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2 **Reinfection could not occur in SARS-CoV-2 infected rhesus macaques**

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18

19     **Abstract**

20     An outbreak of the Corona Virus Disease 2019 (COVID-19), caused by the severe acute  
21     respiratory syndrome CoV-2 (SARS-CoV-2), began in Wuhan and spread globally.  
22     Recently, it has been reported that discharged patients in China and elsewhere were  
23     testing positive after recovering. However, it remains unclear whether the convalescing  
24     patients have a risk of “relapse” or “reinfection”. The longitudinal tracking of re-  
25     exposure after the disappeared symptoms of the SARS-CoV-2-infected monkeys was  
26     performed in this study. We found that weight loss in some monkeys, viral replication  
27     mainly in nose, pharynx, lung and gut, as well as moderate interstitial pneumonia at 7  
28     days post-infection (dpi) were clearly observed in rhesus monkeys after the primary  
29     infection. After the symptoms were alleviated and the specific antibody tested positively,  
30     the half of infected monkeys were rechallenged with the same dose of SARS-CoV-2  
31     strain. Notably, neither viral loads in nasopharyngeal and anal swabs along timeline nor  
32     viral replication in all primary tissue compartments at 5 days post-reinfection (dpr) was  
33     found in re-exposed monkeys. Combined with the follow-up virologic, radiological and  
34     pathological findings, the monkeys with re-exposure showed no recurrence of COVID-  
35     19, similarly to the infected monkey without rechallenge. Taken together, our results  
36     indicated that the primary SARS-CoV-2 infection could protect from subsequent  
37     exposures, which have the reference of prognosis of the disease and vital implications  
38     for vaccine design.

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40 The Corona Virus Disease 2019 (COVID-19) caused by severe acute respiratory  
41 syndrome CoV-2 (SARS-CoV-2), emerged in Wuhan China, has continued to sweep  
42 through South Korea, Japan, Italy and Iran. More than 90,000 people have been infected  
43 worldwide, with nearly 3000 deaths in about 40 countries<sup>1,2</sup>. Since February, it was  
44 estimated that about 14% of discharged patients in Guangdong province and elsewhere  
45 were testing positive after their release from the hospital and had to return to the hospital  
46 for observation<sup>3,4</sup>. Doubts about whether patients have a risk of "relapse" or  
47 "reinfection" after recovery from initial infection have aroused the worldwide concern.  
48 Therefore, in this study, we used the nonhuman primate models with SARS-CoV-2  
49 infection followed by the same virus rechallenge to ascertain the possibility of  
50 reinfection.

51 Four adult Chinese rhesus macaques (No M1-M4, 3-5 kg, 3-5-year-old) were  
52 intratracheally challenged with SARS-CoV-2 at  $1 \times 10^6$  50% tissue-culture infectious  
53 doses (TCID<sub>50</sub>), and the body weight, body temperature, X-ray, sampling of sera,  
54 nasal/throat/anal swabs and all primary tissues were carried out on schedule (Figure 1).  
55 Following the initial infection, three of the four monkeys showed the weight loss  
56 ranging from 200 g to 400 g (Figure 2a), but the changes of rectal temperature in all the  
57 animals were not observed (Figure 2b). Other clinical signs such as reduced appetite,  
58 increased respiration rate and hunched posture were transient after the initial challenge.  
59 We next determined their viral loads in respiratory and anal swabs along the timeline  
60 after the infection. As shown in Figure 2c and 2d, the viral loads in nasal swabs and  
61 pharyngeal swabs reached the highest levels (average, approximately  $6.5 \log_{10}$  RNA  
62 copies/mL) at 3 days post-infection (dpi) and then declined naturally. Similarly, viral  
63 loads from anal swabs reached the peak about  $4.5 \log_{10}$  RNA copies/mL at 3 dpi and  
64 then declined to undetectable level at 14 dpi (Figure 2e). To identify the virus

65 distribution and histopathological changes in SARS-CoV-2 infected monkeys, M1  
66 monkey was euthanized and necropsied at 7 dpi. As shown in Figure 2f (left panel),  
67 viral replication was found in nose ( $10^7$  to  $10^8$  copies/mL), pharynx ( $10^5$  to  $10^6$   
68 copies/mL), lung ( $10^4$  to  $10^7$  copies/mL), gut ( $10^4$  to  $10^6$  copies/mL), spinal cord  
69 (approximately  $10^4$  copies/mL), heart (approximately  $10^4$  copies/mL), skeletal muscle  
70 (approximately  $10^4$  copies/mL) and bladder (approximately  $10^4$  copies/mL).  
71 Furthermore, the lesions occurred mainly in the lung confirmed by the HE staining and  
72 anti-spike protein of SARS-CoV-2 staining, with mild to moderate interstitial  
73 pneumonia characterized by thickened alveolar septa, accumulation of alveolar  
74 macrophages in the alveoli, degeneration of the alveolar epithelia, and infiltration of  
75 inflammatory cells (Figure 3a). In addition, the chest X-ray at 7 dpi showed that the  
76 upper lobe of the right lung had varying degrees of the localized infiltration and  
77 interstitial markings, showing the mild to bilateral ground-glass opacification  
78 (Represented by M4, Figure 3b). For the longitudinal tracking of the characteristics  
79 each monkey, the specific antibody against SARS-CoV-2 of M2, M3 and M4 monkey  
80 was significantly elevated at 14 dpi or 21 dpi and 28 dpi compared to that at 3 dpi and  
81 7dpi (\* $P<0.05$ , \*\* $P<0.01$ , Figure 2g). In the three infected monkeys alive, body weight  
82 and rectal temperature remained relatively stable before rechallenge (Figure 2a and 2b),  
83 viral loads of nasopharyngeal and anal swabs were not detectable along the timeline  
84 before 28 dpi (Figure 2c to 2e), and no common abnormalities were found on a chest  
85 X-ray test at 28 dpi prior to the rechallenge (Represented by M4, Figure 3b). Altogether,  
86 these data suggested that the three animals were considered as recovering from SARS-  
87 CoV-2 infection, similarly meeting the clinical discharge evaluation criteria (absence  
88 of clinical symptoms and radiological abnormalities and 2 negative RT-PCR test  
89 results<sup>5</sup>).

90 Subsequently, two infected monkeys (M3 and M4) were intratracheally re-  
91 challenged at 28 dpi with the same dose ( $1 \times 10^6$  TCID<sub>50</sub>) of SARS-CoV-2 to ascertain  
92 the possibility of reinfection, whereas M2 monkey without any re-treatment was  
93 continuously monitored as the control (Figure 1). None of the monkeys showed the  
94 weight loss after the re-exposure (Figure 2a), but the transient elevation of body  
95 temperature was observed in both re-exposed monkeys (Figure 2b). Viral loads in  
96 nasopharyngeal and anal swabs tested negative after the re-exposure of SARS-CoV-2  
97 along the timeline (Figure 2c to 2e). Correspondingly, M3 monkey was euthanized and  
98 necropsied at 5 days post-reinfection (dpr) to confirm the viral replication and  
99 histopathological changes caused by re-exposure. Compared to M1 monkey at 7 dpi,  
100 no viral replication in all tissues (Figure 2f, right panel), as well as no pathological  
101 damage and viral antigen in lung tissues (Figure 3a, lower panel), were found in M3  
102 monkey at 5 dpr. As shown in Figure 3b, chest X-ray showed that there was no abnormal  
103 in M4 monkey at 5 dpr, similarly prior to the re-exposure (28 dpi). Therefore, our results  
104 suggested that the monkeys with SARS-CoV-2 infection after recovery could not be re-  
105 infected with the same strain. Longitudinally, the monkey undergone single infection  
106 in this study did not appear the recurrence after the recovery either.

107 It has been reported that the high levels of neutralizing antibodies have a protective  
108 effect on SARS-CoV infection, but the low neutralizing antibody are more susceptible  
109 to enhance the SARS-CoV infection and trigger antibody-dependent enhancement  
110 (ADE) effect<sup>6</sup>. As shown in Table 1, the titers of 1:16 (M2, M4) and 1:8 (M3) exhibited  
111 the neutralizing effect at 21 dpi and 28 dpi. After the re-exposure, the titers for M4  
112 elevated 1:40 at 5 dpr and 14 dpr, while M3 maintained the same titer as 1:8 at 5 dpr.  
113 In this study, ADE were not found in infected monkeys that were subsequently exposed  
114 to SARS-CoV-2. Because the neutralization antibody from our animal test is

115 comparable to that from recovered patients, this finding will have important  
116 implications to evaluate vaccine development.

117 From our current longitudinal study of monkeys, the reinfection could not occur if  
118 the monkeys produced the neutralizing antibody at an early stage after the primary  
119 infection. Correspondingly, the convalescing patients won't be contagious when they  
120 build up the enough specific antibody to develop immunity to SARS-CoV-2. On the  
121 other hand, no viral replication in all primary tissues was detectable in re-exposed  
122 monkeys, implying that the coronavirus might not be hidden for a long time. For the  
123 phenomenon on the discharged patients tested positively, it may be attributed to the  
124 "false negative" RT-PCR test results before their discharge or the patients without fully  
125 recovery albeit they met the discharge criteria. Therefore, further refinement of the  
126 diagnostic techniques, antibody monitoring and samples testing from the lower  
127 respiratory tract is essential for the cure of SARS-CoV-2 infection. In this study, our  
128 results indicated that the primary SARS-CoV-2 infection could protect from subsequent  
129 exposures, which have the reference of prognosis of the disease and vital implications  
130 for vaccine design. Importantly, the unsuccessful rechallenge in NHP models suggested  
131 that the re-positivity from discharged patients could not be due to reinfection. It needs  
132 to consider more complicated issues to find out the causes.

133

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- 150

151

152 Table 1 Neutralizing antibody titers to protect of SARS-CoV-2-infected Monkeys from  
153 reinfection.

Animal ID	Primary challenge		Rechallenge	
	21 dpi	28 dpi	5 dpr	14 dpr
M1 <sup>a</sup>	NE	NE	NE	NE
M2 <sup>b</sup>	1:16	1:16	NE	NE
M3 <sup>c</sup>	1:8	1:8	1:8	NE
M4	1:16	1:16	1:40	1:40

154

155 Notes: a M1 was euthanized and necropsied at 7 dpi. NE, not examined.

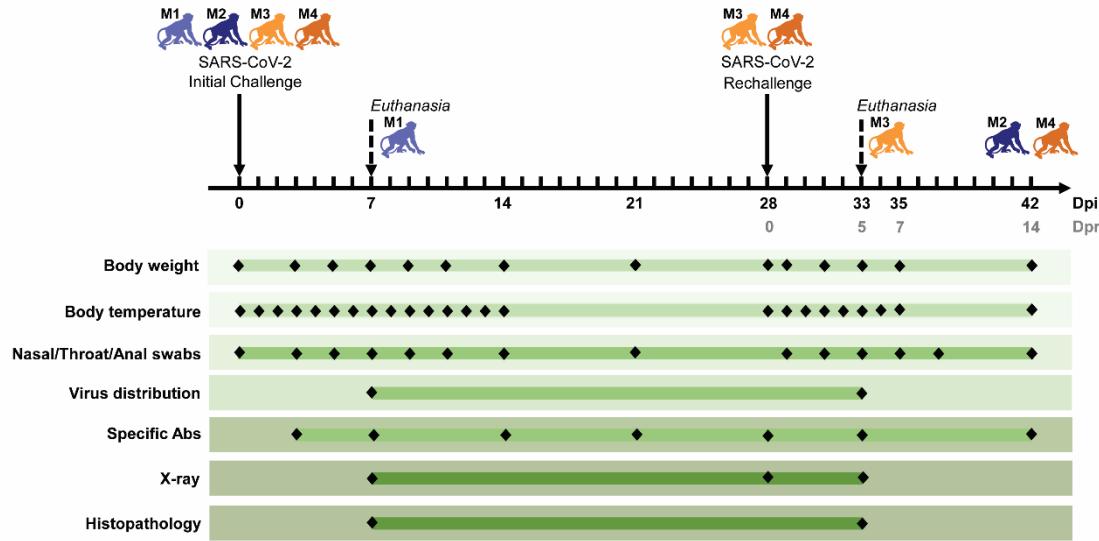
156 b M2 was continuously monitored without rechallenge. NE, not examined.

157 c M3 was euthanized and necropsied at 5 dpr. NE, not examined.

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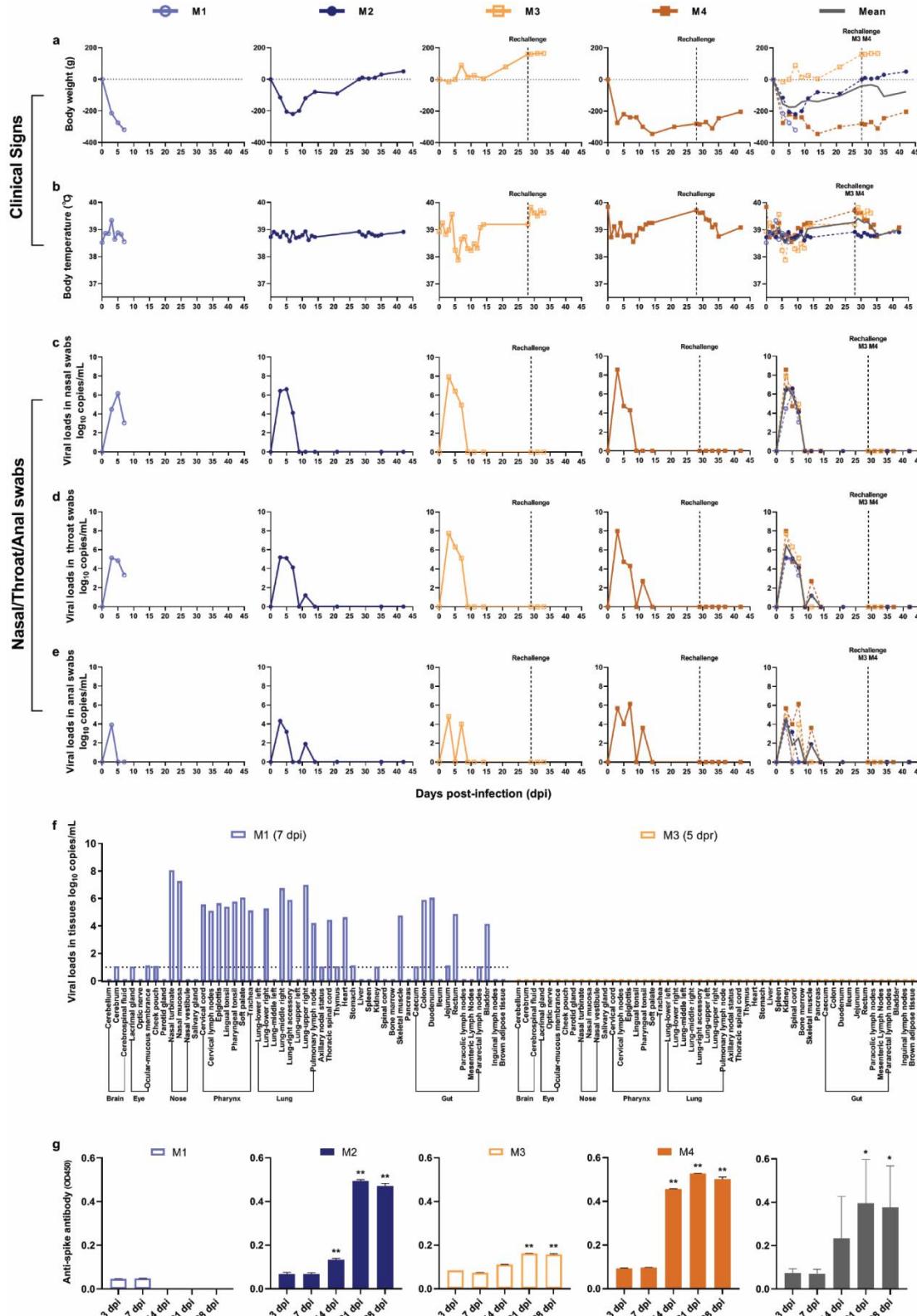


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**Figure 1 Experimental design and sample collection.** Four monkeys were initially challenged with  $1 \times 10^6$  TCID<sub>50</sub> SARS-CoV-2 with the intratracheal route. To investigate the influence of reinfection, M3 and M4 after recovery were intratracheally rechallenged with the same dose of SARS-CoV-2 at 28 days post-infection (dpi). Two animals (M1 and M3) were sacrificed at 7 dpi and 5 days post-rechallenge (dpr), respectively. M2 with single infection and M4 with primary infection followed by secondary challenge were longitudinally monitored during the entire observation. Body weight, body temperature and nasal/throat/anal swabs were measured along the timeline at a short interval. Two measurements of virus distribution and histopathology (HE/IHC stain) were carried out at 7d dpi (M1) and 5 dpr (M3). The specific antibodies against SARS-CoV-2 were detected seven times and X-ray was examined three times.

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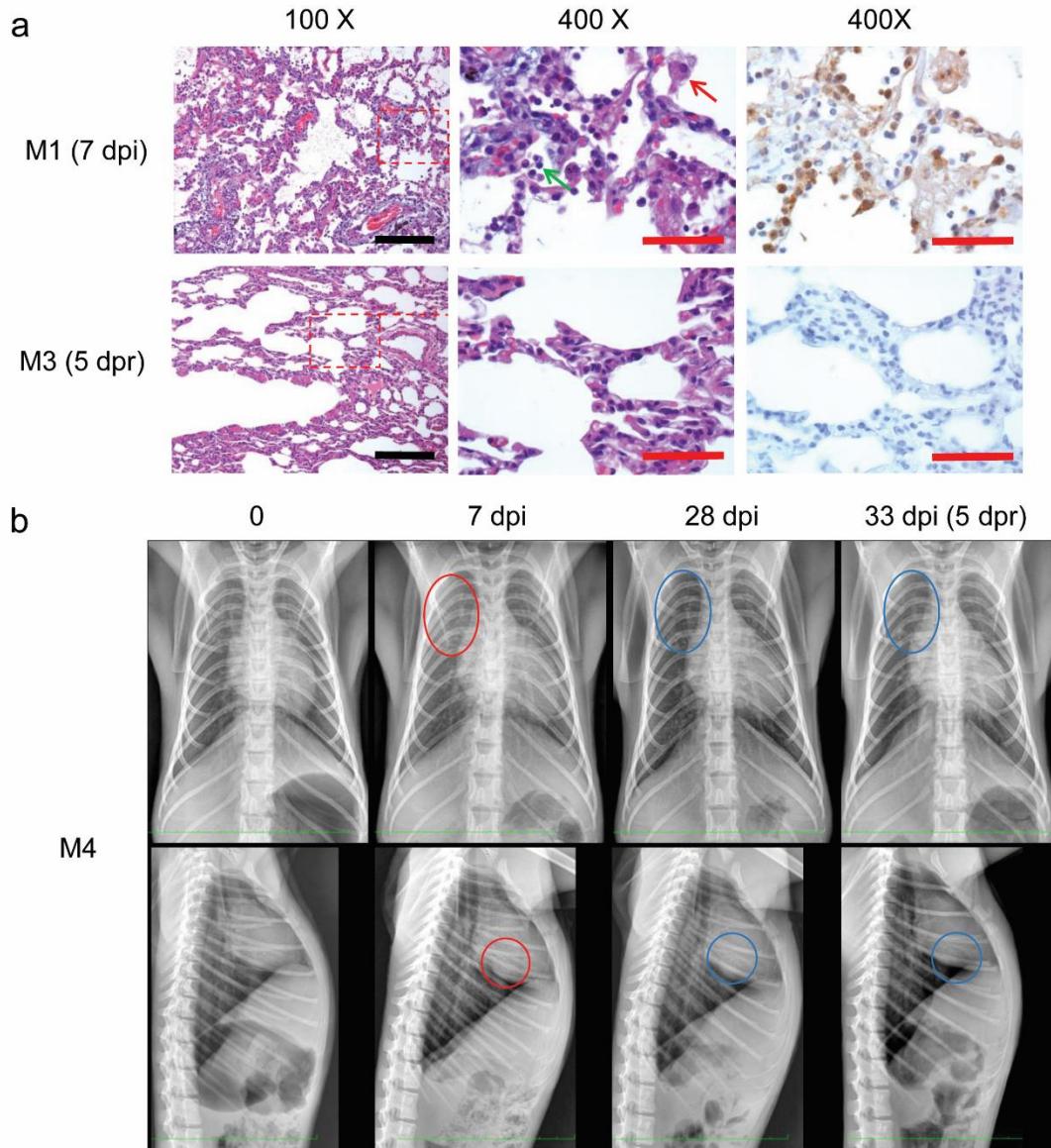
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176 **Figure 2 Longitudinally tracking in clinical signs, viral replication and immune**  
 177 **response. (a and b) Clinical signs in each monkey. Monkeys were recorded daily for**  
 178 **the changes in body weight and rectal temperature along the timeline after the initial**

179 infection followed by the virus rechallenge. Weights were expressed as percentage of  
180 body weight prior to primary infection. **(c, d and e)** Detection of viral RNA in nasal  
181 swabs, throat swabs and anal swabs. SARS-CoV-2 RNA was detected by qRT-PCR in  
182 the swabs from four monkeys at the indicated time points. Two monkeys were  
183 rechallenged at 28 dpi (the dotted line). **(f)** Detection of viral RNA in the mainly organs,  
184 such as brain, eye, nose, pharynx, lung and gut. Compared to M1 with primary infection  
185 at 7 dpi, viral replication tested negatively in the indicated tissues from M3 (at 5 dpr)  
186 with the virus rechallenge. **(g)** Levels of specific IgG against spike protein in each  
187 monkey. The levels of anti-viral antigen specific IgG from each monkey were detected  
188 at 3, 7, 14, 21, 28 dpi. The level of specific IgG at 14 dpi, 21 dpi or 28 dpi was  
189 significantly higher than that at 3 dpi or 7 dpi. The grey lines or bars represented the  
190 average of all animals at the indicated time points. (One-way ANOVA, \* $P<0.05$ ,  
191 \*\* $P<0.01$ )

192

193



194

195 **Figure 3 Longitudinally tracking in histopathology and chest X-ray. (a)**  
196 Histopathology and immunohistochemical examination of lung in M1 monkey (7 dpi)  
197 and M3 monkey (5 dpr). Histopathological examination exhibited that moderate  
198 interstitial pneumonia with infiltration of lymphocytes (green arrow) and swollen  
199 aveolar macrophages (red arrow) in the alveolar cavities at 7 dpi. The antigen of SARS-  
200 CoV-2 were detected with anti-Spike antibody using immunohistochemical  
201 examination. Black scale bar=100  $\mu$ m, red scale bar=50  $\mu$ m (b) Longitudinal  
202 examination of Chest X-ray in M4 monkey. M4 monkey were tested by chest X-ray

203 prior to the challenge, as well as 7 dpi, 28 dpi and 5 dpr. Front chest X-ray (upper panel),

204 Lateral chest X-ray (lower panel).

205

206

207 **Methods**

208 ***Ethics statement***

209 Four 3- to 5-year old rhesus macaques, named as M1 to M4, were housed and cared in  
210 an Association for the Assessment and Accreditation of Laboratory Animal Care  
211 (AAALAC)-accredited facility. All animal procedures and experiments were carried  
212 out in accordance with the protocols approved by the Institutional Animal Care and Use  
213 Committee (IACUC) of the Institute of Laboratory Animal Science, Chinese Academy  
214 of Medical Sciences (BLL20001). All animals were anesthetized with ketamine  
215 hydrochloride (10 mg/kg) prior to sample collection, and the experiments were  
216 performed in the animal biosafety level 3 (ABSL3) laboratory.

217

218 ***Animal experiments***

219 For primary infection, all animals were inoculated intratracheally with SARS-CoV-2  
220 (SARS-CoV-2/WH-09/human/2020/CHN isolated in our laboratory) stock virus at a  
221 dosage of  $10^6$  TCID<sub>50</sub>/1 mL inoculum volume. After the recovery, M3 M4 were  
222 rechallenged intratracheally with the same dose ( $10^6$  TCID<sub>50</sub>/1 mL inoculum volume)  
223 SARS-CoV-2 at 28 dpi. To confirm the virus distribution and pathological changes, M1  
224 at 7 dpi and M3 at 33 dpi (5 dpr) were euthanized and autopsied respectively. All  
225 animals were monitored along the timeline to record body weights, body temperature,  
226 clinical signs, nasal/throat/anal swab, X-ray and specific antibody. The animal  
227 experiment and longitudinal sampling schedule are shown in Figure 1.

228

229 ***Quantification of SARS-CoV-2 RNA***

230 The nasal/throat/anal swab samples and mainly tissue compartments collected from  
231 infected monkeys were tested for SARS-CoV-2 RNA by quantitative real-time reverse

232 transcription-PCR (qRT-PCR). Total RNA was extracted and reverse transcription was  
233 performed as previously described<sup>7</sup>. qRT-PCR reactions were carried out on an ABI  
234 9700 Real-time PCR system (Applied Biosystems Instrument), the cycling protocol and  
235 the primers as follows: 50°C for 2 min, 95°C for 2 min, followed by 40 cycles at 95°C  
236 for 15 s and 60°C for 30 s, and then 95°C for 15 s, 60°C for 1 min, 95°C for 45 s.  
237 Forward primer: 5'-TCGTTTCGGAAGAGACAGGT-3', Reverse primer: 5'-  
238 GCGCAGTAAGGATGGCTAGT-3'.

239

240 **ELISA**

241 Sera were collected from each animal for the measurement of SARS-CoV-2 antibody  
242 by enzyme-linked immunosorbent assay (ELISA) along the detection timeline after the  
243 initial infection. 96-well plates were coated with 0.1μg Spike protein of SARS-CoV-2  
244 (Sino Biological, 40591-V08H) overnight at 4°C and blocked with 2% BSA/PBST for  
245 1 hour at room temperature. 1:100 diluted samples were added to each well and  
246 incubated for 30 minutes at 37 °C , followed by the HRP-labeled goat anti-mouse  
247 antibody (Beijing ZSGB Biotechnology, ZB-2305) incubated for 30 minutes at room  
248 temperature. The reaction was developed by TMB substrate and determined at 450 nm.

249

250 ***Histopathology and Immunohistochemistry***

251 Autopsies were performed according to the standard protocol in ABSL3 laboratory at  
252 7 dpi for M1 and 5 dpr for M3. Lung samples were fixed in 10% neutral-buffered  
253 formalin solution. Then, paraffin sections (3-4 μm in thickness) were prepared and  
254 stained with Hematoxylin and Eosin (H&E) prior to the observation by light  
255 microscopy. For immunohistochemistry (IHC) staining to identify the antigen of  
256 SARS-CoV-2, paraffin dehydrated sections (3-4 μm in thickness) were treated with an

257 antigen retrieval kit (Boster, AR0022) for 1 min at 37°C and quenched for endogenous  
258 peroxidases in 3% H<sub>2</sub>O<sub>2</sub> in methanol for 10 min. After blocking in 1% normal goat  
259 serum for 1 hour at roomtemperature, the slices were stained with 7D2 monoclonal  
260 antibody (laboratory preparation<sup>7</sup>) at 4°C overnight, following with the incubation of  
261 HRP-labeled goat anti-mouse IgG (Beijing ZSGB Biotechnology, ZDR-5307) for 1  
262 hour. Then, the slices were visualized by incubation with 3,30-diaminobenzidine  
263 tetrahydrochloride (DAB) and the image was viewed under an Olympus microscope.

264

265 ***Neutralizing antibody assay***

266 Sera samples were tested for the presence of neutralizing antibody observed by  
267 cytopathic effect (CPE). Briefly, the sera from monkeys were heat-inactivated at 56°C  
268 for 30 min. Then, serially two-fold diluted sera were incubated with 100 TCID<sub>50</sub> SARS-  
269 CoV-2 for 1 h at 37°C, and added into Vero-E6 cells in a 96-well-plate. Cells were  
270 cultured for 1 week to observe for CPE and the serum dilution in which 50% of the  
271 cells were protected from infection was calculated. Each dilution of serum was tested  
272 in triplicates.

273

274 ***Statistical analysis***

275 Comparisons among the groups were determined using One-way ANOVA. All data  
276 were analyzed with GraphPad Prism 8.0 software. The level of statistical significance  
277 is designated as \**p* < 0.05, \*\**p* < 0.01.

278

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284

285 **AUTHOR CONTRIBUTIONS**

286 Conceptualization: C.Q.; Methodology: L.B., W.D., H.G., C.X., J.L., J.X. and Q.L.;  
287 Investigation: L.B., W.D., H.G., C.X., J.L., J.X., Q.L., J.L., P.Y., Y.X., F.Q., Y.Q., F.L.,  
288 Z.X., H.Y., S.G., M.L., G.W., S.W., Z.S., W.Z., Y.H., L.Z., X.L. and Q.W.; Writing –  
289 Original Draft: J.X.; Writing –Review and Editing: C.Q.; Funding Acquisition: C.Q.  
290 and L.B.; Resources: C.Q.; Supervision: C.Q.

291

292 **COMPETING INTERESTS**

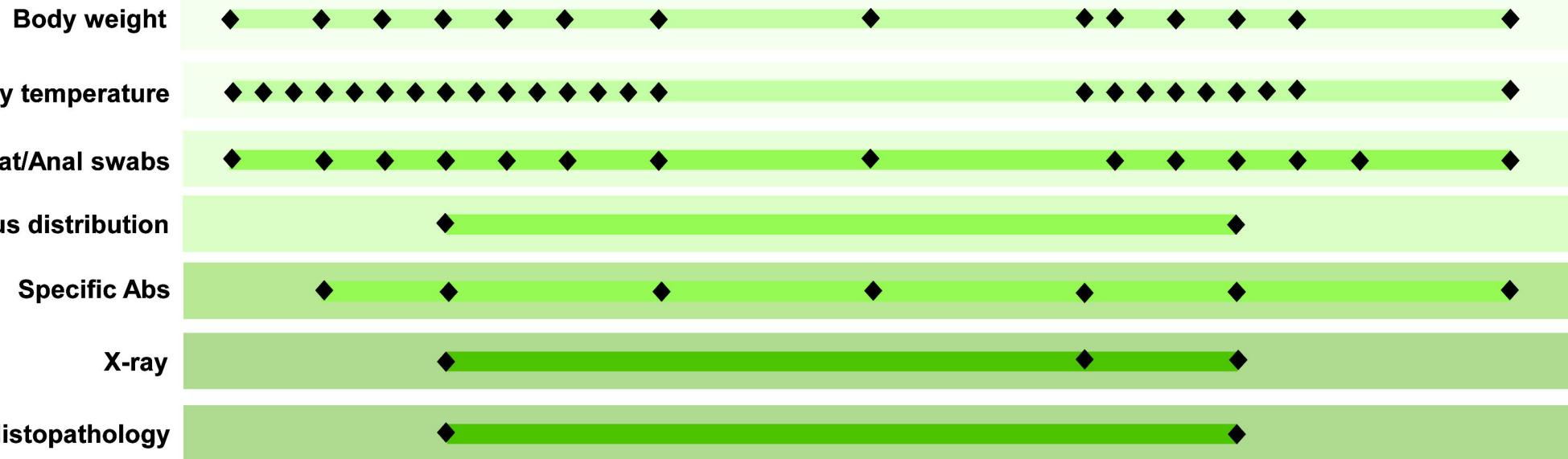
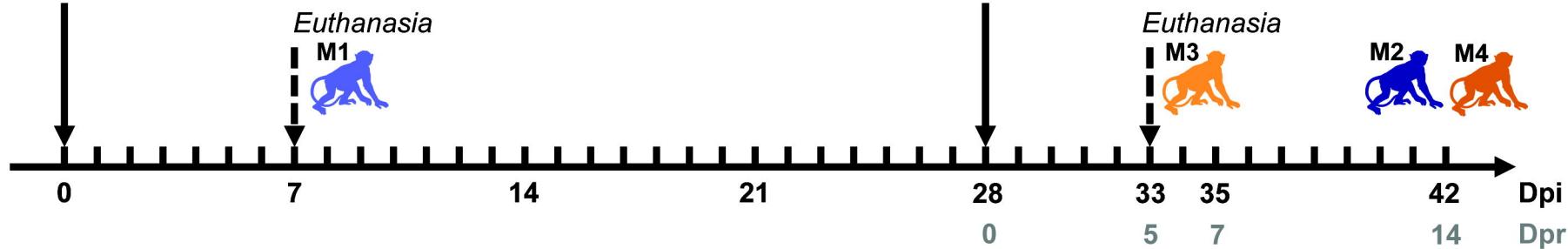
293 The authors have no competing interests to declare.

294

295



SARS-CoV-2  
Initial Challenge



M1 M2 M3 M4 Mean

