

1 **SARS-CoV-2 infection causes transient olfactory dysfunction in mice**

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14

15 **Abstract**

16 Olfactory dysfunction caused by SARS-CoV-2 infection represents as one of the most
17 predictive and common symptoms in COVID-19 patients. However, the causal link
18 between SARS-CoV-2 infection and olfactory disorders remains lacking. Herein we
19 demonstrate intranasal inoculation of SARS-CoV-2 induces robust viral replication in
20 the olfactory epithelium (OE), resulting in transient olfactory dysfunction in humanized
21 ACE2 mice. The sustentacular cells and Bowman's gland cells in OE were identified as
22 the major targets of SARS-CoV-2 before the invasion into olfactory sensory neurons.
23 Remarkably, SARS-CoV-2 infection triggers cell death and immune cell infiltration,
24 and impairs the uniformity of OE structure. Combined transcriptomic and proteomic
25 analyses reveal the induction of antiviral and inflammatory responses, as well as the
26 downregulation of olfactory receptors in OE from the infected animals. Overall, our
27 mouse model recapitulates the olfactory dysfunction in COVID-19 patients, and
28 provides critical clues to understand the physiological basis for extrapulmonary
29 manifestations of COVID-19.

30 **Key words:**

31 SARS-CoV-2; Anosmia; Olfactory dysfunction; Olfactory receptor; Regeneration;
32 Immune response

33

34

35 **Introduction**

36 The Coronavirus disease 2019 (COVID-19) caused by the newly identified severe acute
37 respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused global crisis. The
38 clinical manifestations caused by SARS-CoV-2 predominantly involves the respiratory
39 system, including cough, sore throat, pneumonia, and acute respiratory distress
40 syndrome (ARDS) (**Huang et al., 2020; Wang et al., 2020**). With the wide spreading
41 of the disease, a significant portion of COVID-19 patients developed anosmia,
42 hyposmia or other olfactory dysfunctions according to clinical reports (**Giacomelli et**
43 **al., 2020; Menni et al., 2020; Wölfel et al., 2020**). Accumulated evidence has
44 established the alteration of smell as one of the most predictive symptoms for COVID-
45 19 screening (**Menni et al., 2020; Spinato et al., 2020**).

46 The perception of smell begins with the odorant binding to the olfactory receptors (ORs)
47 of olfactory sensory neurons (OSNs) along the upper surface of olfactory epithelium
48 (OE). Each OSN projects an axon into the glomerulus of the olfactory bulb (OB) and
49 then synapses with the second order neuron to convey the odor information into the
50 olfactory cortex. Previously, upper respiratory tract infections have been considered as
51 a common cause of olfactory disorders. Mouse models have been used to reproduce the
52 olfactory infection and subsequent dysfunction (**Kobayakawa et al., 2007; Papes et**
53 **al., 2018**). For example, the post viral olfactory disorders was observed in Sendai virus
54 infected mice by buried food pellet test (BFPT), as well as the impairment of OE and
55 OB tissues (**Matsunami et al., 2016**). However, the animal model that can recapitulate
56 the olfactory dysfunctions seen in COVID-19 patients has not been established to date.

57 Human nasal respiratory epithelium (RE) cells possess an enriched expression of
58 angiotensin-converting enzyme 2 (ACE2) (**Sungnak et al., 2020; Ziegler et al., 2020**),
59 the functional receptor of SARS-CoV-2 (**Hoffmann et al., 2020; Walls et al., 2020;**
60 **Zhou et al., 2020**). Single-cell RNA sequencing analyses have characterized the
61 expression profile of ACE2 in the OE of mouse and human, mainly in non-
62 neuroepithelium cells (**Brann et al., 2020; Ziegler et al., 2020**), and a recent study
63 based on hamster model has also observed plenty of SARS-CoV-2 infected cells in the

64 OE section (**Bryche et al., 2020; Sia et al., 2020**). Besides, vascular pericytes in OB
65 were validated to possess a high level expression of ACE2 in mouse model (**Brann et**
66 **al., 2020**), which play a key role on the maintenance of blood-brain barrier, as well as
67 the regulation of blood pressure and host immune response (**Armulik et al., 2011**).
68 Interestingly, some respiratory viruses, such as influenza virus, respiratory syncytial
69 virus, are able to invade the OB and other parts of brain to establish infection (**Dubé et**
70 **al., 2018; Netland et al., 2008**). Thus, how SARS-CoV-2 invade the olfactory system
71 and contribute to the observed central nervous system (CNS) diseases remains to be
72 determined. In the present study, we demonstrate that SARS-CoV-2 infection directly
73 cause transient olfactory dysfunction in an established mouse model, and characterized
74 the major target cells and pathological effects attributed to the olfactory dysfunction.

75

76 **Results**

77 **SARS-CoV-2 targets OE and causes transient olfactory dysfunction in hACE2** 78 **mice.**

79 We have previously established a humanized ACE2 (hACE2) mouse model susceptible
80 to SARS-CoV-2 infection (**Sun et al., 2020**). Herein, to determine the impact of SARS-
81 CoV-2 infection on olfactory system, groups of 6-8 weeks old hACE2 mice were
82 intranasally infected with 5.4×10^5 plaque-forming units (PFU) of SARS-CoV-2. Mice
83 inoculated with the same volume of culture media were set as mock infection controls.
84 At 2- and 4-days post infection (dpi), tissues from the respiratory tract and olfactory
85 system were collected from the necropsied mice, respectively, and subjected to
86 virological and immunological assays (**Figure 1A**). As expected, high levels of SARS-
87 CoV-2 RNAs were detected in the nasal respiratory epithelium (RE), trachea and lung
88 at 2 and 4 dpi, and peak viral RNA (2.36×10^{11} RNA copies/mouse) was detected in the
89 lung at 2 dpi (**Figure S1A**). Robust viral nucleocapsid (N) protein was detected in the
90 lung from SARS-CoV-2 infected hACE2 mice, but not from the control animals
91 (**Figure S1B**). Strikingly, high levels of viral RNAs (5.85×10^9 RNA copies/mouse)

92 were also detected in the olfactory mucosa (OM) at 2 dpi and maintained at high level
93 (8.93×10^8 RNA copies/mouse) till 4 dpi (**Figure 1B**), while the viral RNA levels were
94 much lower in the OB and other parts of brain on 2 dpi and decreased to marginal level
95 on 4 dpi. Furthermore, immunofluorescence staining assay detected a large amount of
96 SARS-CoV-2 N proteins in the OE along OM (**Figure 1C**), while no viral N protein
97 was detected in the OB and other parts of brain from SARS-CoV-2 infected hACE2
98 mice (**Figure S1C**). Additionally, *in situ* hybridization (ISH) by RNAscope
99 demonstrated that SARS-CoV-2 RNA was predominantly detected in the OE (**Figure**
100 **S1D**), but no in the OB (**Figure S1E**).

101 To examine whether SARS-CoV-2 infection directly impairs the olfactory function
102 of infected mice, a standard BFPT was conducted on 2 and 4 dpi, respectively.
103 Remarkably, a significantly increased latency (152.8 s *v.s.* 81.8 s; $p=0.022$) to locate
104 food pellets was observed in SARS-CoV-2 infected mice as compared with the control
105 animals on 2 dpi (**Figure 1D**). Of particular note, 2 out of 13 infected mice developed
106 severe symptoms of anosmia as they failed to locate the food pellet within the
107 observation period. Interestingly, recovery from olfactory dysfunction of infected mice
108 was observed at 4 dpi, as the latency to locate food pellets was no difference from that
109 of the control animals (67.1 s *v.s.* 70.2 s; $p=0.992$). Thus, these results demonstrate that
110 SARS-CoV-2 primarily infects OE and leads to olfactory dysfunction in mice.

111 **SARS-CoV-2 primary targets non-neuroepithelial cells in the OE of hACE2 mice.**

112 The OM consists of OE and the underlying lamina propria (LP). The OE is composed
113 of olfactory stem/progenitor cells including the horizontal basal cells (HBCs) and
114 globose basal cells (GBCs) residing in the basal region, the mature and immature OSNs,
115 and a variety of non-neuroepithelial lineage including the sustentacular cells,
116 microvillar cells and Bowman's gland cells. The OSNs lining under the supporting cells
117 project numerous dendritic cilia with ORs into the nasal cavity and intermingle with
118 the microvilli of sustentacular cells and microvillar cells (**Figure S2A**). Due to the
119 asymmetrical expression pattern of ACE2 on the cell membrane as well as the unique
120 organization of OE, it is not easy to determine which cell compartments express ACE2.

121 To overcome this, we took advantage of the tdTomato cassette downstream of hACE2
122 transgene with an internal ribosome entry site (IRES), which allows the detection of
123 hACE2 expression by cytoplasmic fluorescence of tdTomato (**Figure S2B**). An
124 abundant expression of hACE2 along the apical surface of OE as well as within the
125 underlying LP was detected with a human ACE2-specific monoclonal antibody,
126 exhibiting a similar expression pattern of tdTomato (**Figure S2C**). A detailed
127 characterization of hACE2/tdTomato expressing cells in OM revealed that non-
128 neuroepithelial cells, including the sustentacular cells (CK8-positive, **Figure S2D, d1**),
129 the duct and acinus of Bowman's gland cells (Sox9/CK8-positive, **Figure S2D, d2, d4**)
130 in the OE and LP, respectively, and the microvillar cells (CD73/CK8-positive, **Figure**
131 **S2E**), are the primary cell types that harbor human ACE2 expression (**Figure S2D**),
132 whereas little hACE2/tdTomato expression was detected in the neuroepithelial lineage,
133 including HBCs (CK5-positive), GBCs (Sox2-positive at the basal region), immature
134 olfactory sensory neurons (iOSNs) (GAP43-positive) and mature olfactory sensory
135 neurons (mOSNs) (OMP-positive) (**Figure S2D, d1-d4**).

136 To further characterize the primary targets of SARS-CoV-2 in the OE, multiplex
137 immunostaining assays were performed with antibodies against SARS-CoV-2 and
138 specific cell markers. Remarkably, robust expression of SARS-CoV-2 viral N protein
139 was detected in the non-neuroepithelial lineage lining the outer surface of OE at 2 and
140 4 dpi (**Figures 2A and 2C**). The sustentacular cells (58.97%) and Bowman's gland cells
141 (22.76%) represent as the major target cell types at 2 dpi, while some microvillar cells
142 (6.93%) and HBCs (4.11%) were also infected by SARS-CoV-2 (**Figures 2A and 2B**).
143 Additionally, a small population of iOSNs (1.28%) were also infected by SARS-CoV-
144 2, while none mOSN was infected at 2 dpi (**Figures 2A and 2B**). Interestingly, SARS-
145 CoV-2-positive HBCs and iOSNs were found adjacent to infected sustentacular cells
146 (**Fig. 2a**). Additionally, substantial viral protein was detected within the cilia, the
147 cellular bodies and the underlying nerve bundles of mOSNs at 4 dpi (**Figure 2C, c1-**
148 **c2**). These results indicated that SARS-CoV-2 primarily targets the non-neuroepithelial
149 cells lining the outer surface of OE, and subsequently invades the neuroepithelial

150 lineage in hACE2 mice.

151 **SARS-CoV-2 infection triggers apoptosis and immune cell infiltration in OE.**

152 We then characterized the histopathological changes of OE in response to SARS-CoV-
153 2 infection. Strikingly, SARS-CoV-2 infection directly impaired the structural
154 uniformity of OE, as characterized by clusters of remnants on the surface of OE (**Figure**
155 **3A**), as well as disorganized arrangement of supporting cells (**Figure 3B**) and olfactory
156 neurons (**Figure 3C**). The integrity of the cilia layer of mOSNs and the microvilli of
157 supporting cells were severely damaged (**Figures 3B and 3C**). More importantly,
158 compared with mock treated groups, profound cell apoptosis (cleaved-caspase3-
159 positive) was observed in both of the OE and LP section of OM from the SARS-CoV-
160 2 infected mice (**Figure 3D**). Immunofluorescence co-staining indicated the apoptosis
161 can be seen in sustentacular cells, HBCs as well as the cellular bodies and the
162 underlying nerve bundles of iOSNs and mOSNs (**Figure 3D**). Additionally, the
163 infiltrations of immune cells, including the macrophages (CD68-positive), the dendritic
164 cells (CD103-positive) and the neutrophils (Ly-6G-positive) were evident in the
165 infected OE (**Figure 3E**). The profound invasion of CD8 T lymphocytes with high
166 expression of cytotoxic enzymes Perforin and Granzyme B would further deteriorate
167 the cellularity of olfactory epithelial cells (**Figure 3F**). These observed physiological
168 damages upon to SARS-CoV-2 infection probably contribute to the functional loss of
169 olfaction.

170 **SARS-CoV-2 infection induces regeneration of OE.**

171 Without infection, HBCs at the basal region of OE remains quiescent as indicated by
172 little expression of the proliferation marker Ki67 within CK5-positive cells (**Figure 4A,**
173 **a1**). SARS-CoV-2 infection significantly increased the number of CK5/Sox2/Ki67
174 triple-positive cells, strongly suggesting a transition from HBCs to actively cycling
175 GBCs (**Figure 4A, a2**). Of particular note, a prominent upward growth of HBCs from
176 the basal layer into the upper section of OE was observed in infected animals, which
177 also co-express the markers of their lineage offspring such as iOSNs (**Figure 4B, b1**),

178 sustentacular cells (**Figure 4B, b2**) and the microvillar cells (**Figure 4B, b3**). These
179 results suggest that the impaired OE is regenerated through olfactory stem cell-based
180 proliferation and differentiation into olfactory neurons and supporting lineage, thereby
181 restoring the normal function of OE.

182 **SARS-CoV-2 infection induces inflammatory response and suppresses olfactory**
183 **signaling pathway in OE.**

184 To decipher the underlying mechanism of the observed olfactory dysfunction in SARS-
185 CoV-2 infected mice at the molecular level, combined transcriptomic and quantitative
186 proteomic analyses of the OE and OB samples from SARS-CoV-2 infected mice were
187 performed in comparison with that from the control animals. In the OE samples, a total
188 of 939 genes and 507 proteins were regulated upon SARS-CoV-2 infection, and 40 of
189 them were synchronously regulated at both mRNA and protein levels (**Figures S3A**
190 **and S3B**). While in the OB samples, 286 genes and 251 proteins were up/down
191 regulated, and only 4 of them were consistently regulated at mRNA and protein levels
192 (**Figures S3A and S3C**). These results further support that OE represents the major site
193 for SARS-CoV-2 replication. Gene enrichment analyses showed that SARS-CoV-2
194 infection induces strong antiviral defense and inflammatory response in OE at both
195 mRNA and protein levels at 2 dpi, which faded at 4 dpi (**Figures 5A, S4A and S4C**).
196 Moreover, genes related to “positive regulation of cell death” and “regulation of neuron
197 projection development” were also up regulated upon SARS-CoV-2 infection (**Figures**
198 **5A, S4B and S4D**), which was consistent with the immunostaining results (**Figure 3D**
199 **and 4A**). Further integrated omics analysis of the OE samples showed that a total of 30
200 genes were up regulated at both mRNA and protein levels. Of which, antiviral
201 genes/proteins including *Isg15*, *Stat1*, *Stat2*, *Oasl2*, *Ifit2*, *Ifit3*, etc, were found to
202 interact closely. Other genes/proteins involved in neurotransmitter transport including
203 *Erc2*, *Lin7a*, *Slc1a3* and *Slc25a18*, were also observed (**Figure 5B**). While in the OB
204 samples, we did not find any induction of antiviral response related genes, but
205 downregulation of inflammatory response related genes was observed (**Figures S5A**
206 **and S5B**).

207 Of particular note, KEGG pathway enrichment of down regulated transcripts and
208 proteins in OE showed that genes belonging to “olfactory transduction” were
209 significantly enriched (**Figure 5C**). Among all 100 down regulated transcripts at 2 dpi,
210 36 were ORs (**Figures 5D and S3B**), while among 278 down regulated transcripts at 4
211 dpi, 97 were ORs (**Figures S3B and S4E**). Further RT-qPCR assay showed a dozen of
212 OR genes were significantly down regulated in response to SARS-CoV-2 infection
213 (**Figure 5E**), which may also attribute to the observed olfactory dysfunction.

214

215 **Discussion**

216 In the present study, we used an established mouse model to demonstrate that SARS-
217 CoV-2 infection can cause olfactory dysfunction and anosmia, and these experimental
218 evidence support the hypothesis that SARS-CoV-2 infection as the cause of olfactory
219 dysfunction and anosmia in COVID-19 patients (**Iravani et al., 2020; Moein et al.,**
220 **2020**). The SARS-CoV-2 infected mice exhibited damaged OE, immune cell
221 infiltration, down regulated OR expressions and impaired olfactory function, largely
222 mimicking the olfactory abnormalities of COVID-19 patients. Robust viral replication
223 and direct antiviral responses were detected in the OE of the infected mice, but not in
224 OB and other parts of the brain, indicating that SARS-CoV-2 infection may be
225 restricted in OM, instead of spreading to the CNS. A recent study also supports this
226 point of view, for that SARS-CoV-2 protein can be detected in OE, but not in OB, in a
227 hamster model (**Bryche et al., 2020**). One possible explanation for the absence of
228 SARS-CoV-2 in CNS is the IFN-dependent antiviral mechanism, which is an effective
229 barrier to limit the virus from invading into CNS (**Forrester et al., 2018**). In addition,
230 the apoptosis of infected OSNs may contribute to the prevention of virus spreading into
231 CNS after the rapid infection and destruction of OE (**Mori et al., 2002**).

232 Our results show that SARS-CoV-2 initially infects non-neuroepithelial cells, including
233 sustentacular cells, Bowman’s gland cells and microvillar cells, which are involved in
234 OSN support, host immune response, electrolyte balance maintenance, and mucus

235 secretion (**Cooper et al., 2020**). Meanwhile, we observed various levels of damage in
236 OE after SARS-CoV-2 infection, including cilia desquamation, loss of surface
237 microvilli and substantial structural disorganization. In addition, our results showed a
238 certain degree of cell apoptosis and inflammatory infiltration at both cell and molecular
239 level following SARS-CoV-2 infection. All these data indicate that the damaged
240 supporting non-neuroepithelial cells and inflammatory infiltration caused by SARS-
241 CoV-2 infection contribute to the detrimental effects of the virus on olfactory function.
242 Our results are supported by recent findings in mouse and human, showing that the non-
243 neuroepithelial cells of OE express high levels of ACE2 and TMPRSS2 at both mRNA
244 and protein levels (**Brann et al., 2020; Torabi et al., 2020**) (**Trotier et al., 2007**).
245 Interestingly, SARS-CoV-2 positive signals were also observed in mOSNs and HBCs
246 of infected animals, although we didn't detect any hACE2 expression in these cells.
247 The underlying mechanism remains elusive and a hACE2-independent spread of
248 SARS-CoV-2 infection may be considered.

249 We observed many ORs were significantly down regulated at 2 and 4 dpi, suggesting
250 the declined olfaction after SARS-CoV-2 infection. A recent study also showed that
251 induction of anti-viral type I interferon signaling in the mouse OE was associated with
252 diminished odor discrimination and decreased RNA levels of ORs (**Rodriguez et al.,**
253 **2020**). These findings may support what we observed here that SARS-CoV-2 infection
254 causes significant interferon response and dramatic OR decrease simultaneously in OE.
255 We also observed three odorant-binding proteins (OBPs) significantly decreased at
256 protein level with the infection of OE, which are compact globular water-soluble
257 proteins with ligand-binding capabilities and thought to aid in capture and transport of
258 odorants to the ORs (**Matarazzo et al., 2002; Pes and Pelosi, 1995; Sun et al., 2018**).
259 Besides, although no virus infection was observed in OB, we detected some up/down
260 regulated transcripts or proteins by transcriptomic and proteomic analyses. It worth
261 noting that among all 4 proteins co-regulated at both transcriptomic and proteomic
262 levels, Rtp1 (Receptor-transporting protein 1) was down regulated at both levels (Table
263 S1). This protein specifically promotes functional cell surface expression of ORs (**Wu**

264 **et al., 2012**), suggesting that the inhibition of Rtp1 in OB may lead to down regulation
265 of ORs. Therefore, the damage of OE which is closely related to olfactory dysfunction
266 are caused by SARS-CoV-2 infection of non-neuroepithelial cells and OSNs synergizes
267 with the host antiviral immune responses.

268 According to our results, the olfactory dysfunction in SARS-CoV-2 infected animals is
269 recoverable as almost all animals recovered to normal sense of smell at 4 dpi.
270 Additionally, studies focusing on COVID-19 patients with anosmia has shown that
271 most of them would recovery from loss of smell within a few weeks or less (**Hopkins**
272 **et al., 2020; Yan et al., 2020**), indicating a potential mechanism of OE regeneration
273 from injuries. The OE undergoes a lifelong regeneration and replacement depending on
274 two populations of basal stem cells, HBCs and GBCs. HBCs are mitotically quiescent
275 under the normal conditions and convert to be activated and differentiate into other
276 kinds of cells once the damage of OE occurs (**Salazar et al., 2019**). Unlike HBCs, most
277 of GBCs are mitotically activated and responsible for the regeneration of both neuronal
278 and non-neuronal cells (**Gadye et al., 2017; Leung et al., 2007; Yu and Wu, 2017**).
279 Indeed, we observed the regeneration of OE by the significant proliferation and
280 morphological change of HBCs, accompanied by the differentiation of stem cells into
281 iOSNs, sustentacular cells as well as microvillar cells. In this way, the structural basis
282 and function of OE as well as the olfactory function can be restored to normal in SARS-
283 CoV-2 infected animals. Furthermore, it was indicated that the damage and apoptosis
284 of OSNs are closely involved in their regeneration (**Ishimura et al., 2008**), and the
285 occurrence of inflammatory response also facilitates the stem cell differentiation and
286 OE regeneration (**Chen et al., 2019; Lane et al., 2014**). At transcriptomic and
287 proteomic level, we observed up regulated “regulation of neuron projection
288 development” genes/proteins on 2 and 4 dpi, implying the progression of a neuron
289 projection over time from its formation to the mature structure. Interestingly, although
290 there were many significantly down regulated ORs on 4 dpi, the mRNA levels of many
291 ORs rose back slightly compared with that of 2 dpi, indicating the OR expression tends
292 to recover to normal.

293 In summary, our study established a mouse model of olfactory dysfunction induced by
294 SARS-CoV-2. Considering the interspecies discrepancy of olfactory construction
295 between rodent and human, e.g., the relative size of the OB to the brain, the proportion
296 of the brain involved in olfaction and the expression of ORs (**Salazar et al., 2019**),
297 further studies are recommended to reproduce the SARS-CoV-2 caused olfactory
298 dysfunction in other animal models, especially the non-human primates. Also, to
299 validate the targets and biological effects of SARS-CoV-2 infection in human
300 specimens is still ponderable. The animal model of olfactory disorders is available to
301 subsequently evaluate the antiviral drugs as well as vaccines for the inhibition of SARS-
302 CoV-2 and the improvement of post viral olfactory disorders.

303

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316

317 **Author Contributions**

318 C.F.Q., Q.Y., J.Z., and G.Y. conceived the project and designed the experiments. Q.Y.,
319 J.Z., G.Y. and Q.H. performed the majority of the experiments and analyzed the data;
320 R.T.L., Y.Z., Q.C., R.R.Z., H.Q., Y.Q.D., X.F.L., S.J.W., J.H.S., H.M.Z., and T.Z.
321 contributed specific experiments and data analysis. Y.Z. and P.X. contributed to
322 proteomic analysis. C.F.Q., Q.Y., J.Z., G.Y., and R.T.L. wrote the manuscript with all
323 the input from all authors. C.F.Q, X.Y., and P.X. supervised the study. All authors read
324 and approved the contents of the manuscript.

325

326 **Declaration of Interests**

327 None declared.

328

329 **METHODS**

330 **Cell and Virus**

331 The Vero cells were maintained at 37°C under 5% CO₂ in Dulbecco's modified Eagle
332 essential medium (DMEM) supplemented with 10% heat-inactivated fetal bovine
333 serum (FBS, Gibco), 10 mM HEPES and 1% penicillin/streptomycin. The SARS-CoV-
334 2 strain BetaCoV/Beijing/IMEBJ05/2020 (Nos. GWHACBB01000000) was originally
335 isolated from a COVID-19 patient. For virus propagation, Vero cells were incubated
336 with SARS-CoV-2 and the culture supernatants were collected at 3 dpi. The stock of
337 SARS-CoV-2 was serially diluted and titered on monolayers of Vero cells. Studies with
338 infectious SARS-CoV-2 were conducted under biosafety level 3 (BSL3) facilities at the
339 Beijing Institute of Microbiology and Epidemiology, AMMS.

340 **SARS-CoV-2 infection of hACE2 mice**

341 The animal operation procedure was reviewed and approved by the Laboratory Animal
342 Center, AMMS (approval number: IACUC-DWZX-2020-001). For intranasal infection,
343 5.4×10^5 PFU of SARS-CoV-2 was instilled into the nasal cavity of 6-8 weeks old
344 hACE2 mice anaesthetized with sodium pentobarbital at a dose of 50 mg/kg by
345 intraperitoneal route. Mice were monitored daily and euthanized at 2 or 4 dpi to isolate
346 tissues.

347 **RNA Extraction and real-time quantitative PCR**

348 Quantification of SARS-CoV-2 RNA, hACE2 and OR mRNA transcript levels were
349 performed by real-time quantitative PCR (RT-qPCR). Total RNAs were isolated using
350 TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's
351 instructions. SARS-CoV-2 RNA was measured with the primer-probe set: CoV-F3 (5'-
352 TCCTGGTGATTCTTCTTCAGGT-3'), CoV-R3 (5'-TCTGAGAGAGGGTC
353 AAGTGC-3') and CoV-P3 (5'-AGCTGCAGCACCAGCTGTCCA-3'). The relative
354 expression of hACE2 mRNA was measured with the primer set: ehACE2 F1 (5'-
355 CGAAGCCGAAGACCTGTTCTA-3') and ehACE2 R1 (5'-GGGCAAGTGTGG
356 ACTGTTCC-3'). The expression of glyceraldehyde-3-phosphate dehydrogenase
357 (GAPDH) served as the endogenous control, and the following primer set was used: 5'-
358 CCAACCGCGAGAAGATGA-3' and 5'-CCAGAGGCGTACAGGGATAG-3'.
359 Amplification was performed using a One Step PrimeScript RT-PCR Kit (Takara Bio,
360 Otsu, Japan), and the following real-time PCR conditions were applied: 42 °C for 5 min
361 and 95 °C for 10 s followed by 40 cycles of 95°C for 5 s and 60 °C for 20 s on an
362 LightCycler[®] 480 Instrument (Roche Diagnostics Ltd, Rotkreuz, Switzerland). The
363 absolute quantification of SARS-CoV-2 RNA levels was performed by comparison to
364 a standard curve and shown as SARS-CoV-2 RNA copies per mouse. The relative
365 expression of hACE2 and OR mRNA levels was calculated according to the $2^{-\Delta\Delta Ct}$
366 method. Each sample was assayed with three repeats.

367 **The buried food pellet test (BFPT)**

368 The standard BFPT was used to evaluate the olfactory function of SARS-CoV-2-
369 infected mice and DMEM-treated mice as previously described (Lehmkuhl et al., 2014;
370 Yang and Crawley, 2009). Mice were food-restricted to 0.2 g chow per day for 2 days

371 before test and during the experimental period to ensure motivation. The food pellet
372 was buried 1 centimeter below the surface of 3-centimeter-high bedding in a clear test
373 cage (45 cm L × 24 cm W × 20 cm H). One mouse was placed in the center of the cage,
374 and the latency for the mouse to uncover the pellet was recorded. The latency was
375 defined as 300 seconds for the mouse which cannot find the pellet within 5 minutes.

376 **RNAscope in situ hybridization**

377 RNAscope *in situ* hybridization (ISH) for SARS-CoV-2 RNA was performed with the
378 RNAscope assay (Advanced Cell Diagnostics, Newark, CA, USA) according to the
379 manufacturer's instructions. Briefly, the tissues were isolated immediately after
380 euthanasia and fixed in 4% paraformaldehyde (PFA) for 24 hours, and embedded in
381 paraffin after being decalcified using the 10% EDTA solution, 4- μ m-thick formalin-
382 fixed paraffin-embedded (FFPE) slides were warmed at 60 °C for 1 h before they
383 deparaffinized in xylene, rehydrated in a series of graded alcohols and pretreated with
384 RNAscope target retrieval at 95 °C. Slides were detected in situ using 2.5 HD Reagent
385 Kit (BROWN) (Cat: 322310) and sense probe from the RNAscope ISH probe-V-
386 nCoV2019-S (Cat: 848561) at 40 °C in HybEZ hybridization oven and then
387 counterstained with hematoxylin.

388 **Multiplex immunofluorescent staining**

389 The 4- μ m-thick paraffin sections were deparaffinized in xylene and rehydrated in a
390 series of graded alcohols. Antigen retrievals were performed in citrate buffer (pH=6)
391 with a microwave (Sharp, R-331ZX) for 20 min at 95°C followed by a 20 min cool
392 down at room temperature. Multiplex fluorescence labeling was performed using TSA-
393 dendron-fluorophores (NEON 9-color All round Discovery Kit for FFPE, Histova
394 Biotechnology, NEFP950). Briefly, endogenous peroxidase was quenched in 3% H₂O₂
395 for 20 min, followed by blocking reagent for 30 min at room temperature. Primary
396 antibody was incubated for 2-4 h in a humidified chamber at 37°C, followed by
397 detection using the HRP-conjugated secondary antibody and TSA-dendron-
398 fluorophores. Afterwards, the primary and secondary antibodies were thoroughly
399 eliminated by heating the slides in retrieval/elution buffer (Abcracker®, Histova
400 Biotechnology, ABCFR5L) for 10 s at 95°C using microwave. In a serial fashion, each
401 antigen was labeled by distinct fluorophores. Multiplex antibody panels applied in this
402 study were: hACE2 (Abcam, ab108209, 1:200); tdTomato (Rockland, 600-401-379,
403 1:500); SARS-CoV-2 nucleocapsid protein (Sinobiological, 40143-R004, 1:1000);
404 GAP43 (Abcam, ab75810, 1:1000); OMP (Abcam, ab183947, 1:1500); CK5 (Abcam,
405 ab52635, 1:800); CK8 (Abcam, ab53280, 1:800); Sox9 (Abcam, ab185230, 1:500);
406 Sox2 (CST, 23064, :400); CD73 (CST, 13160, 1:500); Furin (Abcam, ab108209, 1:400);
407 Tmprss2 (Abcam, ab92323, 1:500); CD3 (CST, 78588, 1:300); CD8 (CST, 98941,
408 1:300); Cleaved caspase-3 (CST, 9664, 1:1000); CD103 (Abcam, ab224202, 1:300);
409 Ly-6G (CST, 87048, 1:400); CD68 (CST, 97778, 1:300); and Granzyme B (Abcam,
410 ab255598, 1:300). After all the antibodies were detected sequentially, the slices were
411 imaged using the confocal laser scanning microscopy platform Zeiss LSM880.

412 **Histopathological analysis**

413 The structural integrity of the mouse OE was analyzed using hematoxylin and eosin

414 (H&E) staining according to standard procedures. Briefly, after being rehydrated in
415 series of graded alcohols, the 4- μ m-thick slides of mouse OE were stained with
416 hematoxylin for 30 s and washed in water. Slides were then stained in eosin for 15 s
417 and washed again in water.

418 **RNA library construction and sequencing**

419 hACE2 transgenic mice before or after SARS-CoV-2 infection (2 or 4 dpi) as previously
420 described were used for RNA-Seq. Total RNA from OE and OB were extracted using
421 TRIzol (Invitrogen, Carlsbad, CA, USA) and DNase I (NEB, USA) treated, respectively.
422 Sequencing libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit
423 for Illumina® (#E7530L, NEB, USA) following the manufacturer's recommendations
424 and index codes were added to attribute sequences to each sample. The clustering of
425 the index-coded samples was performed on a cBot cluster generation system using
426 HiSeq PE Cluster Kit v4-cBot-HS (Illumina, San Diego, California, USA) according to
427 the manufacturer's instructions. After cluster generation, the libraries were sequenced
428 on Illumina Novaseq6000 platform and 150 bp paired-end reads were generated. After
429 sequencing, perl script was used to filter the original data (Raw Data) to clean reads by
430 removing contaminated reads for adapters and low-quality reads. Clean reads were
431 aligned to the mouse genome (Mus_musculus.GRCm38.99) using Hisat2 v2.1.0. The
432 number of reads mapped to each gene in each sample was counted by HTSeq v0.6.0
433 and TPM (Transcripts Per Kilobase of exon model per Million mapped reads) was then
434 calculated to estimate the expression level of genes in each sample.

435 **Large-scale proteome sample preparation and Tandem Mass Tags (TMT) labeling**

436 The OE and OB tissues were disrupted by a Grinding Mill for six cycles of 5 s each
437 with lysis buffer [9 M Urea, 10 mM Tris-HCl (pH 8.0), 30 mM NaCl, 10 mM
438 iodoacetamide (IAA), 5 mM Na₄P₂O₇, 100 mM Na₂HPO₄ (pH 8.0), 1 mM NaF, 1 mM
439 Na₃VO₄, 1 mM sodium glycerophosphate, 1% phosphatase inhibitor cocktail 2 (Sigma,
440 St. Louis, USA), 1% phosphatase inhibitor cocktail 3 (Sigma, St. Louis, USA), and 1
441 tablet of EDTA-free protease inhibitor cocktail (Roche, Basel, Switzerland) for every
442 10 mL of lysis buffer] and 2 mm steel balls, respectively. The supernatants were
443 obtained after centrifuging at 8,000 rpm for 10 min at 4°C. The protein lysates were
444 inactivated at 56 °C for 30 min then stored at -80°C before further processing. Protein
445 concentration was measured by a short Coomassie blue stained 10% SDS-PAGE as
446 described (Xu et al., 2009). The same amount of protein (130 μ g) from each sample
447 was reduced with 5 mM of dithiothreitol (DTT), alkylated with 20 mM of IAA,
448 precleaned with 10% SDS-PAGE (10%, 0.7 cm), and digested in-gel with a final
449 concentration of 12.5 ng/ μ L for Ac-trypsin combined with endoproteinase lys-C
450 provided by Enzyme & Spectrum (Beijing, China) with a ratio of 2:1 at 37°C for 12-14
451 h (Zhao et al., 2016; Zhao et al., 2015). The extracted peptides from OE and OB
452 groups were labeled with TMT10 reagents according to the manufacturer's instructions
453 (Thermo Scientific, San Jose, CA, USA), respectively. Ten labeled channels were then
454 quenched with 5% hydroxylamine and combined according to normalization value by
455 the ratio checking. The mixed samples were vacuum dried.

456 Peptide fractionation and LC-MS/MS analysis

457 The dried TMT labeled mixture were resuspended in 100 μ L of buffer A [2%
458 acetonitrile (ACN), pH10) and separated by a high pH reverse phase HPLC system
459 (Rigol, L-3120, Beijing, China). The combined samples were injected into a Durashell
460 C₁₈ column (150 Å, 5 μ m, 4.6 \times 250 mm²) and eluted with a linear gradient in 60 min.
461 Briefly, the solvent gradients of buffer B (2% dd H₂O and 98% ACN) were as follows:
462 0% for 5 min, 0-3% for 3 min, 3-22% for 37 min, 22-32% for 10 min, 32-90% for 1
463 min, 90% for 2 min, and 100% for 2 min. The LC flow rate was set at 0.7 mL/min and
464 monitored at 214 nm. The column oven was set at 45 °C. Total 60 fractions were
465 collected and then combined into 15 fractions before vacuum drying according to the
466 peak abundance. The combined samples were dissolved in loading buffer [1% ACN and
467 1% formic acid (FA)] and analyzed using an EASY-nLC 1200 ultra-performance liquid
468 chromatography system (Thermo Fisher Scientific, San Jose, CA, USA) equipped with
469 a self-packed capillary column (75 μ m i.d. \times 15 cm, 3 μ m C₁₈ reverse-phase fused-
470 silica), with a 78 min nonlinear gradient at a flow rate of 600 nL/min. The gradient was
471 comprised of an increase from 6% to 15% solvent B (0.1% FA in 80% ACN) for 15
472 min, 15% to 30% in 40 min, 30% to 40% in 15 min, 40% to 100% in 1 min, and finally
473 holding at 100% for the last 7 min. The eluted peptides were analyzed on Orbitrap
474 Fusion Lumos (Thermo Fisher Scientific, San Jose, CA, USA). MS₁ data were collected
475 in the Orbitrap using a 120 k resolution over an *m/z* range of 300-1500 setting the
476 maximum injection time (MIT) to 50 ms. The automatic gain control (AGC) was set to
477 4 \times 10⁵, determined charge states between 2 and 7 were subjected to fragmentation via
478 higher energy collision-induced dissociation (HCD) with 37% collision energy and a
479 12 s dynamic exclusion window was used with isotopes excluded. For the MS/MS scans,
480 the fractions were detected in the Orbitrap at a resolution of 50 k. For each scan, the
481 isolation width was 1.6 *m/z*, the AGC was 5 \times 10⁴, and the MIT was 86 ms.

482 Database search

483 The raw files from OE and OB groups were searched with MaxQuant (v1.5.5.0) against
484 the mouse reviewed proteome downloaded from UniProt containing 17,478 entries and
485 a canonical SARS-CoV-2 proteome with 30 potentially viral proteins from the SARS-
486 CoV-2 genome (NC_045512.2), and a common contaminant database
487 (<http://www.maxquant.org/contaminants.zip>), respectively. Fully tryptic peptides with
488 as many as 2 missed were allowed. TMT 10 plex (N-Term/K) and cysteine
489 carbamidomethyl were set as fixed modification, whereas oxidation of methionine was
490 set as variable modification. The tolerance of the precursor and fragment ions were set
491 to 20 ppm.

492 Bioinformatic analyses

493 DESeq2 v1.6.3 was used for differential gene expression analysis. Genes with
494 $\text{padj} \leq 0.05$ and $|\text{Log}_2\text{FC}| > 1$ were identified as differentially expressed genes (DEGs).
495 The total proteome quantification datasets were median-normalized, and *pValue* was
496 calculated by Perseus (1.6.6.0). Proteins ratios between control and infection ≥ 1.5 -fold
497 and *pValue* ≤ 0.05 were considered as regulated differentially expressed proteins

498 (DEPs). DEGs and DEPs were used as query to search for enriched biological processes
499 (Gene ontology BP) using Metascape (**Zhou et al., 2019**). KEGG pathway enrichment
500 and protein interaction network were analyzed using STRING (**Szklarczyk et al.,**
501 **2019**). Heatmaps of gene expression levels were constructed using pheatmap package
502 in R (<https://cran.rstudio.com/web/packages/pheatmap/index.html>). Dot plots and
503 volcano plots were constructed using ggplot2 (<https://ggplot2.tidyverse.org/>) package
504 in R.

505 **Statistical analysis**

506 Data were analyzed using GraphPad Prism 8 (GraphPad Software, San Diego,
507 California, USA). The values shown in the graphs are presented as the mean \pm standard
508 deviation of at least three independent experiments. Statistical differences between
509 groups were analyzed using two-tailed unpaired t-tests or a one-way ANOVA statistical
510 test with Dunnett multiple comparisons tests; $p < 0.05$ was considered statistically
511 significant.

512

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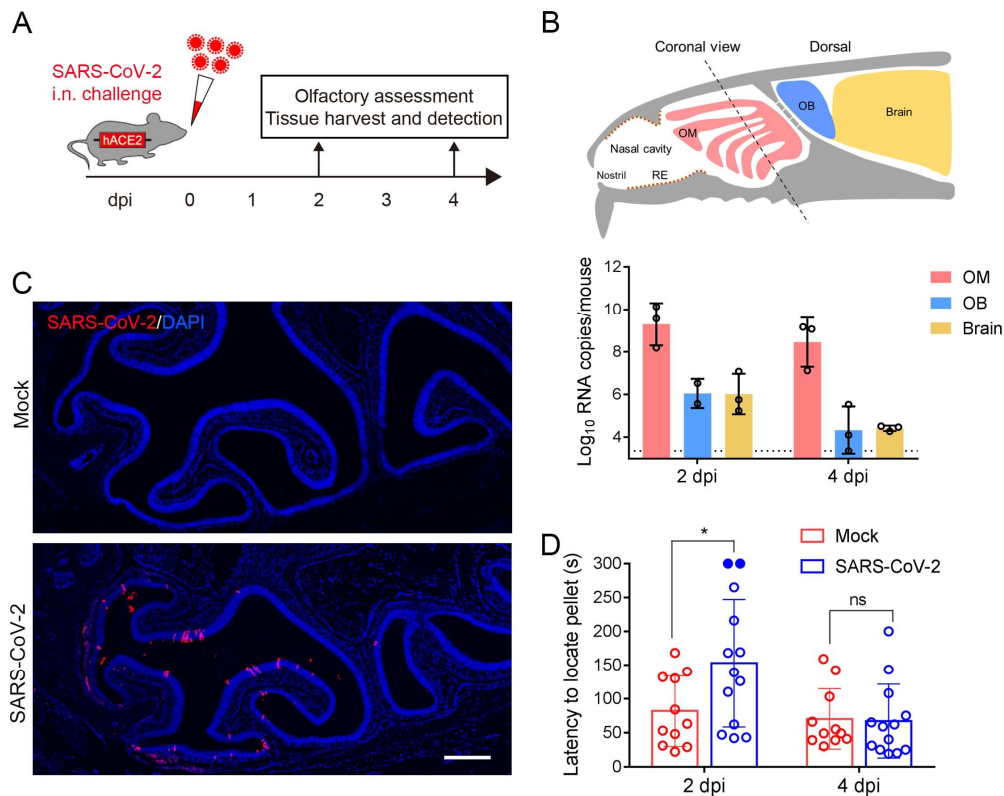
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652

653

654 **Figures and legends**



655

656 **Figure 1. SARS-CoV-2 primarily infects the OE and causes olfactory dysfunction**
657 **in hACE2 mice.**

658 (A) Schematic diagram of experimental design. Briefly, groups of 6-8 weeks old
659 hACE2 mice were infected with 5.4×10^5 PFU of SARS-CoV-2 intranasally. Olfactory
660 function of infected mice was measured by the buried food pellet test at indicated times
661 post inoculation. Mice were sacrificed at 2 dpi and 4 dpi for viral detection and
662 histopathological analysis.

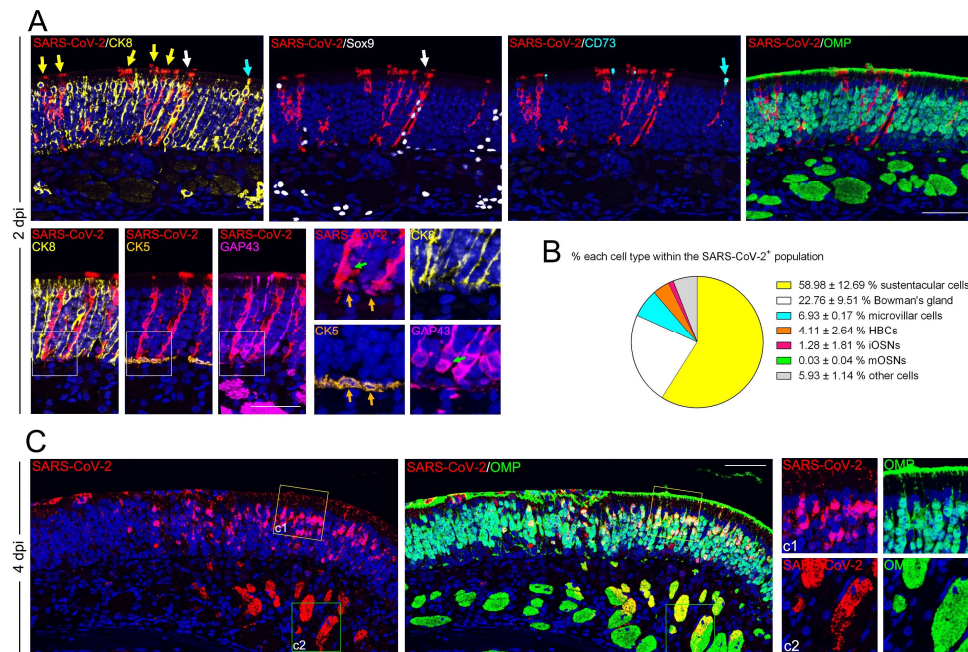
663 (B) Schematic view of the OM in the nasal cavity of mice in a sagittal plane, the dotted
664 line indicated a coronal section (upper). And viral RNA copies were determined by real-
665 time qPCR and shown as mean \pm SD from three independent replicates (lower).

666 (C) Immunostaining of OM from SARS-CoV-2 infected mice for SARS-CoV-2 N
667 protein (red) and DAPI (blue). Scale bar, 400 μ m.

668 (D) Buried food pellet test. Latency to locate the food pellets for mice infected with
669 SARS-CoV-2 (n=13) or DMEM (n=11) was measured at 2 dpi and 4 dpi.

670 See also Figure S1.

671



672

673 **Figure 2. SARS-CoV-2 primarily targets non-neuroepithelial cells in the OE.**

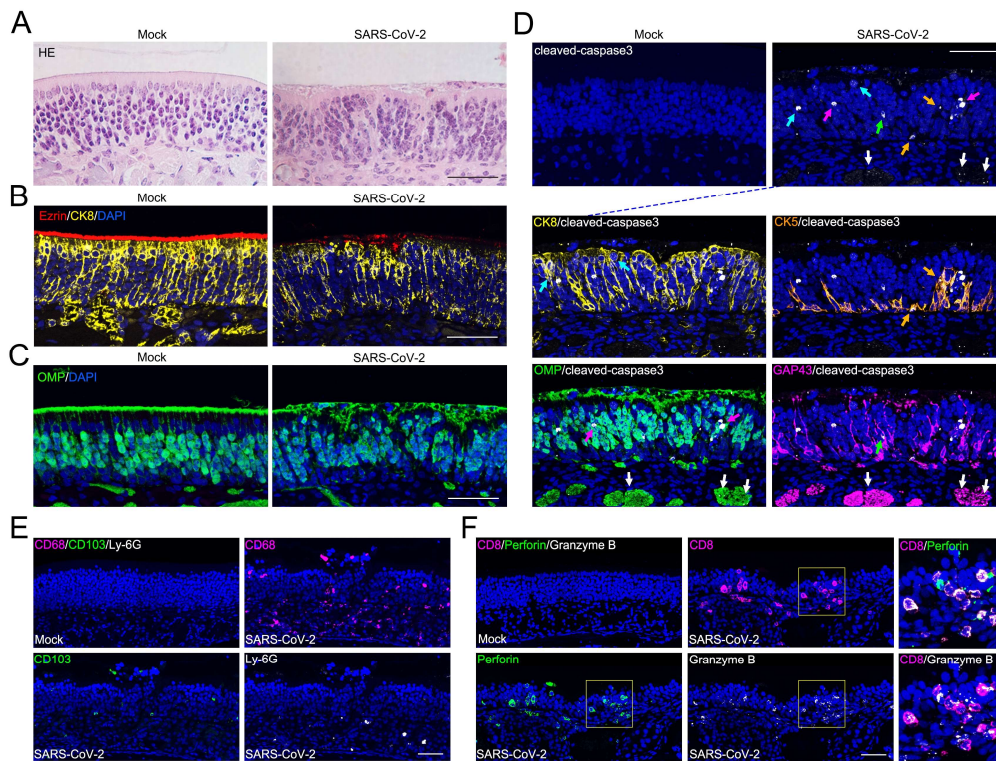
674 (A) Representative multiplex immunofluorescent staining shows SARS-CoV-2
675 (SARS-CoV-2 N protein-positive) infects sustentacular cells (CK8-positive, yellow
676 arrows), Bowman's gland cells (Sox9/CK8-positive, white arrows), microvillar cells
677 (CD73/CK8-positive, cyan arrows), HBCs (CK5-positive, gold arrows) and iOSNs
678 (GAP43-positive, green arrows) at 2 dpi. Little SARS-CoV-2 N protein is detected
679 within OMP-positive mOSNs.

680 (B) Statistical analysis of the percentage of each cell compartment within the SARS-
681 CoV-2-positive cells. Data were presented as mean \pm SD (n = 3).

682 (C) Multiplex immunofluorescent staining shows an OM sample at 4 dpi with SARS-
683 CoV-2 detected in the OMP-positive mOSNs and the underlying nerve bundles. The
684 framed areas labelled as c1 and c2 are shown adjacently at larger magnifications. Scale
685 bar, 50 μ m.

686 See also Figure S2.

687



688

689 **Figure 3. SARS-CoV-2 infection induces apoptosis and immune cell infiltration in**
690 **OE.**

691 (A) Representative hematoxylin-eosin (HE) shows histopathological changes of OE.

692 (B) Representative multiplex immunofluorescent detection of sustentacular cells (CK8-
693 positive) and microvilli (Ezrin-positive) of OE.

694 (C) Representative immunofluorescent detection of mOSNs (OMP-positive) of OE.

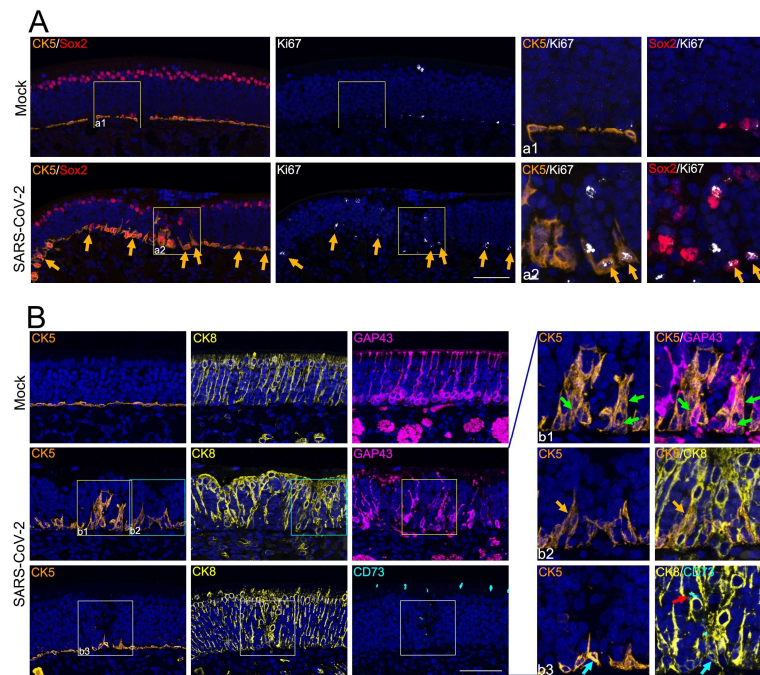
695 (D) Apoptosis of olfactory epithelial cells (cleaved-caspase3-positive, white) after
696 SARS-CoV-2 infection. The panels below shows apoptosis of sustentacular cells (CK8-
697 positive, yellow; indicated by cyan arrows), HBCs (CK5-positive, gold; indicated by
698 gold arrows), mOSN (OMP-positive, green; indicated by magenta arrows), iOSN
699 (GAP43-positive, magenta; indicated by magenta arrows) and olfactory nerve bundles
700 (OMP/GAP43-positive; indicated by white arrows).

701 (E) Representative multiplex immunofluorescent staining shows infiltration of
702 macrophages (CD68-positive, magenta), dendritic cells (CD103-positive, green) and
703 neutrophils (Ly-6G-positive, white) in the OE after infection.

704 (F) Representative multiplex immunofluorescent staining shows infiltration of CD8
705 cytotoxic T lymphocytes (magenta) with expression of Perforin (green) and Granzyme
706 B (white) in the olfactory mucosa after infection. The framed areas are shown
707 adjacently at larger magnifications. Scale bar, 50 μ m.

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711 **Figure 4. SARS-CoV-2 infection triggers regeneration of OE.**

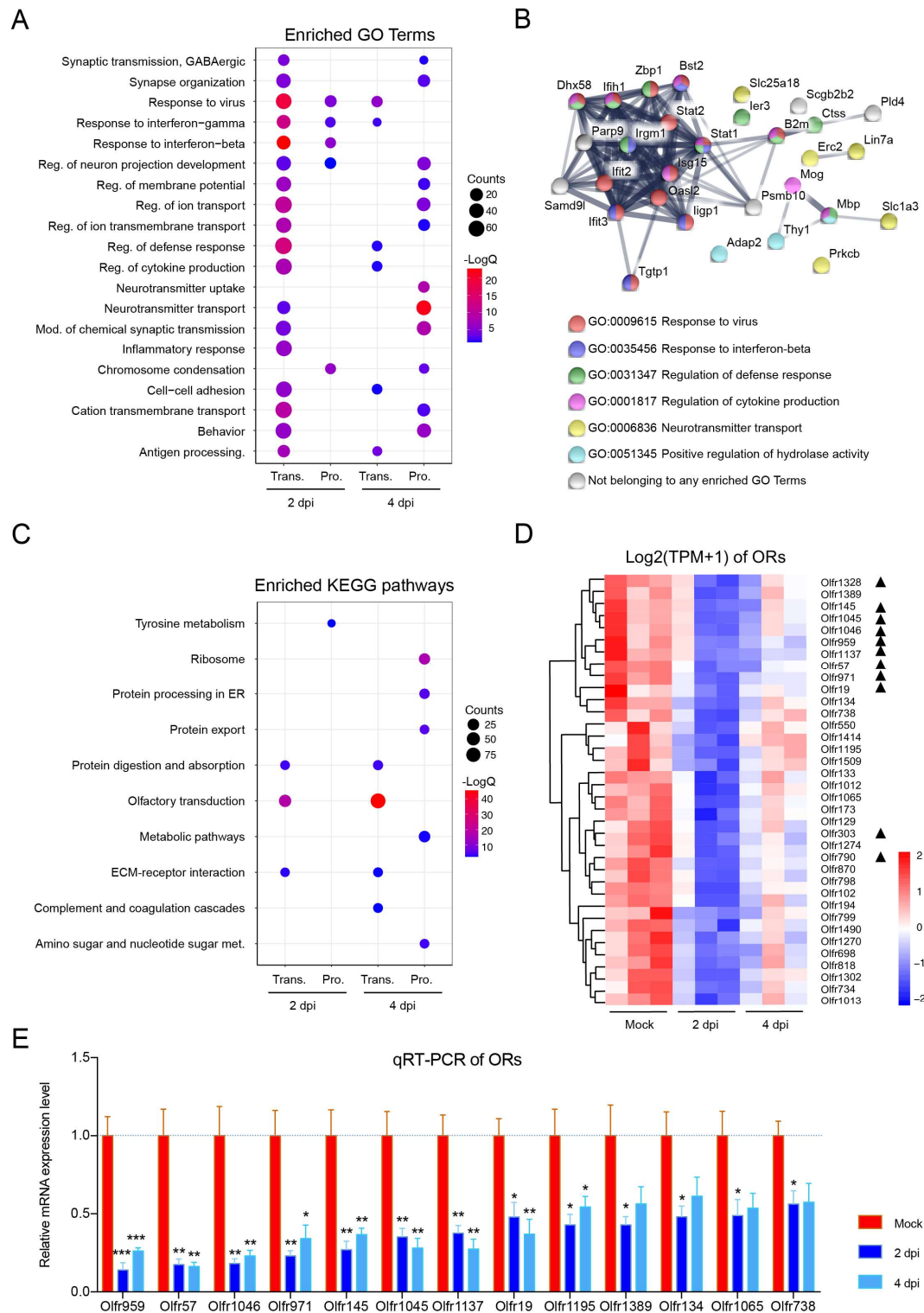
712 (A) Representative immunofluorescent staining of CK5 (gold), Sox2 (red) and Ki67
713 (white) shows the increase of actively cycling olfactory stem cells as labelled
714 CK5/Sox2/Ki67-triple-positive after infection (gold arrows). The framed areas labelled
715 as a1 and a2 are shown adjacently at larger magnifications.

716 (B) Representative immunofluorescent staining of CK5 (gold), CK8 (yellow), CD73
717 (cyan) and GAP43 (magenta) shows the transition states during the differentiation of
718 HBCs. The framed areas labelled as b1–b3 are shown adjacently at larger
719 magnifications. Green arrows in b1 denote CK5/GAP43 double-positive cells. Gold
720 arrows in b2 denote CK5/CK8 double-positive cells. Cyan arrows and red arrow in b3
721 denote CK5/CK8 and CK8/CD73 double-positive cells, respectively. Scale bar, 50 μm.

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 726 **Figure 5. Host response to SARS-CoV-2 in OE at the mRNA and protein levels.**

727 (A) Dotplot visualization of enriched GO terms of up-regulated genes/proteins at 2/4
 728 dpi in OE. Gene enrichment analyses were performed using Metascape against the GO
 729 dataset for biological processes. “Reg.” for regulation, “mod.” for modulation, and
 730 “antigen processing.” for antigen processing and presentation of peptide antigen.

731 (B) Interaction map of 30 proteins which consistently up-regulated at both
 732 transcriptomic and proteomic levels along the course of SARS-CoV-2 infection in OE.

733 Network nodes represent proteins, and their colors indicate different GO Terms they
734 belonging to. Edges represent protein-protein associations, and their thickness indicates
735 the strength of data support.

736 (C) Dotplot visualization of enriched KEGG pathways of down regulated
737 genes/proteins at 2/4 dpi in OE. Gene enrichment analyses were performed using String
738 against the KEGG dataset. “Met.” for metabolism. The color of the dots represents the
739 $-\text{Log}Q$ value for each enriched KEGG pathways, and size represents the gene/protein
740 counts enriched in each term.

741 (D) Heatmap indicating the expression patterns of 36 olfactory receptor genes which
742 were significantly down regulated at 2 dpi. Colored bar represents Z-score of \log_2
743 transformed TPM+1. Total 11 of them also down regulated at 4 dpi were marked with
744 black triangles.

745 (E) RNA expression of 13 representative ORs by qRT-PCR. Columns with *, **, ***
746 indicate ORs significantly down regulated at $p < 0.05$, $p < 0.01$ or $p < 0.001$ relative to their
747 Mock groups (One-way ANOVA followed by post hoc analysis with Turkey test, $n =$
748 3) respectively. Black triangles marked ORs were down regulated at both 2 and 4 dpi
749 based on transcriptome data.

750 See also Figure S3, S4 and S5. Table S1.