-foldon alone. The adjuvant in the recombinant protein vaccine  
307 significantly enhances the immune effect. No inflammation or other adverse effects  
308 were observed in the rabbits.

309 We next measured the effect of different dose of BFA03 adjuvant on immune response  
310 in rabbit. Three groups of rabbit (n=6, male/female=1:1) were intramuscularly  
311 immunized with 40 $\mu$ g NR-foldon adjuvanted with 0.5ml (1HD), 0.25 ml(1/2HD) and

312 0.125 ml(1/4HD) BFA03, respectively, at Day 0 and Day 21, and the levels of immune  
313 responses induced were evaluated two weeks after second immunization. The results  
314 demonstrated that humoral responses declined in a dose-independent manner,  
315 especially the neutralizing GMT, half dose of adjuvant resulted in 2-6 folds reduction  
316 in antibody response. Animals immunized with 1 HD BFA03 adjuvant produced  
317 significantly higher levels of antibodies than 1/2 HD and 1/4 HD BFA03 adjuvant  
318 (Figure3C, 3D). The antibody levels induced in 1/2HD and 1/4HD BFA03 adjuvant  
319 groups were comparable. These results indicated 0.5ml BFA03 in RECOV is a requisite.  
320 We then determined the effect of antigen doses in rabbit. Two groups of rabbit (n=6,  
321 male/female=1:1) were intramuscularly immunized with 20 $\mu$ g, 40 $\mu$ g NR-foldon  
322 adjuvanted with 0.5ml (1HD) BFA03, respectively, at Day 0 and Day 21, and the levels  
323 of immune responses induced were evaluated two weeks after second immunization,  
324 the results demonstrated that 20 $\mu$ g and 40 $\mu$ g antigen, both formulated with 0.5ml  
325 BFA03, induced dose-dependent strong immune response. The neutralizing antibody  
326 GMT based on the VSV pseudovirus detection was 9956 and 30697, respectively, and  
327 the antigen-specific IgG antibody GMT was 2674961 and 4878428, respectively.  
328 (Figure 3E, 3F). The results were comparable to those in mice. The antibody titer  
329 produced by 40  $\mu$ g antigen immunization was higher.  
330 We also evaluated cross-neutralizing capacity of RECOV against the pseudotyped virus  
331 of the main prevalent SARS-CoV-2 variants in rabbit [Figure3G]. Compared with the  
332 neutralizing activity against Wuhan-1 virus (GMT 9148), the vaccinated rabbit sera  
333 from 40 $\mu$ g ReCOV group were able to neutralize the B.1.1.7(Alpha), B.1.351(Beta),

334 P1(Gamma), and B.1.617.2(Delta) variants with a titer of GMT 8354, GMT 1588, GMT  
335 2768, and GMT 17400, respectively. The results demonstrated that the neutralizing  
336 GMT to delta variant is higher but without statistical significance, and the neutralizing  
337 GMT to Beta and Gamma variants displayed a decreasing trend. In general, the  
338 vaccine still has a relatively high neutralizing GMT titers against the main mutant  
339 strains.

340 **Protective effect of RECOV adjuvanted with BFA03 in CAG-hACE2 mice**

341 The CAG-hACE2 transgenic mice were utilized to investigate the protective effect of  
342 RECOV. 36 female CAG-hACE2 transgenic mice, 5-6 weeks old, were divided into 4  
343 groups, namely the negative control group(n=6), adjuvant control group(n=6), low-  
344 dose vaccine group (n=12) (4 $\mu$ g/dose) and high-dose vaccine group(n=12) (16 $\mu$ g/dose).  
345 The latter three groups of animals were intramuscularly immunized with 0.5ml BFA03,  
346 4 $\mu$ g RECOV+ 0.5ml BFA03 and 8 $\mu$ g RECOV+0.5ml BFA03, respectively, at Day 0  
347 and Day 21(Figure4A). Two weeks after the second immunization (D35), RECOV or  
348 BFA03 adjuvant immunized CAG-hACE2 mice were challenged with  $2.5 \times 10^2$  PFU  
349 SARS-CoV-2 virus (Wuhan-1/2020 strain) intranasally (Figure4A). After challenge,  
350 clinical symptoms, including malaise, bristling, arched back, drowsiness, and body  
351 weight reduction were seen in mice received adjuvants only (the model group), and  
352 two-thirds mice of the adjuvant immunized group died four days after challenge, the  
353 remaining mice were euthanized four or five days after challenge according to ethical  
354 principle (Figure4C). In contrast, mice received RECOV, both in 4 $\mu$ g and 16 $\mu$ g groups,  
355 showed no clinical abnormality, with no difference in body weight comparing to the

356 negative control mice (Figure4B). For these groups, half of the mice were euthanized 6  
357 days after challenge, and the other half euthanized 14 days after challenge.  
358 After euthanasia, brain and right lung samples were collected from each animal for viral  
359 load quantification, and left lung were fixed for histopathological evaluation. The  
360 results showed that RECOV vaccination protected the mice from SARS-CoV-2  
361 infection. The viral load in lung tissue of RECOV vaccinated animals, both 6 days post  
362 infection and 14 days post infection, remained at the level of low limit of quantification.  
363 In contrast, a high level of viral load existed in the lung tissue of animals in adjuvant  
364 control group, which up to approximately  $10^{11}$  copy/ml, four- or five-days post  
365 challenge (Figure4F). The viral load in brain tissues among these animal groups  
366 displayed similar patterns as those in lung tissues, since the CAG-hACE2 mice  
367 systematically express hACE2(Figure4G). And RECOV also showed protective effect  
368 on viral dissemination and amplification (Fig 4E, 4F). Pulmonary injury was  
369 histologically scored according to the extent of damage (Figure4E). The lung tissue of  
370 the adjuvant control mice was severely damaged, and the pathological changes  
371 accounted for 50% to 75% of the total tissue. In contrast, the damage of lung tissue of  
372 RECOV inoculated mice were significantly alleviated 50% to 25% at day 6 post  
373 infection, and further to 25% or even recovered at day14 post infection.  
374 The neutralizing activity against the authentic virus was evaluated using sera taken at  
375 day14 post the boost dose, the results showed that both 4 $\mu$ g and 16 $\mu$ g RECOV  
376 inoculated CAG-hACE2 mice elicited robust neutralizing response in (Figure4D).

377 **Protective effect of RECOV adjuvanted with BFA03 in rhesus macaques**

378 The immunogenicity and protective efficacy of RECOV were further evaluated in  
379 rhesus macaques (Figure 5). Six macaques in vaccine group were intramuscularly  
380 immunized twice with 40 $\mu$ g RECOV on day 0 and day14, and another six rhesus  
381 macaques received BFA03 adjuvants alone as placebo control. Four weeks after the  
382 second immunization (D49), all macaques were intranasally and intratracheally  
383 challenged with  $1 \times 10^6$  CCID<sub>50</sub> SARS-COV-2 (Wuhan-1/2020 strain) under anesthesia  
384 (Figure 5A). Body temperature and body weight of macaques in both vaccine group  
385 and adjuvant control group has no obvious abnormal fluctuation from day 0 to day 7  
386 after the first and second vaccine inoculation.

387 The seroconversion rate of the neutralizing antibodies against the authentic virus was  
388 100% after the first immunization with a GMT titer of 14.5, and the neutralizing titer  
389 rise to 886.8 two weeks after the second immunization (Figure 5B). The vaccinated  
390 monkey sera also displayed high cross-neutralizing capacity against the main prevalent  
391 SARS-COV-2 variants in a pseudovirus neutralizing assay with a titer of GMT 8156  
392 against the B.1.1.7 (Alpha), GMT 1048 against B.1.351 (Beta), GMT 3193 against P1  
393 (Gamma), and GMT 8441 against B.1.617.2 (Delta) variants, respectively (Figure 5C).  
394 Compared to the neutralizing activity against Wuhan-1 virus (GMT 5959), the  
395 neutralizing GMT to Delta variant is higher, and the neutralizing GMT to Beta and  
396 Gamma variants displayed a decreasing trend, but all without statistical significance,  
397 similar as those observed in rabbits. In general, the results demonstrated that ReCOV  
398 induced a relatively high neutralizing GMT titers against the main mutant strains in  
399 macaques.

400 To evaluate the protective effect of RECOV on virus propagation and shedding, we  
401 detected the viral load in the nasal and oropharyngeal swabs of the macaques daily after  
402 SARS-CoV-2 challenge. While the control animals displayed and maintained high  
403 level of virus in nasal samples, the viral load in nasal samples in RECOV vaccinated  
404 animals sharply decreased in five days after infection (Figure 5E). We also observed a  
405 remarkable increase of virus quantity in oropharynx swabs in the control animals four  
406 days after infection (Figure 5D), indicating a dissemination to or propagation in this  
407 tissue. In contrast, RECOV could protect such infectious progression.

408 On the 4th day and the 7th day after challenge, half of the animals were euthanized  
409 respectively, to determine the viral load in the tissue samples. The control macaques  
410 had detectable viral load in the BALF, lung lymph nodes, right lower lung, nasal  
411 mucosa, with the highest in trachea (Figure 5F). In contrast, no macaques in RECOV  
412 vaccinated group had a detectable viral load in these tissues, except for one animal  
413 which nasal tissue with a lower viral RNA.

414 Histopathological evaluations were performed after euthanasia, and the lung injury was  
415 scored for each animal. The results showed that four days after infection, macaques in  
416 the placebo group displayed serious damage, in contrast, RECOV vaccination could  
417 prevent such damage (Figure 5G). Seven days after infection, the lesion in control  
418 animals, as well as RECOV vaccinated animals, were relieved to certain extent,  
419 however, benefit from RECOV vaccination was still observed (Figure 5H).  
420 Histopathological examination showed that SARS-CoV-2 infection induced heavily  
421 inflammatory infiltration and disturbed the pulmonary structure in control animals at

422 day 4 after challenge, while RECOV vaccinated animals remained normal in  
423 histological structure with minor infiltration (Figure 5I). The results show that RECOV  
424 vaccination protects rhesus macaques from SARS-CoV-2 infection.

425 **Discussion**

426 Vaccine strategies currently explored for COVID-19 include inactivated whole virus  
427 vaccines, recombinant viral vector vaccines, RNA-and DNA-based vaccines, and  
428 subunit vaccines etc., all these vaccines have been shown to induce neutralizing  
429 antibody responses and to be effective in preventing severe illness in clinical trials.  
430 However, the ongoing emergence of SARS-CoV-2 variants [17] has rendered the  
431 situation confusing, in face of soaring number of infections caused by the highly  
432 contagious SARS-CoV-2 Delta variant, and hints that the immunity triggered by  
433 COVID-19 vaccines might weaken over time, many countries are considering to give  
434 booster doses to those who have been fully vaccinated with the first-generation vaccines.  
435 Therefore, development and/or improvement of more efficacious SARS-CoV-2  
436 vaccines are still a priority worldwide, especially for protein subunit vaccines, which  
437 are known for advantageous production safety, production costs, vaccine storage  
438 temperatures, scale-up manufacturing and global distribution[18].

439  
440 A major goal of protein subunit vaccine development is to rationally design  
441 immunogens that can elicit broad and potent neutralizing antibody responses. Our  
442 proof-of-concept study demonstrated that the combination immunogen of NTD and  
443 RBD elicited more robust neutralizing antibody responses compared with either the

444 RBD or NTD alone, suggesting that integration of the NTD in an RBD-based COVID-  
445 19 vaccine would have the potential of increasing NAb diversity and decreasing viral  
446 escape [14]. Therefore, the NTD-RBD domain of the S protein was chosen as the target  
447 antigen of our subunit vaccine and the NTD-RBD domain was fused with the foldon in  
448 an attempt to mimic the natural trimeric form of the S protein considering that  
449 multimerization of the antigen can enhance the potency of the antibody response [15].  
450 To ensure the right glycosylation, the NTD-RBD-foldon was expressed in CHO cells.  
451 SEC-MALS and the SPR analysis demonstrated that the NTD-RBD-foldon expressed  
452 in CHO cells is a trimer with the comparable affinity to hACE2 as RBD.  
453 The recombinant trimeric two-component SARS-CoV-2 subunit vaccine formulated  
454 with BFA03, could elicit high levels of antigen-specific IgG and neutralizing antibody  
455 responses in mice, rabbit and rhesus macaques. The seroconversion rate of antigen  
456 specific IgG antibody and neutralizing antibody are both 100% in a two-dose regimen  
457 at an interval 21 days in these animals, with high antibody titers after the second  
458 immunization. Meanwhile, the vaccinated sera also showed cross neutralizing activity  
459 against the main VOCs including B.1.1.7(Alpha), B.1.351 (Beta), P.1(Gamma) and  
460 B.1.617.2(Delta), implying that the vaccine is highly immunogenic and may provide  
461 protection against these main prevalent variants.  
462 CAG-hACE2 transgenic mice express hACE2 systematically and is a highly  
463 susceptible model of SARS-CoV-2 infection suitable for evaluating vaccines [19], this  
464 mice model was utilized to evaluate the immunogenicity and protective efficacy of  
465 ReCOV in this study. The results demonstrated that ReCOV immunization elicited

466 robust neutralizing responses against the authentic virus, and fully protected the mice  
467 from SARS-CoV-2 virus challenge. All mice in the adjuvant control group died on the  
468 fourth and fifth days after the challenge, while the vaccinated mice in the low dose and  
469 high dose vaccine groups all survived. The viral loads in the brain and lung tissues in  
470 the vaccinated mice were below or near the limit of detection. In contrast, mice in  
471 adjuvant control group all had extremely high viral loads. The pathological evaluation  
472 also showed that the vaccinated mice had only slight pathological manifestations on the  
473 sixth day after infection and almost no pathological abnormality on the fourteenth day,  
474 while the pathological damage in the control mice was serious. Several mice model  
475 currently are used to evaluate SARS-CoV-2 vaccine protective effect, but no  
476 comparison was performed on their strength to distinguish differences in vaccine  
477 efficacy, this may merit further investigation in future [20,21].

478 We further evaluated the immunogenicity and protective efficacy of ReCOV in rhesus  
479 macaques, since macaques have been established as an effective animal model for  
480 SARS-CoV-2 and to develop upper and lower respiratory track pathology that is similar  
481 to human infection [22,23]. All 6 macaques in the vaccine group were seroconversion  
482 after the first shot in a live virus neutralization assay, and the neutralizing antibody titer  
483 reached to 1825 after the second shot. The vaccinated sera also showed pseudovirus  
484 cross neutralizing activity against the main prevalent variants including B.1.1.7(Alpha),  
485 B.1.351 (Beta), P.1(Gamma) and B.1.617.2(Delta). When challenged with SARS-CoV-  
486 2, ReCOV protects the upper and lower respiratory tract against the presence of viral  
487 RNA on the fifth day after infection, in line with other reports describing vaccine

488 protection studies in non-human primates [24,25 ]. The results demonstrate a potential  
489 of ReCOV vaccine to protect against SARS-CoV-2 virus replication and the caused  
490 disease.

491 In summary, we have designed and developed a recombinant trimeric NTD and RBD  
492 two-component SARS-CoV-2 subunit vaccine, which induced high levels of  
493 neutralizing antibodies against SARS-CoV-2 and the circulating variants in mice,  
494 rabbits and rhesus macaques. Notably, two-dose immunizations of ReCOV provided  
495 complete protection against challenge with SARS-CoV-2 in hACE2 transgenic mice  
496 and rhesus macaques , without observable antibody-dependent enhancement of  
497 infection. These results support further clinical development of ReCOV and the vaccine  
498 is currently being evaluated in a phase I clinical trial in New Zealand (NCT04818801).

499 **FIGURE LEGENDS**

500 **Figure 1. Production and characterization of NTD-RBD-foldon.** (A) Schematic representation  
501 of natural spike protein and NTD-RBD-foldon protein. (B) NTD (green) and RBD (cyan) domain  
502 of NTD-RBD-foldon in one monomer of natural S trimer (gray) (PDB:6VXX). (C) PAGE results  
503 of purified NTD-RBD-foldon protein. Left, native PAGE, non-reduced. Right, SDS-PAGE, non-  
504 reduced. (D) Molecular mass calculation based on SEC-MALS analysis. Black dots under the peak  
505 correspond to the averaged molecular mass. (E) Binding affinity of NTD-RBD-foldon to hACE2  
506 determined by SPR. NTD, N-terminal domain. RBD, receptor-binding domain. S1/S2, S1 and S2  
507 cleave site. FP, fusion peptide. HR1/HR2, heptad repeats. CH, central helix. TM, transmembrane  
508 domain. CT, cytoplasmic tail. Foldon, the C-terminal domain of T4 fibrin.

509

510 **Figure 2. Immunogenicity of RECOV in BALB/c mice.** Animals were intramuscularly  
511 administrated 4 $\mu$ g or 8 $\mu$ g RECOV twice, separated by 21 days, and two weeks after second dose,  
512 blood samples were collected for analysis. (A) GMT of antigen-specific antibody. (B) GMT of  
513 antibody neutralizing authentic SARS-CoV-2. (C) Cross-protection to prevalent variants, analyzed  
514 by pseudo-virus system. (D) Result of splenocyte sorting after antigen stimulation. (E) analysis of  
515 IgG subgroup to further characterize the Th1/Th2 balance.

516

517 **Figure 3. Immunogenicity of RECOV in rabbits.** (A) and (B) BFA03 contribution to the  
518 immunogenicity of NTD-RBD-foldon was evaluated by antigen-specific IgG, as well as  
519 neutralizing GMT to pseudo-virus. (C) and (D) Different amount of BFA03 was introduced to NTD-  
520 RBD-foldon to validate the adjuvant dosage in final formulation of RECOV. (E) and (F) Different  
521 amount of NTD-RBD-foldon was introduced to BFA03 to validate the antigen dosage in final  
522 formulation of RECOV. (G) Cross-protection to prevalent variants, analyzed by pseudo-virus  
523 system.

524

525 **Figure 4. Protective effect of RECOV in CAG-hACE2 mice.** Transgenic mice were  
526 intramuscularly inoculated with 4 $\mu$ g or 8 $\mu$ g RECOV twice, separated by 21 days, and two weeks  
527 after the second dose, 2.5 $\times$ 102 PFU SARS-CoV-2 was administrated to the animals intranasally.  
528 (A) The schematic diagram of the study, (B) Changes in body weight after challenge. (C) Animal  
529 survival curve after challenge, all adjuvant animals died or were euthanized four or five days after  
530 challenge. (D) Neutralizing GMT, induced by RECOV, to authentic SARS-CoV-2. (E)  
531 Histopathological changes, 4 to 5 days after challenge in adjuvant animals, and 6 or 14 days after  
532 challenge in other groups. (F) and (G) Viral load in brain and left lung 4 to 5 days after challenge  
533 in adjuvant animals, and 6 or 14 days after challenge in other groups.

534

535 **Figure 5. Protective effect of RECOV in rhesus monkeys.** (A) The schematic diagram of the  
536 study, (B) GMT of neutralizing antibody to authentic SARS-CoV-2 induced by RECOV. (C)  
537 Cross-protection of RECOV against prevalent variants in monkeys. (D) and (E) Viral load in  
538 oropharyngeal and nasal swabs in monkeys after SARS-CoV-2 challenge. (F) Viral load of  
539 SARS-CoV-2 in monkey tissues 4 days after challenge. (G) and (H) Histopathological scores four  
540 and seven days after challenge. (I) Representative histopathological changes four days after  
541 challenge.



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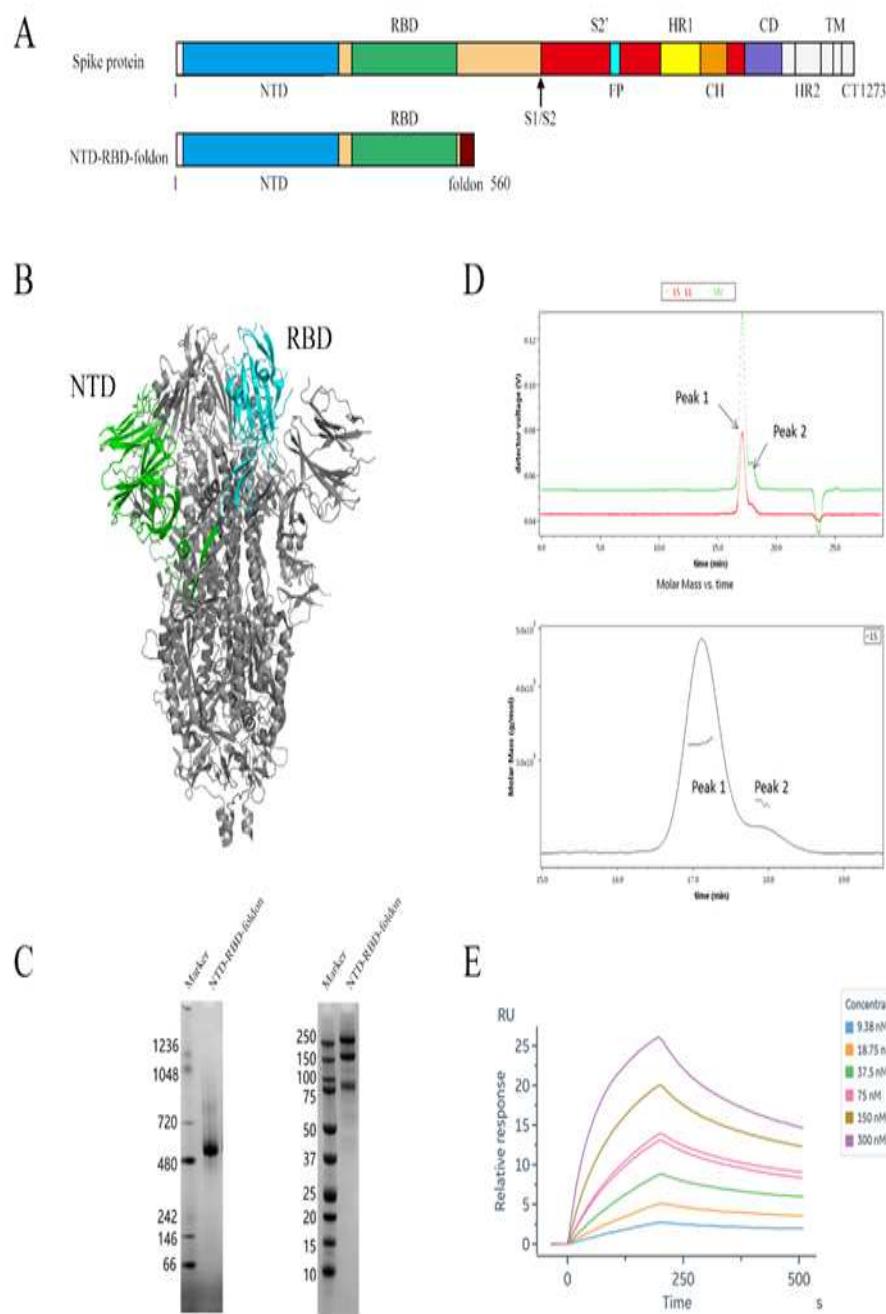
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**Figure 1**

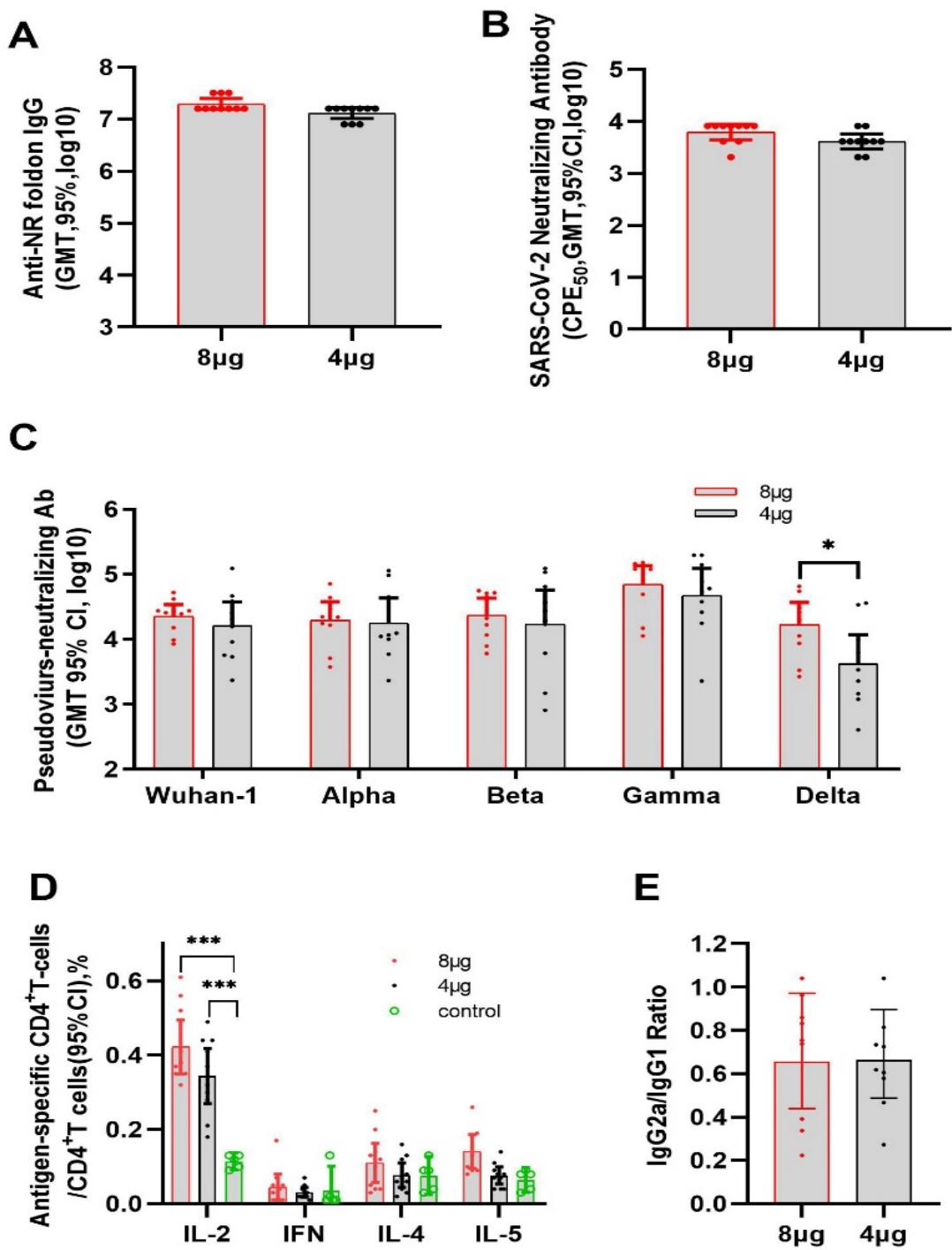
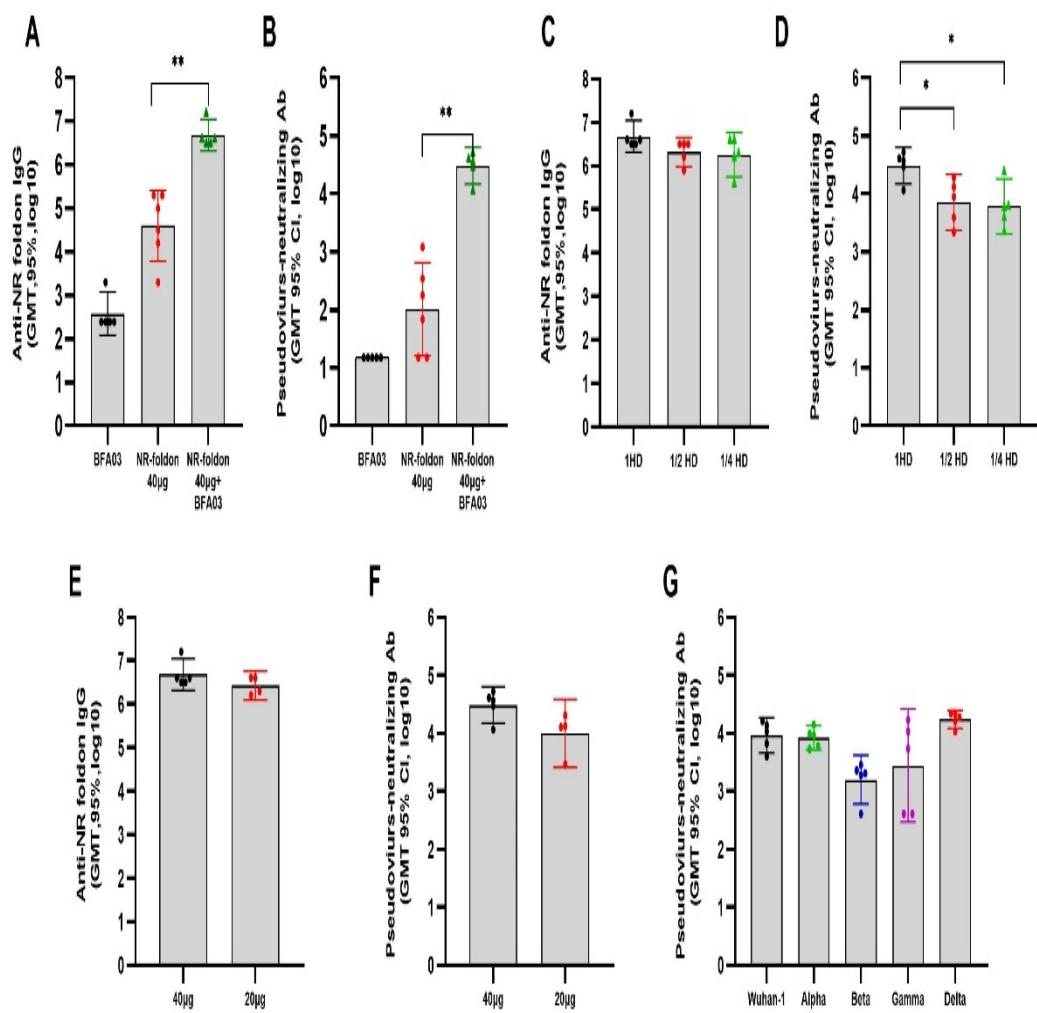
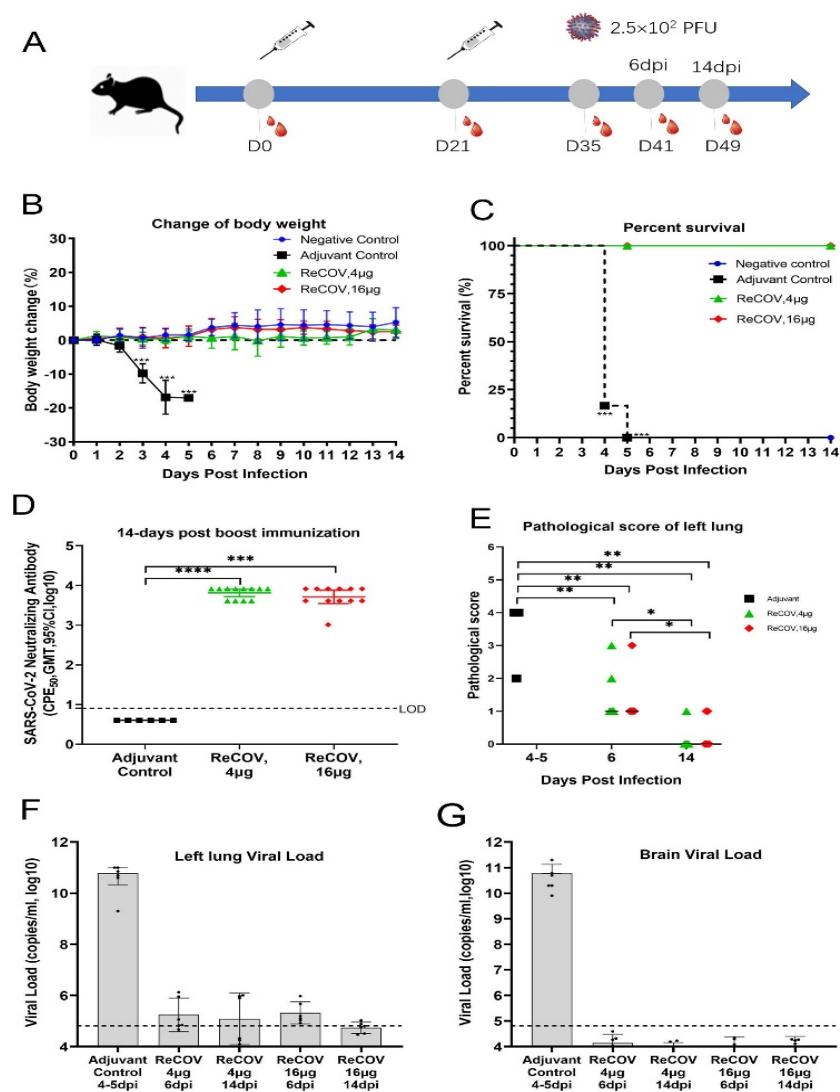


Figure2



**Figure3**



**Figure 4**

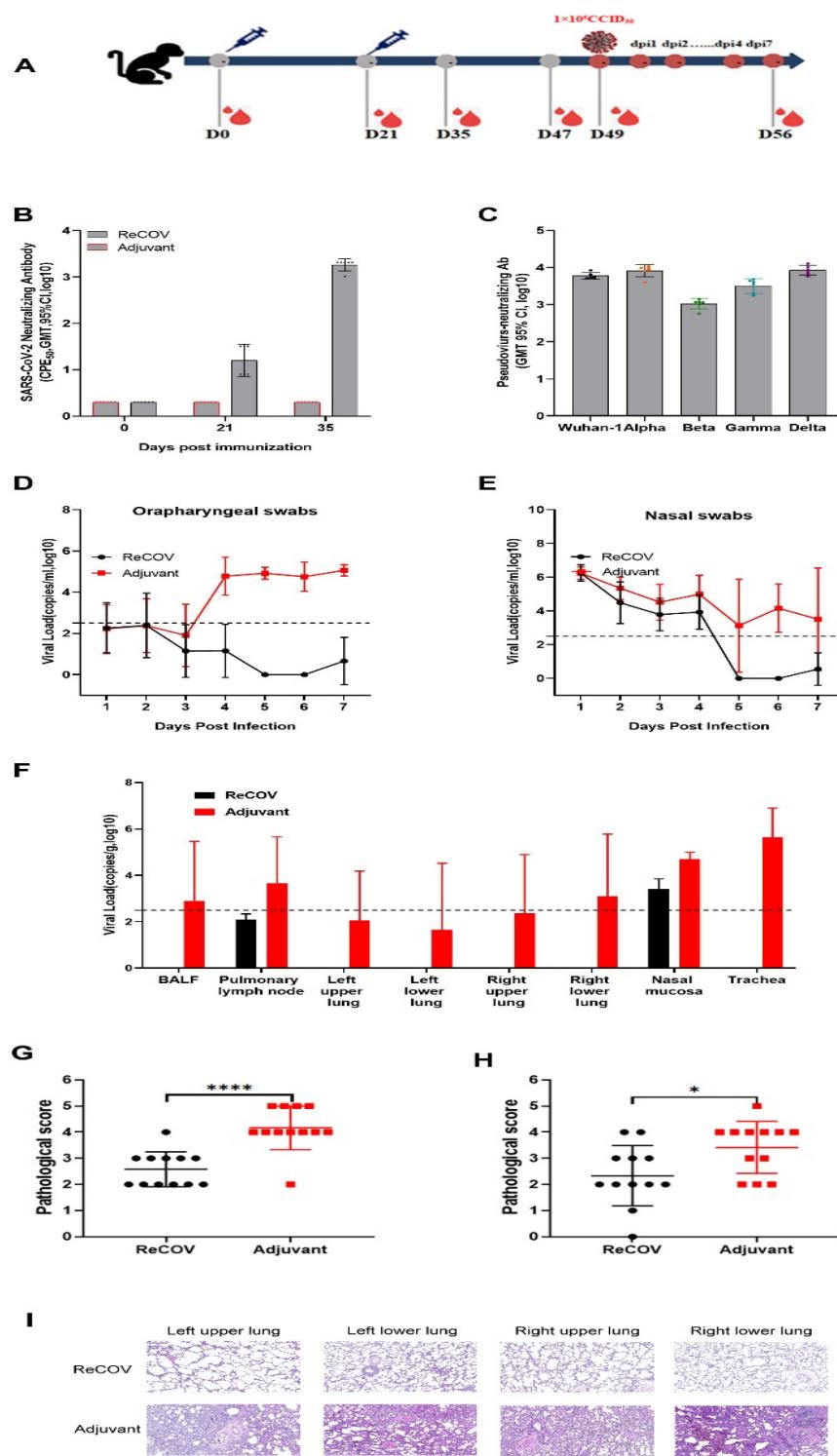


Figure 5