

1 **Mouse Adapted SARS-CoV-2 protects animals from lethal SARS-CoV challenge**

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3 Antonio Muruato^{1,2}, Michelle N. Vu², Bryan A. Johnson², Meredith E. Davis-Gardner⁶, Abigail
4 Vanderheiden⁶, Kumari Lokugmage², Craig Schindewolf², Patricia A. Crocquet-Valdes³, Rose
5 M. Langsjoen¹, Jessica A. Plante^{2,5}, Kenneth S. Plante^{2,5}, Scott C. Weaver^{2,4,5}, Kari Debbink⁸,
6 Andrew L. Routh^{1,4}, David Walker³, Mehul S. Suthar^{6,7}, Xuping Xie¹, Pei-Yong Shi^{1,4#}, Xuping
7 Xie^{1#}, Vineet D. Menachery^{2,4,5#}.

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10 ¹Departments of Biochemistry and Molecular Biology, ²Microbiology and Immunology,
11 ³Pathology, ⁴Institute for Human Infection and Immunity, ⁵World Reference Center of Emerging
12 Viruses and Arboviruses, University of Texas Medical Branch, Galveston, TX, USA
13 ⁶Department of Pediatrics, Emory Vaccine Center, Emory University School of Medicine,
14 Atlanta, GA, USA.
15 ⁷Yerkes National Primate Research Center, Atlanta, GA, USA
16 ⁸Department of Natural Science, Bowie State University, Bowie, MD, USA

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18 #Co-senior authors

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Corresponding Author: Vineet D. Menachery

Address: University of Texas Medical Branch, 301 University Blvd, Route #0610 Galveston, TX
77555

Email: Vimenach@utmb.edu

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26

27 **Abstract**

28 The emergence of SARS-CoV-2 has resulted in a worldwide pandemic causing
29 significant damage to public health and the economy. Efforts to understand the
30 mechanisms of COVID-19 disease have been hampered by the lack of robust mouse
31 models. To overcome this barrier, we utilized a reverse genetic system to generate a
32 mouse-adapted strain of SARS-CoV-2. Incorporating key mutations found in SARSCoV-2
33 variants, this model recapitulates critical elements of human infection including
34 viral replication in the lung, immune cell infiltration, and significant *in vivo* disease.
35 Importantly, mouse-adaptation of SARS-CoV-2 does not impair replication in human
36 airway cells and maintains antigenicity similar to human SARS-CoV-2 strains. Utilizing this
37 model, we demonstrate that SARS-CoV-2 infected mice are protected from lethal challenge with
38 the original SARS-CoV, suggesting immunity from heterologous CoV strains. Together, the
39 results highlight the utility of this mouse model for further study of SARS-CoV-2 infection
40 and disease.

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42 Introduction

43 Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the virus that causes COVID-
44 19 disease, emerged in late 2019 and has since caused an ongoing outbreak with over 153
45 million cases and over 3.2 million deaths in the last 17 months^{1,2}. The novel coronavirus, similar
46 to previous emergent SARS-CoV and Middle East Respiratory Syndrome (MERS)-CoV, can
47 produce severe respiratory disease characterized by fever, labored breathing, pulmonary
48 infiltration and inflammation^{3,4}. In severe cases, SARS-CoV-2 can lead to acute respiratory
49 distress and death. Unlike the earlier pandemic CoVs, SARS-CoV-2 maintains the ability to
50 spread asymptotically and causes a range of disease from mild to severe⁵. These factors
51 have led to a world-wide outbreak that continues to rage over a year after its emergence.

52 In responding to the outbreak, understanding the complexity of SARS-CoV-2 infection
53 has been hampered by the limitations of small animal models⁶. Early on, wild-type SARS-CoV-
54 2 was shown to be unable to utilize mouse ACE2 for entry and infection⁷. Alternative models
55 utilized receptor transgenic mice expressing human ACE2 or Syrian golden hamsters to
56 evaluate SARS-CoV-2 infection and disease *in vivo*⁶. However, the transgenic models, while
57 causing severe disease and lethality, have distinct infection tropism leading to encephalitis in
58 addition to lung disease⁸⁻¹⁰. Similarly, while the hamster model has provided utility in studying
59 disease and transmission¹¹, the absence of genetic knockout and immunological tools limits the
60 types of studies that can be pursued. Without a robust mouse model, many of the resources
61 used to study infection and the immune response are unavailable for SARS-CoV-2 experiments.

62 In order to alleviate these issues, we set out to develop a mouse-adapted strain of
63 SARS-CoV-2 using standard laboratory strains. Building from our infectious clone system¹², we
64 incorporated amino acid changes that facilitated replication in standard Balb/C mice and serially
65 passaged the mutant to create a mouse-adapted strain (CMA3p20) that causes significant
66 weight loss, disease, and lung damage following infection. Notably, virus replication in this
67 model is limited to the respiratory system, thus recapitulating disease observed in most humans.

68 Importantly, the SARS-CoV-2 CMA3p20 strain did not attenuate replication in primary human
69 airway cultures or change the antigenicity of the mouse-adapted strain relative to WT control
70 virus, making it suitable for vaccine and therapeutic studies. Finally, following prior infection with
71 SARS-CoV-2 CMA3p20, mice were protected from lethal challenge with SARS-CoV despite the
72 absence of sterilizing immunity. Together, the results highlight the utility of SARS-CoV-2
73 CMA3p20 to study infection and pathogenesis in standard mouse lines.

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75

76 **Results**

77 The initial, emergent strains of SARS-CoV-2 had spike proteins unable to utilize mouse ACE2
78 and infect standard laboratory mice⁷. To overcome this barrier, we generated a series of
79 mutations in the receptor-binding domain (RBD) of SARS-CoV-2 using our infectious clone¹².
80 Our initial efforts modeled the interaction between SARS-CoV-2 and mouse ACE2 and used
81 previous mouse adapted strains of SARS-CoV (MA15, MA20, and v2163)¹³ to design mutants
82 including changes at Y449H (MA1), Y449H/L455F (MA2), and F486L/Q498Y (MA4) (**S. Fig. 1A-**
83 **C**). We also generated a series of mutants based on a reported natural SARS-CoV-2 isolate
84 (MASCP6) capable of infecting mice¹⁴, which has spike change at N501Y and several additional
85 mutations (**S. Fig. 2A**). Given the capacity of the MASCP6 strain to replicate in mice, we
86 generated mutants that had the spike mutation alone (CMA1), the spike/N protein mutation
87 (CMA2), and all four changes (CMA3) (**S. Fig. 2A**). For each of the six mutants, we utilized site-
88 directed mutagenesis in the WA1 strain clone and rescued virus stocks on Vero E6 cells (**S. Fig.**
89 **2B**). We subsequently infected 10-week-old female Balb/C mice with 10⁵ plaque forming units
90 (PFU) of each mutant virus and evaluated replication in the lung 2 days post infection. For WT,
91 MA1, and MA2, no evidence of viable infection was detected in mouse lung tissues (**S. Fig. 2C**);
92 however, MA4 and CMA1-3 had robust replication in mouse lung suggesting multiple
93 combinations of RBD changes could provide compatibly with mouse ACE2 sufficient for
94 replication in a standard laboratory mouse strain.

95 To further evaluate the mouse adapted strains, we focused on SARS-CoV-2 CMA1,
96 CMA2, and CMA3 mutants over a four-day time course. In female ten-week-old Balb/c mice
97 infected with 10⁵ PFU, none of three mutants induced major disease (**S. Fig. 3A**), although both
98 CMA2 and CMA3 caused more weight loss than CMA1. Examining viral replication in the lung,
99 all three mutants produced ~10⁵ PFU per lobe at day 2 post infection (**S. Fig. 3B**). However, no
100 virus was detected at day 4, suggesting rapid clearance by the host. To determine if type I
101 interferon was the major factor blunting infection, IFNAR^{-/-} SJV129 mice were infected with

102 CMA1, CMA2, and CMA3 at 10^5 PFU. Following infection, all three CMA mutant strains caused
103 significant disease with both CMA2 and CMA3 peaking at ~10% weight loss (**S. Fig. 3C**).
104 However, despite increased disease, viral titers were only slightly higher at day 2 than immune
105 competent Balb/c mice and still cleared by day 4 for all three strains (**S. Fig. 3D**). Together, the
106 results indicate that SARS-CoV-2 CMA1, CMA2, and CMA3 can replicate in both Balb/C and
107 IFNAR^{-/-} mice, but fail to sustain continued replication *in vivo*.

108 **Serial passage of SARS-CoV-2 CMA3**

109 In order to generate a SARS-CoV-2 strain that produced significant disease in an
110 immune competent mouse, we serially passaged SARS-CoV-2 CMA3 in 10-week-old Balb/c
111 mice. A single mouse was infected with 10^5 PFU of CMA3 (p0); the mouse was subsequently
112 euthanized at 1 day post infection with half the lung lobes taken for viral RNA and the other
113 lobes homogenized, clarified, and used to inoculate subsequent passages (**Fig. 1A**); lung
114 samples were titered by plaque assay to verify continued SARS-CoV-2 replication (**Fig. 1B**).
115 After passages (p) 10, 15, and 20, stock viruses were generated on Vero E6 cells, used to infect
116 10-week-old Balb/C mice, and compared to the disease caused by the original CMA3 p0 strain
117 (**Fig. 1C**). Following 10^5 PFU challenge, mice infected with p10 and p15 were found to have
118 augmented weight loss compared to p0; however, mice infected with p20 showed 10% weight
119 loss by day 3 and signs of disease including ruffled fur and hunched posture. We subsequently
120 deep sequenced the passaged virus from the lung RNA and identified two additional spike
121 mutations (K417N and H655Y) and a mutation in the E protein (E8V). Several other mutations
122 were also found as minority variants in the spike and in other parts of the genome. Modeling
123 the receptor binding domain interaction (**Fig. 1D**), K417N and N501Y likely improve binding to
124 mouse ACE2 and facilitate increased *in vivo* disease. Together, mouse adaptation of SARS-
125 CoV-2 CMA3 incorporated three additional fixed mutations that drive increased disease in mice.

126 **Characterization of CMA3p20.**

127 Having observed significant disease in mice infected with CMA3p20 relative to the initial strain
128 of CMA3, we next evaluated weight loss, viral replication, and histopathology in Balb/c mice.
129 First, we tested CMA3p20 for a dose-dependent impact on weight loss (**S. Fig. 4A**); both 10^6
130 and 10^5 PFU caused significant, dose-dependent weight loss with minimal disease observed in
131 the 10^4 challenge. We also compared CMA3p20 infection associated weight loss to a B.1.1.7
132 SARS-CoV-2 variant (UK) which contains the N501Y mutation that permits virus replication in
133 mice (**S. Fig. 3A-D**). After challenge with the 10^6 PFU of the B.1.1.7 variant, female Balb/C
134 mice lost approximately 10% of their starting weight by day 2 and recovered (**S. Fig. 4B**).
135 Together, the results indicate more severe disease with the mouse adapted CMA3p20 than the
136 B.1.1.7 variant.

137 We subsequently used the 10^5 PFU dose of CMA3p20 to examine infection compared
138 to SARS-CoV-2 CMA3 over a seven-day time course. Following infection, 10-week-old female
139 Balb/C mice CMA3p20-infected mice lost significant weight over the first four days, peaking at
140 day 3 with >10% weight loss (**Fig. 2A**). In contrast, the original CMA3 caused minimal weight
141 loss over the course of the seven-day infection. We next examined viral replication in the lung at
142 days 2, 4, and 7 post infection (**Fig. 2B**). CMA3p20 infection had a significant 0.5 log increase
143 in viral load over CMA3 in the lung at day 2; this difference was diminished at day 4 (0.25 log
144 increase, not statically significant) and both virus strains were cleared by day 7 in the lung. We
145 also observed day 2 replication in the trachea of mice that was cleared by day 4 in both CMA3
146 and CMA3p20 infection (**Fig. 2C**). Together, the data demonstrate robust weight loss and clear
147 replication in the mouse respiratory tract.

148 We next evaluated SARS-CoV-2 replication in non-respiratory tissues. Following
149 infection, we noted replication in the heart tissue of a subset of animals at day 2 (**Fig. 2D**).
150 However, infection was transient and not uniform in all animals and no virus was detected in the
151 heart at later time points. We subsequently evaluated viral load in the brain and blood and
152 found no evidence for CMA3 or CMA3p20 infection by plaque assay (**Fig. 2E-F**). To further

153 verify viral replication, we also examined viral RNA expression in the lung, heart, brain, spleen,
154 and liver (**S. Fig. 4C**). While robust viral RNA was observed in the lung, the other tissues had
155 minimal evidence for CMA3p20 replication. Together, the data indicate that the SARS-CoV-2
156 CMA3p20 strain is primarily restricted to and disease driven by virus replication in the
157 respiratory tract.

158 **CMA3p20 induces significant immune infiltration and lung damage.**

159 Further examining lung tissue, histopathology analysis of CMA3p20 infection indicated robust
160 virus replication, immune infiltration, and tissue damage. Utilizing antigen staining against the N
161 protein, we saw evidence for viral replication primarily in the bronchioles with additional staining
162 in the lung parenchyma at day 2 post infection (**Fig. 2G**). We also observed lung infiltration and
163 inflammation following CMA3p20 challenge characterized by peribronchiolitis, perivascular
164 cuffing, and perivascularitis by day 2 post infection (**Fig. 2H**). Similarly, at day 4, we noted
165 collapsed airways and interstitial pneumonia (**Fig. 2I**). Some portions of the day 4 lungs
166 infected with CMA3p20 also had virus induced damage including enlarged and multinucleated
167 alveolar type II cells (**S. Fig. 5A**), loss of cellular polarity (**S. Fig. 5B**), and immune cells in the
168 bronchiolar lumen (**S. Fig. 5D**). Together, the histopathology results demonstrated significant
169 damage, inflammation, and disease in the lung following infection with SARS-CoV-2 CMA3p20.

170 **CMA3p20 retains replication capacity in primary human respiratory cells.**

171 Altering SARS-CoV-2 to be permissive in mice can impact its replication capacity in human cells
172 ¹⁵. Therefore, we examined the ability of CMA3p20 to replicate in primary human airway
173 epithelial (HAE) cultures compared with the WT SARS-CoV-2 WA1 strain. Grown on an air
174 liquid interface, primary HAEs represent a useful *in vitro* model of the human airway ¹⁶.
175 Following infection, CMA3p20 had equal replication to WT SARS-CoV-2 over a 72- hour time
176 course in primary HAE cultures (**Fig. 3A**). Similarly, viral RNA levels at 72 hours post infection
177 were equivalent between CMA3p20 and SARS-CoV-2 WA1 strain (**Fig. 3B**). Together, the

178 results indicate that mouse adaption resulted in no significant replication attenuation of
179 CMA3p20 in primary human airway cells.

180 **CMA3p20 retains antigenicity similar to WT SARS-CoV-2.**

181 In addition to differences in replication in human cells, spike changes in SARS-CoV-2 could alter
182 the overall antigenicity of CMA3p20 as compared to SARS-CoV-2 derived from humans; this
183 result would make it more difficult to interpret vaccine and protection studies derived from mice.
184 Therefore, to evaluate antigenicity, we infected 10-week-old female Balb/C mice with 10^6 PFU of
185 CMA3p20, then, euthanized and harvested sera 28 days post infection. We subsequently used
186 the mouse sera to measure plaque reduction neutralization titer (PRNT₅₀) against the wild-type
187 SARS-CoV-2 WA1 strain (**Fig. 3C**). Mouse sera from mice (n=7) infected with CMA3p20
188 neutralized WT SARS-CoV-2 with a PRNT₅₀ value of $\sim 1/600$. To further evaluate CMA3p20
189 antigenicity, we examined PRNT₅₀ assays utilizing sera from acutely infected COVID-19
190 patients (**Fig. 3D**). Performing neutralization assays in parallel, we found that CMA3p20 had
191 PRNT₅₀ values similar to WT SARS-CoV-2 with each COVID19 patient serum tested. With a R²
192 value of 0.8651 over the 13 samples, the results indicated that CMA3p20 retains similar
193 antigenicity to the WT SARS-CoV-2 and has utility for vaccine and protection studies.

194 To further demonstrate the utility of CMA3p20 to understand *in vivo* protection, we
195 performed a passive transfer experiment with COVID-19 patient sera. One day prior to
196 infection, 10-week-old female BALB/C mice were pre-treated intraperitoneally with either control
197 (PBS) or 100ul of convalescent serum from a COVID-19 patient. Mice were subsequently
198 challenged with 10^5 PFU of CMA3p20 and monitored for weight loss and viral titer. Mice treated
199 with acutely infected COVID-19 patient serum had significantly reduced weight loss at day 3 and
200 4 post infection as compared to control mice (**Fig. 3E**). Similarly, viral titers in the lung were
201 reduced at both day 2 and day 4 in mice receiving COVID-19 patient serum as compared to
202 control. Consistent with the PRNT₅₀ data (**Fig. 3D**), the results from the passive transfer
203 experiment demonstrate that antibody-based immunity generated following human infection can

204 effectively neutralize SARS-CoV-2 CMA3p20. Together, the results confirm similar antigenicity
205 of CMA3p20 and WT SARS-CoV-2.

206 **Prior SARS-CoV-2 infection protects from lethal SARS-CoV challenge.**

207 Having established a SARS-CoV-2 mouse model with significant disease, we next evaluated the
208 capacity of CMA3p20 to protect against heterologous SARS-CoV challenge. Ten-week-old
209 female Balb/c mice were infected with 10^6 PFU of CMA3p20 or control (PBS), monitored for
210 weight loss, and allowed to recover. CMA3p20 infected and control mice were subsequently
211 challenged with a lethal dose of mouse-adapted SARS-CoV (10^4 PFU)¹⁷. Control mice infected
212 with SARS-CoV MA15 had rapid weight loss and lethality with all mice reaching euthanasia
213 criteria by day four post infection (**Fig. 4A & B**). In contrast, mice previously infected with
214 SARS-CoV-2 CMA3p20 had less weight loss compared to controls, only losing approximately
215 10% of their starting weight by day 2. The CMA3p20-infected mice recovered their starting
216 weight at late times demonstrating protection from lethal SARS-CoV infection. Examining
217 disease score, mice previously infect with SARS-CoV-2 CMA3p20 showed some disease at day
218 2, but were generally devoid of ruffled fur, diminished movement, or hunching (**Fig. 4C**). In
219 contrast, the mock infected animals challenged with SARS-CoV showed significant disease that
220 escalated over the course of infection and required euthanasia by day 4 for all animals
221 remaining in the study (n=10). For both CMA3p20 infected and uninfected animals, robust
222 SARS-CoV replication was observed in the lung (**Fig. 4D**). However, mice infected with
223 CMA3p20 has a significant reduction in viral loads as compared to control animals. Yet, the
224 viral replication indicates sterilizing immunity was not achieved. We subsequently evaluated the
225 neutralization capacity of SARS-CoV-2 CMA3p20 sera against SARS-CoV (**Fig. 4E**). CMA3p20
226 mouse sera from pre-challenge, day 2, day 4, and 7 post SARS-CoV infection were able to
227 neutralize SARS-CoV with a low range of PRNT₅₀ values (**Fig. 4F**). Both the pre-challenge and
228 day 2 post challenge sera had neutralization levels $<1/150$, while day 4 and 7 post-challenge
229 sera were only augmented to $\sim 1/200$. While significantly less neutralization than what is

230 observed against WT SARS-CoV-2 (~1/600), the results suggest that SARS-CoV-2 CMA3p20

231 infection induces protection sufficient to protect from lethal challenge with SARS-CoV.

232

233 Discussion

234 In this manuscript, we utilized a reverse genetic system¹⁸ and *in vivo* adaptation to generate a
235 mouse-adapted strain of SARS-CoV-2 (CMA3p20). CMA3p20 induces a dose-dependent
236 disease in young, female Balb/C mice with viral replication limited primarily to the respiratory
237 tract. In addition, CMA3p20 infection causes substantial damage to the mouse lung with
238 significant inflammation, immune infiltration, and pneumonia. Importantly, mutations in
239 CMA3p20 do not alter its ability to infect primary human cell airway cells, and the mouse
240 adapted strain maintains similar antigenicity to wild-type SARS-CoV-2. Utilizing this model, we
241 demonstrated infection with SARS-CoV-2 CMA3p20 provided protection from lethal challenge
242 with the mouse-adapted SARS-CoV. Together, results indicate the utility of CMA3p20 as a
243 model to study SARS-CoV-2 pathogenesis and immunity in standard inbred mice.

244 With the threat posed by SARS-CoV-2 and its emerging variants, questions have been
245 raised on the efficacy and duration of immunity following natural infection^{19,20}. Based on the
246 protection provided by prior SARS-CoV-2 challenge against the heterologous SARS-CoV, our
247 results suggest immunity is more complex than neutralizing serum titer alone. While sera from
248 mice challenged with SARS-CoV-2 CMA3p20 neutralizes SARS-CoV at ~1/150, this level of
249 antibody does not provide sterilizing immunity in the lung. Even after SARS-CoV infection, the
250 serum neutralization is only ~1/200 at day 7 post infection. Yet, in terms of weight loss and
251 disease, the SARS-CoV-2 infected mice showed a significant reduction in severity and complete
252 protection from SARS-CoV-induced lethality.

253 From these initial findings, the exact mechanism of protection is not yet clear. With
254 previous studies with SARS-CoV, antibody-based immunity has led to complete protection from
255 viral replication in the lung^{21,22}. Similar findings have been observed in animal models of
256 SARS-CoV-2, leading to serum neutralization as a primary correlate associated with protection
257²³. However, studies with SARS-CoV have also implicated a role for cellular based immunity²⁴.
258 Notably, initial findings have found non-neutralizing antibody responses against common cold

259 CoV had some level of protection ²⁵. Importantly, the vast majority of T-cell studies have found
260 epitopes directed against the more conserved nucleocapsid protein ²⁵. Absent in most vaccine
261 platforms, it is unclear if the immunity stimulated by natural SARS-CoV-2 infection will mimic
262 vaccine induced immunity and offer protection against heterologous SARS-CoV challenge.

263 In regard to the mouse adapted strain, CMA3p20, several of the key mutations in the
264 spike protein have been observed in novel SARS-CoV-2 variants of concern ²⁶. Starting with the
265 spike mutations, N501Y is represented in several COVID-19 variants of concern, found in 29%
266 of the GSAID database of reported sequences, and has been shown to improve binding to
267 human ACE2 ²⁷. In these studies, N501Y alone (CMA1) permits SARS-CoV-2 to replicate in
268 Balb/c mice. Similarly, spike mutation K417N, also found in several variants of concern, likely
269 augments receptor binding and drives *in vivo* disease ²⁸. In contrast to the other spike
270 mutations, H655Y is outside the RBD, has low penetrance in GSAID sequences (<0.5%), and
271 does not have a clear functional impact ²⁸. Outside the spike changes, the other mutations
272 found in CMA3p20 are not clear in their impact. Mutations in NSP8, NSP10 (<0.5%), and E
273 protein are rarely found in GSAID reported sequences, are not in conserved domains, and may
274 be hitchhiking mutations ²⁹. Notably, NSP6 (L37F) has been observed in 4% of human SARS-
275 CoV-2 isolates, suggesting possible selection ²⁹. Similarly, while N change at 128 is not
276 common ²⁸, differences in disease between CMA2 and CMA1 suggesting a role in
277 pathogenesis.

278 While the mouse-adapted mutations in CMA3p20 render a pathogenic virus in mice, it
279 does not ablate the replication capacity in human cells or antigenicity relative to the wild-type
280 SARS-CoV-2 strains. Previously described mouse adapted strains have been shown to cause
281 significant disease but lose replication capacity in primary human airway cultures ¹⁵. In these
282 studies, CMA3p20 has similar replication levels as SARS-CoV-2 WA1 in primary HAE cultures.
283 In addition, CMA3p20-infected mice generate antibody responses capable of neutralizing wild-
284 type SARS-CoV-2 WA1 and the mouse-adapted strain is similarly neutralized by COVID-19

285 sera. Importantly, passive transfer of human convalescent sera reduced disease in mice
286 challenged with SARS-CoV-2. Together, the results indicate that CMA3p20 induces a robust
287 immune response similar to that seen in humans and useful for understanding immunity in
288 standard animal models.
289

290 **Figure Legends**

291 **Figure 1. Mouse-adaptation of SARS-CoV-2.** a) Schematic of adaptation of SARS-CoV-2
292 CMA3p20. One ten-week-old female Balb/c mice was infected with SARS-CoV-2 CMA3 for 1
293 day, euthanized, and lung tissues harvested for viral RNA and viral titer determination. Lung
294 tissues were homogenized, clarified, and 50ul used to inoculate subsequent animals for 20
295 passages (p). b) Viral replication of CMA3 p1-p20 from lung homogenates isolated from infected
296 mice 1 day post infection. c) Stock virus generated at passages 0, 10, 15, and 20 was used to
297 infect 5 female Balb/c mice at 10^5 PFU and evaluated for weight loss over a 4-day time course.
298 d). Schematic of engineered (red stars) and passage-acquired (blue stars) mutations in CMA3p20
299 stock virus. Table includes Sanger equivalent accumulation of mutations over passages p5, p10,
300 p15, p20, and final stock used for subsequent studies. e) Modeling RBD spike mutations N501Y
301 and K417N found in CMA3p20 with mouse ACE2.

302 **Figure 2. SARS-CoV-2 CMA3p20 induces disease restricted to the lung.** a-f) Ten-week-old
303 Balb/c mice were infected with 10^5 PFU of SARS-CoV-2 CMA3 (black) or CMA3p20 (blue) and
304 followed for a) weight loss and viral titer in the b) lung, c) trachea, d) heart, e) brain, and f) blood.
305 g-i) Histology from CMA3p20 infected mice showed g) viral antigen (N-protein) staining in the
306 airways and parenchyma at day 2. Significant lung infiltration, inflammation and damage was
307 observed at h) day 2 and i) day 4 post infection.

308 **Figure 3. CMA3p20 strain maintains human replication capacity and antigenicity.** a-b)
309 Primary human airway cultures were infected with SARS-CoV-2 WT (black) or CMA3p20 (blue)
310 at an MOI of 0.01 and evaluated for a) viral titer and b) viral RNA. c) Sera collected from female
311 Balb/c mice 28 days post infection with 10^6 PFU of SARS-CoV-2 CMA3p20 were evaluated for
312 capacity to neutralize WT SARS-CoV-2 via PRNT50 assay. d) PRNT50 values from COVID19
313 patient sera plotted against WT virus (y-axis) versus CMA3p20 virus (x-axis). e-f) Ten-week-old
314 female Balb/c mice were treated intraperitoneally with 100ul of human COVID19 sera or control

315 (PBS) one day prior to infection. Mice were subsequently challenged with 10^5 PFU of SARS-
316 CoV-2 CMA3p20 and evaluated for e) weight loss and f) viral titer in the lung.

317 **Figure 4. Prior infection with SARS-CoV-2 protects from lethal SARS-CoV challenge.** a-c)

318 Ten-week-old female Balb/c mice were previously infected with 10^6 PFU of SARS-CoV-2
319 CMA3p20 (blue) or mock (black), monitored for weight loss, and allowed to recover. Twenty-eight
320 days post infection, both groups were challenged with a lethal dose (10^4 PFU) of mouse-adapted
321 SARS-CoV and evaluated for a) weight loss, b) lethality, and c) disease score. d) Mice were
322 subsequently euthanized at day 2, 4, and 7 and lung tissue examined for viral replication. e) Sera
323 from CMA3p20 infected and SARS-CoV challenged were evaluated for virus neutralization
324 (PRNT50) against SARS-CoV-2 (blue) or SARS-CoV over time (no rechallenge- black, day 2 red,
325 day 4-orange, day 7- green).

326 **S. Figure 1. Modeling changes to mouse-adapt SARS-CoV-2.** a) Key amino acid residues

327 found in the receptor binding domain (RBD) of mouse adapted strains of SARS-CoV were aligned
328 to SARS-CoV-2 and used to design mouse-adapted mutations¹³. Key interaction sites between
329 SARS-CoV spike and ACE2 molecules highlight in red³⁰. b-c) Modeling of key RBD residue
330 interactions with mouse ACE2 (PDB:2AJF) comparing b) WT SARS-Cov-2 residues versus c)
331 mutations (green) predicted to improve binding.

332 **S. Figure 2. Construction of mouse-adapted SARS-CoV-2 Mutants.** a) SARS-CoV-2 genome

333 schematic indicating location of amino acid mutations for MA1, MA2, MA4, CMA1, CMA2, and
334 CMA3. b) Viral replication of stock viruses of MA1, MA2, MA4, and CMA1-3 grown on VeroE6
335 cells. c) Viral replication of MA1, MA2, MA4, and CMA1-3 from lung homogenates isolated from
336 infected mice 2 days post infection.

337 **S. Figure 3. SARS-CoV-2 mutants CMA1, CMA2, and CMA3 replicate in laboratory mice.** a-

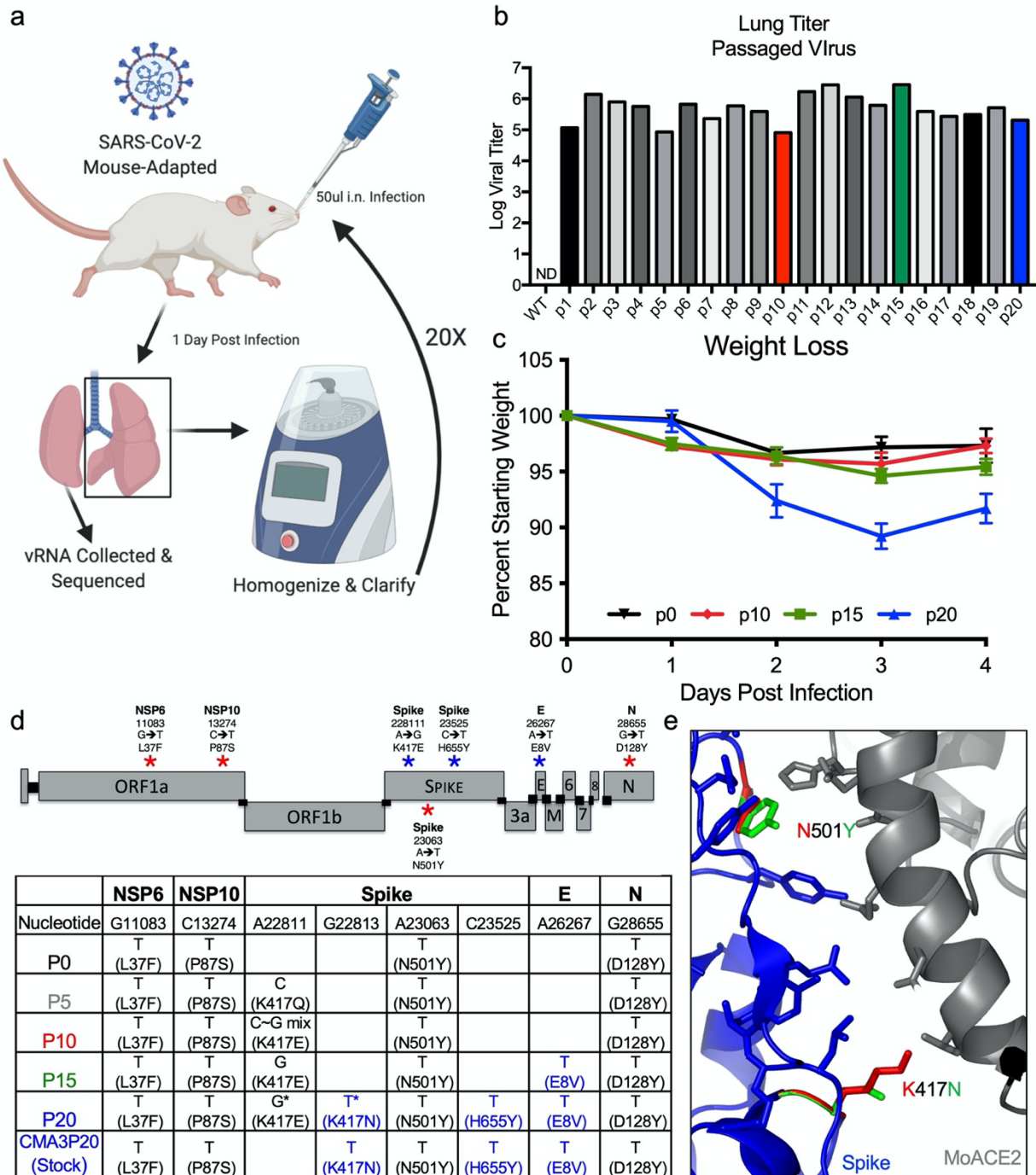
338 b) Ten-week-old female Balb/c mice infected with 10^5 PFU of CMA1 (red), CMA2 (green), or
339 CMA3 (black) were examined for a) weight loss and b) viral lung titer following infection at days 2
340 and 4. c-d) Ten- to twelve-week-old female IFNAR^{-/-} SVJ129 mice infected 10^5 PFU of CMA1

341 (red), CMA2 (green), or CMA3 (black) were examined for c) weight loss and d) viral lung titer
342 following infection at days 2 and 4. ND- non-detected.

343 **S. Figure 4. In vivo characterization of SARS-CoV-2 CMA3p20.** a) Examination of ten-week-
344 old female Balb/c mice infected with SARS-CoV-2 CMA3p20 at 10^4 , 10^5 , and 10^6 PFU (n=5). b)
345 Comparison of weight loss in ten-week old female Balb/c mice infected with 10^6 PFU of SARS-
346 CoV-2 CMA3p20 (blue) or SARS-CoV-2 variant B.1.1.7 (orange). c) RT-PCR of viral RNA load
347 found in lung, heart, brain, spleen, liver, and kidney following 10^5 PFU infection of SARS-CoV-2
348 CMA3p20 2- and 4-days post infection. Dotted line signifies viral RNA value derived from mock
349 infected samples.

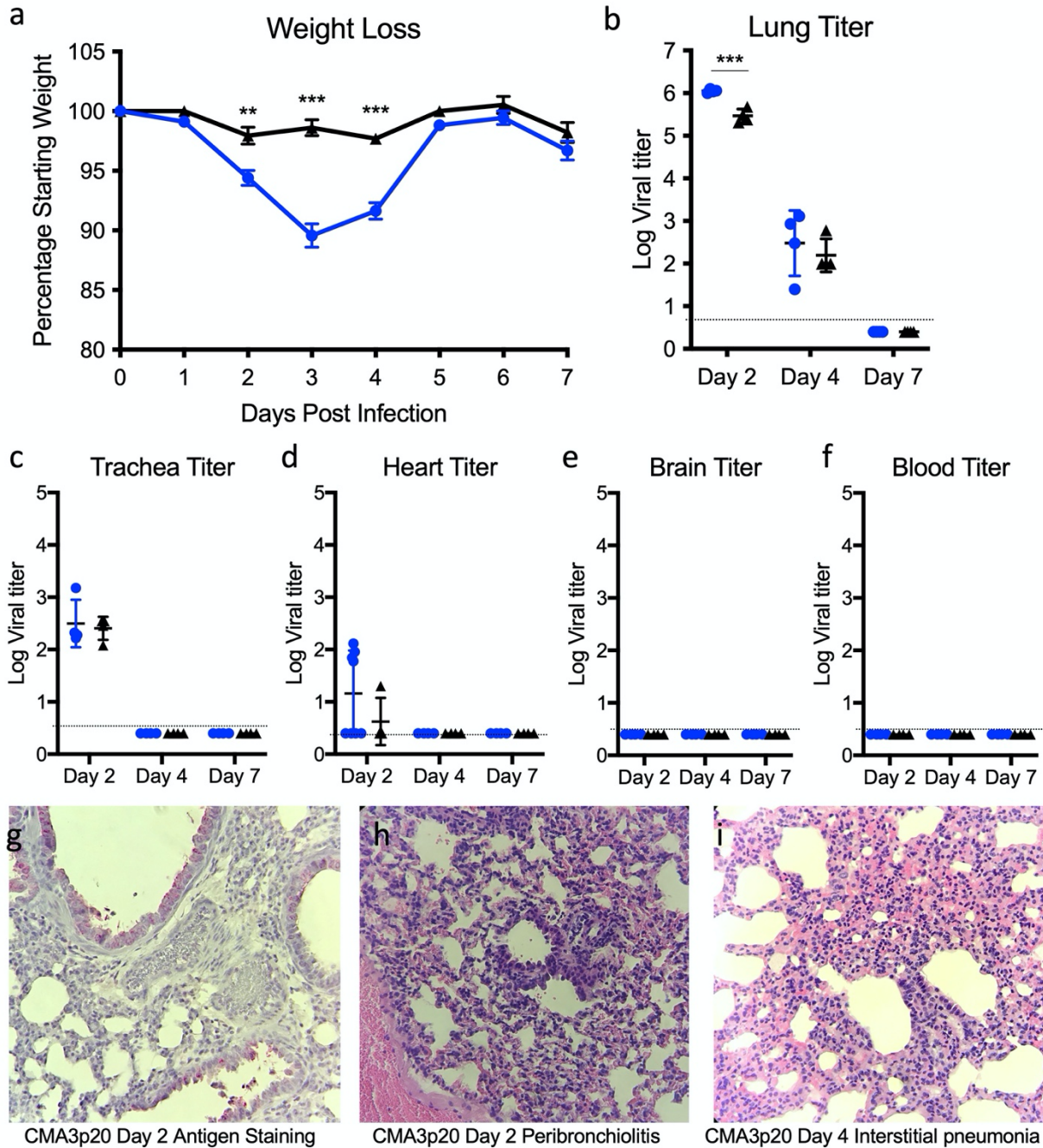
350 **S. Figure 5. SARS-CoV-2 CMA3p20 induces significant lung damage following infection.**
351 a-c) CMA3p20 infected animals 2 days post infection showing a) perivascular cuffing, b)
352 perivascularitis and c) peribronchiolitis. d-f) CMA3p20 induced lung inflammation and damage 4
353 days post infection including d) cytopathic effect of the virus, e) loss of cellular polarity, and f)
354 inflammatory cells in the lumen.

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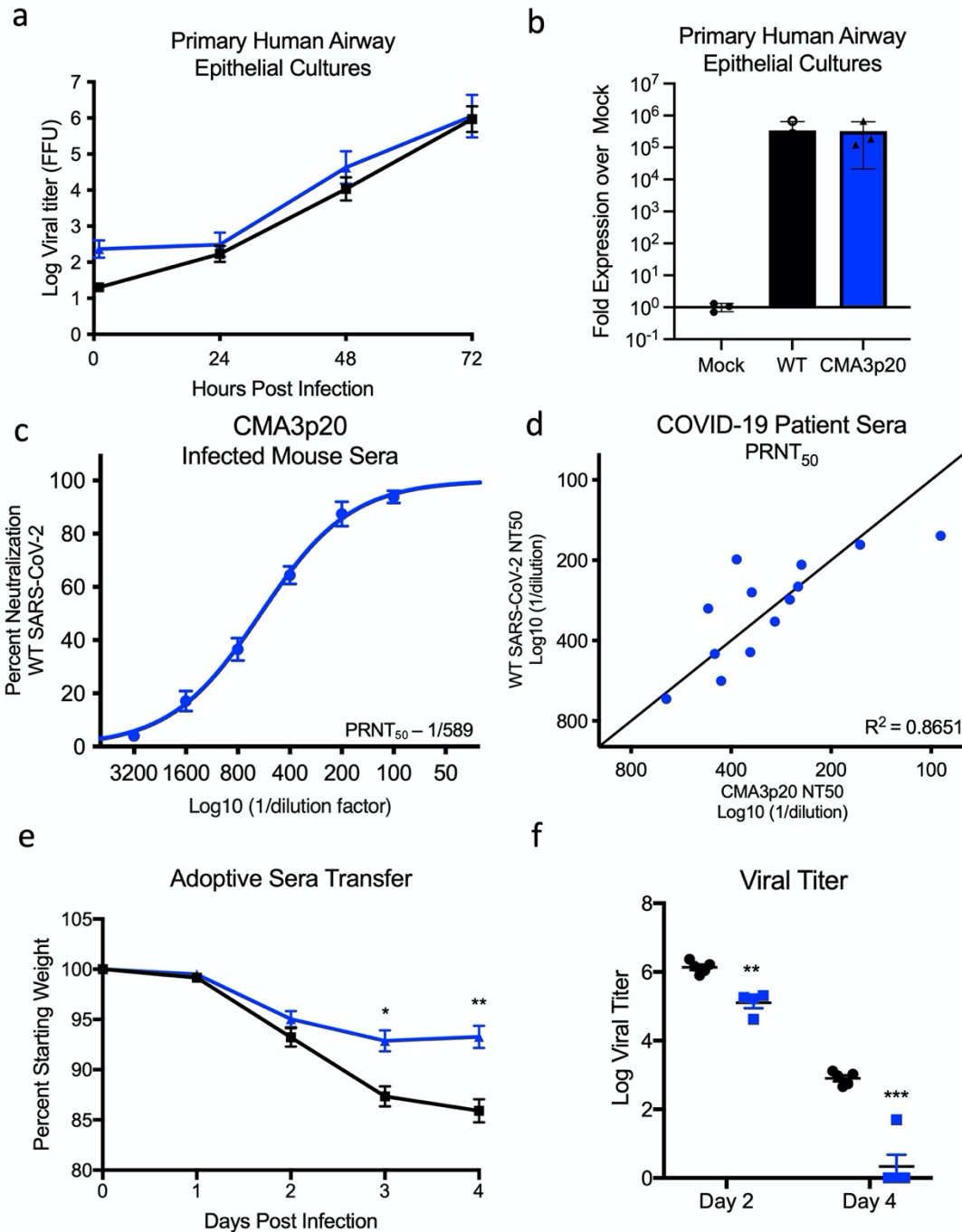
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Figure 1. Mouse-adaptation of SARS-CoV-2. a) Schematic of adaptation of SARS-CoV-2 CMA3p20. One ten-week-old female Balb/c mice was infected with SARS-CoV-2 CMA3 for 1 day, euthanized, and lung tissues harvested for viral RNA and viral titer determination. Lung tissues were homogenized, clarified, and 50ul used to inoculate subsequent animals for 20 passages (p). b) Viral replication of CMA3 p1-p20 from lung homogenates isolated from infected mice 1 day post infection. c) Stock virus generated at passages 0, 10, 15, and 20 was used to infect 5 female Balb/c mice at 105 PFU and evaluated for weight loss over a 4-day time course. d). Schematic of engineered (red stars) and passage-acquired (blue stars) mutations in CMA3p20 stock virus. Table includes Sanger equivalent accumulation of mutations over passages p5, p10, p15, p20, and final stock used for subsequent studies. e) Modeling RBD spike mutations N501Y and K417N found in CMA3p20 with mouse ACE2.



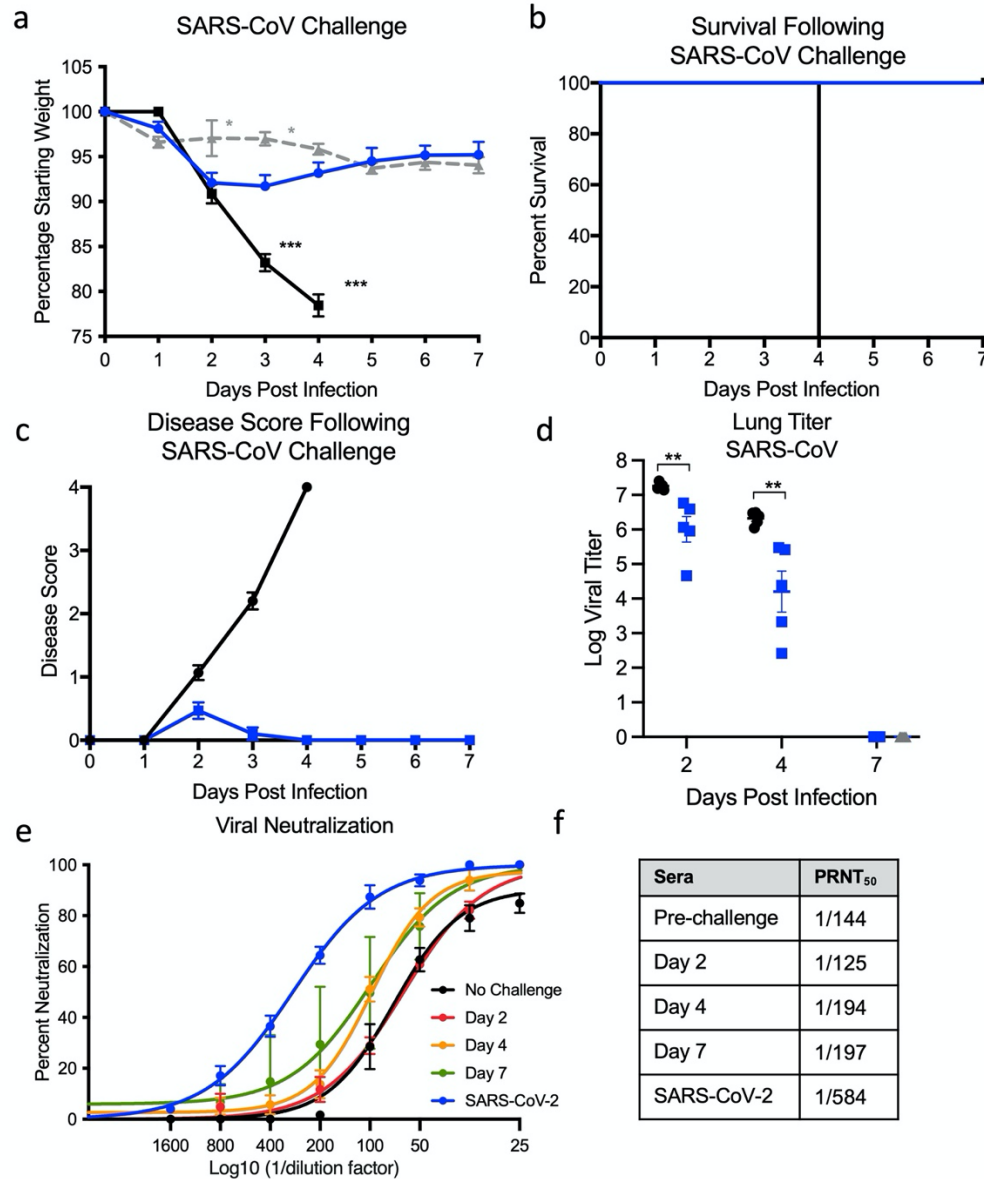
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Figure 2. SARS-CoV-2 CMA3p20 induces disease restricted to the lung. a-f) Ten-week-old Balb/c mice were infected with 105 PFU of SARS-CoV-2 CMA3 (black) or CMA3p20 (blue) and followed for a) weight loss and viral titer in the b) lung, c) trachea, d) heart, e) brain, and f) blood. g-i) Histology from CMA3p20 infected mice showed g) viral antigen (N-protein) staining in the airways and parenchyma at day 2. Significant lung infiltration, inflammation and damage was observed at h) day 2 and i) day 4 post infection. Magnification at 10x for g-i.



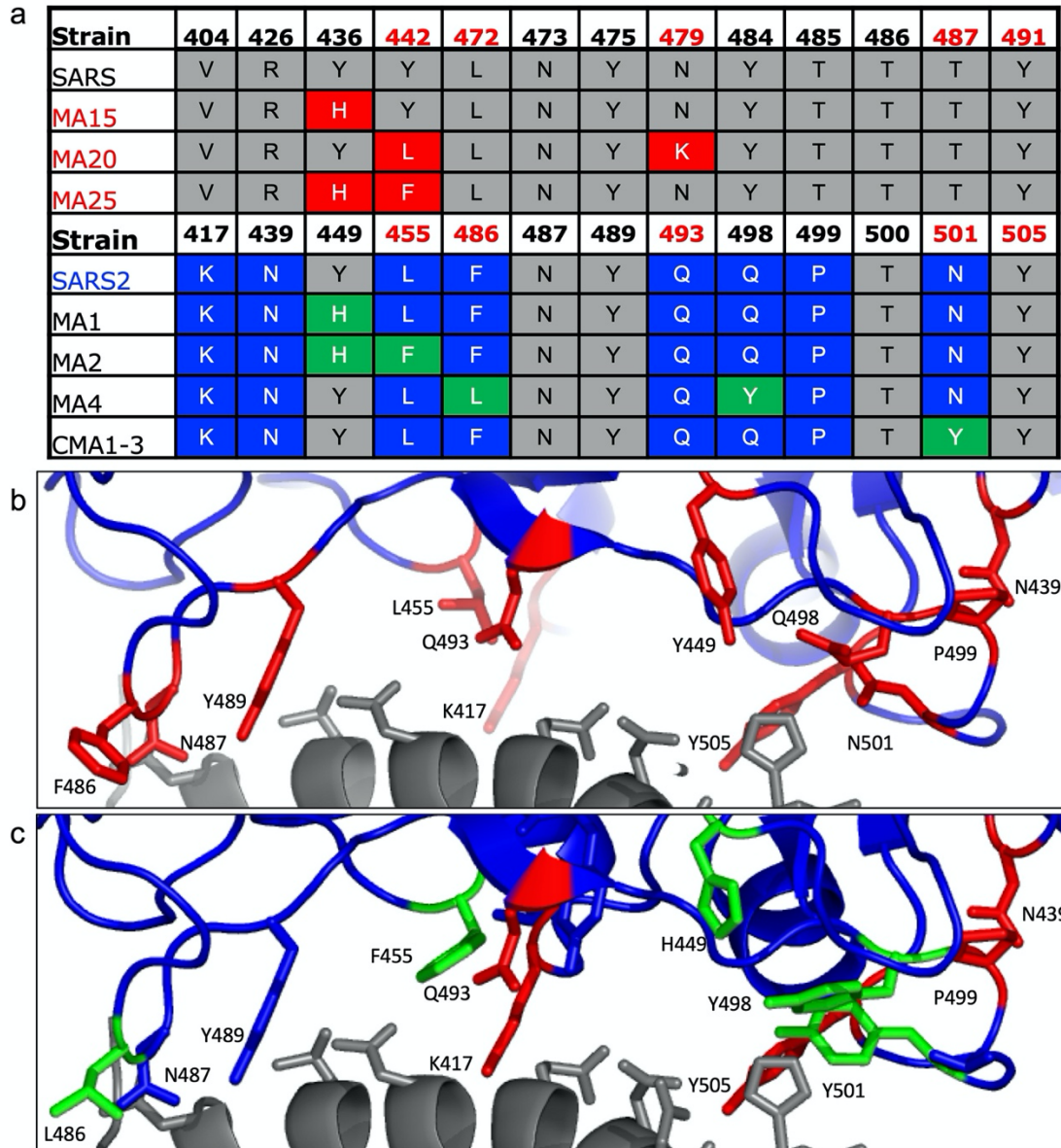
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Figure 3. CMA3p20 strain maintains human replication capacity and antigenicity. a-b) Primary human airway cultures were infected with SARS-CoV-2 WT (black) or CMA3p20 (blue) at an MOI of 0.01 and evaluated for a) viral titer and b) viral RNA. c) Sera collected from female Balb/c mice 28 days post infection with 106 PFU of SARS-CoV-2 CMA3p20 were evaluated for capacity to neutralize WT SARS-CoV-2 via PRNT₅₀ assay. d) PRNT₅₀ values from COVID19 patient sera plotted against WT virus (y-axis) versus CMA3p20 virus (x-axis). e-f) Ten-week-old female Balb/c mice were treated intraperitoneally with 100ul of human COVID19 sera or control (PBS) one day prior to infection. Mice were subsequently challenged with 105 PFU of SARS-CoV-2 CMA3p20 and evaluated for e) weight loss and f) viral titer in the lung.

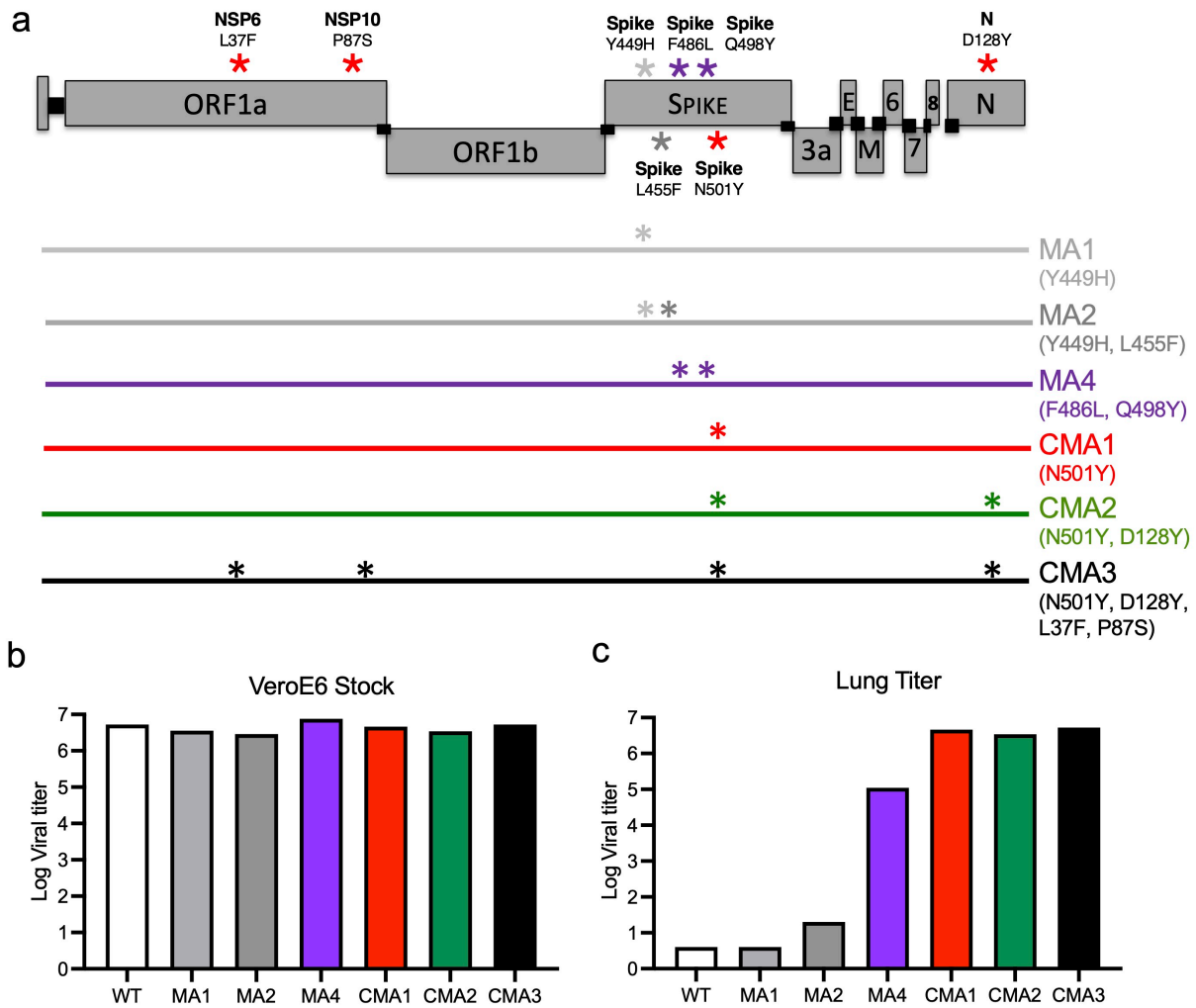


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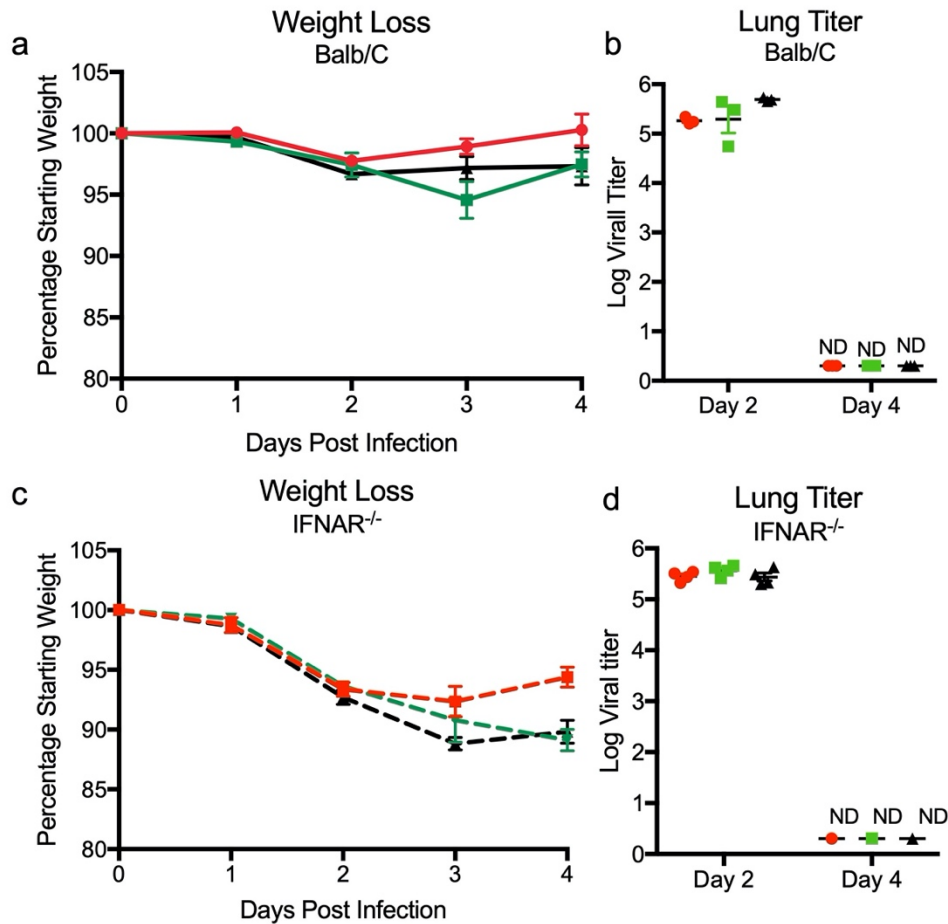
385 **Figure 4. Prior infection with SARS-CoV-2 protects from lethal SARS-CoV challenge.** a-c)
 386 Ten-week-old female Balb/c mice were previously infected with 10⁶ PFU of SARS-CoV-2
 387 CMA3p20 (blue) or mock (black), monitored for weight loss, and allowed to recover. Twenty-
 388 eight days post infection, both groups were challenged with a lethal dose (10⁴ PFU) of mouse-
 389 adapted SARS-CoV and evaluated for a) weight loss, b) lethality, and c) disease score. d) Mice
 390 were subsequently euthanized at day 2, 4, and 7 and lung tissue examined for viral replication.
 391 e) Sera from CMA3p20 infected and SARS-CoV challenged were evaluated for virus
 392 neutralization (PRNT₅₀) against SARS-CoV-2 (blue) or SARS-CoV over time (no rechallenged-
 393 black, day 2 red, day 4-orange, day 7- green).



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 395 **S. Figure 1. Modeling changes to mouse-adapt SARS-CoV-2.** a) Key amino acid residues found in the
 396 receptor binding domain (RBD) of mouse adapted strains of SARS-CoV were aligned to SARS-CoV-2
 397 and used to design mouse-adapted mutations 13. Key interaction sites between SARS-CoV spike and
 398 ACE2 molecules highlight in red 30. b-c) Modeling of key RBD residue interactions with mouse ACE2
 399 (PDB:2AJF) comparing b) WT SARS-Cov-2 residues versus c) mutations (green) predicted to improve
 400 binding.

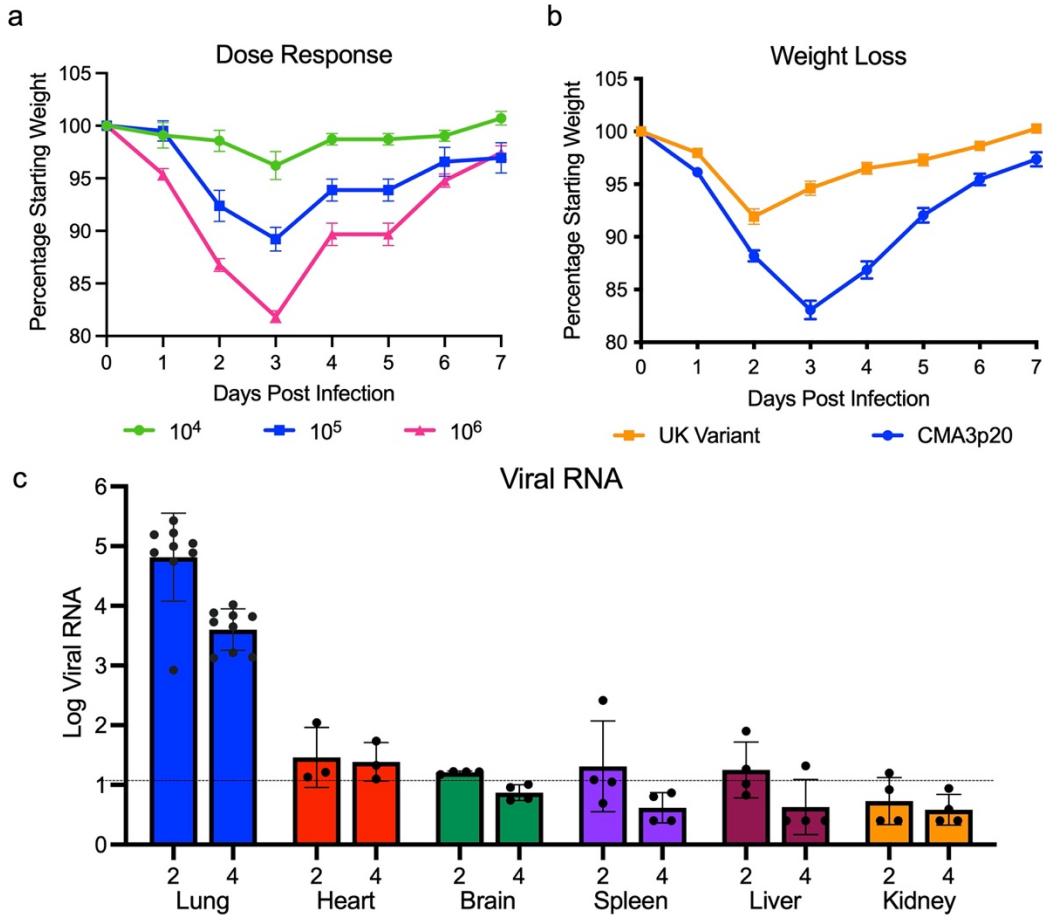


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 402 **S. Figure 2. Construction of mouse-adapted SARS-CoV-2 Mutants.** a) SARS-CoV-2 genome
 403 schematic indicating location of amino acid mutations for MA1, MA2, MA4, CMA1, CMA2, and
 404 CMA3. b) Viral replication of stock viruses of MA1, MA2, MA4, and CMA1-3 grown on VeroE6
 405 cells. c) Viral replication of MA1, MA2, MA4, and CMA1-3 from lung homogenates isolated from
 406 infected mice 2 days post infection.
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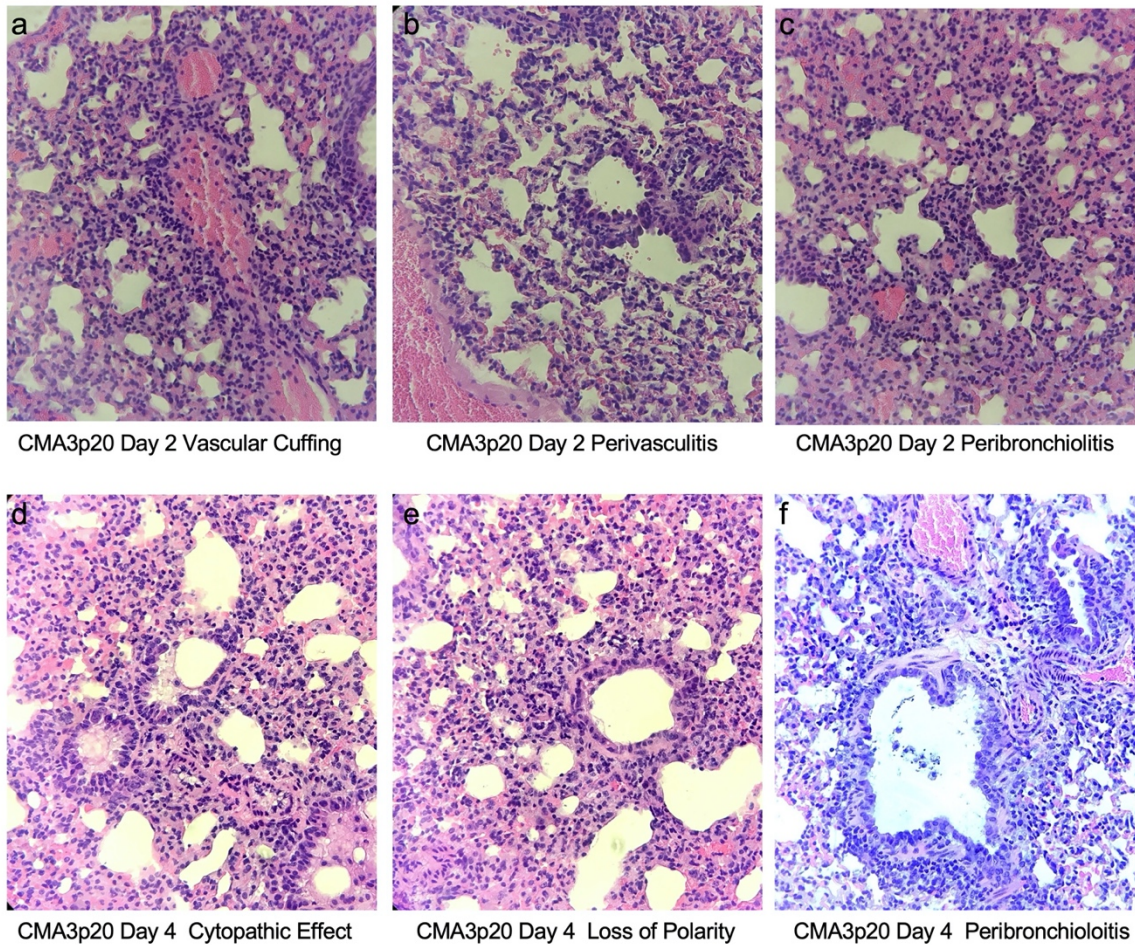
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S. Figure 3. SARS-CoV-2 mutants CMA1, CMA2, and CMA3 replicate in laboratory mice. a- b) Ten-week-old female Balb/c mice infected with 105 PFU of CMA1 (red), CMA2 (green), or CMA3 (black) were examined for a) weight loss and b) viral lung titer following infection at days 2 and 4. c-d) Ten- to twelve-week-old female IFNAR^{-/-} SVJ129 mice infected 105 PFU of CMA1 (red), CMA2 (green), or CMA3 (black) were examined for c) weight loss and d) viral lung titer following infection at days 2 and 4. ND- non-detected.



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S. Figure 4. In vivo characterization of SARS-CoV-2 CMA3p20. a) Examination of ten-week-old female Balb/c mice infected with SARS-CoV-2 CMA3p20 at 10⁴, 10⁵, and 10⁶ PFU (n=5). b) Comparison of weight loss in ten-week old female Balb/c mice infected with 10⁶ PFU of SARS-CoV-2 CMA3p20 (blue) or SARS-CoV-2 variant B.1.1.7 (orange). c) RT-PCR of viral RNA load found in lung, heart, brain, spleen, liver, and kidney following 10⁵ PFU infection of SARS-CoV-2 CMA3p20 2- and 4-days post infection. Dotted line signifies viral RNA value derived from mock infected samples.



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S. Figure 5. SARS-CoV-2 CMA3p20 induces significant lung damage following infection.

a-c) CMA3p20 infected animals 2 days post infection showing a) perivascular cuffing, b) perivasculitis and c) peribronchiolitis. d-f) CMA3p20 induced lung inflammation and damage 4 days post infection including d) cytopathic effect of the virus, e) loss of cellular polarity, and f) inflammatory cells in the lumen. Magnification at 10x for a-f.

429 **Methods**

430 **Viruses and cells.** The recombinant wild-type and mouse-adapted strains of SARS-CoV-2 are
431 based on the sequence of USA-WA1/2020 isolate provided by the World Reference Center for
432 Emerging Viruses and Arboviruses (WRCEVA) and was originally obtained from the USA Centers
433 for Disease Control and Prevention as described ³¹. Wild-type and mutant SARS-CoV-2 as well
434 as recombinant mouse-adapted recombinant SARS-CoV ¹⁷ were titrated and propagated on Vero
435 E6 cells, grown in DMEM with 5% fetal bovine serum and 1% antibiotic/antimycotic (Gibco).
436 Standard plaque assays were used for SARS-CoV and SARS-CoV-2 ^{32,33}. All experiments
437 involving infectious virus were conducted at the University of Texas Medical Branch (Galveston,
438 TX) or Emory University (Atlanta, Georgia) in approved biosafety level 3 (BSL) laboratories with
439 routine medical monitoring of staff.

440 **Phylogenetic tree, sequence identity heat map, and structural modeling.** Spike receptor
441 binding tables were constructed from a set of representative group 2B coronaviruses by using
442 alignment data paired with neighbor-joining phylogenetic trees built in Geneious (v.9.1.5) using
443 the spike amino acid sequences derived the following accession numbers: QHU79204 (SARS-
444 CoV-2 WA1), AGZ48806 (RsSHC014), ALK02457 (WIV16), and AYW99817.1(SARS-CoV
445 Urbani). Mouse-adapted SARS-CoV-2 structural homology models were generated using
446 SWISS-Model ^{34,35} with the 6LZG crystal structure (RCSB Protein Data Bank) as the template
447 structure for the spike protein and the 2AJF crystal structure (RCSB Protein Data Bank) as the
448 template for ACE2. Homology models were visualized and manipulated in PyMOL (version 2.4.2).

449 **Construction of mouse-adapted mutant SARS-CoV-2.** Both wild-type and mutant viruses were
450 derived from the SARS-CoV-2 USA-WA1/2020 infectious clone as previously described ¹². For
451 mouse adapted virus construction, the individual mutations were synthesized and introduced into
452 the appropriate plasmids (F1-F7) via PCR-based mutagenesis with synthesized specific primers
453 containing corresponding mutations. The resulted plasmid was validated by further restriction

454 enzyme digestion and Sanger sequencing. Thereafter, plasmids containing wild-type and mutant
455 SARS-CoV-2 genome fragments were amplified and digested by restriction enzyme. The SARS-
456 CoV-2 genome fragments were purified and ligated *in vitro* to assemble the full-length cDNA
457 according to the procedures described previously^{12,36}. *In vitro* transcription reactions then were
458 performed to synthesize full-length genomic RNA. To recover the viruses, the RNA transcripts
459 were electroporated into Vero E6 cells. The medium from electroporated cells as harvested at 40
460 hours post transfection and served as seed stocks for amplifying one passage on Vero E6 cells
461 (P1 stock). Viral mutants were confirmed by sequence analysis prior to use. Synthetic
462 construction of SARS-CoV-2 mouse adapted strains were approved by the University of Texas
463 Medical Branch Institutional Biosafety Committee.

464 ***In vitro* infection.** Viral infections in primary human airway cells were performed as previously
465 described³⁷. Briefly, the apical side of the HAE cultures were washed 3 times with PBS. Cultures
466 were infected with SARS-CoV-2 WT (WA1) or CMA3p20 at MOI 0.01 and allowed to adsorb for
467 1 hr at 37°C. After adsorption, the apical side was washed 3 times with PBS, and the basolateral
468 media replaced. Viral washes were collected adding PBS to the apical side and incubated for 30
469 minutes at 37°C. Viral titer was evaluated by focus forming assay as previously described³⁷.
470 RNA from HAE were collected at 48 hours post infection. RNA was harvested from mock-infected
471 and infected HAE cultures by treating with RNA lysis buffer for >5 min and gently pipetting to
472 recover cells. Total RNA was extracted using the Zymo Quick-RNA miniprep kit (VWR; R1055)
473 according to the manufacturer's protocol. Purified RNA was reverse transcribed into cDNA using
474 the high-capacity cDNA reverse transcription kit (Thermo Fisher, 43-688-13). RNA levels were
475 quantified using the IDT Prime Time gene expression master mix and TaqMan gene expression
476 Primer/Probe sets (IDT) and run on a QuantStudio5 qPCR system using SARS-CoV-2 RDRP-
477 specific primers (forward [F], GTGARATGGTCATGTGTGGCGG; reverse [R],
478 CARATGTTAAASACACTATTAGCATA) and probe (56-6-carboxyfluorescein

479 [FAM]/CAGGTGGAA/ZEN/CCTCATCAGGAGATGC/3IABkFQ) were used. Three or more
480 biological replicates were harvested at each described time and results are representative of
481 multiple experiments. No blinding was used in any sample collections, nor were samples
482 randomized. Microsoft Excel for Mac 2011 was used to analyze data.

483 **Deep sequencing analysis.** RNA libraries of SARS-CoV-2 mutants were prepared with 300 ng
484 of RNA using the Tiled-ClickSeq protocol as previously described^{38,39} using tiled primers cognate
485 to the SARS-CoV-2 genome (accession number NC_045512.2) and the TruSeq i7 LT adapter
486 series and i5 hexamer adaptors containing a 12N unique molecular identifier (UMI). Libraries were
487 sequenced on the Illumina MiSeq platform with MiSeq Reagent Kit v2 using paired-end reads
488 (R1:250 cycles, R2:50 cycles). Raw data was de-multiplexed using TruSeq indexes using the
489 MiSeq Reporter Software. Demultiplexed read data were quality filtered, adaptor-trimmed and
490 primer-trimmed as previously described³⁹. Reads were mapped to the WA-1 reference
491 (NC_045512.2) using *ViReMa*⁴⁰. Reads were de-duplicated with *umi_tools*⁴¹ using 12N unique
492 molecular identifiers (UMIs) embedded in the i5 click-adaptor. A consensus reference sequence
493 was generated using Pilon⁴² ensuring that read coverage was greater than 25x across 99.5% of
494 the reference genome. Pileup files were generated using Samtools v1.9⁴³ and minority variants
495 were extracted by nucleotide voting (PHRED>=30) using a custom python3 script previously
496 described³⁹.

497 **Plaque reduction neutralization test.** Neutralization assays were performed using conventional
498 plaque reduction neutralization assay (PRNT50) as previously described⁴⁴. Briefly, 100 PFU of
499 SARS-CoV-2, mouse-adapted SARS-CoV-2, or SARS-CoV MA15 was incubated with serially
500 diluted serum from mice or COVID-19 patients (total volume of 200 µl) at 37°C for 1 h. The virus-
501 serum mixture was added to the pre-seeded Vero E6 cells. After 1 h 37°C incubation, 2 ml of 2%
502 high gel temperature agar (SeaKem) in DMEM containing 5% FBS and 1% P/S was overlaid onto
503 infected cells. After 2 days of incubation, 2 ml neutral red (1 g/l in PBS; Sigma) was added to the

504 agar-covered cells. After another 5-h incubation, neutral red was removed. Plaques were counted
505 for NT50 calculation. The PRNT assay was performed at the BSL-3 facility at UTMB.

506 **Ethic Statement.** This study was carried out in accordance with the recommendations for care
507 and use of animals by the Office of Laboratory Animal Welfare, National Institutes of Health. The
508 Institutional Animal Care and Use Committee (IACUC) of University of Texas Medical Branch
509 (UTMB) approved the animal studies under protocol 1711065 and 1707046. For samples Emory
510 University, collection and processing were performed under approval from the University
511 Institutional Review Board (IRB #00001080 and #00022371). Adults ≥ 18 years were enrolled who
512 met eligibility criteria for SARS-CoV-2 infection (PCR or rapid antigen test confirmed by a
513 commercially available assay) and provided informed consent.

514 **Human Serum samples**

515 For Emory University, acute peripheral blood samples were collected from hospitalized patients
516 at the time of enrollment. Infected patients were randomly selected from a convenience sample
517 and no data was collected on the number of patients that were pre-screened or declined
518 participation. All patients enrolled in July 2020 and had a mean age of 57 (range: 26-85; 50%
519 male). Samples were collected in the first 9 days (range: 2-9) of their hospital stay (range: 3-33
520 days) and mostly 1-2 weeks after symptom onset (range 5-19 days), the majority of the patients
521 had comorbid conditions (n=16) with 19 out of 20 having severe disease and one patient had
522 moderate disease. All of these patients had radiological evidence of pneumonia; 19 out of the 20
523 patients required supplemental oxygen, and 4 out of 20 patients were admitted to the intensive
524 care unit (ICU). Three enrolled patients died of COVID-19.

525 **Mice and *in vivo* infection.** Ten-week-old BALB/C mice were purchased from Charles River
526 Laboratories and were maintained in Sealsafe™ HEPA-filtered air in/out units. Prior to infection,
527 animals were anesthetized with isoflurane and infected intranasally (IN) with 10^4 to 10^6 plaque
528 forming units diluted 50 μ l of phosphate-buffered saline (PBS). Infected animals were monitored
529 for weight loss, morbidity, and clinical signs of disease, and lung titers were determined as

530 described previously⁴⁵. Infected animals were weighed daily, and lung tissue collected 2-, 4- and
531 7-days post infection for downstream analysis by plaque assay.

532 **Real-Time PCR for Viral RNA.** RNA from tissues were collected using RNA later (). Samples
533 were subsequently homogenized with Trizol reagent (Invitrogen). RNA was then extracted from
534 Triazol using the Direct-zol RNA Miniprep Plus kit (Zymo Research #R2072) per the
535 manufacturer's instruction. Extracted RNA was then converted to cDNA with the iScript cDNA
536 Synthesis kit (BioRad #1708891). Quantitative real time PCR (qRT-PCR) was performed with the
537 Luna Universal qPCR Master Mix (New England Biolabs #M3003) on a CFX Connect instrument
538 (BioRad #1855200). Primer 1 (Forward - AAT GTT TTT CAA ACA CGT GCA G and Primer 2
539 (Reverse - TAC ACT ACG TGC CCG CCG AGG) were used to detect SARS-CoV-2 genomes. A
540 primer annealing temperature of 63°C was used for all assays.

541 **Histological Analysis.** The left lung was removed and submerged in 10% buffered formalin
542 (Fisher) without inflation for 1 week. Lungs from mice sacrificed on day 2 and day 4 were fixed in
543 formalin and paraffin embedded. Five-micron serial sections were taken and used for
544 histopathological staining with hematoxylin and eosin and Immunohistochemistry (IHC) assay.
545 IHC was conducted using rabbit polyclonal antibodies raised against the SARS-CoV nucleocapsid
546 protein (clone NB100-56576 [1:100], Novus Biologicals, Littleton, CO) and biotinylated anti-rabbit
547 IgG, streptavidin AP (Vector Labs, Burlingame, CA) with Permanent Red chromogen
548 (Dako/Agilent, Santa Clara CA). Normal rabbit serum was used as primary antibody for the
549 negative control. Briefly, the sections were deparaffinized by immersion in three xylene baths for
550 5 minutes each. The slides were rehydrated by immersion in a series of alcohol baths ranging
551 from 100 to 95% for 5 minutes each. The slides were pretreated with a citrate-based buffer, pH
552 6.0 at 98 °C for heat-induced epitope retrieval. Endogenous avidin and biotin sites were blocked
553 using avidin/biotin blocking kit (Abcam, Cambridge, MA). The slides were incubated with anti-
554 SARS-CoV antibody for one hour at room temperature, washed in 1X Tris-buffered saline (TBS),
555 0.05% Tween 20 (wash buffer), and incubated with goat biotinylated anti-rabbit IgG (H + L)

556 antibody for 30 min at room temperature followed by rinse in wash buffer. Slides were then
557 incubated with streptavidin-AP conjugate for 30 min at room temperature and washed, followed
558 by incubation with Permanent Red for 5 minutes. Counterstaining with Mayer's hematoxylin
559 solution was performed and the slides mounted with coverslip using Permount mounting medium.

560 **Data Availability.** The raw data that support the findings of this study are available from the
561 corresponding author upon reasonable request.⁴⁶

562 **Biological Materials.** Recombinant wild-type and mutant SARS-CoV-2 described in this
563 manuscript will be made available through the World Reference Center for Emerging Viruses and
564 Arboviruses (WRCEVA) at UTMB through material transfer agreement. The SARS-CoV-2 B.1.1.7
565 strain was provided by Dr. Natalie Thornburg and colleagues at the Centers for Disease Control
566 and Prevention.

567 **Competing interests**

568 XX, P-YS, and VDM have filed a patent on the reverse genetic system and reporter SARS-CoV-
569 2. Other authors declare no competing interests.

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584 **Author Contributions**

585 Conceptualization, AM, MV, BAJ, ALR, MSS, XX, P-YS, VDM ; Methodology, AM, MV, BAJ, MED,
586 AV, PV, KD, ALR, DW, MSS, XX, P-YS; Investigation, AM, MV, BAJ, MED, AV, KL, CS, PV, RML,
587 KD, ALR, DW, MSS, XX, PY-S; Resources, JAP, KSP, SCW, KD, ALR, DW, MSS, XX, P-YS,
588 VDM; Data Curation, AM, MV, BAJ, MED, AV, PV, KD, ALR, DW, MSS, XX, PY-S, VDM.; Writing-
589 Original Draft, AM, VDM; Writing-Review & Editing, AM, P-YS, VDM; Data Visualization, AM, KD,
590 VDM; Supervision, SCW, ALR, DW, MSS, P-YS, VDM.; Funding Acquisition, SCW, MSS, P-YS,
591 VDM

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