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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 challenge. 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.

39

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^{1,2}. 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^{3,4}. 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 1×10^6 50% tissue-culture infectious
53 doses (TCID₅₀), 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 results⁵).

90 Subsequently, two infected monkeys (M3 and M4) were intratracheally re-
91 challenged at 28 dpi with the same dose (1×10^6 TCID₅₀) 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) effect⁶. 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 ^a	NE	NE	NE	NE
M2 ^b	1:16	1:16	NE	NE
M3 ^c	1:8	1:8	1:8	NE
M4	1:16	1:16	1:40	1:40

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

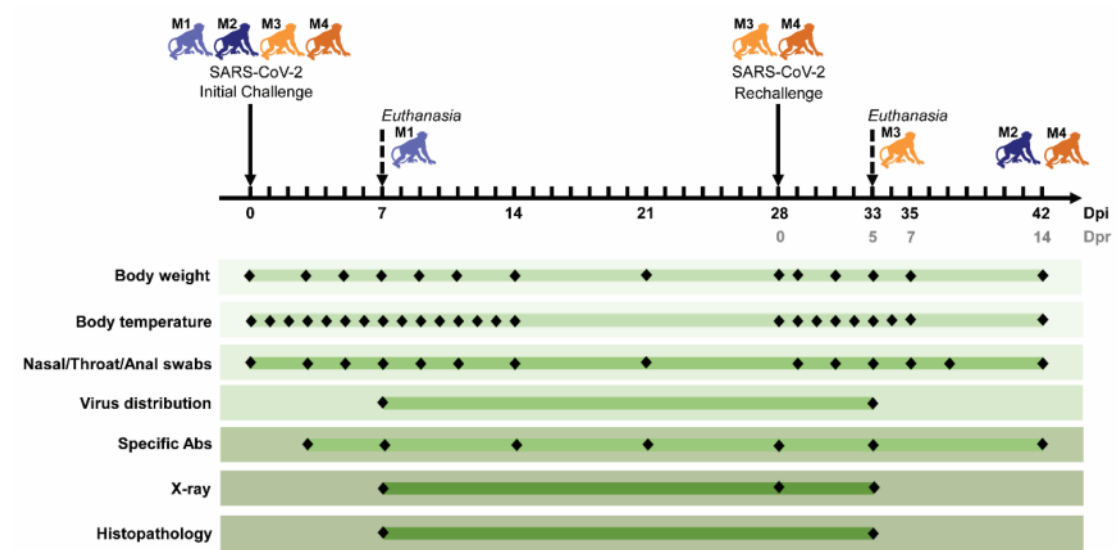
156 b M2 was continuously monitored without rechallenge. NE, not examined.

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

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161

162 **Figure 1 Experimental design and sample collection.** Four monkeys were initially
163 challenged with 1×10^6 TCID₅₀ SARS-CoV-2 with the intratracheal route. To investigate
164 the influence of reinfection, M3 and M4 after recovery were intratracheally
165 rechallenged with the same dose of SARS-CoV-2 at 28 days post-infection (dpi). Two
166 animals (M1 and M3) were sacrificed at 7 dpi and 5 days post-rechallenge (dpr),
167 respectively. M2 with single infection and M4 with primary infection followed by
168 secondary challenge were longitudinally monitored during the entire observation. Body
169 weight, body temperature and nasal/throat/anal swabs were measured along the
170 timeline at a short interval. Two measurements of virus distribution and histopathology
171 (HE/IHC stain) were carried out at 7d dpi (M1) and 5 dpr (M3). The specific antibodies
172 against SARS-CoV-2 were detected seven times and X-ray was examined three times.

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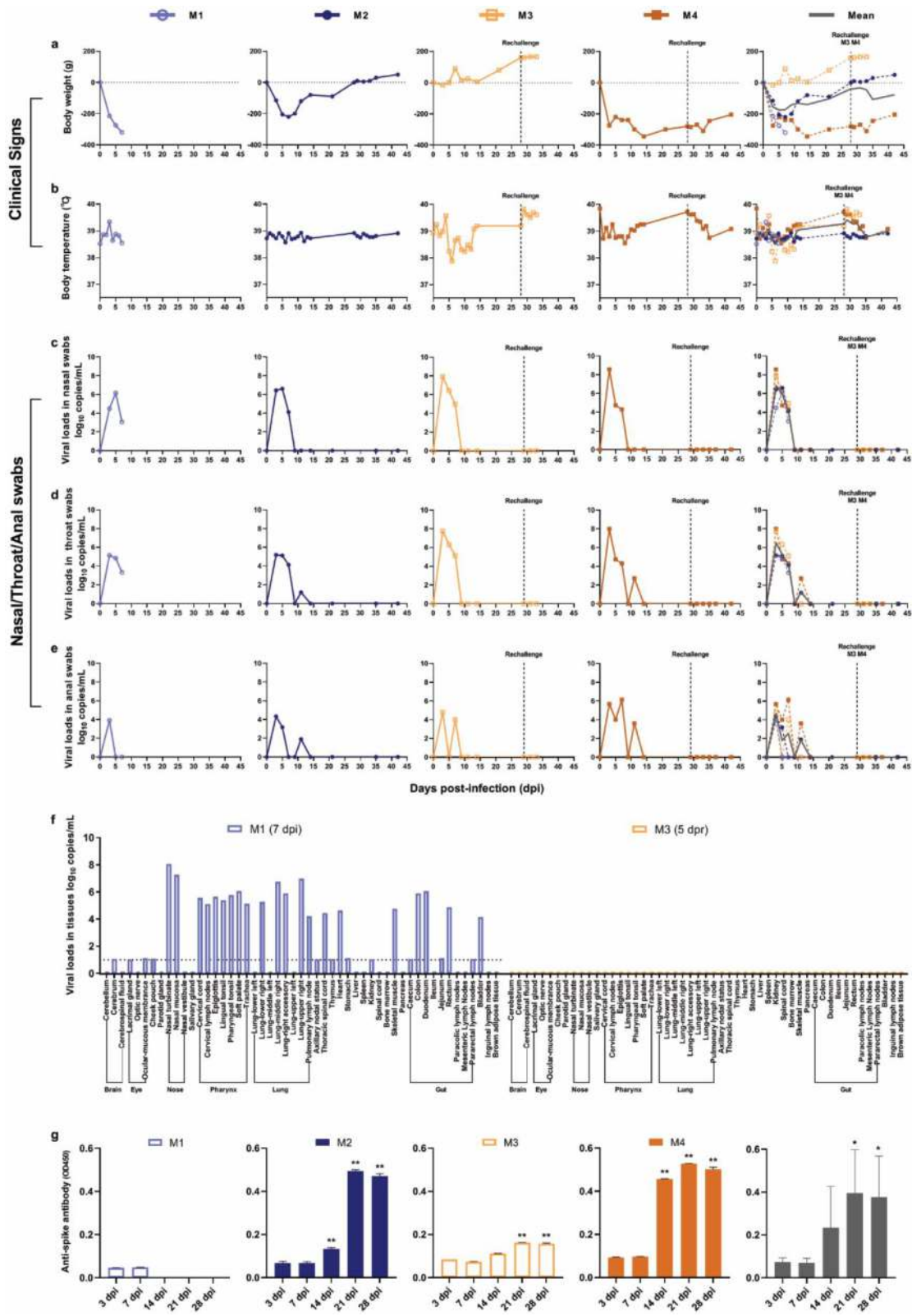
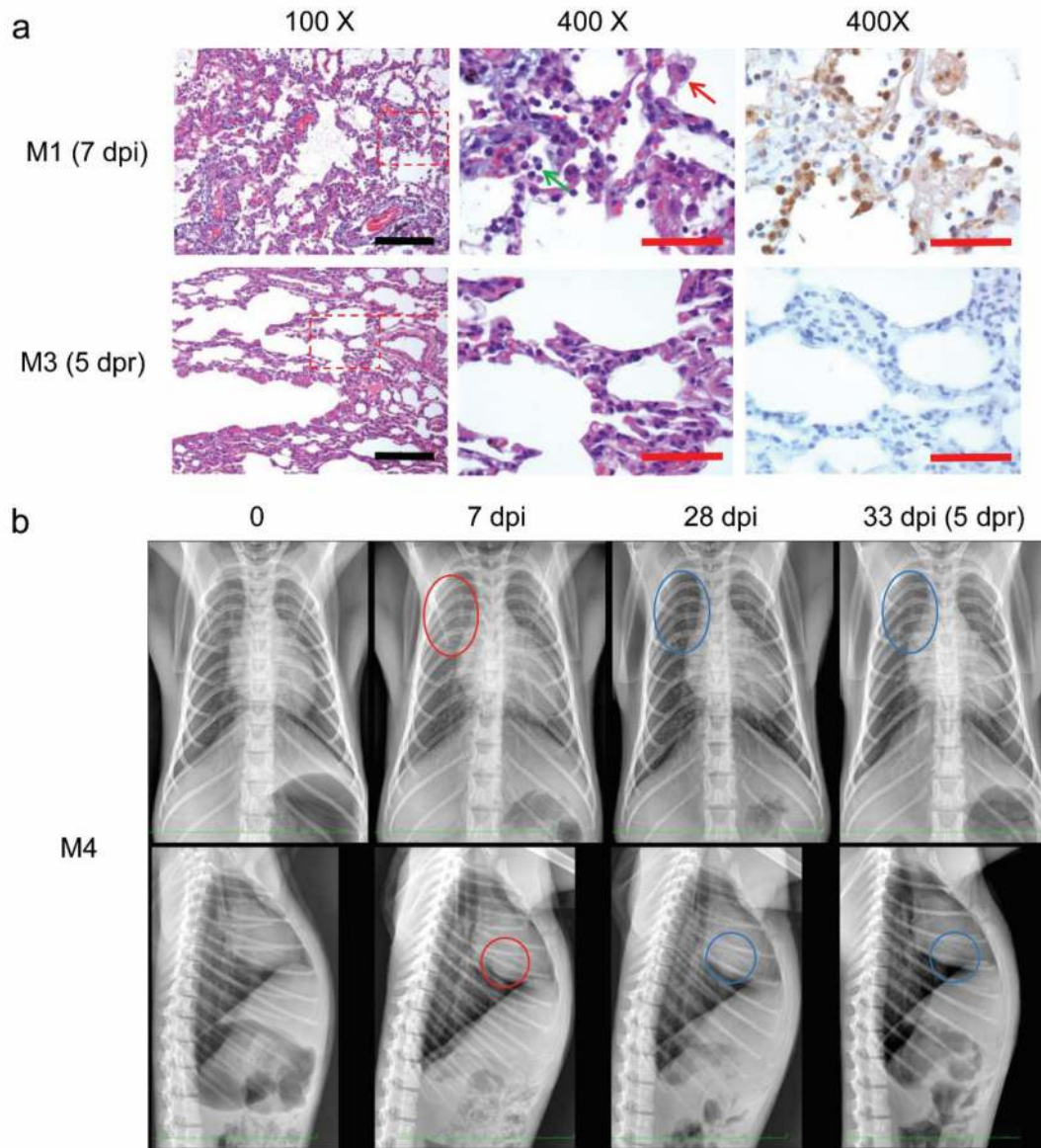


Figure 2 Longitudinally tracking in clinical signs, viral replication and immune response. (a and b) Clinical signs in each monkey. Monkeys were recorded daily for the changes in body weight and rectal temperature along the timeline after the initial

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 μm, red scale bar=50 μm (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 TCID₅₀/1 mL inoculum volume. After the recovery, M3 M4 were
222 rechallenged intratracheally with the same dose (10^6 TCID₅₀/1 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 euthanasia 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 described⁷. 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% H₂O₂ 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 preparation⁷) 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 TCID₅₀ 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.

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292 **COMPETING INTERESTS**

293 The authors have no competing interests to declare.

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SARS-CoV-2
Initial Challenge

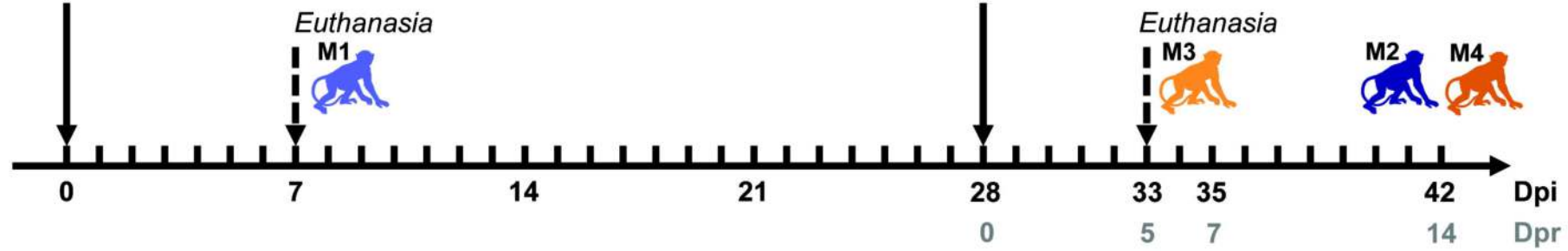


SARS-CoV-2
Rechallenge

Euthanasia



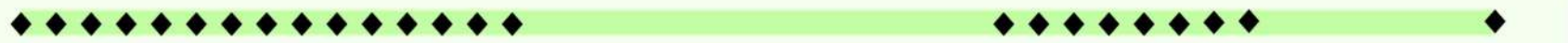
Euthanasia



Body weight



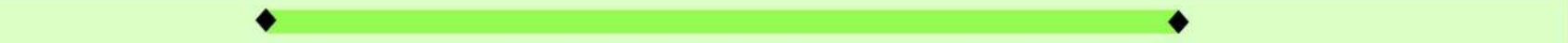
Body temperature



Nasal/Throat/Anal swabs



Virus distribution



Specific Abs



X-ray



Histopathology



