

1 **Title:** Fatal neuroinvasion and SARS-CoV-2 tropism in K18-hACE2 mice is partially
2 independent on hACE2 expression

3

4 **Running Title:** Spatiotemporal analysis of SARS-CoV-2 in K18-hACE2

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38

39 **ABSTRACT**

40 Animal models recapitulating distinctive features of severe COVID-19 are critical to
41 enhance our understanding of SARS-CoV-2 pathogenesis. Transgenic mice expressing
42 human angiotensin-converting enzyme 2 (hACE2) under the cytokeratin 18 promoter
43 (K18-hACE2) represent a lethal model of SARS-CoV-2 infection. The precise
44 mechanisms of lethality in this mouse model remain unclear. Here, we evaluated the
45 spatiotemporal dynamics of SARS-CoV-2 infection for up to 14 days post-infection.
46 Despite infection and moderate pneumonia, rapid clinical decline or death of mice was
47 invariably associated with viral neuroinvasion and direct neuronal injury (including brain
48 and spinal neurons). Neuroinvasion was observed as early as 4 dpi, with virus initially
49 restricted to the olfactory bulb supporting axonal transport via the olfactory
50 neuroepithelium as the earliest portal of entry. No evidence of viremia was detected
51 suggesting neuroinvasion occurs independently of entry across the blood brain barrier.
52 SARS-CoV-2 tropism was not restricted to ACE2-expressing cells (e.g., AT1
53 pneumocytes), and some ACE2-positive lineages were not associated with the presence
54 of viral antigen (e.g., bronchiolar epithelium and brain capillaries). Detectable ACE2
55 expression was not observed in neurons, supporting overexpression of ACE2 in the nasal
56 passages and neuroepithelium as more likely determinants of neuroinvasion in the K18-
57 hACE2 model. Although our work incites caution in the utility of the K18-hACE2 model to
58 study global aspects of SARS-CoV-2 pathogenesis, it underscores this model as a unique
59 platform for exploring the mechanisms of SARS-CoV-2 neuropathogenesis that may have

60 clinical relevance acknowledging the growing body of evidence that suggests COVID-19

61 may result in long-standing neurologic consequences.

62

63 **IMPORTANCE**

64 COVID-19 is predominantly a respiratory disease caused by SARS-CoV-2 that has
65 infected more than 191 million people with over 4 million fatalities (2021-07-20). The
66 development of animal models recapitulating distinctive features of severe COVID-19 is
67 critical to enhancing our understanding of SARS-CoV-2 pathogenesis and in the
68 evaluation of vaccine and therapeutic efficacy. Transgenic mice expressing human
69 angiotensin-converting enzyme 2 (hACE2) under the cytokeratin 18 promoter (K18-
70 hACE2) represent a lethal model of SARS-CoV-2 infection. Here, we show lethality of this
71 model is invariably associated with viral neuroinvasion linked with viral replication and
72 assembly. Importantly, pneumonia albeit invariably present was generally moderate with
73 the absence of culturable infectious virus at peak neuroinvasion. The dynamics of viral
74 neuroinvasion and pneumonia were only partially dependent on hACE2. Overall, this
75 study provides an in-depth sequential characterization of the K18-hACE2 model following
76 SARS-CoV-2 infection, highlighting its significance to further study the mechanisms of
77 SARS-CoV-2 neuropathogenesis.

78

79 INTRODUCTION

80 The world is experiencing the devastating effects of the Coronavirus Disease 2019
81 (COVID-19) pandemic, a highly contagious viral respiratory disease caused by the newly
82 emerged betacoronavirus, Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-
83 CoV-2) (1-3). The initial index case was reported at a seafood market in Wuhan, Hubei
84 Province, China in late 2019 (1). While still under investigation, it has been postulated
85 that the progenitor of SARS-CoV-2 may have originated from horseshoe bats
86 (*Rhinolophus affinis*) or Malayan pangolins (*Manis javanica*) that, following spill over into
87 humans, acquired the genomic features leading to adaptation and human-to-human
88 transmission (1). SARS-CoV-2 has a high transmissibility rate, and, to date, it has
89 infected nearly 194 million people, resulting in over 4 million fatalities (2021-07-28) (4).
90 COVID-19 causes respiratory disease of variable severity, ranging from mild to severe,
91 with the development of acute respiratory distress syndrome requiring intensive care and
92 mechanical ventilation (3, 5-7). Numerous comorbidities including hypertension, obesity,
93 and diabetes, among others, are affiliated with an increased risk of developing severe
94 COVID-19 (5, 6, 8-10). Furthermore, a proportion of infected patients go on to develop
95 poorly understood neurological signs and/or symptoms mostly associated with the loss of
96 smell and taste (anosmia and ageusia), headache, dizziness, encephalopathy (delirium),
97 and ischemic injury (stroke), in addition to a range of less common symptoms (5, 7, 11-
98 17). Multiple studies have identified either SARS-CoV-2 RNA and/or protein in the brain
99 of COVID-19 patients with the olfactory neuroepithelium postulated as a portal of entry
100 (18, 19). COVID-19 has severely challenged health care systems around the globe, with
101 the urgent need for medical countermeasures including the development of efficacious
102 vaccines and therapeutics.

103 Animal models permissive to SARS-CoV-2 that could serve as suitable models to help
104 better understand the pathogenesis of COVID-19, while simultaneously assisting in the
105 development and evaluation of novel vaccines and therapeutics to combat this disease,
106 are critically needed (20-22). While various animal models (mice, hamsters, non-human
107 primates, ferrets, minks, dogs, and cats) have been evaluated to date (22-30), none
108 faithfully recapitulates all the pathological features of COVID-19. The main limitation in
109 the development of suitable murine models of COVID-19 is related to the virus entry
110 mechanism: SARS-CoV-2 binds to target cells via interaction between the viral spike
111 protein (S) and the host angiotensin-converting enzyme 2 (ACE2), considered to be the
112 major host entry receptor (31). The low binding affinity between the S protein and murine
113 ACE2 (mACE2) renders conventional mouse strains naturally resistant to infection,
114 posing a challenge in the development of murine models of COVID-19 (31-34). These
115 difficulties have been circumvented by the development of transgenic murine models that
116 express human ACE2 (hACE2) under different promoters including hepatocyte nuclear
117 factor-3/forkhead homologue 4 (HFH4), and cytokeratin 18 (K18) (30, 35-38). The
118 transgenic murine model expressing hACE2 under a K18 promoter (namely K18-hACE2)
119 was developed by McCray et al in 2007 to study SARS-CoV-1 (36), which shares the
120 same host receptor as SARS-CoV-2 (39).

121 SARS-CoV-2 infection of K18-hACE2 mice results in up to 100% lethality, analogous to
122 that reported for SARS-CoV (30, 36, 38). Early reports communicated lethality to be
123 associated primarily with severe lung inflammation and impaired respiratory function,
124 suggesting that this model can recapitulate features of the respiratory disease observed
125 in severe cases of COVID-19 (30, 38). However, the confounding impact of neuroinvasion

126 and its role in the clinical decline of SARS-CoV-2 infected K18-hACE2 mice is becoming
127 more readily acknowledged (19, 40, 41).

128 Under K18 regulation, the expression of hACE2 is reported to be limited mainly to airway
129 epithelial cells and enterocytes lining the colonic mucosa, to a lower degree within kidney,
130 liver, spleen, and small intestine, and to a relatively minor level of expression in the brain
131 (36). However, the cellular distribution of ACE2, and particularly hACE2, in tissues of K18-
132 hACE2 mice remains largely undetermined. We hypothesized that the nature, severity,
133 and outcome of disease in K18-hACE2 mouse model is not solely dictated by the
134 expression and tissue distribution of hACE2 and that increased lethality in this model is
135 ultimately related to neuroinvasion, in part driven by regional ACE2 overexpression in the
136 nasal passages promoting retrograde axonal transport through the olfactory nerve. To
137 investigate this hypothesis, we undertook a comprehensive spatiotemporal analysis of
138 histologic and ultrastructural changes, cellular distribution of viral protein and RNA, viral
139 loads, and antibody titers, along with a detailed analysis of the distribution of *hACE2*
140 mRNA, ACE2 protein, and its correlation with SARS-CoV-2 tropism as it pertains to this
141 model.

142 Although SARS-CoV-2 protein and RNA were detected in ACE2-expressing cells such as
143 olfactory neuroepithelium (ONE) and alveolar type 2 (AT2) cells, we found that SARS-
144 CoV-2 primarily infected neurons and alveolar type 1 (AT1) pneumocytes, which lacked
145 detectable ACE2 protein. Our results support neuroinvasion as the primary cause of
146 mortality in the K18-hACE2 mouse model. This claim was supported by the observation
147 that viral load and titers peaked in the brain when animals began to meet euthanasia
148 criteria or succumb to disease. This was clinically reflected by onset of profound

149 hypothermia and onset of neurological signs including tremors. Neurons in terminal
150 animals display prominent spongiotic degeneration and necrosis with concurrent
151 detection of abundant viral protein, RNA, and virus particle assembly. Although
152 pneumonia was uniformly observed and peaks at 7 dpi, it was of moderate severity with
153 declining viral loads compared to that of the brain. We found that several histologic
154 hallmarks of severe COVID-19 were lacking in this model, (i.e., lack of diffuse alveolar
155 damage and microthrombi), suggesting pneumonia plays a contributing role rather than
156 the primary determinant of lethality in this model. Interestingly, the absence of detectable
157 ACE2 expression in neurons suggested that viral neuroinvasion is a mechanism partially
158 independent of ACE2.

159 Altogether, this study expands the current knowledge on the K18-hACE2 murine model
160 to study severe COVID-19. Our findings will help refine utilization of this model for
161 providing a relevant understanding of the molecular mechanisms driving
162 neuropathogenesis and pulmonary pathology.

163

164 **RESULTS**

165 **SARS-CoV-2 is invariably fatal in infected K18-hACE2 mice with evidence of**
166 **neuroinvasion.**

167 K18-hACE2 mice inoculated intranasally with SARS-CoV-2 (1×10^6 plaque-forming units
168 [PFU]; n=35 [n=19 male and n=16 female) began losing weight as early as 4 days post-
169 infection (dpi) irrespective of sex, with maximum weight loss occurring at 6-7 dpi (18.4%
170 \square 6.8% in male mice, 21.4% \square 1.8% in female, and combined 19.7% \square 5.2%; Fig. 1A).
171 Trends in weight loss paralleled increasing clinical scores and declines in core body

172 temperature, with the latter two precipitously increasing or decreasing respectively near
173 the time of death (Fig. 1B, C). SARS-CoV-2-infected K18-hACE2 mice exhibited
174 neurological signs starting 6 dpi, characterized by profound stupor, tremors,
175 proprioceptive defects, and abnormal gait, with most animals euthanized or found dead
176 in their cage at 6 and 7 dpi (~94%; 33/35 [Fig. 1D]). At the time of death (6-7 dpi), the
177 median clinical score was 3 (interquartile range = 1) and the mean body temperature was
178 30.9 ± 3.0 °C. Two male mice survived to the end of the 14-day observation period and
179 did not display hypothermia during the course of the observation period, a feature that
180 was consistently observed in animals that succumbed to disease or met euthanasia
181 criteria.

182 Peak of lethality was associated with significant increases in viral loads (viral RNA and
183 infectious virus particles) in the brain of the K18-hACE2 mice (Fig. 1E,F), as previously
184 reported (19, 38, 41). In the lung, viral RNA copies were detectable at the earliest
185 experimental timepoint (2 dpi) and remained stable over time, consistently within the
186 value range reported in previous studies (30). While viral RNA remained high, viral titers
187 however gradually declined over time, with no infectious virus recovered by 7 dpi (Fig.
188 1E-F). Although the absence of recoverable infectious virus in the lungs at 7 dpi was
189 unexpected, this sharp decline mirrors published work that illustrated a 100-fold decline
190 in PFUs at 7 dpi compared to 4 dpi (30). In contrast, viral RNA and infectious particles in
191 the brain were low to undetectable at 4 dpi, but dramatically increased at 7 dpi (Fig 1E-F)
192 representing the highest mean viral RNA load and infectious virus particles during the
193 study. A small amount of viral RNA was detected in the serum (Fig.1G); however,
194 incubation of SARS-CoV-2 permissive cell lines with serum samples did not result in any

195 detectable productive infection *in vitro*, confirming an absence of viremia in intranasally-
196 inoculated K18-hACE2 mice (Fig. 1H). Altogether, our data illustrate that lethality was
197 associated with increasing viral RNA loads and infectious virus particles in the brain,
198 which were simultaneously declining in the lung.

199

200 **SARS-CoV-2 results in transient mild infection in the nasal cavity of K18-hACE2**
201 **mice.**

202 We next performed detailed histologic analysis of various tissues to uncover the
203 morphologic correlates of lethality in K18-hACE2 mice. For this, we first focused on the
204 spatial and temporal dynamics of SARS-CoV-2 infection in the upper respiratory tract and
205 analyzed the anterior/rostral nasal cavity (Fig. 2A-F) and olfactory neuroepithelium (Fig.
206 2G-L) for disease-associated lesions. To do so, we performed a thorough sequential
207 histologic analysis combined with immunohistochemistry (IHC) and RNAscope® *in situ*
208 hybridization (42) to determine the cellular localization and abundance of SARS-CoV-2
209 protein and RNA, using an anti-spike monoclonal antibody and an S-specific RNA probe,
210 respectively.

211 At 2 dpi, the anterior/rostral nasal cavity was characterized by mild, multifocal neutrophilic
212 inflammation (rhinitis) with segmental degeneration and necrosis of transitional and
213 respiratory epithelium (Fig. 2B), which colocalized with intracytoplasmic SARS-CoV-2
214 protein and RNA (Fig. 2E). Adjacent nasal passages were partially filled with small
215 amounts of cellular debris, degenerate neutrophils, and small numbers of erythrocytes.
216 The lamina propria underlying affected areas was infiltrated by low to mild numbers of
217 neutrophils and fewer lymphocytes (Fig. 2B). At 4 dpi, epithelial degeneration and

218 necrosis in the rostral and intermediate turbinates was no longer observed, replaced by
219 mild residual lymphocytic rhinitis and rare neutrophils within the lamina propria (Fig. 2C),
220 and absence of exudate within nasal passages. SARS-CoV-2 protein and RNA were less
221 commonly observed and restricted to rare positive cells in the respiratory epithelium (Fig.
222 2F and Table 1 and Table S1). By 7 dpi, the anterior/rostral nasal cavity was histologically
223 within normal limits and no SARS-CoV-2 protein or RNA were detectable (Fig. 2C, F and
224 Table 1 and Table S1).

225 The posterior nasal cavity, olfactory neuroepithelium (ONE) (Fig.2G-L), displayed mild
226 segmental degeneration and necrosis at 2 dpi, which colocalized with abundant SARS-
227 CoV-2 protein and RNA (Fig. 2K and Table 1 and Table S1). By 4 dpi, histopathologic
228 lesions in the ONE had resolved, but rare SARS-CoV-2 protein and RNA were observed
229 both at 4 and 7 dpi (Fig. 2L and Table 1). No SARS-CoV-2 protein or RNA were detected
230 in the ONE by 14 dpi (Table 1 and Table S1).

231

232 **SARS-CoV-2 induces moderate interstitial pneumonia in K18-hACE2 mice.**

233 In the lower respiratory tract, histologic alterations in the pulmonary parenchyma mainly
234 involved the alveoli, interstitium and perivascular compartment (Fig. 3A-K). Overall,
235 pathologic alterations in the lungs were characterized by moderate progressive
236 lymphohistiocytic and neutrophilic interstitial pneumonia that peaked at 7 dpi (Fig. 3G, H).
237 We quantitatively analyzed the total % of pneumonia using a machine learning classifier
238 to differentiate normal vs. pneumonic lung tissue. Peak disease was confirmed to occur
239 at 7 dpi, with a mean of ~10% of total lung area affected, with one outlier of ~40% of total
240 lung area, suggesting that more severe disease is possible, albeit uncommon (Fig. 3K).

241 Of note, data from our 2 dpi animals were excluded from tissue classification analysis as
242 lungs were sub-optimally insufflated and the classifier algorithm falsely labeled areas of
243 atelectasis as pneumonia. Pneumonia was interpreted to be minimal at this timepoint and,
244 thus, considered negligible.

245 At 2 dpi, minimal perivascular and peribronchiolar inflammation, consisting primarily of
246 lymphocytes and histiocytes, and occasional perivascular edema were observed (Fig.
247 3C). Pulmonary vessels were frequently reactive and lined by a plump endothelium with
248 marginating leukocytes (Fig. 3D). SARS-CoV-2 protein and RNA (Fig. 4A-J) were
249 observed in proximity to areas of interstitial pneumonia and localized within the cytoplasm
250 of alveolar type (AT) 1 (squamous epithelium) and fewer AT2 cells (cuboidal epithelium)
251 (Fig. 4B, G).

252 At 4 dpi, peak in viral protein and RNA abundance were observed (correlating with the
253 highest viral titer and RNA load as determined by RT-qPCR and plaque assays) (Fig 4C,
254 H) along with increasing lymphohistiocytic and neutrophilic infiltrate (Figs. 3E, F). SARS-
255 CoV-2 cellular tropism did not differ from that described at 2 dpi, but SARS-CoV-2 protein
256 and RNA were more abundant and routinely observed in histologically normal
257 pneumocytes (Fig 4C, H).

258 At 7 dpi, lymphohistiocytic and neutrophilic interstitial pneumonia peaked in severity,
259 which on average was moderate to regionally severe, affecting ~10-40% of the
260 parenchyma (Fig. 3G, H, K). Additional unique findings at 7dpi included, rare alveolar
261 septal necrosis, mild proliferation of AT2 cells, and sporadic regional pulmonary edema
262 (Fig. 3G, H). SARS-CoV-2 protein and RNA were occasionally still abundant in several
263 animals, but predominated in histologically normal parenchyma, with minimal to rare

264 detection in areas of prominent inflammation (Fig. 4D, I and Table 1, and Table S1).
265 Taken together, this suggested progressive resolution of viral infection by the host
266 consistent with declining viral loads and absence of culturable infectious virus at this
267 timepoint (Fig. 1E, F).

268 In the two survivors euthanized at 14 dpi, persistent mild to moderate lymphohistiocytic
269 interstitial pneumonia was observed, with formation of sporadic lymphoid aggregates and
270 mild persistence of AT2 hyperplasia (Fig. 3I, J). SARS-CoV-2 protein or RNA were no
271 longer detectable at 14 dpi (Fig 4E, J).

272 Of note, no evidence of SARS-CoV-2 infection was observed in bronchiolar epithelium
273 and pulmonary vasculature at any time during the study (Figs. 3 and 4, and Table 1, and
274 Table S1). Similarly, hyaline membranes, vascular thrombosis, and syncytial cells were
275 not observed at any time point across all animals, which contrasts with severe disease
276 described in human autopsies (43) and non-human primate studies (44, 45). In one
277 animal (7 dpi), there was localized flooding of bronchioles by degenerate neutrophils and
278 cellular debris mixed with birefringent foreign material consistent with aspiration
279 pneumonia, a rare complication previously reported in K18 hACE2 mice infected with
280 SARS-CoV-1 that was ultimately attributed to pharyngeal and laryngeal dysfunction
281 secondary to central nervous system (CNS) disease (36).

282 Altogether, our data displays evidence of a significant but moderate lymphohistiocytic
283 interstitial pneumonia in SARS-CoV-2 infected K18-hACE2 mice. Histopathological
284 features contrast with those observed in severe cases of COVID-19 in humans and
285 suggest that the lethality observed in this model is in part independent of virally induced
286 lung injury and resultant pneumonia.

287

288 **Pulmonary SARS-CoV-2 replication occurs exclusively in AT1 and AT2 cells.**

289 Subsequently, we aimed to further investigate SARS-CoV-2 tropism in the lower
290 respiratory tract of K18-hACE2 mice. We first performed qualitative multiplex IHC to probe
291 the localization of SARS-CoV-2 protein in AT1 cells (cell marker: receptor for advanced
292 glycation end-products [RAGE]), AT2 cells (cell marker: surfactant protein C [SPC]), and
293 endothelial cells (cell marker: CD31). SARS-CoV-2 protein was restricted within RAGE+
294 AT1 and SPC+ AT2 pneumocytes, but not with CD31+ endothelial cells (Fig. 5A-C).
295 Transmission electron microscopy (TEM) corroborated our IHC and ISH data, where we
296 observed viral protein and RNA exclusively within the cytoplasm of squamous and
297 cuboidal pneumocytes. Double membrane-bound vesicles (DMVs) and virus particles
298 were exclusively observed in cells containing abundant caveolae (AT1 cells) or lamellar
299 bodies (AT2 cells) by transmission electron microscopy (Fig. 5D-F). No viral particles or
300 replication intermediates were observed in bronchiolar epithelial cells (Fig. 5G, H) or
301 vascular endothelium. Of note, cubic membranes were a distinctive feature only observed
302 in AT1 pneumocytes (Fig 5E).

303

304 **Effective control of SARS-CoV-2 infection in the lower respiratory tract is**
305 **associated with recruitment of macrophage and to a lesser degree cytotoxic T**
306 **cells.**

307 Next, we quantitatively characterized the cell density of inflammatory cells (cells/ μm^2)
308 targeting macrophages (Iba1), cytotoxic T cells (CD8), B cells (CD19) and total area
309 immunoreactivity (% area μm^2) of viral protein (Spike) in the lungs of SARS-CoV-2

310 infected K18-hACE2 mice (Fig. 6A-H). SARS-CoV-2 S immunoreactivity peaked between
311 4-7 dpi (Fig. 6A), supporting a positive correlation between viral infection and the
312 progressive inflammatory cell infiltrate, but was not statistically significant across groups.
313 We attribute this finding to our low sample size for quantitative whole slide analysis,
314 especially at 2 and 4 dpi, and individual animal variability likely represented by the
315 inherent heterogeneity of viral pneumonia. Iba1+ macrophages represented the
316 predominant inflammatory infiltrate across all time points with a temporal increase
317 peaking at 7 dpi ($p=0.0044$ compared to sham inoculated mice, Fig. 6B, G). Cytotoxic T
318 cells were the second most abundant inflammatory infiltrate quantified, which also
319 displayed a temporal increase peaking around 4-7 dpi (Fig. 6C, F-G); however, these
320 cells were present at a ~10-fold reduced frequency compared to macrophages and
321 statistical significance was not observed across timepoints, suggesting an early and
322 plateaued response of this inflammatory population. B cells were elevated by 7 dpi but
323 reached peak cell density at 14 dpi (Fig. 6D, H), the only time point where discrete
324 lymphoid aggregates were observed histologically ($p\leq 0.0001$ compared to sham
325 inoculated mice). Altogether, our data suggest that a strong and persistent myeloid
326 infiltrate and, to a lesser degree, cytotoxic T cells are important contributors to the decline
327 of viral load that occurs in the lungs between 4-7 dpi, with B cells potentially being involved
328 if animals survive the acute stage of disease. Of note, minimal inflammatory cells were
329 observed in sham-inoculated K18-hACE2 mice supporting that the two survivors were de-
330 facto infected with SARS-CoV-2 (Fig. 6E), which was further supported by the presence
331 of neutralizing antibodies in their serum as compared to naïve mice (Fig. 6I).

332

333 **SARS-CoV-2 exhibits extensive neuroinvasion with resultant neuronal**
334 **degeneration and necrosis in K18-hACE2 mice.**

335 Pursuing our hypothesis that the lethality of the K18-hACE2 mice is associated with
336 neuroinvasion, we analyzed sagittal sections of the whole head to characterize
337 progression of histologic lesions and distribution of viral protein and RNA at different
338 timepoints post-infection (2, 4, 6-7 and 14 dpi). Histologic alterations in the brain were
339 severe and widespread by 7 dpi with involvement of the olfactory bulb and of the cerebrum
340 (Fig.7), as well as of the cerebral cortex (most predominantly somatosensory and
341 somatomotor areas), hippocampus (mainly CA1 region), midbrain (thalamus and
342 hypothalamus), brainstem, and the dentate nucleus. Histologic findings included
343 moderate to marked neuronal spongiosis, multifocal shrunken, angular, hypereosinophilic
344 and pyknotic neuronal bodies with loss of Nissl substance/chromatolysis (neuronal
345 degeneration and necrosis, Fig. 7J) and occasionally delimited by multiple glial cells
346 (satellitosis). In the olfactory bulb, delicate lymphocytic perivascular cuffing (Fig. 7K) and
347 a general increase in the number of reactive glial cells (gliosis) were evident in the
348 neuroparenchyma neighboring areas of neuronal degeneration and necrosis. Notably, the
349 cerebellum (cortical layers and associated white matter of the cerebellar folia) was spared
350 of histologic changes (data not shown).

351 Neuronal morphologic changes directly correlated with abundant neuronal
352 immunoreactivity for SARS-CoV-2 S protein and viral RNA, which was observed
353 exclusively within neuronal cell bodies and processes (Figs. 7C, F, I, L, O, and 8A).
354 SARS-CoV-2 protein and RNA had a widespread distribution throughout the brain in
355 roughly 85% (11/13) of infected K18-hACE2 mice at 7 dpi, including neuronal bodies

356 within the cerebral cortex, CA1, CA2 and CA3 regions of the hippocampus, anterior
357 olfactory nucleus, caudoputamen, nucleus accumbens, thalamic nuclei including
358 hypothalamus, midbrain, pons and medulla oblongata nuclei (Fig. 8A). Few
359 vestibulocochlear nerve fascicles showed immunoreactivity for viral protein; while no viral
360 S protein or RNA was detected in areas spared of histological changes including the
361 cerebellar cortex and white matter, optic nerve and retina, and the spiral ganglion of the
362 inner ear (albeit the eye and inner ear were not present in most sections examined).
363 SARS-CoV-2 S protein and RNA preceded histological findings with rare detection as
364 early as 4 dpi in mitral and inner nuclear neurons of the olfactory bulb, as well as small
365 clusters of neurons within the anterior olfactory nucleus and orbital area of the cerebral
366 cortex (Figs. 7C, F, I, L, O, and 8A).

367 Using a NanoLuc expressing recombinant SARS-CoV-2 virus (rSARS-CoV-2 NL), we
368 observed significant detection of bioluminescence in the brain of a representative K18-
369 hACE2 mice at day 6 post-infection, which was associated with lower bioluminescence
370 signal in the lungs at the same time point, consistent with our previous findings (Fig. 8B)
371 and other reports (46). To better characterize the gliosis that was observed histologically
372 in animals with abundant neuronal degeneration and necrosis we quantitatively
373 characterized the total area immunoreactivity (% area μm^2) of microglia (Iba-1),
374 astrocytes (GFAP), and SARS-CoV-2 (S) in sagittal sections of whole brain (Fig. 9A-C).
375 Total % area immunoreactivity for astrocytes (GFAP) and microglia (Iba1) dramatically
376 increased at 7 dpi compared to sham inoculated negative controls (GFAP, $p=0.0101$;
377 Iba1, $p=0.0327$), paralleling peak expression of SARS-CoV-2 S, which was also
378 significantly increased compared to Sham inoculated negative controls ($p=0.0351$; Fig.

379 9A-C). Notably, the morphology of microglial, which had delicate cytoplasmic processes
380 in sham inoculated (Fig. 9B), 2 and 4 dpi mice, was replaced by broad shortened
381 processes at 7 dpi (Fig. 9C). Morphological differences in astrocyte processes were more
382 subtle, but still possessed broader and a more extensive branching pattern compared to
383 2dpi, 4dpi, and sham inoculated negative controls (Fig. 9B, C). To confirm exclusive
384 neuronal tropism, we performed TEM on an animal euthanized at 6 dpi that exhibited
385 neurologic signs of disease in the form of tremors. Viral assembly was observed
386 exclusively in neurons, with no detection in glial cells. The prominent histologic phenotype
387 of neuronal spongiosis was characterized by profound accumulation of double membrane
388 vesicles (DMVs) and virus particles, with loss of Nissl substance (degeneration) or
389 nuclear pyknosis, karyolysis, and global electron dense transformation of the cytoplasm
390 (necrosis) (Fig. 9D, E). Although viral assembly and/or particles were not observed in
391 microglia or astrocytes, quantitative immunohistochemical analysis supports reactive
392 microgliosis and astrogliosis that is temporally linked with peak neuroinvasion, suggesting
393 that activation of these cells contributes to neuronal injury either through direct neurotoxic
394 and/or loss of normal homeostatic neurotrophic mechanisms.

395 Considering the severe bladder distention noted at necropsy and proprioceptive deficits
396 observed clinically, we examined the cervicothoracic and lumbosacral segments of the
397 spinal cord. In 9/11 animals that died or were euthanized due to terminal disease, similar
398 histologic findings were observed as those described in the brain, albeit with milder gliosis
399 and lymphocytic perivascular cuffing (Fig. 10A). We also observed mild-to-moderate
400 detection of viral protein in the spinal cord that predominated within neurons of the
401 cervicothoracic segments (Fig. 10B, and Table 1 and Table S1). Finally, Luxol Fast Blue

402 was utilized to visualize the integrity of myelin following SARS-CoV-2 invasion in the brain
403 and spinal cord at 7 dpi, with no evidence of demyelination noted (Fig. 10C).

404 Taken together, our data illustrate that SARS-CoV-2 infection of K18-hACE2 results in
405 severe neuronal invasion of the CNS, via transport to the olfactory bulb originating from
406 axonal processes traversing the ONE. Alternative and concurrent routes such as
407 retrograde transport of other cranial nerves as well as parasympathetic and sympathetic
408 sensory nerves cannot be ruled out, especially acknowledging the near diffuse distribution
409 of virus in the brain at terminal stages of disease, with the exception of the cerebellum.
410 Viral neuroinvasion resulted in extensive neuronal cytopathic effect in infected cells that
411 ultimately resulted in cell death, comprising not only the brain but also the spinal cord.
412 Further research is warranted to characterize the role of uninfected but reactive microglia
413 and astrocytes in SARS-CoV-2 neuronal injury using a multidimensional approach
414 including molecular and functional testing.

415

416 **ACE2 expression and distribution does not fully reflect SARS-CoV-2 tissue tropism**
417 **in K18-hACE2 mice.**

418 To further explore the mechanism driving lethal SARS-CoV-2 infection in K18-hACE2
419 mice, we first investigated the tissue and cellular distribution of the ACE2 receptor in both
420 C57BL/6J and K18-hACE2 mice by IHC (Fig. 11A-L) using a cross-reactive anti-ACE2
421 antibody (cross-reactive to hACE2 and mACE2) (Table S2). In the lower respiratory tract
422 (lungs), ACE2 was ubiquitously expressed along the apical membrane of bronchiolar
423 epithelium and, less commonly, in rare and scattered AT2 pneumocytes (Fig. 11A-C). No
424 ACE2 expression was found in AT1 pneumocytes. No evident differences in the

425 distribution and abundance of ACE2 expression were identified between uninfected
426 C57BL/6J, sham-inoculated K18-hACE2, and terminal (7 dpi) K18-hACE2 mice
427 inoculated with SARS-CoV-2.

428 We therefore aimed at analyzing expression and distribution of *hACE2* mRNA using
429 RNAscope® ISH (Fig.12). Although no expression of *hACE2* mRNA was detected in the
430 lungs of non-transgenic C57BL/6J mice (Fig. 12A), expression of *hACE2* mRNA was
431 detectable, but of low expression in the lungs of K18-hACE2 mice, and mostly involved
432 bronchiolar epithelial cells with sporadic expression in AT2 pneumocytes (Fig. 12B,C).
433 These findings therefore suggest that *hACE2* expression might not be the sole host factor
434 determinant of susceptibility to SARS-CoV-2. This is clearly exemplified by the following:
435 1) certain cell types that, while expressing *hACE2*, were non-permissive to SARS-CoV-2
436 infection throughout the experiment (i.e. bronchiolar epithelial cells); and 2) the near
437 diffuse infection of AT1 cells by 4 dpi despite absent expression of *hACE2* in these cells.
438 Altogether, these observations then support evidence for an ACE2-independent viral
439 entry mechanism playing a major role in the pulmonary dissemination of K18-hACE2
440 mice.

441 In contrast to the lung, ACE2 protein was clearly overexpressed in the nasal cavity of
442 K18-hACE2 mice compared to C57BL/6J mice. We assessed ACE2 protein expression
443 on the rostral transitional epithelium, respiratory epithelium at the level of the intermediate
444 turbinates, as well as in the ONE and olfactory bulb (Fig. 11D-F, G-I). Unlike C57BL/6J
445 mice, in which ACE2 was undetectable within the nasal cavity, ACE2 protein was diffusely
446 expressed within the apical membrane of transitional and respiratory epithelium, and
447 segmentally within the apical surface of the ONE in both sham-inoculated and SARS-

448 CoV-2-infected K18-hACE mice (Fig. 11D-F). For the olfactory bulb, olfactory
449 neuroepithelium and respiratory epithelium of rostral turbinates, estimation of *hACE2*
450 abundance and distribution could not be accurately assessed since the decalcification
451 procedure is believed to have had a significant impact in the quality of cellular mRNA as
452 demonstrated by the low detection of the housekeeping mRNA, *Ppib* (data not shown).

453 In the brain of both C57BL/6J and K18-hACE2 mice, ACE2 protein was observed in the
454 vascular endothelium lining blood vessels (Fig. 11J-L), as well as ependymal and choroid
455 plexus epithelium. In contrast, distribution of *hACE2* mRNA expression involved clusters
456 of neurons within the cerebral cortex, hippocampus, midbrain, brainstem, and Purkinje
457 cells from the cerebellum, with no expression noted in non-transgenic C57BL/6J mice
458 (Fig. 12D-F). There was no expression of *hACE2* mRNA in vascular endothelial cells.

459 Taken together, our data show a discrepancy between ACE2 protein and RNA expression
460 and distribution within the CNS. This is partly attributable to the fact that the ACE2
461 antibody we utilized cross reacts with both hACE2 and mACE2 proteins, while the ACE2
462 probe employed was human specific. The absence of *hACE2* hybridization with
463 simultaneous ACE2 immunoreactivity in the capillary endothelium supports the notion that
464 ACE2 expression in these cells is of murine origin. The absence of ACE2
465 immunoreactivity in neurons is suggestive of a potential restriction in the translation (or
466 post-translation) of the ACE2 protein in these cells. This, in addition to the fact that
467 Purkinje cells of the cerebellum do not appear permissive to SARS-CoV-2 infection
468 despite the low expression of *hACE2* mRNA, suggests that ACE2 is likely not the sole
469 host factor associated with neuroinvasion and that other ACE2-independent entry
470 mechanisms contribute to neuroinvasion and spread by SARS-CoV-2 in this murine

471 model. Alternatively, and/or in parallel, the overexpression of ACE2 protein within the
472 nasal passages may be sufficient to enhance neuroinvasion by enhancing axonal
473 transport via the ONE.

474

475 **Absence of infection and histologic lesions in extrapulmonary and extraneural**
476 **tissues despite ACE2 expression.** Other tissues examined included heart, kidney,
477 stomach, duodenum, jejunum, ileum, cecum, and colon. All of these were histologically
478 within normal limits (data not shown). No SARS-CoV-2 S protein was detected in any of
479 these tissues at any time point (Table 1). ACE2 distribution was evaluated in sections of
480 the heart, stomach, small intestine, and colon. While ACE2 expression was limited to the
481 capillary vascular endothelium in the heart and glandular stomach, intense expression
482 was noted in the non-glandular mucosa of the stomach (Fig.13 A-C) and apical surface
483 of enterocytes lining the small intestinal mucosa (Fig.13 D-F). Colonic enterocytes rarely
484 expressed ACE2 (Fig. 13G-I).

485

486 **DISCUSSION**

487 The K18-hACE2 transgenic mouse model has become a widespread laboratory animal
488 model suitable for studying SARS-CoV-2 pathogenesis as well as medical
489 countermeasures against COVID-19 (20). The suitability of this model relies on the
490 common host entry receptor shared between SARS-CoV and SARS-CoV-2 (20, 39), and
491 transgenic mice expressing hACE2 under the K18 promoter develop lethal clinical
492 disease associated with pulmonary pathology and neuroinvasion, with high viral titers (30,
493 36-38, 40, 47-49). In contrast, other murine models of SARS-CoV-2 (e.g. adenovirus-
494 transduced hACE2 mice and hACE2 knock-in mice) develop only mild disease with

495 limited and short-lived viral replication and pulmonary pathology, and low to no lethality
496 (37, 50). While the K18-hACE2 murine model has been critical in shedding light on
497 mechanisms of lung injury and dysfunction, it fails to faithfully recapitulate several key
498 histologic features of severe and lethal cases of COVID-19 in humans, such as diffuse
499 alveolar damage (DAD) with hyaline membrane formation and multi-organ failure
500 associated with hypercoagulability and widespread microthrombi formation (43, 51).

501 To better understand the pathogenesis of SARS-CoV-2, well-characterized animal
502 models are critically needed (22). Even though the K18-hACE2 murine model is currently
503 under extensive use, several aspects associated with the temporospatial dynamics of
504 SARS-CoV-2 infection remain poorly characterized, including the expression and cellular
505 distribution of hACE2. In this work we further characterized pathological aspects related
506 to viral pathogenesis in this unique murine model and hypothesized that the
507 temporospatial distribution of SARS-CoV-2 and pathological outcomes following infection
508 in the K18-hACE2 murine model is partially but not solely associated with hACE2 and
509 that increased lethality in this model is related to neuroinvasion. The study presented
510 herein provides additional novel information regarding the temporal and spatial aspects
511 of SARS-CoV-2 infection in the K18-hACE2 mouse model with emphasis on pathological
512 outcomes as well as a thorough and methodical characterization of ACE2 expression in
513 this transgenic mouse model, which contributes to our understanding of this critical model
514 used for preclinical evaluation of vaccines and antiviral therapeutics. Our findings not only
515 demonstrate that lethality of this murine model is associated with neuroinvasion and
516 subsequent neuronal cytopathic effect, but that SARS-CoV-2 tropism is not solely

517 restricted to ACE2-expressing cells in K18-hACE2 mice. Thus, the neuropathogenic
518 potential of SARS-CoV-2 is dependent on other currently unknown host factors.

519 Herein, we utilized a large cohort of K18-hACE2 mice enrolled in either a 14-day natural
520 history or pre-determined serial euthanasia study to sequentially evaluate SARS-CoV-2
521 tropism and pathological alterations, spatial and temporal analysis of host factors
522 including inflammatory response and *ACE2/hACE2* expression, and several clinical
523 indices. Survival curve analysis demonstrated that lethality in infected mice only occurs
524 at or after 6 dpi, and in most mice (~94%), coincided with the initiation of neurologic signs
525 and/or symptoms, neuronal cytopathic effect, and abundance of viral S protein, RNA, and
526 infectious viral particles in the CNS. These observations clearly indicate neuroinvasion as
527 a key determinant in the fatal outcome affiliated with this model. Our study also
528 demonstrates that SARS-CoV-2 has a tropism for neurons within the spinal cord
529 (predominantly within the cervicothoracic segments), which was only observed in mice
530 with severe concurrent brain involvement. This observation could reflect descending
531 progression originating from the brain, or alternatively axonal transport via motor or
532 sympathetic sensory fibers. Concurrent brain and spinal cord disease rationalize the
533 neurologic signs observed with this model, which included decreased
534 mobility/responsiveness and decreased urine voiding, reflective of severe urinary bladder
535 dilation and accumulation of concentrated urine. Given the spinal cord involvement, the
536 latter is potentially attributed to altered spinal reflexes and/or decreased intervention of
537 the detrusor muscle, which is required for normal micturition. An additional striking clinical
538 feature in infected K18-hACE2 mice at 7 dpi was hypothermia, which is likely a
539 consequence of hypothalamic (controls thermoregulation) and generalized neuronal

540 dysfunction associated with SARS-CoV-2 neurotropism and serves as a clear clinical
541 indicator of CNS involvement in this model. Our results unequivocally demonstrate that
542 neuroinvasion is a major driver of fatality in this animal model compared to others such
543 as Syrian hamsters, which display more severe pulmonary disease and infection of the
544 ONE but lack evidence of neuroinvasion (52). Furthermore, these animals invariably
545 recover within 14 days following intranasal infection with SARS-CoV-2 (24, 29, 52-54).
546 Very few infected K18-hACE2 mice (2/30) from our survival curve study (14 dpi) survived
547 and, while residual pulmonary inflammation was observed, these animals did not exhibit
548 any evidence of neuroinvasion. Uniquely, both survivors developed pulmonary interstitial
549 aggregates of B lymphocytes which were not observed at earlier time points, suggestive
550 of the development of protective adaptive humoral response further supported by
551 presence of neutralizing antibodies in these two animals, when compared to naïve
552 animals. The absence of any overt neurological clinical signs, normal histologic
553 appearance of the CNS and, absence of detectable SARS-CoV-2 protein or RNA in the
554 two surviving mice supports the notion that animals can either fully recover from a milder
555 form of neuroinvasion, or more likely, that in rare instances neuroinvasion fails to occur
556 for unknown reasoning. Furthermore, we acknowledge that extensive neurobehavior
557 testing, which is beyond the expertise of the authors, would be required to rule out any
558 long-term neurological sequelae in the rare instance of survivors. Overall, these findings
559 are of importance to researchers with a particular interest in studying SARS-CoV-2-
560 associated neuropathogenesis, as premature euthanasia due to other clinical features
561 (i.e., weight loss, ruffled fur, and/or respiratory distress) have the potential to precede
562 CNS disease. Such terminal endpoints, if elected, may preclude evaluation of the effects

563 of SARS-CoV-2 in the CNS. Instead, decreased responsiveness/mobility, tremors, and
564 hypothermia should be interpreted to reflect better clinical findings supportive of
565 neuroinvasive disease.

566 To date, the precise mechanism(s) enabling neuroinvasion in the K18-hACE2 model is
567 poorly understood (11, 13, 15, 16, 52). Here, we determined that K18-hACE2 transgenic
568 mice show a significant upregulation in the expression of ACE2 in the nasal cavity
569 compared to wild-type C57BL/6J mice, in which ACE2 expression is undetectable by IHC.
570 This difference between K18-hACE2 and C57BL/6J mice is clearly attributed to the
571 expression of the *hACE2* transgene and is a key feature to the neuropathogenesis of this
572 model. Interestingly, temporal analysis of SARS-CoV-2 S protein and RNA in the ONE of
573 transgenic mice preceded and/or occurred simultaneously with infection of neurons within
574 the glomerular and mitral layers of the olfactory bulb, supporting axonal transport through
575 the cribriform plate as a primary portal of neuroinvasion. Expression of *hACE2* within
576 neurons in the CNS is overall low and does not directly correlate with our
577 immunohistochemical findings, where ACE2 protein was restricted to capillary
578 endothelium, ependymal and choroid epithelium with sparing of neurons and their
579 processes. These findings suggest the ACE2 expression in these anatomical
580 compartments could be attributed to *mACE2* and/or indicative of a post-transcriptional
581 event that could be limiting neuronal expression of *hACE2*. These along with the fact that
582 *hACE2* mRNA is not abundantly and equally expressed among different neuronal
583 populations and that Purkinje cells in the cerebellum express *hACE2* mRNA but are not
584 permissive to SARS-CoV-2 infection, suggest that entry of SARS-CoV-2 into neurons is
585 likely mediated by other host receptors independent of ACE2. Alternatively,

586 overexpression at the interface of the ONE and neuronal synapses may be sufficient to
587 rationalize the severe neuroinvasion observed in this model.

588 Infection of brain organoids has been shown to be inhibited using anti-ACE2 antibodies
589 (19). However, brain organoids do not recapitulate the complexity of the entire CNS, and
590 axonal transport of viral particles into the CNS can hardly be modeled *in vitro*. Altogether,
591 this suggests that while ACE2 is assuredly an important mediator of CNS neuroinvasion,
592 studying mechanisms of SARS-CoV-2 neuroinvasion likely require the use of more
593 complex experimental systems. Neuropilin-1, a transmembrane glycoprotein serving as
594 cell surface receptor for semaphorins and other ligands, as well as Tetraspanin 8
595 (TSPAN8), have recently been proposed as alternative host receptors for SARS-CoV-2
596 entry (55, 56). Even though we analyzed the expression of neuropilin-1 in this study (data
597 not shown), we observed ubiquitous expression in the nasal passages, brain, kidneys,
598 liver, and lungs, precluding any definitive conclusions in support or against these claims
599 (55).

600 Anosmia and ageusia (loss of smell and taste, respectively) represent the earliest and
601 most common but transient neurologic symptoms in people with COVID-19, being
602 reported in $\geq 50\%$ of cases (12, 13, 17). Hyposmia or anosmia has also been clearly
603 characterized in K18-hACE2 mice, occurring between 2-3 dpi, which was characterized
604 through a series of unique behavioral tests requiring a normal sense of smell (38). Other
605 neurologic manifestations of COVID-19 have been attributed to acute cerebrovascular
606 disease, with cohort studies reporting strokes in 2–6% of hospitalized patients (7, 13).
607 Long-term neurologic sequelae associated with COVID-19 or its effect on
608 neurodegenerative diseases remain unclear (7). Very little is known about the

609 pathogenesis of these neurologic manifestations and whether they are directly or
610 indirectly associated with SARS-CoV-2. ACE2 expression has been described in humans
611 both in health and with chronic rhinosinusitis, with expression noted in sustentacular cells
612 of the ONE, but not within immature and mature olfactory neurons (57). This observation
613 led the authors to suggest that anosmia in COVID-19 is likely attributable to an indirect
614 effect of SARS-CoV-2 infection. However, recent studies evaluating the brain and nasal
615 autopsies from patients who died of COVID-19, detected SARS-CoV-2 protein and RNA
616 in cells of neural origin within the ONE and cortical neurons occasionally associated with
617 locally ischemic regions (18, 19). These studies provide evidence that the K18-hACE2
618 mice could have translational significance, even though ischemic lesions have not been
619 reported including results from our study. Even though SARS-CoV-2 infects sustentacular
620 cells within the neuroepithelium of Syrian hamsters (52), the K18-hACE2 and a transgenic
621 mice expressing hACE2 under the HFH4 promoter are the only published models that
622 consistently develop neuroinvasion with wild-type virus and, thus, will be particularly
623 useful for studying SARS-CoV-2 neuropathogenesis, particularly the mechanisms of viral
624 trafficking of into the CNS through the ONE (35).

625 Another important observation of the K18-hACE2 model is that SARS-CoV-2 tropism
626 extensively involves infection of ACE2 and *hACE2* negative cells, including certain
627 population of neurons and the vast majority of AT1 pneumocytes. Similarly, sole
628 expression of *hACE2* in some cell types (i.e., CNS capillaries and bronchiolar epithelial
629 cells) clearly does not render these cells susceptible to SARS-CoV-2 even following
630 intranasal exposure and underscores the notion that other undetermined host factors are

631 likely required to allow viral entry. Therefore, this model is relevant for investigating the
632 role of alternative ACE2-independent entry mechanisms.

633 In conclusion, this study provides a comprehensive spatiotemporal analysis of SARS-
634 CoV-2 infection in the K18-hACE2 transgenic murine model along with an analysis of the
635 contribution of ACE2 in the permissiveness of the model. Our work provides extensive
636 evidence that SARS-CoV-2 exhibits a marked neurotropism that is associated with
637 lethality, and that this process likely occurs through mechanisms that are in part hACE2-
638 independent. Although we documented significant reactive microgliosis and astrogliosis
639 in terminal neuroinvasive disease, the exact role and molecular determinants of these
640 observations, and their role in neuronal injury of the K18hACE2 model warrants further
641 research. Lethal CNS invasion, combined with the absence of severe pulmonary
642 hallmarks associated with lethal COVID-19, therefore calls for attentive caution when
643 utilizing the K18-hACE2 mouse model to investigate certain aspects of SARS-CoV-2
644 pulmonary pathogenesis. Furthermore, due to the acute and fulminant neuroinvasion of
645 this model, the protective ability of anti-viral therapies and T-cell based vaccines against
646 lethal challenge in this model might indeed be underestimated, which is reflected in
647 several studies that have utilized terminal timepoints preceding neuroinvasion as their
648 efficacy endpoints (58-60). Regardless, the K18-hACE2 mouse model represents a
649 promising model for understanding the mechanisms governing SARS-CoV-2
650 neuroinvasion, ACE2-independent virus entry, and evaluating potent and fast-acting
651 prophylactic countermeasures. Lastly, this model may serve useful in evaluating efficacy
652 of therapeutics to block development of reactive/injurious microglial and/or astrocyte
653 phenotypes if determined to play a key role in the neuronal injury observed in this model.

654

655 **MATERIALS AND METHODS**

656 **Biosafety.** All aspects of this study were approved by the Institutional Biosafety
657 Committee and the office of Environmental Health and Safety at Boston University prior
658 to study initiation. Work with SARS-CoV-2 was performed in a biosafety level-3 laboratory
659 by personnel equipped with powered air-purifying respirators.

660 **Cells and viruses.** African green monkey kidney Vero E6 cells (ATCC[®] CRL-1586[™],
661 American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's
662 minimum essential medium (DMEM; Gibco, Carlsbad, CA [#11995-065]) containing 10%
663 fetal bovine serum (FBS, ThermoFisher Scientific, Waltham, MA), 1X non-essential amino
664 acids (ThermoFisher Scientific), penicillin and streptomycin (100 U/ml and 100 µg/ml),
665 and 0.25 µg/ml of amphotericin B (Gibco[®], Carlsbad, CA), and incubated at 37 °C and
666 5% CO₂ in a humidified incubator.

667 **SARS-CoV-2 isolate stock preparation and titration.** All replication-competent SARS-
668 CoV-2 experiments were performed in a biosafety level 3 laboratory (BSL-3) at the Boston
669 University' National Emerging Infectious Diseases Laboratories. 2019-nCoV/USA-
670 WA1/2020 isolate (NCBI accession number: MN985325) of SARS-CoV-2 was obtained
671 from the Centers for Disease Control and Prevention (Atlanta, GA) and BEI Resources
672 (Manassas, VA). To generate the passage 1 (P1) virus stock, Vero E6 cells, pre-seeded
673 the day before at a density of 10 million cells, were infected in T175 flasks with the master
674 stock, diluted in 10 ml final volume of Opti-MEM (ThermoFisher Scientific). Following virus
675 adsorption to the cells at 37 °C for 1 h, 15 ml DMEM containing 10% FBS and 1X
676 penicillin/streptomycin was added to the flask. The next day, media was removed, the cell

677 monolayer was rinsed with 1X phosphate buffered saline (PBS) pH 7.5 (ThermoFisher
678 Scientific) and 25 ml of fresh DMEM containing 2% FBS was added. Two days later, when
679 the cytopathic effect of the virus was clearly visible, culture medium was collected, filtered
680 through a 0.2 μm filter, and stored at $-80\text{ }^{\circ}\text{C}$. Our P2 working stock of the virus was
681 prepared by infecting Vero E6 cells with the P1 stock, at a multiplicity of infection (MOI)
682 of 0.1. Cell culture media was harvested at 2 and 3 dpi, and after the last harvest,
683 ultracentrifuged (Beckman Coulter Optima L-100k; SW32 Ti rotor) for 2 h at 25,000 rpm
684 ($80,000\text{ }X\text{ }g$) over a 20% sucrose cushion (Sigma-Aldrich, St. Louis, MO). Following
685 centrifugation, the media and sucrose were discarded, and pellets were left to dry for 5
686 minutes at room temperature. Pellets were then resuspended overnight at $4\text{ }^{\circ}\text{C}$ in 500 μl
687 of 1X PBS. The next day, concentrated virions were aliquoted and stored at $-80\text{ }^{\circ}\text{C}$.
688 The titer of our viral stock was determined by plaque assay. Vero E6 cells were seeded
689 into a 12-well plate at a density of 2.5×10^5 cells per well and infected the next day with
690 serial 10-fold dilutions of the virus stock for 1 h at $37\text{ }^{\circ}\text{C}$. Following virus adsorption, 1 ml
691 of overlay media, consisting of 2X DMEM supplemented with 4% FBS and mixed at a 1:1
692 ratio with 1.2% Avicel (DuPont; RC-581), was added in each well. Three days later, the
693 overlay medium was removed, the cell monolayer was washed with 1X PBS and fixed for
694 30 minutes at room temperature with 4% paraformaldehyde (Sigma-Aldrich). Fixed cells
695 were then washed with 1X PBS and stained for 1h at room temperature with 0.1% crystal
696 violet (Sigma-Aldrich) prepared in 10% ethanol/water. After rinsing with tap water, the
697 number of plaques were counted, and the virus titer was calculated. The titer of our P2
698 virus stock was 4×10^8 plaque forming units (PFU)/ml.

699 **Recombinant SARS-CoV-2 NanoLuciferase stock.** Recombinant SARS-CoV-2 virus
700 expressing a NanoLuciferase reporter (rSARS-CoV-2 NL) (61) was generously provided
701 by the Laboratory of Pei-Yong Shi. A day prior to propagation 10 million Vero E6 cells
702 were seeded in a T-175 flask. To grow virus, 10 μ l of rSARS-CoV-2 NL virus stock was
703 diluted in 10 ml of OptiMEM media (ThermoFisher Scientific, #51985091) and then added
704 to cells. Virus was incubated on cells for 1 hour at 37°C then 15 mL of DMEM containing
705 10% FBS and 1% penicillin/streptomycin was added. The morning after infection, media
706 was removed, cells were washed once with 1X PBS and 25 ml of fresh DMEM containing
707 2% FBS and 1% penicillin/streptomycin was added to the flask. Virus was incubated for
708 an additional 48 hours, supernatant was collected, filtered through a 0.22 μ M filter, and
709 stored at -80°C. To concentrate virus, the stock was thawed and concentrated by
710 ultracentrifugation (Beckman Coulter Optima L-100k; SW32 Ti rotor) at 25,000 x g for 2
711 hours at 4 °C on a 20% sucrose cushion (Sigma-Aldrich, St. Louis, MO). Media and
712 sucrose were decanted, pellets were allowed to dry for 5 minutes at room temperature,
713 then viral pellets were suspended in 100 μ l of 1X PBS and left at 4 °C overnight. The next
714 day, concentrated virus was aliquoted and stored at -80 °C.

715 **Mice.** Mice were maintained in a facility accredited by the Association for the Assessment
716 and Accreditation of Laboratory Animal Care (AAALAC). All protocols were approved by
717 the Boston University Institutional Animal Care and Use Committee (PROTO202000020).
718 Heterozygous K18-hACE2 C57BL/6J mice of both sexes (strain: 2B6.Cg-Tg(K18-
719 ACE2)2PrImn/J) were obtained from the Jackson Laboratory (Jax, Bar Harbor, ME).
720 Animals were group-housed by sex in Tecniplast green line individually ventilated cages

721 (Tecniplast, Buguggiate, Italy). Mice were maintained on a 12:12 light cycle at 30-70%
722 humidity and provided ad-libitum water and standard chow diets (LabDiet, St. Louis, MO).
723 **Intranasal inoculation with SARS-CoV-2.** At 4 months of age, K18-hACE2 mice of both
724 sexes were intranasally inoculated with 1×10^6 PFU of SARS-CoV-2 in 50 μ l of sterile 1X
725 PBS (n=61 [n=34 male and n=27 female], or sham inoculated with 50 μ l of sterile 1X PBS
726 (n=3; female). Inoculations were performed under 1-3% isoflurane anesthesia. Thirty-
727 eight of these animals were enrolled in a 14-day survival curve study. For histologic
728 analysis, twenty-six animals were examined (n=15 male and n=11 female), which
729 included three female Sham/PBS inoculated controls and predetermined euthanasia
730 timepoints at 2 and 4 dpi prior to animals reaching euthanasia criteria.

731 **Clinical monitoring.** Animals included in the 14-day survival curve study were
732 intraperitoneally implanted with an RFID temperature-monitoring microchip (Unified
733 Information Devices, Lake Villa, IL, USA) 48-72 hours prior to inoculation. An IACUC-
734 approved clinical scoring system was utilized to monitor disease progression and
735 establish humane endpoints (Table 2). Categories evaluated included body weight,
736 general appearance, responsiveness, respiration, and neurological signs for a maximum
737 score of 5. Animals were considered moribund and humanely euthanized in the event of
738 the following: a score of 4 or greater for 2 consecutive observation periods, weight loss
739 greater than or equal to 20%, severe respiratory distress, or lack of responsiveness.
740 Clinical signs and body temperature were recorded once per day for the duration of the
741 study. For design of the survival curve, animals euthanized on a given day were counted
742 dead the day after. Animals found dead in cage were counted dead on the same day.

743 **In vivo 3D-imaging and analysis.** K18-hACE2 mice were infected with 1×10^6 PFU of
744 SARS-CoV-2 NL in 50 μ l of 1X PBS via intranasal inoculation. To image, mice were
745 administered two, 75 μ l subcutaneous injections of 1X PBS containing 0.65 μ M
746 Fluorofurimazine (FFz) substrate (Promega) for a total of 1.3 μ M FFz per mouse. Mice
747 were then imaged using a 3D-imaging mirror gantry isolation chamber (InVivo Analytics)
748 and an IVIS spectrum imager (PerkinElmer). To perform imaging, mice were anesthetized
749 with 2.5% isoflurane, placed into a body conforming animal mold (BCAM) (InVivo
750 Analytics), and then imaged within 5 minutes of FFz injection. Images were acquired
751 using a sequence imaging as followed; 60 seconds (s) open filter, 240 s 600 nm, 60 s
752 open, 240 s 620 nm, 60 s open, 240 s 640 nm, 60 s open, 240 s 660 nm, 60 s open, 680
753 nm, 60 s open. Data analysis was performed using the cloud-based InVivoPlot software
754 (InVivo Analytics).

755 **Tissue processing and viral RNA isolation.** Tissues were collected from mice and
756 stored in 600 μ l of RNA $later$ (Sigma-Aldrich; # R0901500ML) and stored at -80 $^{\circ}$ C. For
757 processing, 20 – 30 mg of tissue were placed into a 2 ml tube with 600 μ l of RLT buffer
758 with 1% β -mercaptoethanol and a 5 mm stainless steel bead (Qiagen, Valencia, CA;
759 #69989). Tissues were then dissociated using a Qiagen TissueLyser II (Qiagen) with the
760 following cycle parameters: 20 cycles/s for 2 min, 1 min wait, 20 cycles/s for 2 min.
761 Samples were centrifuged at 17,000 X g (13,000 rpm) for 10 minutes and supernatant
762 was transferred to a new 1.5 ml tube. Viral RNA isolation was performed using a Qiagen
763 RNeasy Plus Mini Kit (Qiagen; #74134), according to the manufacturer's instructions, with
764 an additional on-column DNase treatment (Qiagen; #79256). RNA was finally eluted in
765 30 μ l of RNase/DNase-free water and stored at -80 $^{\circ}$ C until used.

766 **Quantification of infectious particles by plaque assay.** Quantification of SARS-CoV-
767 2 infectious particles were quantified by plaque assay. After euthanizing mice, tissues
768 were collected in 600 μ L of RNA $later$ (ThermoFisher Scientific, AM7021) and stored at -
769 80 C until analysis. The day prior to experiments, 24-well plates containing 8×10^4 Very
770 E6 cells per well were plated. Between 20-40 mg of tissue was weighed out and placed
771 into a 2 ml tube containing 500 μ l of OptiMEM (ThermoFisher) and a 5mm Steal Bead
772 (Qiagen #69997). Tissues were then homogenized using a Qiagen TissueLyser II
773 (Qiagen; Germantown, MD) by two dissociations cycles (two-minutes at 1,800
774 oscillations/minute) with a one-minute rest in between. Samples were then subject to
775 centrifugation with a benchtop centrifuge at 13,000 rpm for 10 minutes and supernatant
776 was transferred to a new 1.5 ml tube. From this, 1:10 – 1:10⁶ dilutions were made in
777 OptiMEM and 200 μ l of each dilution were plated onto 24-well plates. Media was
778 incubated at 37 °C for 1 hour with gentle rocking of the plate every 10 minutes. After viral
779 adsorption, 800 μ l of a 1:1 mixture of 2X DMEM containing 4% FBS 1%
780 penicillin/streptomycin and 2.4% Avicel (Dupont) was overlaid into each well. Cells were
781 then incubated for 72 hours at 37°C with 5% CO₂. After incubation, Avicel was removed,
782 cells were washed with 1X PBS, and cells were fixed in 10% formalin for 1 hour. After
783 fixation, formalin was removed, cells were stained with 0.1% crystal violet in 10%
784 ethanol/water for 30 minutes and washed with tap water. Plates were then dried, the
785 number of plaques were counted, and infectious particles (PFU/mg of tissue) were
786 calculated.

787 **RNA isolation from serum.** Total viral RNA was isolated from serum using a Zymo
788 Research Corporation Quick-RNA™ Viral Kit (Zymo Research, Tustin, CA; #R1040)

789 according to the manufacturer's instructions. RNA was eluted in 15 µl of RNase/DNase-
790 free water and stored at -80 °C until used.

791 **SARS-CoV-2 E-specific reverse transcription quantitative polymerase chain**
792 **reaction (RT-qPCR).** Viral RNA was quantitated using single-step RT-quantitative real-
793 time PCR (Quanta qScript One-Step RT-qPCR Kit, QuantaBio, Beverly, MA; VWR;
794 #76047-082) with primers and TaqMan® probes targeting the SARS-CoV-2 E gene as
795 previously described (62). Briefly, a 20 µl reaction mixture containing 10 µl of Quanta
796 qScript™ XLT One-Step RT-qPCR ToughMix, 0.5 µM Primer E_Sarbeco_F1
797 (ACAGGTACGTTAATAGTTAATAGCGT), 0.5 µM Primer E_Sarbeco_R2
798 (ATATTGCAGCAGTACGCACACA), 0.25 µM Probe E_Sarbeco_P1 (FAM-
799 AACTAGCCATCCTTACTGCGCTTCG-BHQ1), and 2 µl of template RNA was
800 prepared. RT-qPCR was performed using an Applied Biosystems QuantStudio 3
801 (ThermoFisher Scientific) and the following cycling conditions: reverse transcription for
802 10 minutes at 55 °C, an activation step at 94 °C for 3 min followed by 45 cycles of
803 denaturation at 94 °C for 15 seconds and combined annealing/extension at 58 °C for 30
804 seconds. Ct values were determined using QuantStudio™ Design and Analysis software
805 V1.5.1 (ThermoFisher Scientific). For absolute quantitation of viral RNA, a 389 bp
806 fragment from the SARS-CoV-2 E gene was cloned onto pIDTBlue plasmid under an SP6
807 promoter using NEB PCR cloning kit (New England Biosciences, Ipswich, MA). The
808 cloned fragment was then *in vitro* transcribed (mMessage mMachine SP6 transcription
809 kit; ThermoFisher) to generate an RT-qPCR standard.

810 **Serum infectivity assay.** One day prior to the experiment, 5×10^4 Vero E6 cells were
811 plated into a 24-well plate. Cells were then dosed with 200 µl of OptiMEM containing 20

812 μ l of serum or SARS-CoV-2 WA-isolate (MOI=0.001 [positive control]), incubated for 1
813 hour at 37°C, media was removed and fresh DMEM containing 2% FBS and 1%
814 penicillin/streptomycin was added. Cells were incubated at 37°C with 5% CO₂ for 48
815 hours, 100 μ l of supernatant was collected and RNA was extracted using a Quick-RNA
816 Viral Kit as per manufacturer' instructions (Zymo Research) for analysis by RT-qPCR.

817 **Serum neutralization assay.** One day prior to the experiment, 1×10^4 VeroE6 cells were
818 plated into a 96-well plate. Serum was de-complemented at 56°C for 30 minutes. Serum
819 was diluted 1:10 in OptiMEM and then serially diluted 2-fold for a total of ten-dilutions.
820 Serum dilutions were mixed with SARS-CoV-2 NL virus (MOI=1), incubated for 1 hour at
821 room temperature and then plated onto cells. After a 1-hour incubation at 37°C inoculum
822 was removed and 200 μ l of fresh DMEM containing 2% FBS and 1%
823 penicillin/streptomycin was added. After a 24h incubation at 37°C with 5% CO₂ media
824 was removed and cells were fixed with 10% formalin for 1 hour. A SARS-CoV-2 spike
825 neutralizing antibody (Sino Biological Inc.; 2 μ g/ μ L) was used as a positive control for
826 neutralization. After fixation formalin was removed, cells were washed with 1X PBS and
827 20 μ M furimazine (MedChem Express) luciferin substrate was added onto cells. Cells
828 were then imaged using an IVIS spectrum imager (PerkinElmer) and analyzed using
829 LivingImage software (PerkinElmer).

830 **Histology.** Animals were anesthetized with 1-3% isoflurane and euthanized with an
831 intraperitoneal overdose of ketamine and xylazine before harvest and fixation of tissues.
832 Lungs were insufflated with ~1.5mL of 1% low melting point agarose (Sigma-Aldrich)
833 diluted in 1X PBS using a 24-gauge catheter placed into the trachea. The skull cap was
834 removed and the animal decapitated and immersed in 10% neutral buffered formalin.

835 Additional tissues harvested included the heart, kidneys, and representative sections of
836 the gastrointestinal tract, which included the duodenum, jejunum, ileum, cecum, and
837 colon. Tissues were inactivated in 10% neutral buffered formalin at a 20:1 fixative to tissue
838 ratio for a minimum of 72 hours before removal from BSL-3 in accordance with an
839 approved institutional standard operating procedure. Following fixation, the whole head
840 was decalcified in Immunocal™ Decalcifier (StatLab, McKinney, TX) for 7 days before
841 performing a mid-sagittal section dividing the two hemispheres into even sections.
842 Tissues were subsequently processed and embedded in paraffin following standard
843 histological procedures. Five-micron sections were obtained and stained with
844 hematoxylin and eosin or Luxol Fast Blue (myelin stain).

845 **Immunohistochemistry and RNAscope® *in situ* hybridization.** Immunohistochemistry
846 (IHC) was performed using a Ventana BenchMark Discovery Ultra autostainer (Roche
847 Diagnostics, Indianapolis, IN). Specific IHC assay details including antibodies, protein
848 retrieval, sequence of multiplex assays, and incubation periods are found in Table 2
849 SARS-CoV-2 S was semiquantitatively scored as follows: 0, no viral protein observed; 1,
850 up to 5% positive cells per 400X field examined; 2, 5-25% positive cells per 400X field
851 examined; and 3, up to 50% positive cells per 400X field examined.

852 For SARS-CoV-2 RNAscope® ISH, an anti-sense probe targeting the spike (S; nucleotide
853 sequence: 21,563-25,384) of SARS-CoV-2, USA-WA1/2020 isolate (GenBank accession
854 number MN985325.1) was used as previously described (23, 42). The RNAscope® ISH
855 assay was performed using the RNAscope 2.5 LSx Reagent Kit (Advanced Cell
856 Diagnostics, Newark, CA) on the automated BOND RXm platform (Leica Biosystems,
857 Buffalo Grove, IL) as described previously (23). Briefly, four-micron sections of formalin-

858 fixed paraffin-embedded (FFPE) tissue was subjected to automated baking and
859 deparaffinization followed by heat-induced epitope retrieval (HIER) using a ready-to-use
860 EDTA-based solution (pH 9.0; Leica Biosystems) at 100 °C for 15 min. Subsequently,
861 tissue sections were treated with a ready-to-use protease (RNAscope® 2.5 LSx Protease)
862 for 15 min at 40 °C followed by a ready-to-use hydrogen peroxide solution for 10 min at
863 room temperature. Slides were then incubated with the ready-to-use probe mixture for 2
864 h at 40 °C, and the signal amplified using a specific set of amplifiers (AMP1 through AMP6
865 as recommended by the manufacturer). The signal was detected using a Fast-Red
866 solution for 10 minutes at room temperature. Slides were counterstained with a ready-
867 to-use hematoxylin for 5 min, followed by five washes with 1X BOND Wash Solution
868 (Leica Biosystems) for bluing. Slides were finally rinsed in deionized water, dried in a 60
869 °C oven for 30 min, and mounted with Ecomount® (Biocare, Concord, CA, USA). A SARS-
870 CoV-2-infected Vero E6 cell pellet was used as a positive assay control. For all assays,
871 an uninfected mouse was used as a negative control.

872 For *hACE2* mRNA RNAscope® ISH, an anti-sense probe targeting *hACE2* (GenBank
873 accession number NM_021804.3; Cat. No. 848038) with no cross-reactivity to murine
874 *Ace2* was used in a similar manner as described above with the exception that AMP5 and
875 AMP6 were incubated for 45 min and 30 min, respectively. Murine *peptidylprolyl*
876 *isomerase B (Ppib)* mRNA was used as a housekeeping gene to determine RNA quality
877 and a Vero E6 cell pellet was used as a positive assay control.

878 **Multispectral microscopy.** Fluorescently labeled slides were imaged using a Mantra
879 2.0™ or Vectra Polaris™ Quantitative Pathology Imaging System (Akoya Biosciences,
880 Marlborough, MA). To maximize signal-to-noise ratios, images were spectrally unmixed

881 using a synthetic library specific for the Opal fluorophores used for each assay and for
882 4',6-diamidino-2-phenylindole (DAPI). An unstained lung or brain section were used to
883 create a tissue specific autofluorescence signature that was subsequently removed from
884 whole-slide images using InForm software version 2.4.8 (Akoya Biosciences).

885 **Quantitative Image analysis of multiplex immunohistochemistry.** Digitized whole
886 slide scans were analyzed using the image analysis software HALO (Indica Labs, Inc.,
887 Corrales, NM). Slides were manually annotated to include only the brain and/or lung
888 parenchyma depending on the panel being evaluated. Visualization threshold values
889 were adjusted in viewer settings to reduce background signal and fine-tune visibility of
890 markers within each sample. For the CNS panel, area quantification (AQ) was performed
891 to determine percentages of SARS-CoV-2 Spike, Iba1 (microglia) and GFAP (astrocyte)
892 immunoreactivity. For the lung panel, we employed the HALO Highplex (HP) module
893 which allows for simultaneous analysis of multiple fluorescent markers within a cellular
894 compartment. Individual cells were identified using DAPI to segment individual nuclei.
895 Minimum cytoplasm and membrane thresholds were set for each dye to detect positive
896 staining within a cell. Parameters were set using the real-time tuning mechanism that was
897 tailored for each individual sample based on signal intensity. Phenotypes were
898 determined by selecting inclusion and exclusion parameters relating to stains of interest.
899 We used the following phenotypes: CD8+ (cytotoxic T-cells), CD20+ (B-cells), and Iba1+
900 (macrophages). The algorithm produces a quantitative output for each cell phenotype as
901 well as total cells per total area analyzed for an output of cells/ μm^2 . The AQ module was
902 also used the lung panel for quantification of SARS-CoV-2-Spike immunoreactivity.

903 **Quantitative image analysis of brightfield microscopy.** Digitized whole slide scans of
904 hematoxylin & eosin (H&E) stained k18 mouse lungs were analyzed using the Halo
905 Tissue Classifier module. TC is a train-by-example machine learning algorithm used to
906 identify dissimilar areas of tissue based on contextual features. For these lung samples,
907 a classifier was created to distinguish areas of pneumonic lung from normal stroma. The
908 classifier was run on whole lung images to determine the percentage of pneumonia.
909 Quantitative outputs are given as total classified area (mm²), normal lung area (mm²),
910 and pneumonia area (mm²). We divided pneumonic area by total classified area to
911 generate a percentage of pneumonia for statistical analysis.

912 **Transmission electron microscopy.** Tissue samples were fixed for 72 hours in a
913 mixture of 2.5% Glutaraldehyde and 2% formaldehyde in 0.1 M sodium cacodylate buffer
914 (pH 7.4). Samples were then washed in 0.1M cacodylate buffer and postfixed with
915 1% Osmiumtetroxide (OsO₄)/1.5% Potassiumferrocyanide (KFeCN₆) for 1 hour at room
916 temperature. After washes in water and 50mM Maleate buffer pH 5.15 (MB), the samples
917 were incubated in 1% uranyl acetate in MB for 1hr, washed in MB and water, and
918 dehydrated in grades of alcohol (10min each; 50%, 70%, 90%, 2x10min 100%). The
919 tissue samples were then put in propyleneoxide for 1 hr and infiltrated ON in a 1:1 mixture
920 of propyleneoxide and TAAB Epon. The following day the samples were embedded in
921 fresh TAAB Epon and polymerized at 60°C for 48 hrs. Semi-thin (0.5um) and ultrathin
922 sections (50-80nm) were cut on a Reichert Ultracut-S microtome (Leica). Semi-thin
923 sections were picked up on glass slides and stained with Toluidine blue for examination
924 at the light microscope level to find affected areas in the tissue. Ultrathin sections from
925 those areas were picked up onto formvar/carbon coated copper grids, stained with 0.2%

926 lead citrate and examined in a JEOL 1200EX transmission electron microscope
927 (JOEL, Akishima, Tokyo, Japan). Images were recorded with an AMT 2k CCD camera.

928 **Statistical analysis.** Descriptive statistics and graphics as well as Kaplan-Meier
929 (survival) curves and statistical tests were performed using GraphPad Prism v9.1.2
930 statistical analysis software (GraphPad, San Diego, CA). Clinical parameters and
931 quantitative pathology results were analyzed using a one-way ANOVA with Dunnett post-
932 hoc analysis with means of groups compared to the Sham-inoculated negative controls.
933 Viral load data were evaluated using either a one-way (serum qPCR) or two-way ANOVA
934 (tissue qPCR and PFU data) with Tukey post hoc analysis. Significance levels were set
935 at p -value <0.05 in all cases. Statistical significance on figures and supplemental figures
936 is labelled as follow: $*p\leq 0.05$, $**p\leq 0.01$, $***p\leq 0.001$, $****p\leq 0.0001$.

937

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960

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968

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1258

1259 FIG. LEGENDS

1260 **Fig. 1. SARS-CoV-2 caused lethal disease in K18-hACE2 mice.** K18-hACE2 mice
1261 (n=35) were inoculated intranasally with 1×10^6 plaque forming units (PFU). Body weight
1262 (A), clinical signs (B), temperature (C), and survival (D) were monitored daily in sham/PBS
1263 animals (black, up to 7 dpi) and in infected animals (male, red; female, blue; up to 14 dpi).
1264 Animals meeting euthanasia criteria were counted dead the following day. Viral loads
1265 (genome copy numbers/mg) or infectious virus particles (PFU/mg of tissue) were
1266 monitored in the lung and brain (E-F). RNA copies were also examined in the serum
1267 (genome copies/mL) either directly on serum (G) or via a re-infectivity assay (H) using
1268 Vero E6 cells. The limit of detection is shown with a dashed line. n=3 (sham/PBS, n=19
1269 (male), n=16 (female). One-way or two-way ANOVA. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$,
1270 **** $p \leq 0.0001$. For A-D, blue and green asterisks compare sham group vs. male group,
1271 and sham group vs. female group respectively. NA, non-applicable statistical test due
1272 extensive animal death at 6 dpi and limited n at 7 dpi.

1273 **Fig. 2. Temporal analysis of SARS-CoV-2 infection in the nasal cavity of K18-hACE2**
1274 **mice.** Histological changes, and viral protein (brown) and RNA (red) distribution and
1275 abundance were assessed in non-infected (mock: A, D, G, J) and infected mice at 2 (B,

1276 E, H ,K) and 4 (C, F, I, L) days following intranasal inoculation. At 2 dpi, suppurative
1277 rhinitis in the rostral and intermediate turbinates (B, arrow) correlated with abundant
1278 intraepithelial SARS-CoV-2 protein (E) and RNA (E, inset). Abundant viral protein and
1279 RNA were detected in the olfactory neuroepithelium (ONE, K and inset) in the absence
1280 of histologic lesions (H). At 4 dpi, only sporadically infected cells were noted in the
1281 epithelium lining the nasal turbinates and ONE (F and L, arrow and insets) in the absence
1282 of histologic lesions (C and I). Mock-infected are depicted in A, D, G and J. H&E and Fast
1283 Red (viral RNA), 200X total magnification. Bar = 100 μ m.

1284 **Fig. 3. Temporal analysis of SARS-CoV-2 infection in the lungs of K18-hACE2 mice.**

1285 Lung tissues from non-infected mice (Mock: A, B) and from infected mice at 2 (C, D), 4
1286 (E, F), 7 (G, H) and 14 (I, J) days following intranasal inoculation were analyzed. Subgross
1287 histological images of the lungs and corresponding pneumonia classifiers for each
1288 timepoint are depicted in panel K (green = normal; yellow = pneumonia). Mild to moderate
1289 interstitial pneumonia was evident starting at 2 dpi with frequently reactive blood vessels
1290 (D, arrow). At 7 dpi, alveolar type 2 (AT2) cell hyperplasia was observed (H, arrows).
1291 Residual mild pneumonia was observed in the rare animals that survived to 14 dpi, with
1292 rare sporadic lymphoid aggregates (J-arrows). H&E, 50X (A, C, E, G, and I; bar = 500
1293 μ m), 200X (B, D, F, H and J; bar = 100 μ m) and 1X (K) total magnification. One-way
1294 ANOVA; ns, non-significant.

1295 **Fig. 4. Temporal analysis of SARS-CoV-2 RNA and protein distribution in the lungs**

1296 **of K18-hACE2 mice.** Presence of viral RNA (A-E) and Spike protein (F-J) was assessed
1297 by ISH and IHC in non-infected (mock: A, F) and infected lung tissues from K18-hACE2
1298 mice at 2 (B, G), 4 (C, H), 7 (D, I) and 14 (E, J) days following intranasal inoculation.

1299 Peak viral RNA and protein occurred at 4 dpi (C,H), with an evident decline by 7 dpi (D,
1300 I). No viral RNA or protein were detected at 14 dpi (E, J). Fast Red, viral RNA (A-E) and
1301 DAB viral protein (F-J), 100X total magnification. Bar = 200 μ m. Insets (I&J), 400x total
1302 magnification.

1303 **Fig. 5. SARS-CoV-2 tropism following intranasal inoculation in K18-hACE2.** (A-C)

1304 At 4 dpi, SARS-CoV-2 (yellow) showed tropism for RAGE⁺ alveolar type 1 (AT1, magenta)
1305 and scattered SPC⁺ alveolar type 2 (AT2, red) cells (B and C, arrowheads, and arrows,
1306 respectively) but not for CD31⁺ endothelial cells. B and C represent magnification of inset
1307 1 and 2 from A, respectively. (D-F) 6 dpi, virus particles (VPs) were bound by double
1308 membrane vesicles (DMVs) in AT1 (E) and AT2 (F) cells. AT1 contained abundant
1309 caveolae. Another unique feature observed in AT1 cells was the presence of cubic
1310 membranes (CM). AT2 pneumocytes were characterized by presence of lamellar bodies.
1311 E and F represent magnification of inset 1 and 2 from D, respectively. (G, H) Viral particles
1312 were not identified in ciliated or non-ciliated club bronchiolar epithelium. H represents an
1313 inset magnification of G. Multiplex fluorescent IHC, 100x (A; bar= 100 μ m) and 200x (B,C;
1314 bar = 50 μ m) total magnification. TEM, bar = 2 μ m (B), 100 nm (B1, B2 and C1), and 3
1315 μ m (C). A, alveolar lumen; BM, basement membrane; C, capillary; Cav, caveolae; Ci,
1316 ciliated epithelium; Cl, club epithelium; CM, cubic membranes; DMVs, double-membrane
1317 vesicles; END, endothelium; J; cell-cell junction; VPs, viral particles.

1318 **Fig. 6. Temporal immunoprofiling of the pulmonary host inflammatory response to**

1319 **SARS-CoV-2.** (A-H) Quantification and 4-plex fluorescent IHC targeting SARS-CoV-2

1320 Spike (A, E-H), and macrophage Iba-1⁺ (B, E-H), CD8⁺ (C, E-H) and CD19⁺ cell (D, E-
1321 H) infiltration in the lung of sham/PBS mice and in inoculated mice (2, 4, 7 and 14 dpi). In

1322 inoculated mice, SARS-CoV-2 Spike peaked between 4-7 dpi (A, F-G). Iba-1+
1323 macrophages (red) increased significantly peaking at 7 dpi (B, G), along with a lower
1324 infiltration of CD8+ T lymphocytes-magenta that peaked between 4-7 dpi (C,F-G) while
1325 Sham/PBS mice had low residual inflammatory cells (E). CD19+ B cells arranged in
1326 clusters were only evident in the two survivors euthanized at 14 dpi (D, H). Insets depict
1327 immune cell phenotyping outputs that were applied across the entire whole slide.
1328 Multiplex fluorescent IHC (E-H): 100X and 400X (insets) total magnification, bar=50µm.
1329 I. Neutralizing activity of serum isolated from a naïve/non-infected K18-hACE2 (purple)
1330 and from the two 14 dpi survivors at 14 dpi (survivor 1 and 2, red and black respectively).
1331 An anti-SARS-CoV-2 Spike RBD antibody (anti-RBD, blue) was used as a positive
1332 control. Serum was serially diluted by 2-fold. One-way ANOVA. ** $p \leq 0.01$.

1333 **Fig. 7. Temporal neuronal damage in K18-hACE2 mice following intranasal**
1334 **inoculation with SARS-CoV-2.** Histologic changes in the cerebrum (A, D, G, J, M) or
1335 olfactory bulb (B, E, H, K, N) in non-infected or infected K18-hACE2 mice. SARS-CoV-2
1336 Spike protein was also probed in the olfactory bulb of non-infected and infected K18-
1337 hACE2 mice by IHC (C, F, I, L, O). SARS-CoV-2 protein (brown) was evident as early as
1338 4 dpi (I, arrow), but no histologic changes were noted until 7 dpi (J-K). At that time point,
1339 mild (J, arrowheads) to marked (J, inset) spongiosis with neuronal degeneration and
1340 necrosis involving multiple areas within the cerebral cortex and elsewhere were observed.
1341 Similar changes were evident in the olfactory bulb, with occasional perivascular
1342 cuffs/gliosis (K, arrowhead) and abundant viral protein (L, arrows). No histologic
1343 alterations or viral protein were detected in survivor mice euthanized at 14 dpi (M-O).

1344 H&E and DAB (viral protein), 100X (A, B, D, E, G, H, J, K, M, N; bar = 200 μ m) and 200X
1345 (C, F, I, L, O; bar = 100 μ m) total magnification.

1346 **Fig. 8. Invasion of SARS-CoV-2 into the central nervous system.** (A) Sagittal sections
1347 of the head of non-infected (mock, top panel) and infected (4 and 7 dpi, middle and bottom
1348 panel respectively) were analyzed for viral protein and RNA distribution. At 4 dpi (middle
1349 panel), SARS-CoV-2 infected neurons within the mitral layer of the olfactory bulb (1,
1350 arrow) as well as small clusters of neuronal bodies within the cerebral cortex (2, SARS-
1351 CoV-2 RNA in inset). At 7 dpi (bottom panel), SARS-CoV-2 protein was widespread along
1352 the mitral layer of the olfactory bulb (1) and throughout the central nervous system (2,
1353 SARS-CoV-2 RNA in inset) with exception of the cerebellum. EPL, external plexiform
1354 layer; GCL, granular cell layer; GL, glomerular layer; ML, mitral layer. DAB (viral protein)
1355 and Fast Red (viral RNA). 7.5X (bar = 2.5 mm) and 200X (bar = 100 μ m) total
1356 magnification. On the right of each panel, pictures labelled 1 and 2 are 266X total
1357 magnification insets represented by the hashed squares labeled in the lower (7.5X)
1358 magnification images. (B) Representative three-dimensional profile view (right side) of a
1359 K18-hACE2 mouse following inoculation with a rSARS-CoV-2 NL virus (10^6 PFU).
1360 NanoLuc bioluminescent signal was detected and quantified at 6 dpi following
1361 fluorofurimazine injection (Sub-cutaneous) using the InVivoPLOT (InVivoAx) system and
1362 an IVIS Spectrum (PerkinElmer) optical imaging instrument. Location of the lungs and
1363 brain are indicated.

1364 **Fig. 9. SARS-CoV-2 replication and assembly of virus particles in hippocampal**
1365 **neurons and glial response.** (A-C) Quantification (A) of 3-plex fluorescent IHC (B,C)
1366 targeting SARS-CoV-2 Spike, astrocyte (GFAP) and Iba-1+ microglial infiltration in the

1367 brain of sham/PBS mice and in inoculated mice (2, 4, 7 and 14 dpi). The amount of viral
1368 protein rapidly and markedly increases by 7 dpi, along with an intense astrocytic and
1369 microglial response. (D-E) Ultrastructural examination of infected neighboring
1370 hippocampal neurons illustrated cytoplasmic swelling by numerous double membrane
1371 bound vesicles (DMVs) and free virus particles. Karyolysis and global electron dense
1372 transformation of the cytoplasmic compartment was also observed indicative of neuronal
1373 necrosis (NN). E is an inset magnification from D. Multiplex IHC, 200X total magnification,
1374 bar = 100 μ m. TEM, bar = 100 nm (D) or 50nm (E). One-way ANOVA; * $p \leq 0.05$.

1375 **Fig. 10. Histological and immunohistochemical findings in the cervicothoracic**
1376 **spinal cord of SARS-CoV-2-infected K18-hACE2 mice at 7 dpi.** (A) At 7 dpi, multifocal
1377 neuronal bodies within the grey matter are shrunken, angular and hyperchromatic
1378 (neuronal degeneration and necrosis), and the neuroparenchyma has multiple clear
1379 spaces filled with small amounts of debris (spongiosis) with a slight increase in the
1380 number of glial cells (gliosis). H&E, 100X total magnification. (B) Abundant SARS-CoV-2
1381 spike protein localized within the perikaryon and processes of neurons within the spinal
1382 cord. DAB, 200X total magnification. Bar = 100 μ m. (C) No myelin loss was noted in the
1383 white matter. Luxol Fast Blue, 200X total magnification. Bar = 100 μ m.

1384 **Fig. 11. Distribution of ACE2 in lungs, nasal cavity, brain, and olfactory bulb of wild-**
1385 **type C57BL/6J and transgenic K18-hACE2 mice.** Lung (A-C), nasal
1386 (rostral/intermediate turbinates [R/I]) and olfactory epithelium (ONE) (D-F), olfactory bulb
1387 (G-I) and brain (J-L) from non-infected C57BL/6J, and from non-infected and infected (7
1388 dpi). K18-hACE2 mice were analyzed via immunohistochemistry using a cross-reactive
1389 anti-ACE2 antibody. In the lungs (A-C), ACE2 expression (brown) was mostly restricted

1390 to the apical membrane of bronchiolar epithelial cells with scattered positive AT2 cells
1391 (inset arrows). Nasal (rostral/intermediate turbinates [R/I]) and olfactory epithelium (ONE)
1392 were devoid of ACE2 in C57BL/6J mice (D) but expression was enhanced in K18-hACE2
1393 mice with intense apical expression (E and F). ACE2 expression within the olfactory bulb
1394 (G-I) and the brain (J-L) was restricted to capillary endothelium with no neuronal
1395 expression. DAB, 200X total magnification. Bar = 100 μ m.

1396 **Fig. 12. Expression and distribution of *hACE2* mRNA in the brain and lungs of**
1397 **C57BL/6J and K18-hACE2 transgenic mice via RNAscope® ISH.** (A-C) *hACE2* lung
1398 expression. While no expression of *hACE2* was noted in the lungs of wild-type C57BL/6J
1399 mice (A), *hACE2* was expressed in the bronchiolar epithelium (arrowheads) and sporadic
1400 AT2 cells (arrows) in transgenic K18-hACE2 mice (B and C), which correlated with
1401 immunohistochemical findings. (D-F) *hACE2* brain expression. *hACE2* was not
1402 expressed in the Cerebrum of C57BL/6J mice (D) but in clusters of neurons within the
1403 cerebrum (E) and hippocampus (F). Fast Red, 400X total magnification. Bar = 50 μ m.

1404 **Fig. 13. Expression of ACE2 in the gastrointestinal tract.** Immunohistochemistry was
1405 performed using a cross-reactive anti-ACE2 antibody in the stomach (A-C), small
1406 intestine (D-F) and colon (G-I). In the stomach, ACE2 expression (red) was intense in the
1407 non-glandular mucosa and capillaries of the glandular mucosa (A-C, arrows). Enterocytes
1408 lining the small intestine of C57BL/6J and K18-hACE2 mice displayed prominent apical
1409 cytoplasmic ACE2 expression (D-F). In the colon, scattered enterocytes expressed ACE2
1410 (G-I, arrows). Fast Red, 100X (A-C; bar = 200 μ m) and 200X (D-I; bar = 100 μ m) total
1411 magnification.

1412

1413

1414 **Table 1.** SARS-CoV-2 viral protein abundance in tissues derived from SARS-CoV-2-infected K18-hACE2 mice. Median
 1415 scores are represented along with ranges between brackets when applicable.

DPI	AT1/AT2	Bronchioles	Rostral turbinates	Intermediate turbinates	ONE	Olf. bulb	Brain	Spinal cord (CT)	Spinal cord (LS)	GI*	Kidneys
Mock	0	0	0	0	0	0	0	0	0	0	0
2	2 (1-2)	0	1 (0-2)	2 (1-2)	1 (1-2)	0	0	0	0	0	0
4	2 (1-3)	0	0 (0-1)	0 (0-1)	1 (0-1)	0 (0-1)	0 (0-1)	0	0	0	0
6-8	2 (1-3)	0	0	0	1 (0-1)	1 (0-2)	3 (0-3)	1 (0-2)	0 (0-1)	0	0
14	0	0	0	0	0	0	0	0	0	0	0

1416 0, no SARS-CoV-2 protein observed; 1, 0 to 5% of cells within a high magnification (400X) field are positive for viral
 1417 protein; 2, 5 to 25% of cells within a high magnification (400X) field are positive for viral protein; 3, >25 to <50% of cells
 1418 within a high magnification (400X) field are positive for viral protein. NA, not available. AT1, alveolar type 1 pneumocytes;
 1419 AT2, alveolar type 2 pneumocytes; ONE, olfactory neuroepithelium; CT, cervicothoracic segment; LS, lumbosacral
 1420 segment; GI, gastrointestinal tract.

1421 *Sections examined included stomach, small intestine (duodenum, jejunum, and ileum) and large intestine (cecum and
 1422 colon).

1423

1424 **Table 2.** Clinical scoring system used for clinical monitoring of SARS-CoV-2-infected K18-hACE2 mice.

Category	Score = Criteria
Body weight	1 = 10-19% loss
Respiration	1 = rapid, shallow, increased effort
Appearance	1 = ruffled fur, hunched posture
Responsiveness	1 = low to moderate unresponsiveness
Neurologic signs	1 = tremors

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1428 **Table 3.** Antibodies and antigen retrieval conditions for the assays performed in this study.

	Sequence	Antigen Target	Species Origin	Clone	Manufacturer	Catalog	Primary Antibody Dilution	Antigen Retrieval (Ventana)	Chromogen or Fluorophore
Assay 1	NA	SARS-CoV-2 Spike (S)	Mouse	E7U6O	Cell Signaling Technology	Pre-commercialization	1:1000	CC1 (Tris)	DAB
Assay 2	NA	Angiotensin converting enzyme 2 (ACE2)	Rabbit	EPR34435	Abcam	ab108252	1:200	CC1 (Tris)	Discovery Red and DAB
Assay 3	1	SARS-CoV-2 S	Mouse	E7U6O	Cell Signaling Technology	Pre-commercialization	1:1000	CC1 (Tris)	Opal 480
	2	Iba-1	Rabbit	Polyclonal	WAKO	019-19741	1:2000	CC2 (Citrate)	Opal 570
	3	GFAP	Rabbit	Polyclonal	DAKO	Z0334	1:500	CC1 (Tris)	Opal 690
Assay 4	1	CD8	Rabbit	D4W2Z	Cell Signaling Technology	98941	1:200	CC1 (Tris)	Opal 620
	2	SARS-CoV-2 S	Mouse	E7U6O	Cell Signaling Technology	Pre-commercialization	1:1000	CC1 (Tris)	Opal 570
	3	CD19	Rabbit	D4V4B	Cell Signaling Technology	90176	1:600	CC2 (Citrate)	Opal 520
	4	Iba-1	Rabbit	Polyclonal	WAKO	019-19741	1:2000	CC2 (Citrate)	Opal 690
Assay 5	1	RAGE	Rat	EPR21171	R&D	MAB1179q-100	1:50	CC1 (Tris)	Opal 480
	2	SARS-CoV N	Rabbit	Polyclonal	Novus biologicals	NB100-56576	1:200	CC1 (Tris)	Opal 570
	3	Prosurfactant C Protein	Rabbit	Polyclonal	Seven Hills Bioreagents	WRAB-9337	1:800	CC2 (Citrate)	Opal 690
	4	CD31	Rabbit	D8V9E	Cell Signaling Technology	77699S	1:100	CC2 (Citrate)	Opal 520

1429

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Figure 1

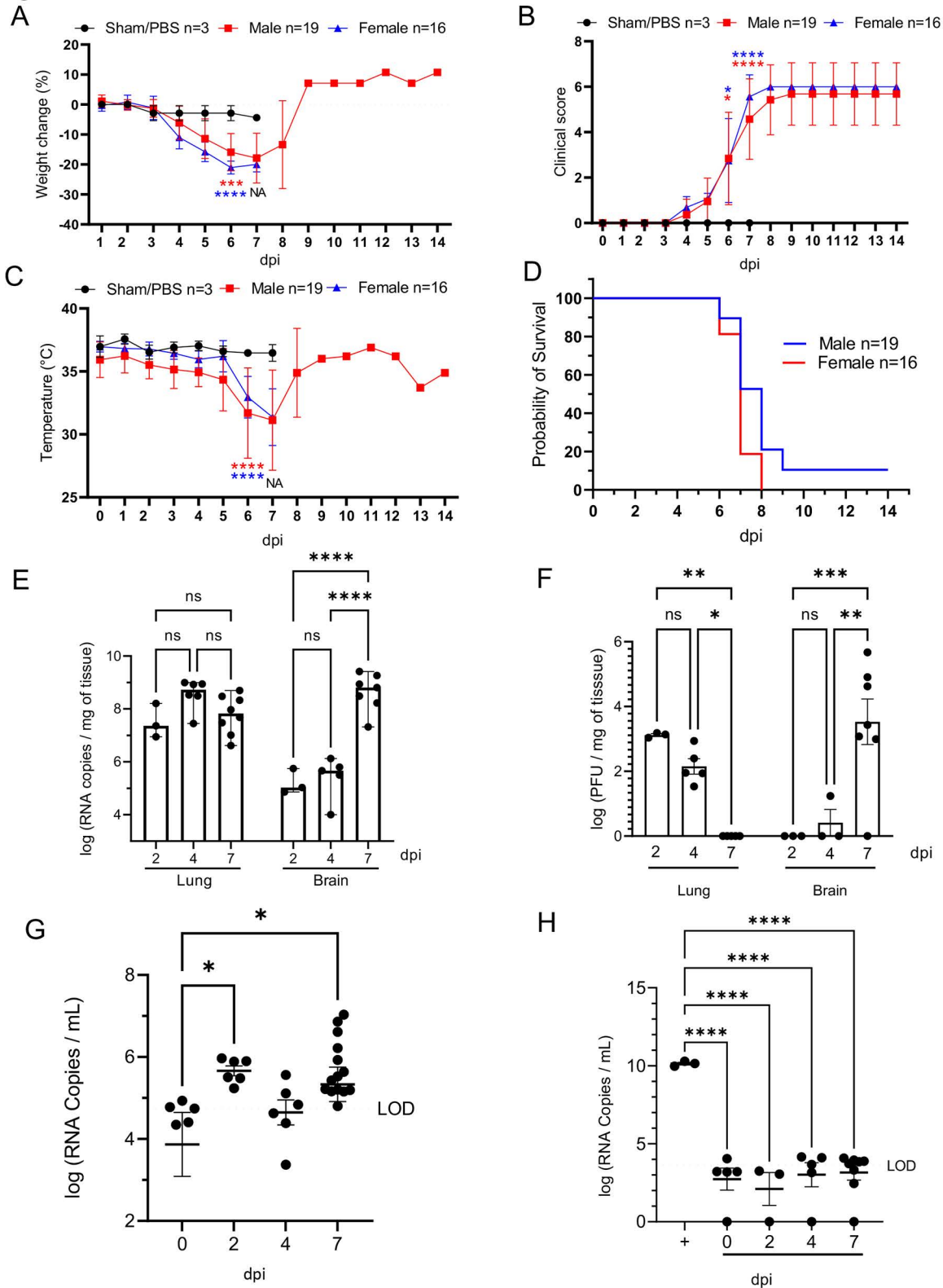
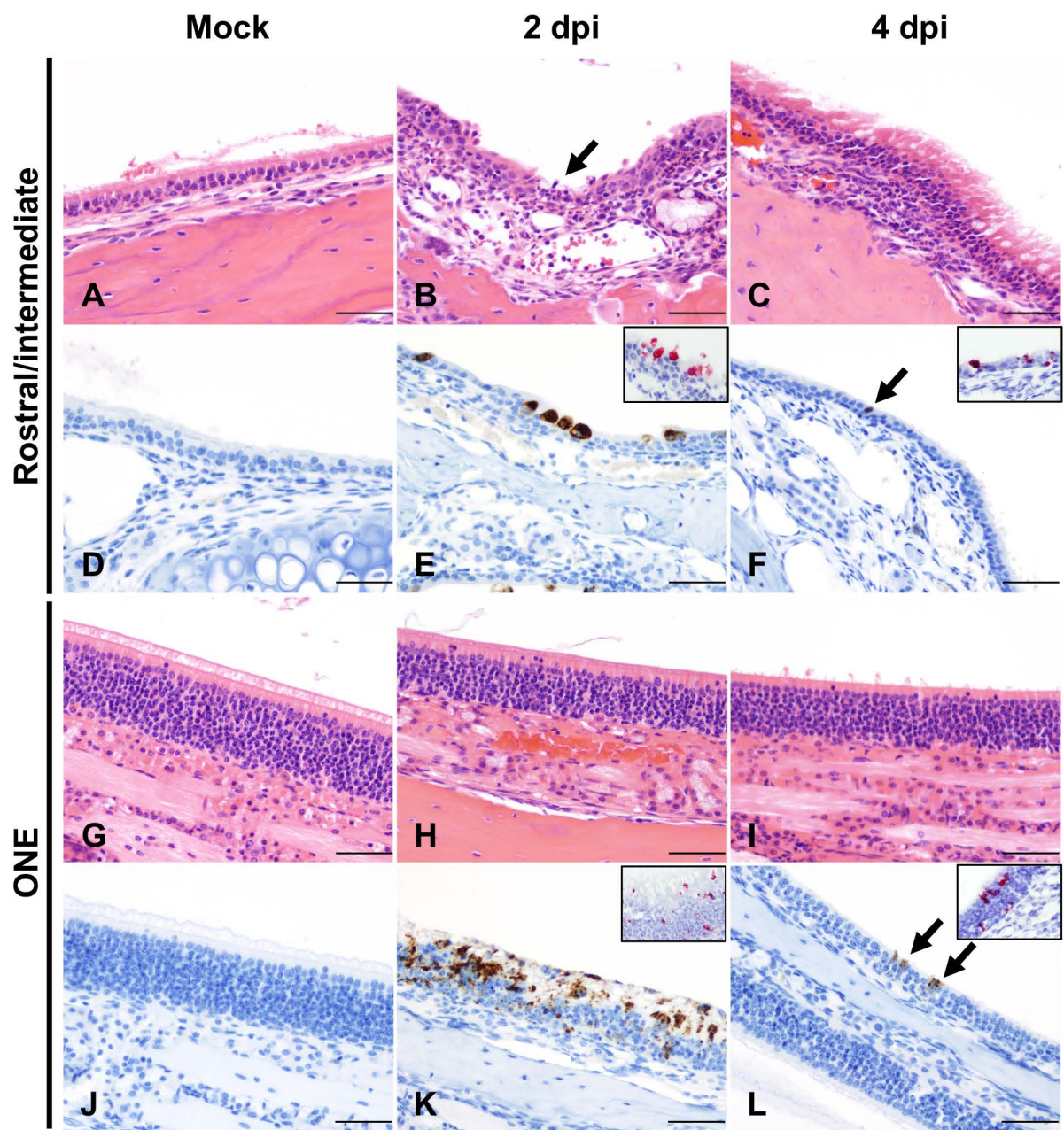


Figure 2



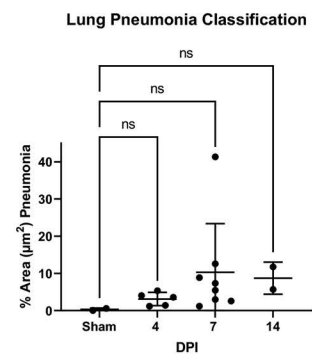
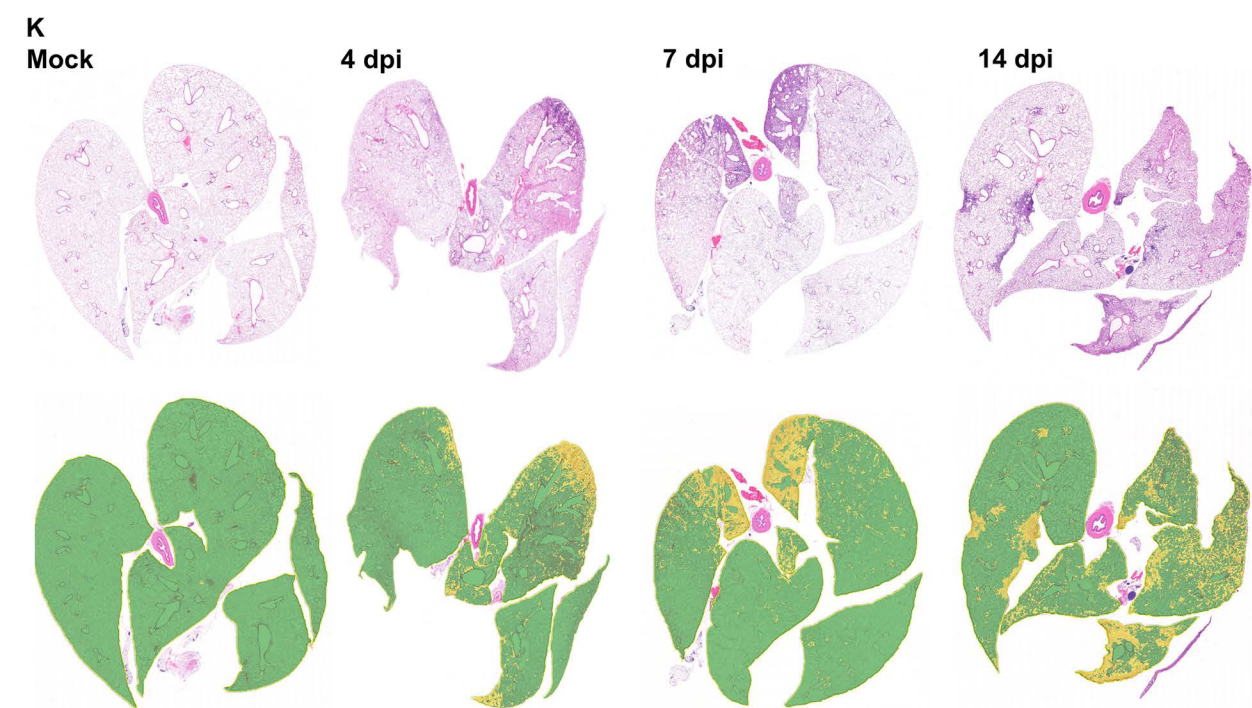
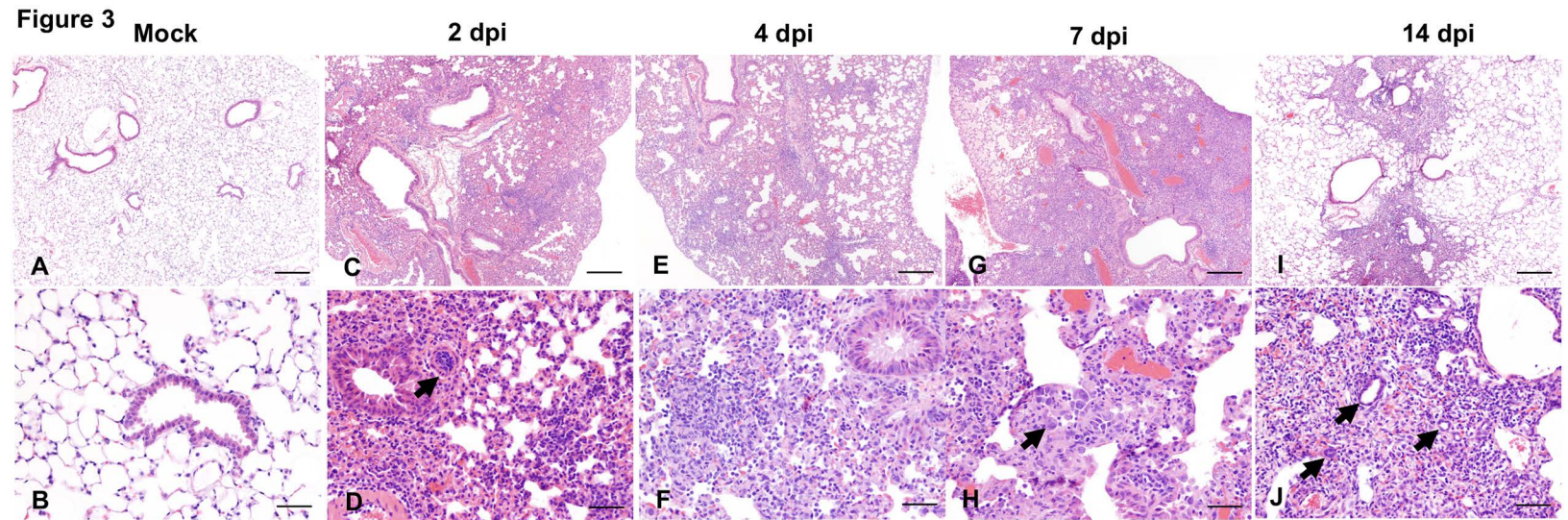


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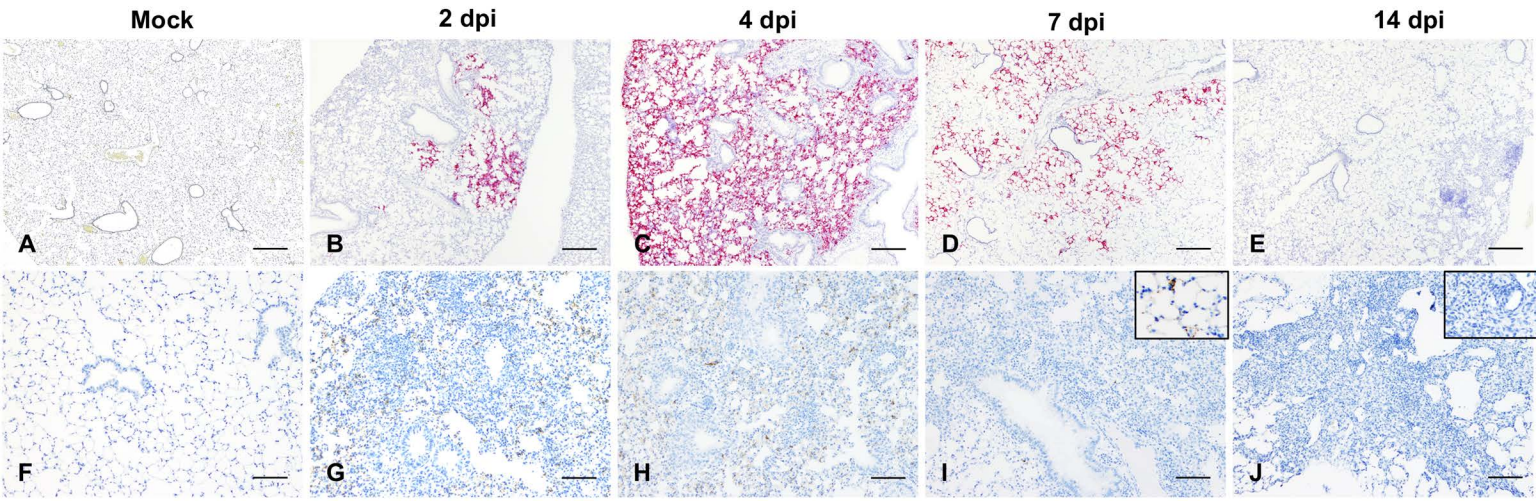


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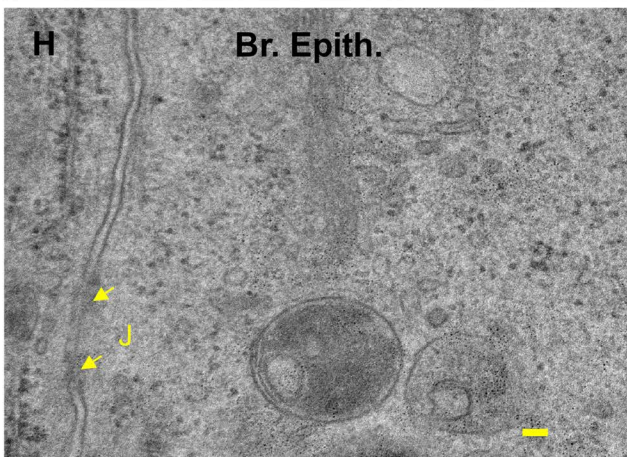
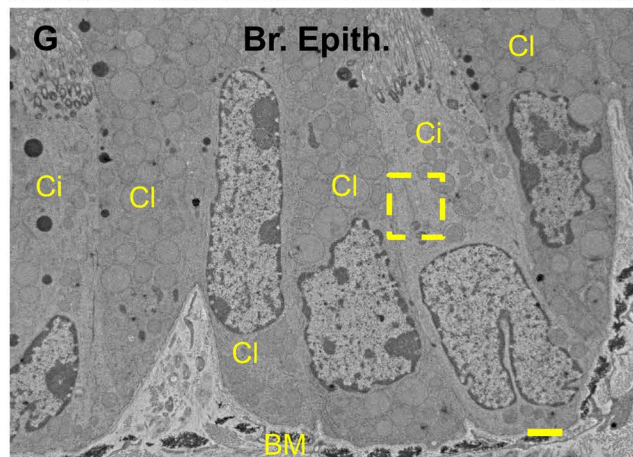
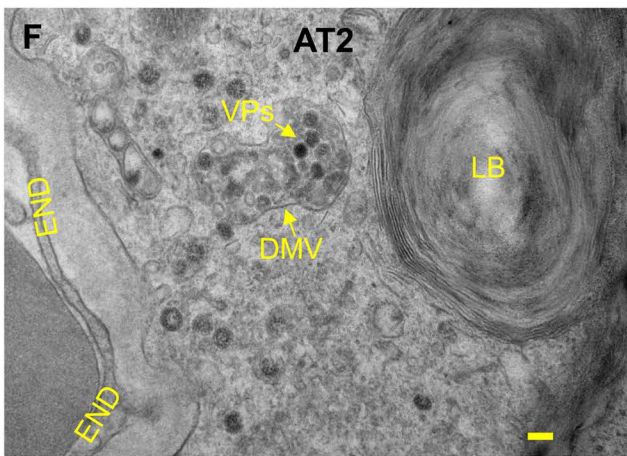
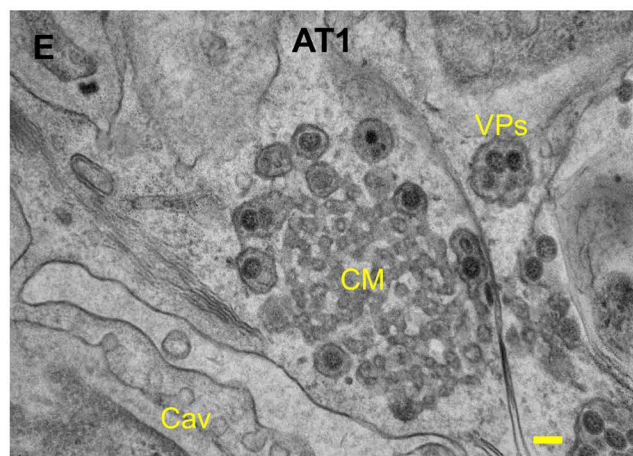
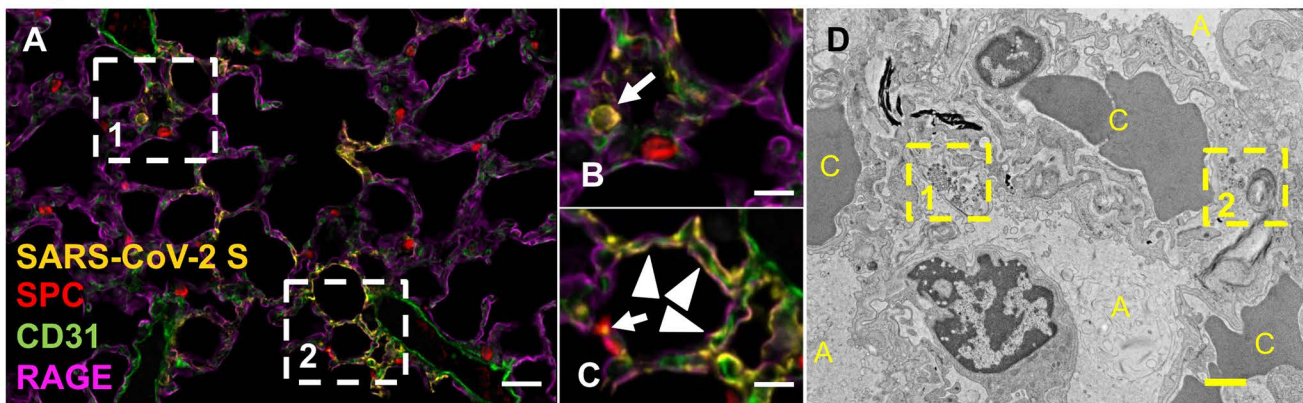


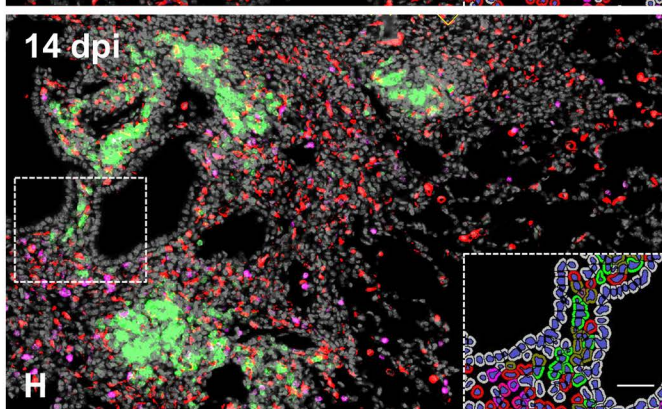
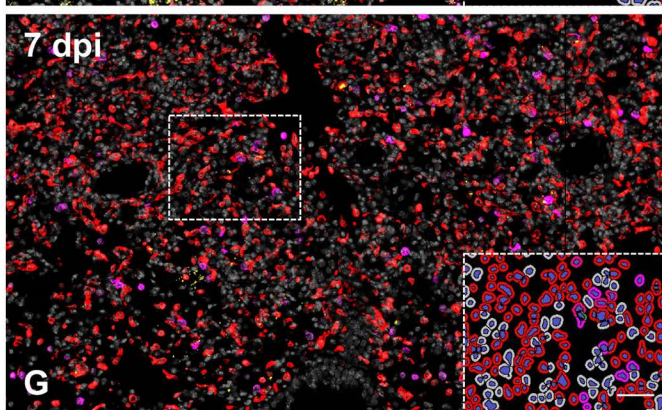
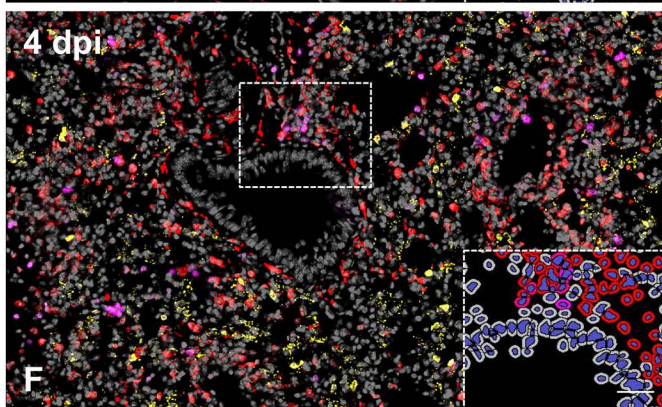
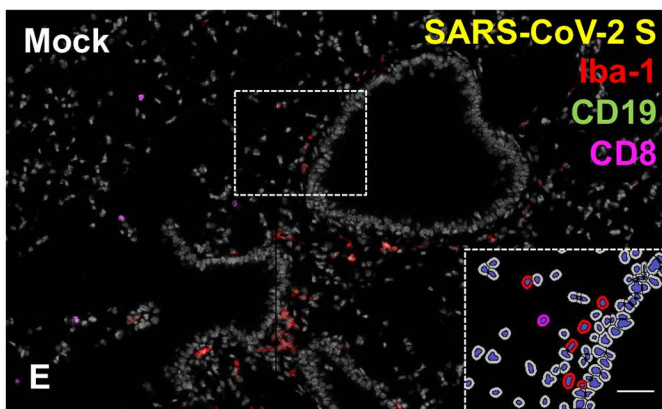
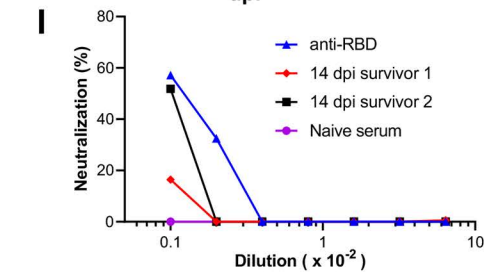
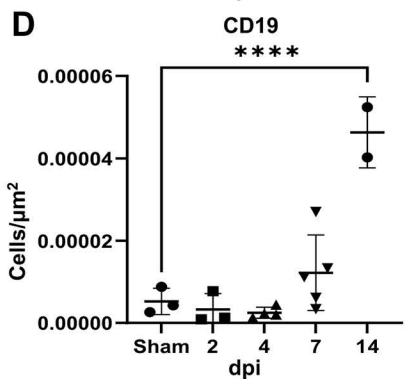
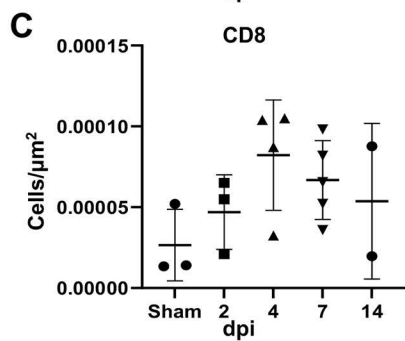
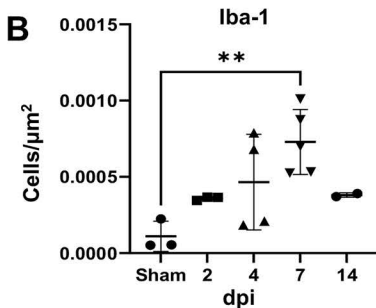
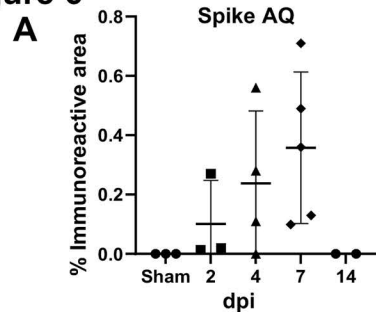
Figure 6

Figure 7 Cerebrum

Olfactory bulb

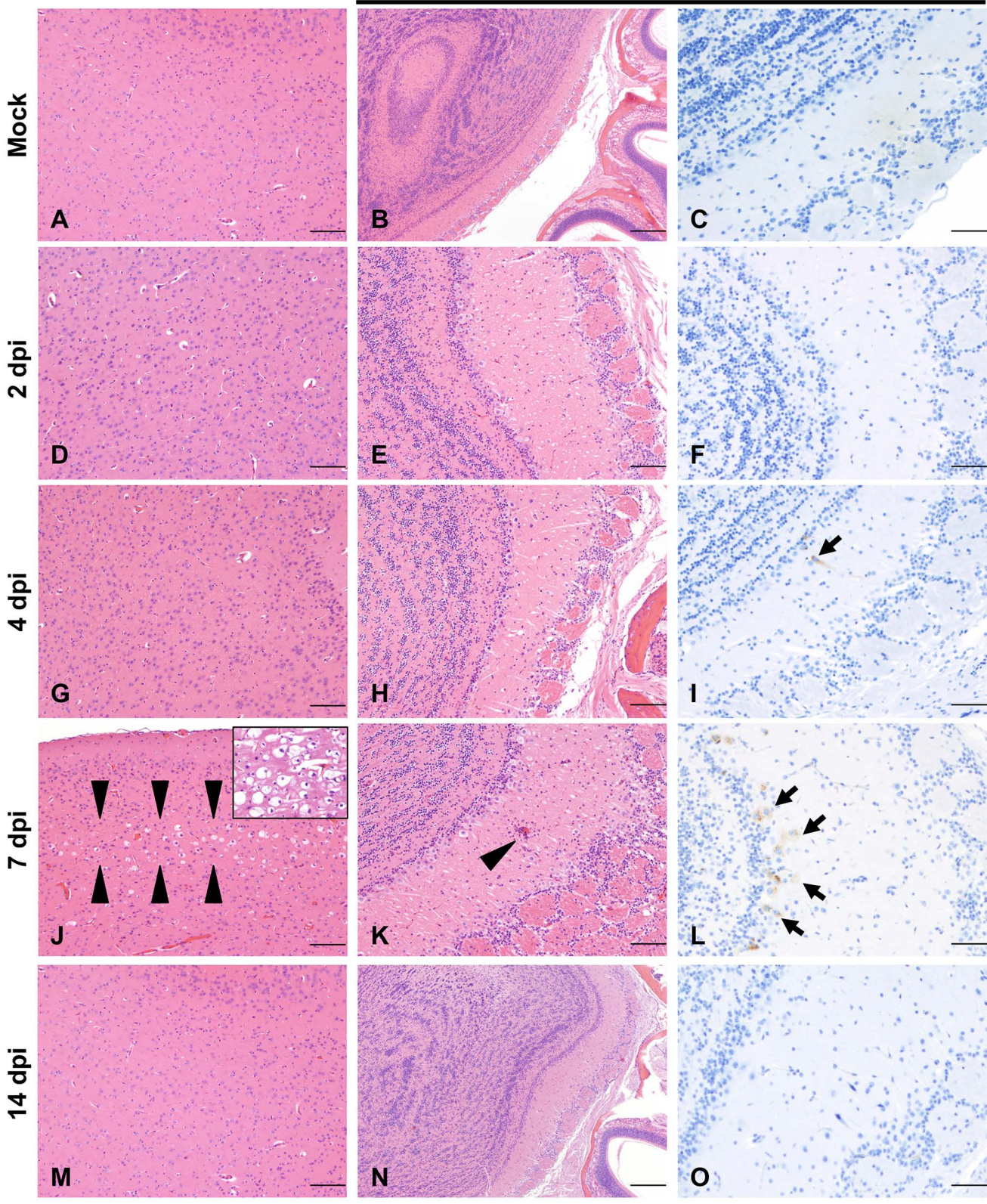


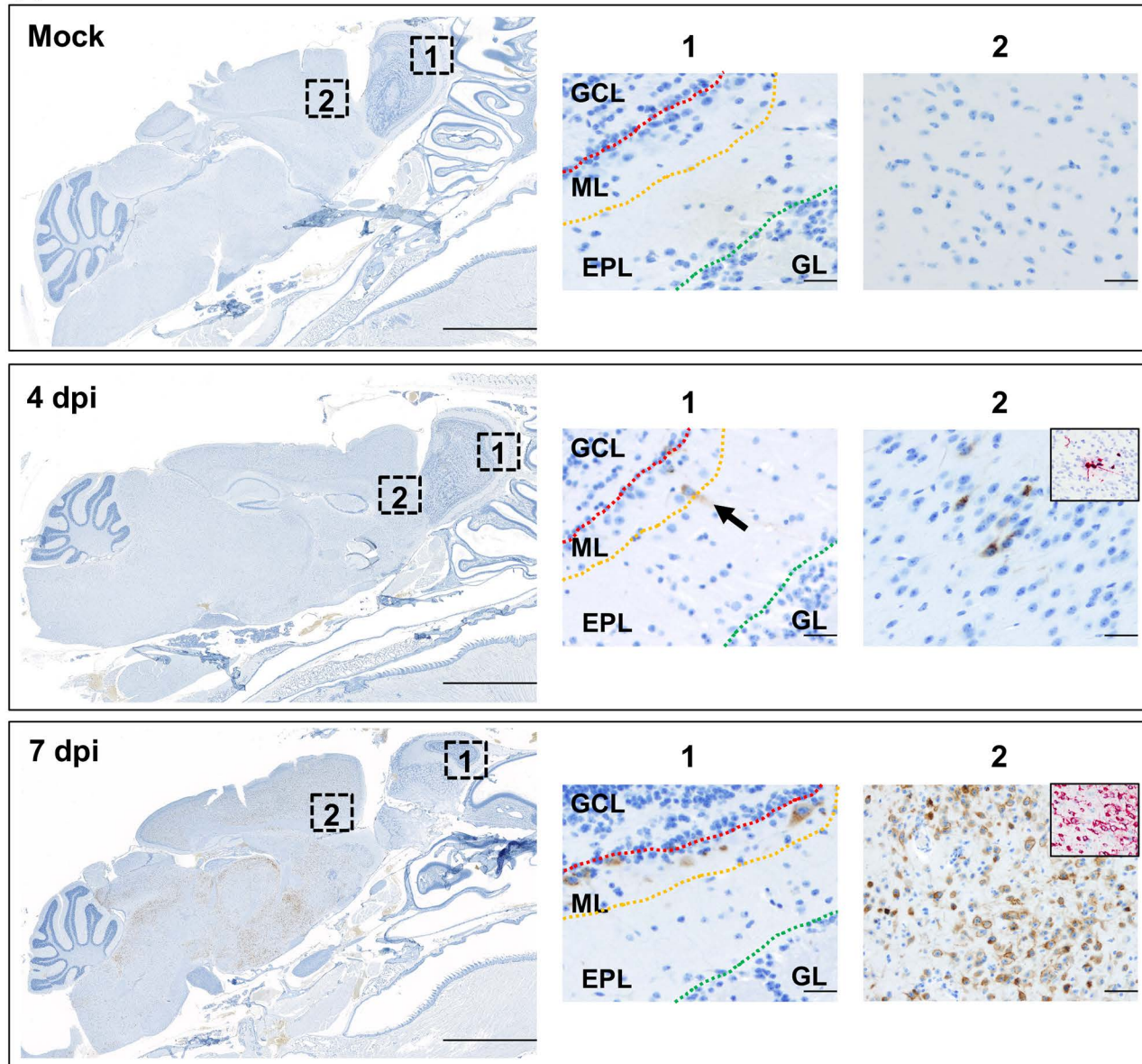
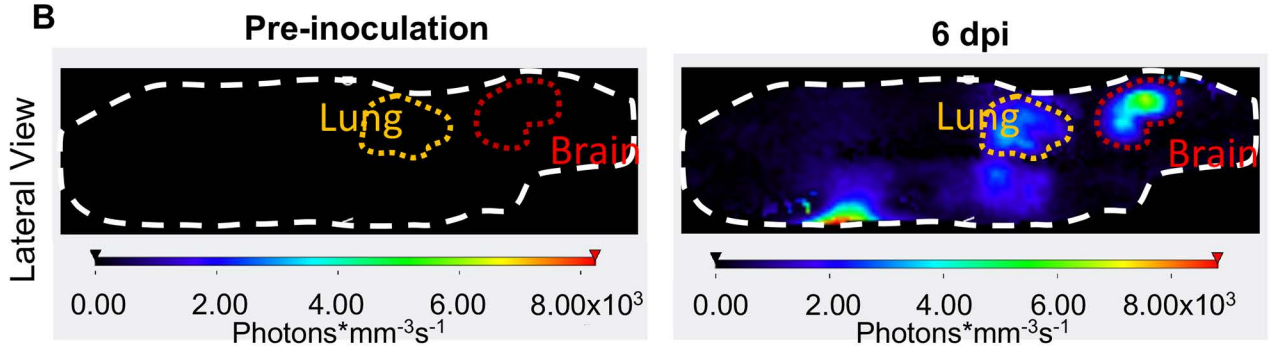
Figure 8**A****B**

Figure 9

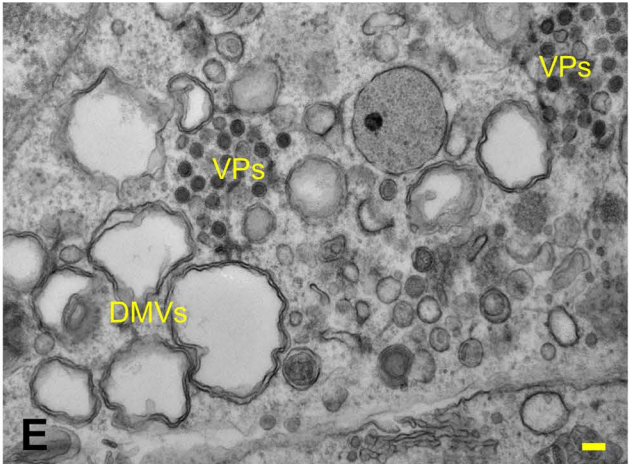
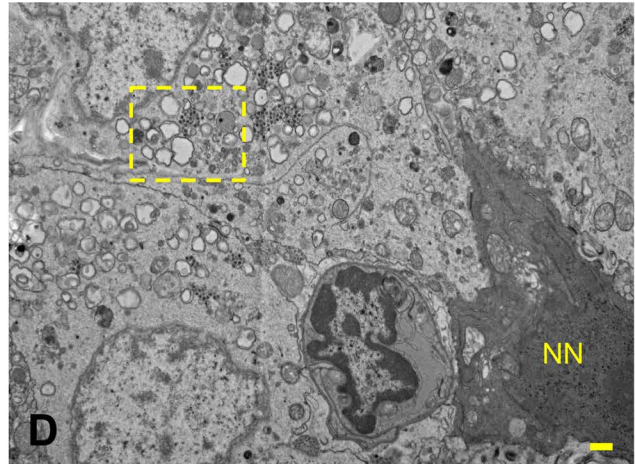
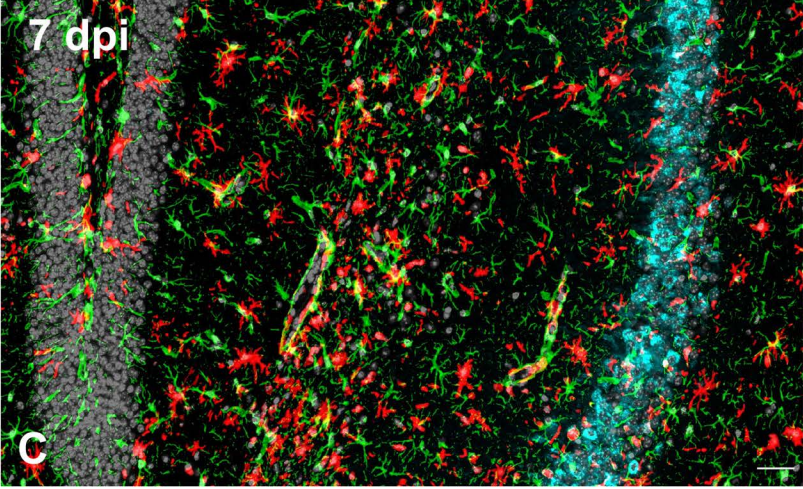
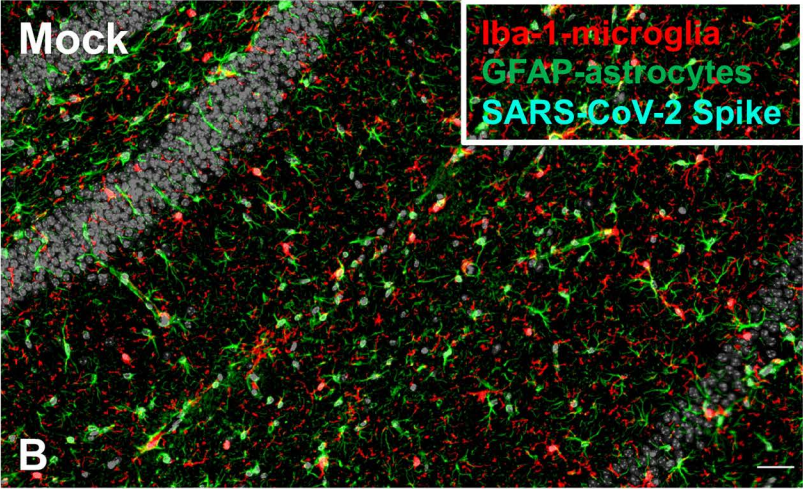
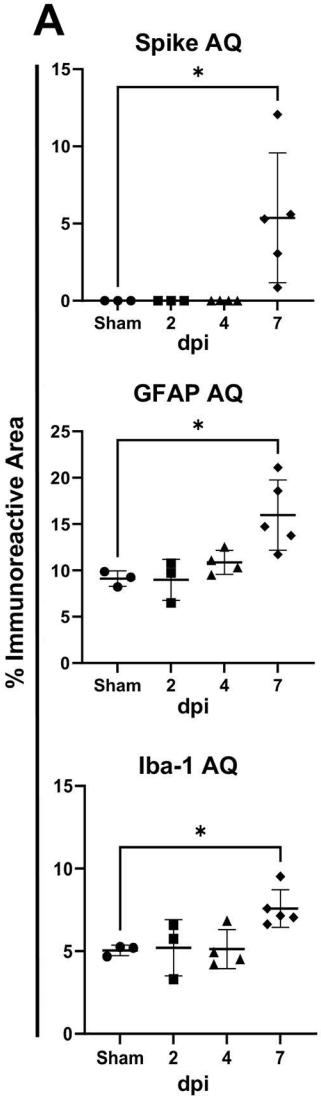


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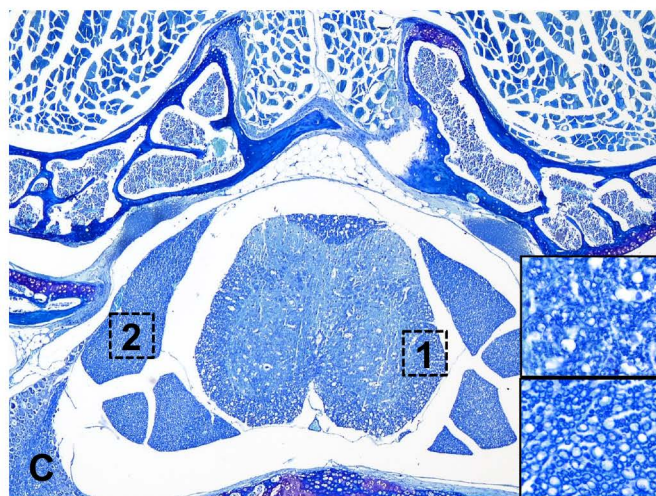
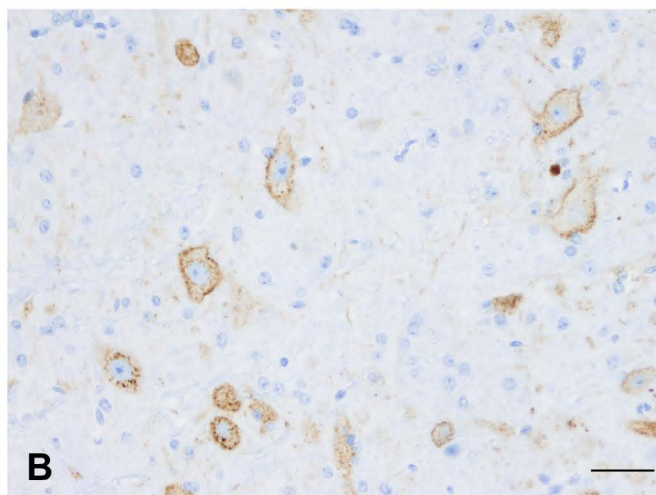
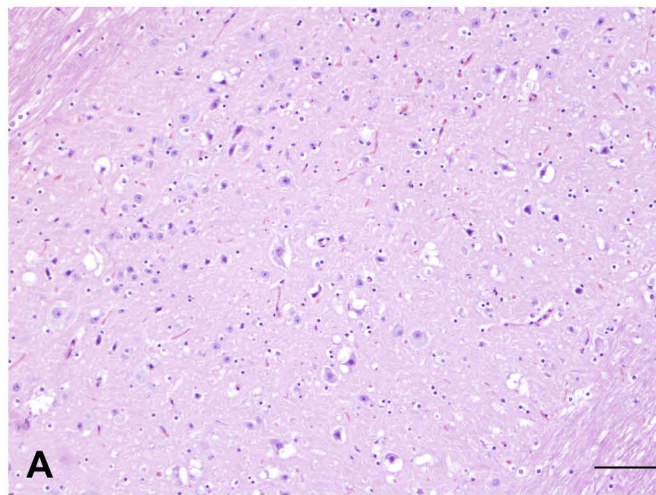


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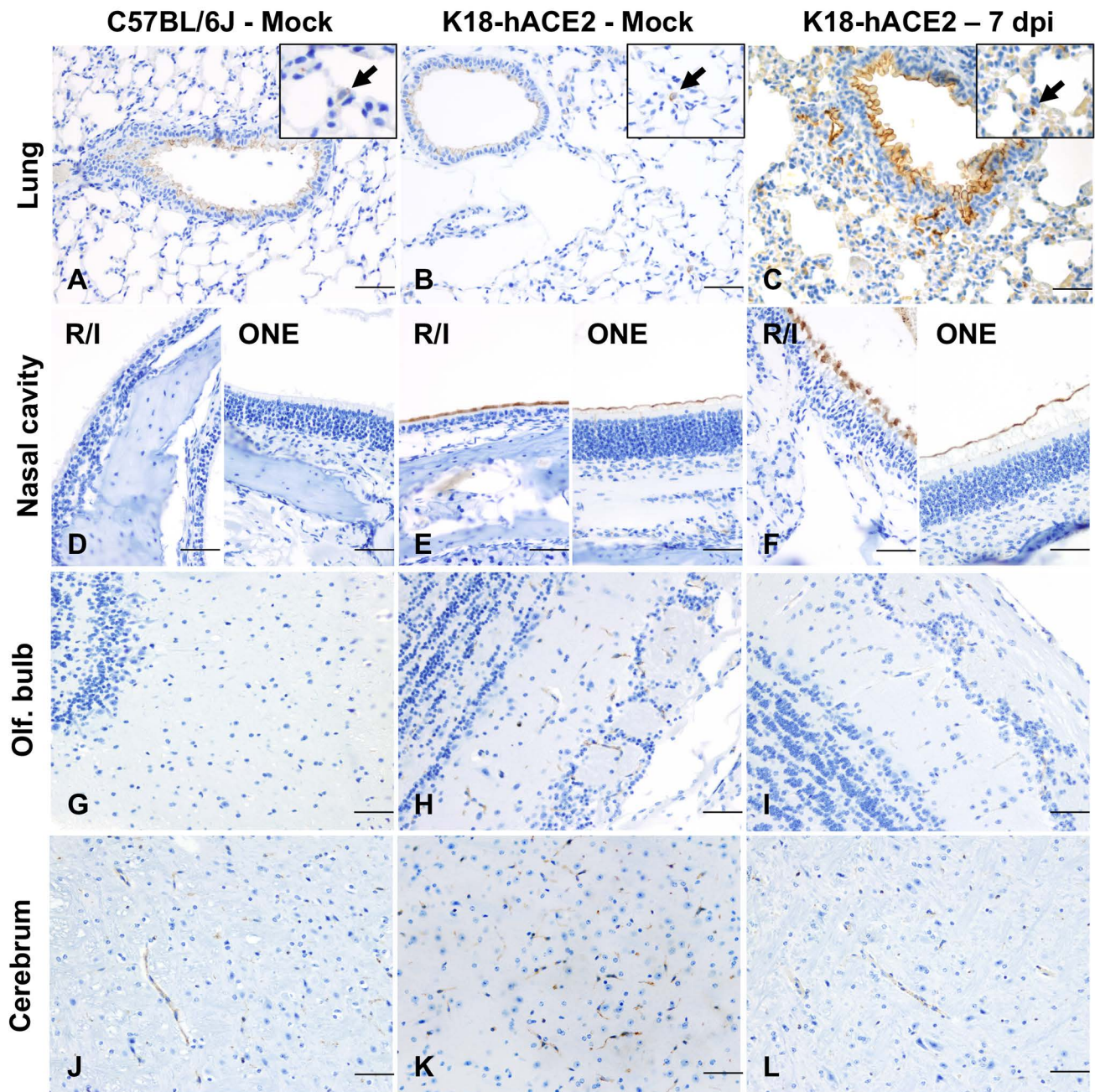


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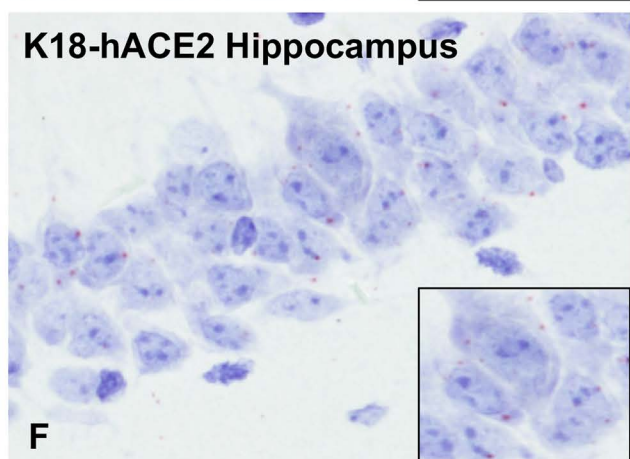
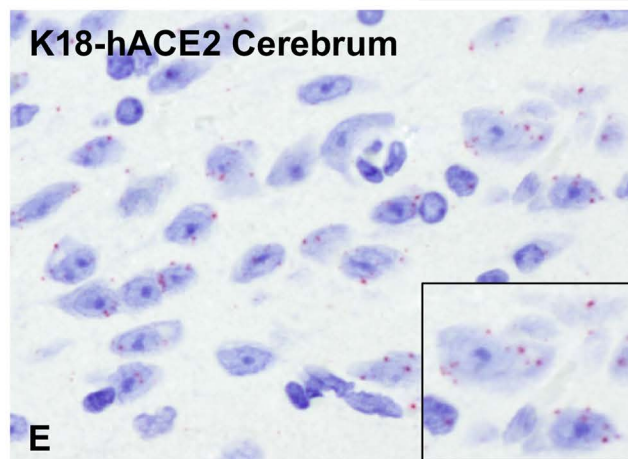
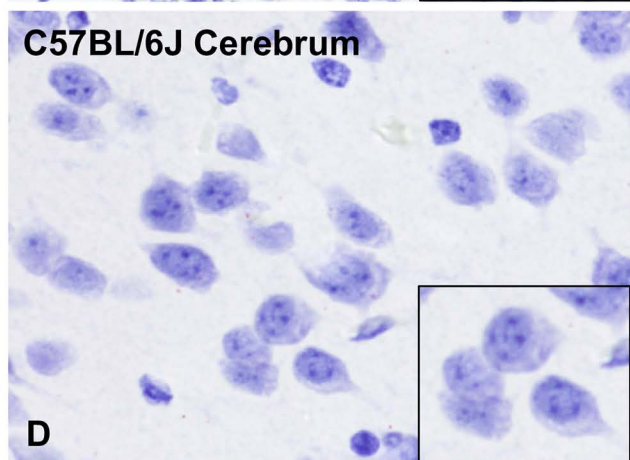
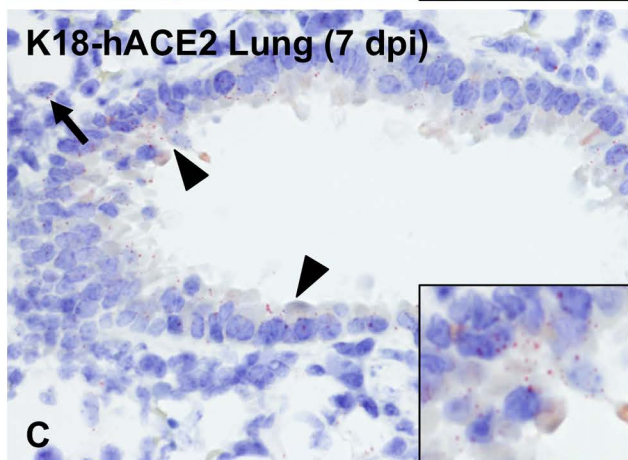
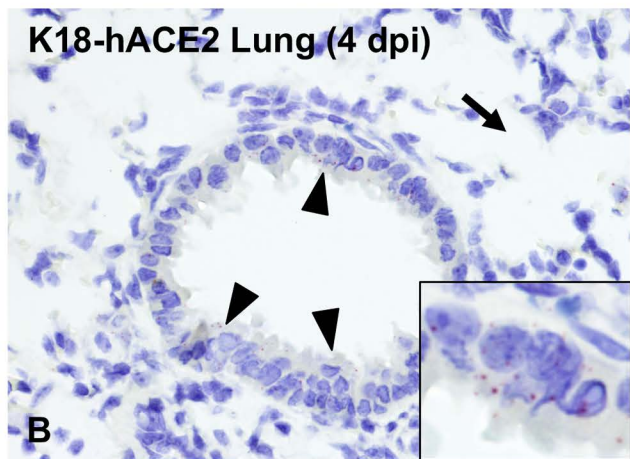
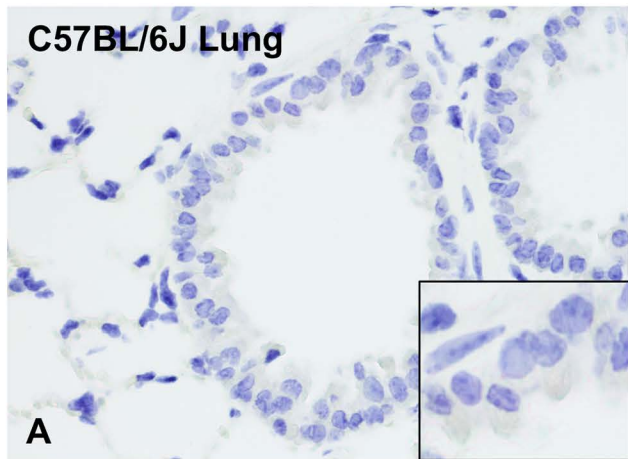


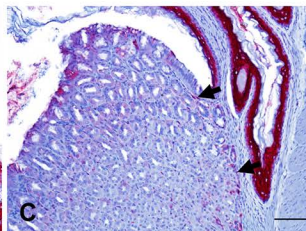
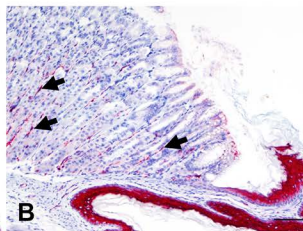
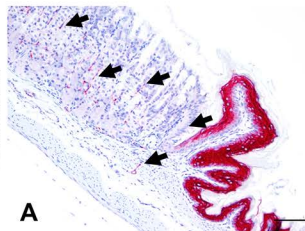
Figure 13

C57BL/6J - Mock

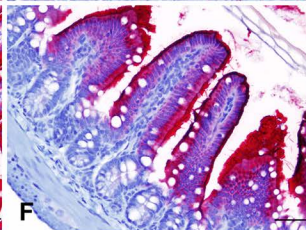
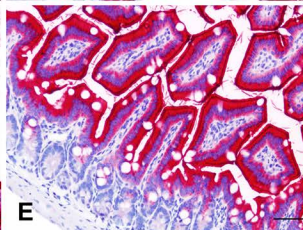
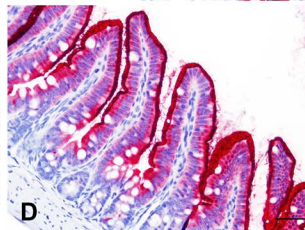
K18-hACE2 - Mock

K18-hACE2 - 7 dpi

Stomach



Small intestine



Colon

