1 **Subacute SARS-CoV-2 replication can be controlled in the absence of CD8+ T cells in**

2 **cynomolgus macaques**

3 Short title: Subacute SARS-CoV-2 replication can be controlled without CD8+ T cells

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5 Takushi Nomura[1]*, Hiroyuki Yamamoto[1], Masako Nishizawa[1], Trang Thi Thu Hau[1],

6 Shigeyoshi Harada[1], Hiroshi Ishii[1], Sayuri Seki[1], Midori Nakamura-Hoshi[1], Midori Okazaki[1],

7 Sachie Daigen[1], Ai Kawana-Tachikawa[1,2,3], Noriyo Nagata[4], Naoko Iwata-Yoshikawa[4], Nozomi

8 Shiwa[4], Shun Iida[4], Harutaka Katano[4], Tadaki Suzuki[4], Eun-Sil Park[5], Ken Maeda[5], Yuriko

9 Suzaki[6], Yasushi Ami[6], and Tetsuro Matano[1,2,3]*

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11 [1]AIDS Research Center, National Institute of Infectious Diseases, Tokyo, Japan

12 [2]Institute of Medical Science, University of Tokyo, Tokyo, Japan

13 [3]Joint Research Center for Human Retrovirus Infection, Kumamoto University, Kumamoto, Japan

14 [4]Department of Pathology, National Institute of Infectious Diseases, Tokyo, Japan

15 [5]Department of Veterinary Science, National Institute of Infectious Diseases, Tokyo, Japan

16 [6]Division of Laboratory Animal Research, National Institute of Infectious Diseases, Tokyo,

17 Japan

18

19 *Equally-contributed corresponding authors: nomutaku@nih.go.jp (TN) and

20 tmatano@nih.go.jp (TM)

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Abstract

SARS-CoV-2 infection presents clinical manifestations ranging from asymptomatic to fatal respiratory failure. Despite the induction of functional SARS-CoV-2-specific $CD8^+$ T-cell responses in convalescent individuals, the role of virus-specific $CD8^+$ T-cell responses in the control of SARS-CoV-2 replication remains unknown. In the present study, we show that subacute SARS-CoV-2 replication can be controlled in the absence of $CD8^+$ T cells in cynomolgus macaques. Eight macaques were intranasally inoculated with 10^5 or 10^6 $TCID_{50}$ of SARS-CoV-2, and three of the eight macaques were treated with a monoclonal anti-CD8 antibody on days 5 and 7 post-infection. In these three macaques, $CD8^+$ T cells were undetectable on day 7 and thereafter, while virus-specific $CD8^+$ T-cell responses were induced in the remaining five untreated animals. Viral RNA was detected in nasopharyngeal swabs for 10-17 days post-infection in all macaques, and the kinetics of viral RNA levels in pharyngeal swabs and plasma neutralizing antibody titers were comparable between the anti-CD8 antibody treated and untreated animals. SARS-CoV-2 RNA was detected in the pharyngeal mucosa and/or retropharyngeal lymph node obtained at necropsy on day 21 in two of the untreated group but undetectable in all macaques treated with anti-CD8 antibody. $CD8^+$ T-cell responses may contribute to viral control in SARS-CoV-2 infection, but our results indicate possible containment of subacute viral replication in the absence of $CD8^+$ T cells, implying that $CD8^+$ T-cell dysfunction may not solely lead to viral control failure.

42   **Author Summary**

43   SARS-CoV-2 infection presents a wide spectrum of clinical manifestations ranging from

44   asymptomatic to fatal respiratory failure. The determinants for failure in viral control and/or

45   fatal disease progression have not been elucidated fully. Both acquired immune effectors,

46   antibodies and CD8$^+$ T cells, are considered to contribute to viral control. However, it remains

47   unknown whether a deficiency in either of these two arms is directly linked to failure in the

48   control of SARS-CoV-2 replication. In the present study, to know the requirement of CD8$^+$ T

49   cells for viral control after the establishment of infection, we examined the effect of CD8$^+$ cell

50   depletion by monoclonal anti-CD8 antibody administration in the subacute phase on SARS-

51   CoV-2 replication in cynomolgus macaques. Unexpectedly, our analysis revealed no significant

52   impact of CD8$^+$ cell depletion on viral replication, indicating that subacute SARS-CoV-2

53   replication can be controlled in the absence of CD8$^+$ T cells. CD8$^+$ T-cell responses may

54   contribute to viral control in SARS-CoV-2 infection, but this study suggests that CD8$^+$ T-cell

55   dysfunction may not solely lead to viral control failure or fatal disease progression.

56

Introduction

The coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has rapidly spread resulting in a major pandemic [1]. SARS-CoV-2 transmission occurs via the respiratory route, and the average incubation period from infection to symptom onset has been estimated to be 5 days [2]. SARS-CoV-2 infection presents a wide spectrum of clinical manifestations ranging from asymptomatic to fatal respiratory failure [3]. Multiple cofounding factors such as age and underlying diseases are associated with COVID-19 severity [4-8]. For instance, auto-antibodies against type I interferon have been reported to be associated with life-threatening COVID-19 pneumonia [9,10]. However, the exact determinants for failure in viral control and/or fatal disease progression have not been elucidated fully.

Most non-fatal COVID-19 cases show a limited period of detectable virus production in pharyngeal swabs peaking at around one week post-infection [11]. Host acquired as well as innate immune responses are involved in the control of viral replication [8,12-14]. Anti-SARS-CoV-2 neutralizing antibodies are induced in most infected individuals [13-16]. Recent clinical studies including those on convalescent plasma and/or monoclonal antibody administration have indicated efficacy of neutralizing antibodies against SARS-CoV-2 infection [17-20]. Animal studies have confirmed *in vivo* efficacy of neutralizing antibodies against infection [21-25]. Also, SARS-CoV-2-specific T-cell responses are induced in most non-fatal COVID-19 cases [26-28]. Current studies have indicated induction of functional virus-specific CD8+ T-cell responses in convalescent COVID-19 individuals, implying suppressive pressure of CD8+ T cells on SARS-CoV-2 replication [29,30]. Thus, both acquired immune effectors, antibodies and CD8$^+$ T cells, are considered to contribute to viral control. However, it remains unknown whether a deficiency in either of these two arms is directly linked to failure in the control of SARS-CoV-2 replication. It has been reported that COVID-19 patients with

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82   agammaglobulinemia controlled disease progression, suggesting viral control even in the

83   absence of antibody responses [31].

84   A previous study of anti-CD8 antibody administration prior to re-infection in rhesus

85   macaques has indicated partial contribution of CD8+ T cells to protection against SARS-CoV-

86   2 re-infection [25]. However, the requirement of CD8$^+$ T cells for the control of virus replication

87   after the establishment of infection remains unclear. In the present study, we investigated the

88   effect of CD8$^+$ cell depletion by monoclonal anti-CD8 antibody administration in the subacute

89   phase on SARS-CoV-2 replication in cynomolgus macaques. Unexpectedly, our analysis

90   revealed no significant impact of CD8$^+$ cell depletion on viral replication, indicating that

91   subacute SARS-CoV-2 replication can be controlled in the absence of CD8$^+$ T cells.

92

93   **Results**

94   **Kinetics of SARS-CoV-2 infection in cynomolgus macaques after intranasal inoculation**

95   Previous studies have shown that intranasal and intratracheal inoculation with 10^5 TCID$_{50}$

96   (50% tissue culture infective doses) of SARS-CoV-2 results in the establishment of infection in

97   rhesus macaques, with viral RNA detectable for more than a week post-infection in pharyngeal

98   swabs [32,33]. In the present study, we first examined whether intranasal SARS-CoV-2

99   inoculation only can result in viral infection in cynomolgus macaques. In the first experiment,

100   cynomolgus macaques were intranasally inoculated with 10^6 (exactly 7.5 x 10^5 in macaque

101   N011), 10^5 (exactly 7.5 x 10^4 in macaques N012 and N013), or 10^4 (exactly 7.5 x 10^3 in macaque

102   N014) TCID$_{50}$ of SARS-CoV-2 (Table 1). Macaques N011, N012, and N013 showed similar

103   levels of viral RNA in nasopharyngeal swabs on day 2, at the peak (Fig 1A). Viral RNA was

104   also detected in throat swabs with a lower peak (Fig 1B). Viral RNA in nasopharyngeal swabs

105   was detectable for approximately two weeks (up to: day 17 in N011, day 12 in N012, and day

106   14 in N013) after virus inoculation (Fig 1A, 1C, and 1D). Subgenomic RNAs (sgRNAs) were

107   also detected in nasopharyngeal and throat swabs, indicating viral replication (Fig 2A and 2B).

108   SARS-CoV-2 sgRNAs were detected in nasopharyngeal swabs until day 9 in N011, day 7 in

109   N012, and day 5 in N013 (Fig 2A, 2C, and 2D). However, in macaque N014, which was

110   inoculated with 10^4 TCID$_{50}$ of SARS-CoV-2, sgRNAs were undetectable, and viral RNAs were

111   detectable albeit at lower levels, only until day 5 in nasopharyngeal swabs (Fig 1A and Fig 2A),

112   indicating that 10^4 TCID$_{50}$ is below the virus inoculum threshold to consistently induce

113   detectable viral replication. N014 was subsequently excluded from further analyses.

114   In the second experiment, two (N021 and D023) and three (N022, D024, and D025)

115   macaques were intranasally inoculated with 10^6 and 10^5 TCID$_{50}$ of SARS-CoV-2, respectively

116   (Table 1). Monoclonal anti-CD8 antibody was administered intravenously on days 5 and 7 to

117   three (D023, D024, and D025 in Group D) of the five macaques. All of the five macaques in

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118   the second experiment showed comparable levels of viral RNAs and sgRNAs in

119   nasopharyngeal swabs on day 2 compared to the three macaques inoculated with 10^6 or 10^5

120     $TCID_{50}$ of SARS-CoV-2 in the first experiment (Fig 1A and Fig 2A). Indeed, no significant

121   difference in RNA levels in nasopharyngeal swabs on day 5 was observed between the first

122   three and the second five animals (Fig 1E). No clear difference in viral loads in either

123   nasopharyngeal or throat swabs on days 2 and 5 was observed between macaques inoculated

124   with 10^6 (n = 3) and 10^5 (n = 5) $TCID_{50}$ of SARS-CoV-2 (Fig 1C and 1D and Fig 2C and 2D).

125   Viral RNA in nasopharyngeal swabs was detectable until day 14 in N021 and day 10 in N022

126   following inoculation (Fig 1A, 1C, and 1D). SARS-CoV-2 sgRNAs in nasopharyngeal swabs

127   were detected until day 5 in N021 and day 7 in N022 following inoculation (Fig 2A, 2C, and

128   2D). Collectively, in the first and second experiments, intranasal inoculation of cynomolgus

129   macaques with 10^6 or 10^5 $TCID_{50}$ of SARS-CoV-2 resulted in viral replication with viral RNA

130   detectable for 10-17 days in nasopharyngeal swabs.

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132   **Kinetics of SARS-CoV-2 infection after CD8$^+$ cell depletion**

133   We then investigated the effect of CD8$^+$ cell depletion on viral replication in the subacute

134   phase of SARS-CoV-2 infection. In the three Group D macaques administered with anti-CD8

135   antibody on days 5 and 7, CD8$^+$ T cells were undetectable in peripheral blood on day 7 and

136   thereafter (Fig 3). These three macaques showed comparable levels of viral RNA in

137   nasopharyngeal swabs before (day 5) and after (day 7) anti-CD8 antibody treatment compared

138   to the five untreated Group N macaques (Fig 1F). Viral RNA in nasopharyngeal swabs was

139   detectable until day 10 in D025 and day 14 in macaques D023 in D024 after virus inoculation

140   (Fig 1A, 1C, and 1D). Viral sgRNAs in nasopharyngeal swabs were detected until day 2 in

141   D023, day 5 in D024, and day 7 in D025 (Fig 2A, 2C, and 2D). Collectively, no clear difference

142   in viral RNA levels in swabs was observed for the three anti-CD8 antibody-treated Group D

143   versus the five untreated Group N macaques.

144   We also examined whether virus could be recovered from individual swab samples (Table

145   2). SARS-CoV-2 was recovered from nasopharyngeal and throat swabs from all eight animals

146   intranasally inoculated with either 10^6 or 10^5 $TCID_{50}$. Virus was recovered for 2-12 days in anti-

147   CD8 antibody-untreated macaques (until day 2 in N021, day 5 in N013 and N022, day 7 in

148   N011, and day 12 in N012) and for 2-7 days in anti-CD8 antibody-treated macaques (until day

149   2 in D024 and day 7 in D023 and D025). There was no indication of enhanced virus recovery

150   after CD8 cell depletion.

151   Macaque N021 was euthanized on day 14, while the remaining animals were euthanized

152   on day 21 post-infection (Table 1). Examination of body temperature showed transient slight

153   fever in some animals (on day 2 in N021 and D025; on day 6 in D023 and D024; on days 13-

154   19 in N012) (S1 Fig). Histopathological analysis of the lung obtained at necropsy on day 14 in

155   macaque N021 revealed mild or moderate pulmonary inflammation (S2 Fig), whereas no

156   significant pathology in the lung was detected on day 21 in other animals.

157   RNA was extracted from the pharyngeal mucosa, retropharyngeal lymph nodes (RPLN),

158   lung, intestine, and spleen obtained at necropsy, and subjected to RT-PCR for detection of viral

159   RNA (Table 3). Viral RNA was undetectable in tissues from macaques N012, N014, N021,

160   D023, and D024. However, viral RNA was detected in the RPLN of N011, in the pharyngeal

161   mucosa, RPLN, and spleen of N013, and in the spleens of N022 and D025. Additionally, viral

162   sgRNAs were also detectable in pharyngeal mucosa, RPLN, and spleen of N013. There was no

163   evidence of enhanced viral replication in anti-CD8 antibody-treated macaques.

164

165   **Antibody and T-cell responses in macaques after intranasal SARS-CoV-2 inoculation**

166   Anti-SARS-CoV-2 neutralizing antibody (NAb) responses were induced in all the

167   macaques after intranasal SARS-CoV-2 inoculation (Fig 4) NAb responses were detected on

168   day 7 in macaques N021 and D025 only, and in all animals on day 14. Macaques D025 and

169   D024 exhibited the highest and lowest NAb titers, respectively. No clear difference in NAb

170   responses was observed between the three anti-CD8 antibody-treated and the five untreated

171   macaques.

172   Finally, we examined $CD8^+$ T-cell responses specific for SARS-CoV-2 spike (S),

173   nucleocapsid (N), and membrane-and-envelope (M&E) antigens in the five anti-CD8 antibody-

174   untreated macaques inoculated with 10^6 or 10^5 $TCID_{50}$ of SARS-CoV-2. In the analysis using

175   peripheral blood mononuclear cells (PBMCs), SARS-CoV-2-specific $CD8^+$ T-cell responses

176   were undetectable in macaque N013 but detected in the remaining four macaques (Fig 5A and

177   5B). Macaque N022 exhibited $CD8^+$ T-cell responses on day 7 while the remaining three

178   macaques (N011, N012, and N021) showed initial SARS-CoV-2 specific responses on day 14.

179   Analysis using submandibular lymph nodes (SMLN) obtained at necropsy found SARS-CoV-

180   2-specific $CD8^+$ T-cell responses in macaques N011, N013, and N021 (Fig 5C). Interestingly,

181   SARS-CoV-2-specific $CD8^+$ T-cell responses were undetectable in PBMCs but detected in

182   SMLN in macaque N013. Thus, SARS-CoV-2-specific $CD8^+$ T-cell responses were detected in

183   all the five anti-CD8 antibody-untreated Group N macaques inoculated with 10^6 or 10^5 $TCID_{50}$

184   of SARS-CoV-2.

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Discussion

Host T-cell and B-cell responses have been reported to contribute to the control of SARS-CoV-2 replication [8,12,13,28]. In a murine model of infection with a mouse-adapted strain of SARS-CoV, depletion of CD4+ T cells resulted in reduced neutralizing antibody responses and delayed virus clearance from the lung [34]. Furthermore, SARS-CoV replication was controlled in the absence of CD4+ T and B cells, implicating CD8+ T cells in viral control [35]. Recent studies in humans have shown that functional virus-specific CD8+ T-cell responses are induced in convalescent COVID-19 individuals [29,30]. These reports suggest contribution of CD8$^+$ T cells in the control of SARS-CoV-2 replication. However, it remains unclear whether SARS-CoV-2 replication can be controlled in the absence of CD8$^+$ T cells. In the present study, we investigated the impact of depletion of CD8$^+$ cells (including CD8$^+$ T cells) by anti-CD8 antibody administration on SARS-CoV-2 replication in the subacute phase after establishment of virus infection. We found no significant enhancement of viral replication or delay in viral clearance after CD8$^+$ cell depletion, indicating that subacute SARS-CoV-2 replication can be controlled in the absence of CD8$^+$ T cells.

Our findings do not deny the contribution of CD8$^+$ T cells in the control of SARS-CoV-2 replication or the possibility of viral protection by vaccine-induced CD8$^+$ T cells. What is indicated in the present study is that CD8$^+$ T-cell dysfunction is not directly linked to failure in viral control, possibly implying that there may be multiple arms of host immune mechanisms involved in containing SARS-CoV-2 replication.

An animal model for SARS-CoV-2 infection is necessary for analysis of pathogenesis and transmission and the evaluation of vaccines and anti-viral drugs. Non-human primate models are recognized as being the most clinically relevant because of their genetic and physiological similarities to humans. Recent studies have shown that rhesus and cynomolgus macaques can be infected with SARS-CoV-2 and exhibit clinical manifestations resembling

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211 human COVID-19 [32,36-38]. Both macaque species present mild to moderate forms of

212 COVID-19, which is observed in the majority of the human population. We thus used a model

213 of SARS-CoV-2 infection in cynomolgus macaques for analysis of the effect of CD8$^+$ cell

214 depletion on virus replication.

215 We attempted SARS-CoV-2 inoculation via the intranasal route only without

216 intratracheal inoculation, because it may more closely reflect viral transmissions in humans.

217 The geometric means of peak viral RNAs and sgRNAs in nasopharyngeal swabs (on day 2)

218 were 2.7×10^8 (range: 1.1×10^8 to 5.2×10^8) and 1.0×10^6 (range: 1.8×10^5 to 1.8×10^6)

219 copies/swab, respectively, which are equivalent to those in rhesus macaques inoculated both

220 intranasally and intratracheally with 10^5 TCID$_{50}$ of SARS-CoV-2 [32,33]. In macaques

221 inoculated with 10^5 TCID$_{50}$ (1.4×10^8 RNA copies) of SARS-CoV-2, viral RNA copies in

222 nasopharyngeal swabs on day 2 were comparable (N012) to or greater (N013, N022, D024, and

223 D025) than the total viral RNA copies in the inoculum, confirming viral replication in macaques

224 even with 10^5 TCID$_{50}$. The three macaques inoculated with 10^6 TCID$_{50}$ showed similar levels

225 of viral RNA in nasopharyngeal swabs on days 2 and 5 compared to the five macaques with 10^5

226 TCID$_{50}$. All the eight macaques intranasally inoculated with 10^6 or 10^5 TCID$_{50}$ of SARS-CoV-

227 2 developed efficient anti-SARS-CoV-2 NAb responses, and the five anti-CD8 antibody-

228 untreated macaques induced SARS-CoV-2-specific CD8$^+$ T-cell responses. Taken together, our

229 results show that intranasal inoculation of cynomolgus macaques with 10^6 or 10^5 TCID$_{50}$ of

230 SARS-CoV-2 results in viral replication in the pharyngeal mucosa. Containment of viral

231 replication in the pharyngeal mucosa would be important for the control of further viral

232 transmission as well as disease progression.

233 Our cynomolgus macaque model of intranasal but not intratracheal SARS-CoV-2

234 inoculation is considered to represent asymptomatic or mild COVID-19. However,

235 histopathological analysis of the lung detected pulmonary inflammation in one animal (N021)

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236   on day 14 post-infection (S2 Fig), suggesting the potential of intranasal SARS-CoV-2

237   inoculation to induce moderate pulmonary diseases. Other animals may also have developed

238   mild pulmonary inflammation detectable on day 14, which was resolved by day 21. Macaque

239   N013 showed a unique phenotype with undetectable viral RNAs in swabs after day 14 (Fig 1D)

240   but relatively higher levels of viral RNA in pharyngeal mucosa and submandibular lymph nodes

241   on day 21 post-infection (Table 3). Virus-specific $CD8^+$ T-cell responses were undetectable in

242   PBMCs but efficiently detected in the submandibular lymph nodes on day 21 (Fig 5),

243   suggesting localized virus replication in the pharyngeal mucosa.

244   The sample size used in this study is relatively limited (three anti-CD8 antibody treated

245   animals and five untreated controls). However, these three animals exhibited similar levels of

246   pharyngeal viral loads before the anti-CD8 antibody treatment, and again showed similar levels

247   of viral loads after $CD8^+$ cell depletion. Neither enhancement of viral replication nor delay in

248   viral control was observed. Therefore, this study provides sufficient evidence for our conclusion.

249   In summary, the present study showed that subacute SARS-CoV-2 replication can be

250   controlled even in the absence of $CD8^+$ T cells. In SARS-CoV-2 infection, $CD8^+$ T-cell

251   responses may contribute to viral control, but our results suggest that $CD8^+$ T-cell dysfunction

252   does not solely lead to viral control failure or disease progression.

253

Materials and Methods

Ethics statement

Animal experiments were performed in the National Institute of Infectious Diseases (NIID) after approval by the Committee on the Ethics of Animal Experiments in NIID (permission number: 520001) under the guidelines for animal experiments in accordance with the Guidelines for Proper Conduct of Animal Experiments established by the Science Council of Japan (http://www.scj.go.jp/ja/info/kohyo/pdf/kohyo-20-k16-2e.pdf). The experiments were in accordance with the "Weatherall report for the use of non-human primates in research" recommendations (https://royalsociety.org/topics-policy/publications/2006/weatherall-report/). Each macaque was housed in a separate cage and received standard primate feed and fresh fruit daily. Virus inoculation, blood collection, nasopharyngeal and throat swab collection, and anti-CD8 antibody treatment were performed under ketamine anesthesia. Macaques were euthanized by whole blood collection under deep anesthesia on day 14 or 21 post-infection.

Animal experiments

SARS-CoV-2 wk-521 strain [39] (2019-nCoV/Japan/TY/WK-521/2020, GenBank Accession LC522975) was expanded in Vero E6/TMPRSS2 cells [39] and harvested to prepare a virus inoculum stock. Virus infectivity was assessed by detection of cytopathic effect (CPE) on Vero E6/TMPRSS2 cells and determination of endpoint titers. Nine cynomolgus macaques (*Macaca fascicularis*, 3-6 years old) were intranasally inoculated with the same stock of SARS-CoV-2 wk-521 at a dose of 10^6 (exactly 7.5×10^5) $TCID_{50}$ (1.4×10^9 RNA copies) (n = 3), 10^5 (exactly 7.5×10^4) $TCID_{50}$ (n = 5), or 10^4 (exactly 7.5×10^3) $TCID_{50}$ (n = 1) (Table 1). Three (Group D) of the nine macaques were intravenously administrated with 5 mg/kg body weight of anti-CD8α antibody clone MT807 (NIH Nonhuman Primate Reagent Resource) on days 5 and 7 post-infection. Body temperature was measured with a small implantable thermo logger

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279   (DST micro-T; Star-Oddi) that was set intraperitoneally under ketamine anesthesia at least five

280   days prior to virus inoculation. Macaques were euthanized and subjected to necropsy on day 14

281   or 21 post-infection (Table 1).

282

283   **Detection of SARS-CoV-2 RNAs**

284   Swab RNA was extracted from 0.2 ml of swab solutions (1ml of DMEM with 2% fetal

285   bovine serum [Cytiva]) using QIAamp Viral RNA Minikit (QIAGEN) and subjected to real-

286   time RT-PCR for viral RNA quantitation [40] using QuantiTect Probe RT-PCR Kit (Qiagen)

287   and QuantStudio 5 (Thermo Fisher Scientific). Swab RNAs were also subjected to real-time

288   RT-PCR for measurement of viral subgenomic RNA (sgRNA) levels [32,33,41] using the

289   following primers: SARS2-LeaderF60 (5'-CGATCTCTTGTAGATCTGTTCTCT-3'), SARS2-

290   N28354R (5'-TCTGAGGGTCCACCAAACGT-3'), and SARS2-N28313Fam (FAM-

291   TCAGCGAAATGCACCCCGCA-TAMRA). Tissue RNAs were extracted from homogenized

292   tissues by using TRIzol™ Plus RNA Purification Kit (Thermo Fisher Scientific) with phenol-

293   chloroform extraction and subjected to real-time RT-PCR for detection of viral RNAs.

294

295   **Virus recovery from swabs**

296   Vero E6/TMPRSS2 cells in 96-well plates were added with 10-fold serially diluted swab

297   solutions and cultured for 4 days without medium change. Virus recovery was assessed by

298   detection of CPE and determination of endpoint titers. Swab samples with virus titers greater

299   than 1×10^2 $TCID_{50}$/swab were considered positive.

300

301   **Analysis of anti-SARS-CoV-2 NAb responses**

302   Plasma samples were heat inactivated for 30 min at 56 °C. Serial two-fold dilutions of

303   heat-inactivated plasma were tested in quadruplicate. In each mixture for quadruplicate testing,

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304   40 μl of diluted plasma were incubated with 40 μl of 80 TCID$_{50}$ SARS-CoV-2 wk-521. After

305   incubation for 45 min at room temperature, 20 μl of the mixture was added to each of four wells

306   (1 x 10^4 Vero cells/well) in a 96-well plate. Three days later, virus infectivity was assessed by

307   detection of CPE to determine the endpoint titers. The lower limit of detection was 1:10.

308

309   **Analysis of cell surface markers**

310   Whole blood samples were treated with Lysing Solution (BD) and subjected to surface

311   staining using anti-CD3 APC-Cy7 (SP34-2; BD), anti-CD4 FITC (M-T477; BD), anti-CD8

312   PerCP (SK1; BD), and anti-CD20 PE (2H7; BD) antibodies. Alternatively, whole blood

313   samples from anti-CD8 antibody-treated animals were stained with anti-CD3 APC-Cy7, anti-

314   CD4 PerCP (L200; BD), anti-CD8 FITC (DK25; FUJIFILM), and anti-CD20 PE. Stained cells

315   were analyzed by BD FACS Canto II.

316

317   **Analysis of SARS-CoV-2 antigen-specific CD8$^+$ T-cell responses**

318   Virus-specific CD8$^+$ T-cell frequencies were measured by flow cytometric analysis of

319   gamma interferon (IFN-γ) induction after specific stimulation as described previously [42].

320   PBMCs were prepared from whole blood by density gradient centrifugation using Ficoll-Paque

321   PLUS (Cytiva). Lymph node-derived lymphocytes were prepared from minced lymph nodes by

322   density gradient centrifugation using Ficoll-Paque PLUS. Cells were pulsed and cocultured

323   with peptide pools (at a final concentration of more than 0.1 μM for each peptide) using panels

324   of overlapping peptides spanning the SARS-CoV-2 S, N, M, and E amino acid sequences (PM-

325   WCPV-S-1, PM-WCPV-NCAP-1, PM-WCPV-VME-1, and PM-WCPV-VEMP-1; JPT Peptide

326   Technologies) in the presence of GolgiStop (monensin, BD), 1 μg/ml of anti-CD28 (CD28.2,

327   BD) and 1 μg/ml anti-CD49d (9F10, BD) for 6 hours. Intracellular IFN-γ staining was

328   performed with a CytofixCytoperm kit (BD) and anti-CD3 APC-Cy7, anti-CD4 FITC, anti-

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329   CD8 PerCP, and anti-IFN-γ PE (4S.B3; BioLegend). Stained cells were analyzed by BD FACS

330   Lyric. A representative gating schema for flow cytometric analysis is shown in Fig. 5A. Specific

331   T-cell frequencies were calculated by subtracting nonspecific IFN-γ^{+} T-cell frequencies from

332   those after peptide-specific stimulation. Specific T-cell frequencies less than 0.03% of CD8^{+} T

333   cells were considered negative.

334

335   **Statistical analysis**

336   Statistical analyses were performed using Prism software (GraphPad Software, Inc.) with

337   significance set at p values of < 0.05. Comparisons were performed by Mann-Whitney U test.

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340   **Acknowledgments**

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473

Supporting Information

S1 Fig. Changes in body temperatures pre- and post-infection in macaques.

S2 Fig. Histopathology of the lung in macaque N021.

Representative histopathology with hematoxylin and eosin staining (H&E) of the lung obtained from macaque N021 at autopsy on day 14 post-infection, indicating mild or moderate pulmonary inflammation. Infiltration of mononuclear cells were observed around blood vessels and bronchiole (upper left panel). Lymphocytes, eosinophils, and macrophages were observed in pulmonary alveoli (upper right and lower panels).

484 **Figure Captions**

485 **Fig 1. Viral RNA levels in swabs.**

486 (A-D) Changes in viral RNA levels in nasopharyngeal (A, C, D) and throat (B) swabs after

487 SARS-CoV-2 infection in all animals (A, B) or those infected with 10^6 (C) or 10^5 (D) $TCID_{50}$

488 of SARS-CoV-2. The lower limit of detection was approximately 3 x 10^3 copies/swab. (E)

489 Comparison of viral RNA levels in nasopharyngeal swabs at day 5 post-infection between 10^6

490 $TCID_{50}$-infected and 10^5 $TCID_{50}$-infected macaques. No significant difference was observed.

491 (F) Comparison of viral RNA levels in nasopharyngeal swabs at days 5 (left), 7 (middle), and

492 9-12 (right) post-infection between Group N and D animals infected with 10^6 or 10^5 $TCID_{50}$ of

493 SARS-CoV-2. No significant difference was observed.

494

495 **Fig 2. Viral subgenomic RNA levels in swabs.**

496 Changes in viral sgRNA levels in nasopharyngeal (A, C, D) and throat (B) swabs after SARS-

497 CoV-2 infection in all animals (A, B) or those infected with 10^6 (C) or 10^5 (D) $TCID_{50}$ of SARS-

498 CoV-2. The lower limit of detection was approximately 3 x 10^3 copies/swab.

499

500 **Fig 3. Peripheral blood B- and T-cell frequencies.**

501 Changes in %$CD3^+$, %$CD3^+CD4^+$, %$CD3^+CD8^+$, and %$CD3^-CD20^+$ T cells in macaque

502 PBMCs after SARS-CoV-2 infection.

503

504 **Fig 4. SARS-CoV-2-specific neutralizing antibody responses.**

505 Changes in plasma anti-SARS-CoV-2 neutralizing antibody titers post-infection in all animals

506 (left) or those infected with 10^6 (middle) or 10^5 (right) $TCID_{50}$ of SARS-CoV-2.

507

508 **Fig 5. SARS-CoV-2-specific $CD8^+$ T-cell responses.**

24

509   (A) Representative gating schema for detection of IFN-γ induction after stimulation with

510   overlapping M&E peptide pools in macaque N012 on day 14 post-infection. (B) Frequencies

511   of CD8$^+$ T cells targeting S, N, and M&E in PBMCs on days 7, 14, and 21 post-infection in

512   Group N animals infected with 10^6 (middle) or 10^5 (right) TCID$_{50}$ of SARS-CoV-2. (C)

513   Frequencies of CD8$^+$ T cells targeting S, N, and M & E in submandibular lymph nodes obtained

514   at necropsy in macaques N011, N013, N021, and N022. Samples were unavailable for analysis

515   in macaque N012.

516

Table 1. Macaque experimental protocol

Group	Experiment[a]	Macaques	Gender	Age (yrs)	SARS-CoV-2 dose[b] ($TCID_{50}$)	anti-CD8 Ab Tx[c]	Necropsy[d]
N	1	N011	male	6	10^6	NT	d21
N	1	N012	male	6	10^5	NT	d21
N	1	N013	male	6	10^5	NT	d21
-[e]	1	N014	male	6	10^4	NT	d21
N	2	N021	female	3	10^6	NT	d14
N	2	N022	female	3	10^5	NT	d21
D	2	D023	male	6	10^6	d5 & d7	d21
D	2	D024	male	6	10^5	d5 & d7	d21
D	2	D025	female	3	10^5	d5 & d7	d21

[a]Two sets of experiments were performed using the same SARS-CoV-2 inoculum stock.

[b]Macaques were intranasally inoculated with the indicated doses (10^6 [exactly 7.5 x 10^5], 10^5 [exactly 7.5 x 10^4], or 10^4 [exactly 7.5 x 10^3] $TCID_{50}$) of SARS-CoV-2 on day 0.

[c]Macaques in Group D were treated intravenously with anti-CD8 antibody on days 5 and 7 post-infection. NT, not treated.

[d]Macaques were euthanized and necropsied on day 14 or 21 post-infection.

[e]N014 was excluded from comparisons between groups N and D.

Table 2. Virus recovery from pharyngeal swabs after SARS-CoV-2 infection

Macaques	Virus recovery from swabs[a]								
	d0	d2	d5	d7	d9/10	d12	d14	d17	d21
N011	-	+	+	+	-	-	-	-	-
	-	+	+	-	-	-	-	-	-
N012	-	+	+	+	-	+	-	-	-
	-	+	-	-	-	-	-	-	-
N013	-	+	-	-	-	-	-	-	-
	-	+	+	-	-	-	-	-	-
N014	-	-	-	-	-	-	-	-	-
	-	+	-	-	-	-	-	-	-
N021	-	+	-	-	-	-	-	-	-
	-	+	-	-	-	-	-	-	-
N022	-	+	+	-	-	-	-	-	-
	-	+	-	-	-	-	-	-	-
D023	-	+	-	+	-	-	-	-	-
	-	+	-	-	-	-	-	-	-
D024	-	+	-	-	-	-	-	-	-
	-	+	-	-	-	-	-	-	-
D025	-	+	+	+	-	-	-	-	-
	-	+	+	-	-	-	-	-	-

[a]Swab samples were added to Vero-E6 cell culture to recover infectious virus. + indicates successful virus recovery from nasopharyngeal (upper row) or throat (lower row) swabs for each animal.

531 **Table 3. Detection of viral RNA in tissues obtained at necropsy**

Macaques	Autopsy	Detection of viral RNA in tissues[a]				
		Pharyngeal mucosa	Retropharyngeal lymph node	Lung	Intestine	Spleen
N011	d21	-	+	-	-	-
N012	d21	-	-	-	-	-
N013	d21	+	+	-	-	+
N014	d21	-	-	-	-	-
N021	d14	-	-	-	-	-
N022	d21	-	-	-	-	+
D023	d21	-	-	-	-	-
D024	d21	-	-	-	-	-
D025	d21	-	-	-	-	+

532 [a]RNA was extracted from individual tissues and subjected to RT-PCR to detect SARS-CoV-2
533 RNA. + indicates detection of viral RNA.

28

Fig 1

A nasopharyngeal swab (all)

B throat swab (all)

C nasopharyngeal swab (10^6 $TCID_{50}$ in)

D nasopharyngeal swab (10^5 $TCID_{50}$ in)

E d5 pi

F d5 pi / d7 pi / d9-12 pi

N011, N012, N013, N014, N021, N022, D023, D024, D025

Fig 2

A nasopharyngeal swab (all)

B throat swab (all)

C nasopharyngeal swab (10^6 $TCID_{50}$ in)

D nasopharyngeal swab (10^5 $TCID_{50}$ in)

N011
N012
N013
N014
N021
N022
D023
D024
D025

Fig 3

days post-infection

Fig 4

Fig 5

A

B

Specific CD8⁺ T-cell frequencies (% of CD8⁺ T cells)

S   N   M&E

N011   N012   N013   N021   N022

C

Specific CD8⁺ T-cell frequencies (% of CD8⁺ T cells)

S   N   M&E

N011   N013   N021   N022