1   **Evolution of nasal and olfactory infection characteristics of SARS-CoV-2 variants**

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Abstract

SARS-CoV-2 infection of the upper airway and the subsequent immune response are early, critical factors in COVID-19 pathogenesis. By studying infection of human biopsies in vitro and in a hamster model in vivo, we demonstrated a transition in tropism from olfactory to respiratory epithelium as the virus evolved. Analyzing each variants revealed that SARS-CoV-2 WA1 or Delta infects a proportion of olfactory neurons in addition to the primary target sustentacular cells. The Delta variant possesses broader cellular invasion capacity into the submucosa, while Omicron displays longer retention in the sinonasal epithelium. The olfactory neuronal infection by WA1 and the subsequent olfactory bulb transport via axon is more pronounced in younger hosts. In addition, the observed viral clearance delay and phagocytic dysfunction in aged olfactory mucosa is accompanied by a decline of phagocytosis related genes. Furthermore, robust basal stem cell activation contributes to neuroepithelial regeneration and restores ACE2 expression post-infection. Together, our study characterized the nasal tropism of SARS-CoV-2 strains, immune clearance, and regeneration post infection. The shifting characteristics of viral infection at the airway portal provides insight into the variability of COVID-19 clinical features and may suggest differing strategies for early local intervention.

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative pathogen in the worldwide pandemic of coronavirus disease 2019 (COVID-19), is readily transmitted via respiratory droplets during close contact. The nasal cavity is the entry point of respiratory tract, and the high viral load detected there indicates that this is the principal initial site of SARS-CoV-2 infection and immune response[1,2]. The loss of the sense of smell is common in patients infected

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60 with the SARS-CoV-2 original WA1 and later Delta strains. Pathological studies visualizing

61 olfactory viral infection in postmortem samples of nasal respiratory and olfactory epithelium

62 partially explain the smell loss in COVID-19 patients[3]. Unlike the previous strains, Omicron

63 rarely causes olfactory loss, possible suggesting a change in cellular tropism as the virus evolved.

64 Systematic characterization of the SARS-CoV-2 infection pattern in the nose is important for

65 understanding COVID-19 pathogenesis and for developing early local intervention.

66 The cellular tropism of SARS-Cov-2 in the nasal cavity is relevant to pathologic tissue damage

67 and to COVID testing. Cellular entry of SARS-CoV-2 depends on the binding of the virus spike

68 S protein to angiotensin-converting enzyme 2 (ACE2) in host tissue[4,5]. The level of viral

69 receptors and its subcellular localization is a key determinant of susceptibility to infection. In

70 parallel with the gradually decreased ACE2 RNA expression pattern from the upper airway to

71 distal intrapulmonary regions[6,7], *in vitro* SARS-CoV-2 infection of human respiratory epithelial

72 cell cultures shows a gradient of diminishing infectivity (olfactory epithelial cells were not

73 included in these studies)[7]. Notably, we have observed up to 700-fold higher expression of ACE2

74 in the sustentacular cells of olfactory epithelium in comparison to respiratory epithelial cells in

75 human nose and trachea[8]. Whether the enrichment of ACE2 in the olfactory epithelium correlates

76 with more susceptibility to SARS-CoV-2 infection than respiratory cells, and how the infection

77 affects the olfactory sensory neurons are largely unknown.

78 Despite the substantially reduced COVID-19 incidence in adults after the rapid

79 vaccination program rollout, the unvaccinated population including young children, as well

80 as break-through infections by new variants, now comprise the majority cases. SARS-CoV-

81 2 infection typically causes mild acute airway illness in approximately 81% of COVID-19

82 patients; however, 14-17% of hospitalized cases experience severe symptoms and require

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83   intensive care[9]. The severity of COVID-19 is highly age-related, with a fatality rate that can

84   reach 30.5% in patients of 85 years or older[10], suggesting compromised anti-viral immunity with

85   aging. The correlation between aging and the cellular damage and subsequent immune response

86   to SARS-CoV-2 requires further investigation.

87   In this study, we perform *in vitro* infection of human nasal explants and show an extremely

88   high infection rate of SARS-CoV-2 WA1 strain in olfactory epithelium relative to the adjacent

89   respiratory epithelium. By comparing the infection patterns of WA1, Delta, and Omicron strains

90   in the hamster nasal cavity, we demonstrated a transition in tropism from olfactory to respiratory

91   epithelium as the virus evolved, providing insight into COVID-19 pathogenesis and diagnosis.

92   Using a WA1 strain infected hamster model, our additional findings demonstrate an age-

93   associated infection of olfactory neurons and impaired macrophage phagocytosis. These

94   findings indicate that the nasal viral replication and local immune defense could be a

95   potential target of early intervention.

96

97   **Results**

98   **WA1 strain primarily targets human olfactory neuroepithelium**

99   The human olfactory mucosa is located in the superior part of the nasal cavity contains

100   sustentacular cells and olfactory sensory neurons that is responsible for the sense of smell. To

101   examine the precise cellular tropism of SARS-CoV-2 in the nasal cavity including both

102   respiratory and olfactory epithelium, we initially performed WA1 strain *in vitro* infection

103   experiments using human nasal tissue discarded in endonasal sinus and skull base surgery in

104   COVID-19-negative individuals. To establish a reliable protocol to detect SARS-CoV-2 antigen,

105   we screened and verified 4 different antibodies for visualizing spike (S) or nucleocapsid protein

106   (NP) in the infected tissue sections (Extended Data Fig. 1a). The staining pattern of antibodies

107   predominant located in apical sustentacular cells is consistent with the viral RNA detected by

108   RNAscope analysis (Extended Data Fig.1b).

109   Because the olfactory mucosa is irregularly distributed and surrounded by respiratory

110   epithelium in the human nasal cavity [11], we included the neuronal marker Tuj1 for the

111   verification of olfactory epithelium. By immunostaining with SARS-CoV-2 NP, we observed

112   substantial viral antigen in the $Tuj1^+$ olfactory region at 9 hours post infection, but very little NP

113   in the adjacent Tuj1 negative respiratory epithelium (Fig. 1a,b). The vast majority of NP^+ cells

114   co-localized with $Krt18^+$ olfactory sustentacular cells (Extended Data Fig. 1c,d). Viral infection

115   caused extensive sustentacular cell death, with rapid detachment and sloughing into the nasal

116   lumen (Fig. 1a,b, and Extended Data Fig. 1c,d). Compared to the mock control (Extended Data

117   Fig. 1e), structural damage was readily detected in the olfactory mucosa but not in the respiratory

118   epithelium (Fig. 1a, b). A high viral infection ratio was also found in human olfactory cleft

119   specimens obtained from skull base surgery (Fig. 1c). Low viral infection was observed in 24

120   explants that only contained respiratory epithelium (Fig. 1d,e). We quantified the number of NP^+

121   cells in 7 tissue explants, revealing 100-300-fold more infected cells in olfactory epithelium

122   compared to adjacent respiratory epithelium (Fig. 1e). These results together our earlier observed

123   enrichment of ACE2 expression illustrated an olfactory specific tropism of SARS-CoV-2 WA1

124   strain and explained the common symptom of anosmia in COVID-19 patients.

125   **Omicron variant shows transition in tropism from olfactory to respiratory epithelium**

126   As SARS-CoV-2 evolved, new variants including Delta and Omicron caused surges in

127   cases worldwide. The tropism of these different strains in the nasal cavity has not been

128   clarified. We next characterized the cellular tropism of WA1, Delta, and Omicron in nasal

129 mucosa using a hamster model. These experiments allowed us to determine whether the

130 observed cellular tropism of WA1 in human olfactory epithelium is applicable to new

131 variants in animal models and relates to subsequent disease pathogenesis.

132 The olfactory mucosa in the hamster nasal cavity is located in the posterior and dorsal

133 aspect, while the anterior and ventral areas are respiratory. We confirmed that the

134 expression of the neuronal marker Tuj1 in olfactory epithelium was mutually exclusive

135 with the respiratory marker Foxj1 in the nasal cavity (Extended Data Fig. 2a). Therefore,

136 the olfactory epithelium can be identified based on Tuj1 positive staining, the presence of

137 axon bundles, and the relatively increased thickness of the neuroepithelium. After SARS-

138 CoV-2 inoculation (1×10^5 TCID50), we captured confocal images of the entire nasal cavity

139 in coronal sections at three different levels (Fig. 2a). At 4 days post infection (dpi), we verified

140 the extremely high viral antigen NP in the Tuj1$^+$ olfactory epithelium of WA1 or Delta-infected

141 hamsters, with a sharp decline in the adjacent respiratory epithelium (Fig. 2b). About 79.2% or

142 70.3% length of Tuj1$^+$ olfactory epithelium was infected by WA1 or Delta, respectively (Fig. 2b,

143 c). We observed that the expression of ACE2 in some OMP$^+$ olfactory areas is low or

144 undetectable, interpretating the uninfected areas in WA1 or Delta treated groups (Extended Data

145 Fig. 2b).

146 In contrast to WA1 or Delta strain, the infected olfactory epithelium in Omicron group was

147 dramatically reduced to 6.7%, which is consistent with earlier reports of a comparatively lower

148 pathogenicity in lung of Omicron infected hamsters[12,13]. The low infection rate of Omicron in

149 olfactory epithelium (Fig. 2b, c) seems to correlate with the low incidence of smell loss in

150 patients. Interestingly, we observed the Omicron infected NP$^+$ nasal and sinus respiratory cells

151 was increased 7-10-fold when compared to WA1 or Delta, suggesting an olfactory to respiratory

152 tropism transition with the Omicron variant (Fig. 2b, d). These tropism patterns were further

153 demonstrated in sections of the anterior or posterior nasal cavity where the proportion of

154 respiratory epithelium is much greater or less, respectively (Extended Data Fig. 2c, 3a). Together,

155 these results identify that the SARS-Cov-2 variants have different tropism in nasal mucosa that

156 may play a role in the shifting pathogenic features of COVID-19 as the virus evolved.

157 To support the olfactory epithelial tropism of WA1 and Delta, we further performed qPCR

158 analysis of ACE2 expression in the entire nasal respiratory or olfactory mucosa in C57BL/6J

159 wildtype mouse at the ages of 2 weeks, 2 months, and 19 months. Compared to the nasal

160 respiratory epithelium, ACE2 mRNA transcription in adult olfactory epithelium was increased 5-

161 7-fold in 2m or 19m old animals (Fig. 2e). It should be noted that ACE2 mRNA levels in whole

162 olfactory mucosa are greatly diluted by the larger proportion of ACE2-low-to-negative cells

163 (neurons), relative to respiratory mucosa. In addition, ACE2 protein in human[14,15] or mouse[16]

164 epithelial tissue is predominantly expressed at the apical surface. The more diffuse cellular

165 pattern of ACE2 staining in human autopsy specimens may result from post-mortem degradation.

166 In any putative human olfactory tissue sample, a neuronal marker must be utilized for

167 verification because the olfactory mucosa is irregularly distributed and surrounded by respiratory

168 epithelium. Consistent with previous data, we observed a gradually increased ACE2 expression

169 in olfactory mucosa from 2 weeks through adulthood[17,18]. The level of ACE2 expression in nasal

170 respiratory mucosa was comparable between young and old animals (Fig. 2e). Together, the

171 ACE2 expression pattern supports the olfactory epithelium as a site of SARS-CoV-2 replication

172 especially for WA1 or Delta variants. The decreased olfactory tropism in the Omicron variant is

173 consistent with the recently reported endocytic entry pathway[19,20].

174 **Delta variant demonstrates greater infection of cells in the nasal submucosa**

175 In the lamina propria, we frequently detected NP$^+$ cells in Delta inoculated hamsters at 4dpi.

176 Co-staining of NP and Pan-cytokeratin revealed that some of those infected cells were Bowman's

177 glands (Fig. 3a), the producer of specialized mucus critical for odor perception[21]. These results

178 are in line with our previously reported ACE2 expression in human biopsies[8] and the observation

179 that SARS-CoV-2 targets Bowman's glands in postmortem samples by other groups[3]. The

180 number of NP$^+$ Bowman's glands in Delta infected hamsters increased 21-fold when compared to

181 WA1, and is sharply decreased in Omicron group (Fig. 2b, 3b). Additionally, NP$^+$ elongated

182 submucosal cells can be readily detected in olfactory and respiratory mucosa of Delta-infected

183 animals (Fig. 3c) but is dramatically reduced in Omicron treated hamsters. These NP$^+$ cells are

184 aSMA$^+$ but negative for Iba1 (macrophage marker) and Vimentin (mesenchymal and olfactory

185 ensheathing cell marker), suggesting the contractile myofibroblasts/mesenchymal cell lineage

186 (Fig. 3c, Extended Data Fig. 3b). The broader cell types targeted in the submucosa by the Delta

187 variant may increase the severity of tissue damage.

188 We next asked whether the infected submucosal cells are rapidly cleared or instead serve as an

189 ongoing viral reservoir. At 7dpi, we observed almost all the NP$^+$ olfactory epithelial cells had

190 been lost, other than those in sloughed off debris in the nasal lumen. In the submucosa, except

191 NP$^+$ axon in WA1 group, NP$^+$ cell was barely detectable in animals infected with any of the 3

192 strains (Fig. 3d). These results in agreement with the reported viral titer analysis at 7dpi[12,13].

193 However, in the paranasal sinuses, an area was not examined in earlier studies[12,13], we detected a

194 small number of NP$^+$ respiratory epithelial cells in WA1 but rarely in Delta treated hamsters at

195 7dpi. In parallel with the tropism transition from olfactory to respiratory epithelium, more

196 pronounced NP$^+$ sinonasal epithelial cells (3.3 positive cells/mm epithelium) were observed in

197 Omicron variant-treated hamsters (Fig. 3d, e), suggesting a longer duration of the Omicron

198   variant infection in sinus epithelium relative to the ancestral SARS-CoV-2 strains. It is unknown

199   whether those Omicron -infected cells in the sinuses are actively transmitting virus at 7dpi.

200   Age associated SARS-CoV-2 WA1 infection of olfactory sensory neurons

201   While neurological symptoms, including headache, encephalitis, and altered mental status

202   have been reported in COVID-19 patients [22,23], the evidence of SARS-CoV-2 olfactory neuronal

203   infection is controversial[3,24]. Earlier studies have shown SARS-CoV-2 RNA or viral antigen in

204   postmortem brain tissue samples[25,26], and rare infection observed in olfactory neurons in autopsy

205   tissue hints towards transmucosal invasion[27]. The reported data have indicated SARS-CoV-2

206   infection affects neurons in the hamster model[28,29]; however, Tuj1$^+$ immature neurons are

207   normally located next to the basal layer, and the long foot-like processes of infected

208   sustentacular cells surrounding olfactory neurons could be mis-interpreted in earlier reports.

209   The direct evidence of olfactory neuronal infection and the factors that affect the frequency of

210   infection and entry to the brain remain to be clarified[3,24].

211   Given the high tropism of SARS-CoV-2 WA1 or Delta in olfactory mucosa, we took

212   advantage of a hamster model to examine WA1 or Delta infection in the olfactory neuronal

213   population. The hamster model allowed us to avoid the significant limitations of autopsy tissue,

214   including an often prolonged and severe disease course and tissue degradation during the

215   postmortem interval. We utilized a higher viral inoculum (1×10^7 TCID50) to generate more

216   uniform infections that would allow us to identify variation across age groups[30]. As expected, we

217   observed the vast majority of NP$^+$ cells were apical sustentacular cells[31] in WA1 infected

218   hamsters (Extended Data Fig.1a,b) at 4 dpi. Interestingly, in the superior turbinate of posterior

219   nasal cavity, we observed NP labeling of a small portion of cells located in the olfactory sensory

220   neuronal layer and their axon bundles (Fig. 4a). Co-staining of NP with neuronal markers

221   Tuj1(immature) and OMP (mature) revealed viral infection in a subset of cells from the neuronal

222   lineage (Fig. 4b, c). NP[+]/OMP[+] infected olfactory neurons were also detected in Delta variant

223   treated hamsters (Extended Data Fig. 4a). We detected viral antigen travel along the Tuj1^{+} axon

224   from epithelium to the lamina propria (Fig. 4d, e). In axon bundles, NP co-localized with Tuj1^{+}

225   or OMP[+] axons (Extended Data Fig. 4b, c) but did not colocalize with Vimentin[+] ensheathing

226   cells (Extended Data Fig. 4d). In addition, we confirmed the olfactory neuronal infection by

227   WA1 or Delta at 1×10^5 TCID50 (Extended Data Fig. 4e, f). Precise quantification of the number

228   of infected olfactory neurons is a challenge because the intensity of marker staining in infected

229   and dying cells subsides[3] compared to normal cells (Extended Data Fig. 4g) and because the

230   epithelium sloughs off in some areas. We observed at least 20 NP[+]/OMP[+] or Tuj1^{+} neurons in

231   each section of hamster infected with the WA1 at 1×10^5 TCID50. Compared to WA1, olfactory

232   neuronal infection is sharply decreased in Delta and rare in Omicron group. These data suggested

233   that WA1 or Delta can also infect a proportion of olfactory sensory neurons, in addition to

234   sustentacular cells that are the primary target in the upper airway. We therefore used WA1 strain

235   for the following aging-related experiments.

236   The rare expression of ACE2 in olfactory sensory neurons[8,16] suggests that neuronal entry may

237   mediated by other receptors such as Neuropilin-1(Nrp1)[32,33]. In the olfactory epithelium, Nrp1

238   was expressed in the olfactory nerve in the embryonic stage and in immature neurons after

239   birth[34,35]. By using qPCR analysis, we detected 2.7-fold reduction of Nrp1 mRNA in the

240   olfactory epithelium of 19-month-old compared to 2 weeks young mice (Extended Data Fig. 5a).

241   Age related Nrp1 reduction in the olfactory epithelium was also verified by

242   immunohistochemistry. About 34.2% of Tuj1^{+} olfactory neurons express Nrp1 in young mice but

243   only 9.7% of Tuj1^{+} neurons in the aged group display a low level of Nrp1(Extended Data Fig.

244   5b-d). A few mature olfactory neurons in young mice also express Nrp1 (Extended Data Fig. 5b).

245   In addition, Nrp1 can be detected in the axon bundles and periglomerular cells in young olfactory

246   bulb, but are sharply declined in aged mice (Extended Data Fig. 5b,e).

247   The age-related pattern of Nrp1 expression indicated a potential higher efficiency of SARS-

248   CoV-2 infection in olfactory neurons in young population. To assess whether age could be a

249   factor mediating neuronal infection in the olfactory epithelium, we performed SARS-CoV-2

250   WA1 (1×10^7 TCID50) infection experiments using young (1-month) and aged (8-month)

251   hamsters. At 6 dpi, viral antigen (NP) could be readily detected in the axon bundles in young

252   hamsters, but infected axons were dramatically decreased in older hamsters (Fig. 4 f-h). We also

253   examined the WA1-infected human explants and identified a remarkable increase of viral load in

254   Tuj1$^+$ neurons and axon bundles in tissue from young individuals (<30 years old) (Fig. 4i, j). As

255   expected, we observed 38.9% of Tuj1$^+$ olfactory neurons co-express Nrp1 in younger human

256   biopsies, but the proportion of Tuj1$^+$/Nrp1$^+$ neurons dramatically reduced (7.2%) in older adults

257   (Fig. 4k, l). Together, these results support age-dependent olfactory neuron infection and axonal

258   transport.

259   **Increased olfactory bulb axonal transport of WA1 in young hamsters**

260   The increased frequency of viral NP in the axons of younger animals indicated that SARS-

261   CoV-2 WA1 may be prone to accessing the brain in this population. To verify this hypothesis,

262   we examined the olfactory bulbs of 1 and 8-month old hamsters. At 6 dpi. we detected NP$^+$

263   axons located in the olfactory nerve layer (ONL) in young hamsters (Fig. 5a,b), suggesting the

264   viral transport to olfactory bulb. Compared to the young hamsters, infected axons are rarely

265   detected in the older group (Fig. 5a-c). Co-staining analysis of serial sections verified that the NP

266   signal is located in the Tuj1$^+$ olfactory nerve layer (Fig. 5d). In the leptomeningeal layer where

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267   the viral RNA signal was detected in postmortem samples[3], the NP antibody staining was not

268   detectable in hamster (Fig. 5a-d). In addition, the observed leptomeningeal viral RNA staining

269   was speculated to be extracellular virions instead of intracellular viral RNA synthesized by

270   infected cells[3]. In parallel to the greater olfactory bulb viral transport, the number of Iba1$^+$

271   microglia cells in young olfactory bulb was increased 1.7-fold compared with older group (Fig.

272   5e). No viral antigen could be detected in the mock control.

273   Immunostaining of horizontal sections crossing the olfactory mucosa and forebrain region

274   revealed a massive number of NP$^+$ axons traveling from the lateral olfactory epithelium to

275   olfactory bulb in young, but not aged, hamsters at 6 dpi (Extended Data Fig. 6a-f). In line with

276   the reported Nrp1 expression in lateral olfactory nerve, which contains axons from turbinate

277   neurons[36], the infected axon in the septum nerve was rare. NP$^+$ axons could also be detected in

278   glomeruli where the olfactory sensory neuron axon terminal projections synapse with OB mitral

279   cells (Fig. 5b, d) at 6dpi. As a consequence of olfactory viral transport, we observed Caspase-3$^+$

280   apoptotic cells and virus RNA in the glomerular layer at 4dpi (Fig. 5f, and Extended Data Fig.

281   6g,h) in the young group. These Caspase-3$^+$ cells were negative for Iba1 or the neuronal marker

282   NeuN. The transported virus in olfactory bulb appears to lose the capacity for replication based

283   on the restriction of NP signal to axons in the outer olfactory nerve layer and glomeruli at 6dpi

284   (Fig. 5b-d). Despite the close anatomic relationship between the olfactory mucosa and the nearby

285   OB axons, no obvious transmucosal viral antigen NP was displayed except within axons.

286   Similar to ACE2 expression in lung vascular endothelial cells[37], ACE2 in the mouse or

287   hamster olfactory bulb is mainly located in the blood vessels (Extended Data Fig. 6i,j). We

288   observed CD45$^+$/Iba1$^-$ immune cells infiltrating into the olfactory bulb in SARS-Cov2 infected

289   hACE2 mice (Extended Data Fig. 6k,l), indicating passage of leukocytes across an impaired

290   blood-brain barrier. Given the lack of lymphatic vessels in brain parenchyma, it is unlikely that

291   viral infection of the olfactory bulb occurs via this route[38]. The inflammatory response in the

292   hamster brain is not as severe as in the hACE2 mouse model, therefore the vascular damage is

293   also likely much milder in hamster. Together, these results support that SARS-CoV-2 WA1 can

294   gain access to the olfactory bulb region in the brain mainly through olfactory neuronal axons

295   with higher frequency in younger population, while virus replication is limited.

Age-related viral clearance delay and phagocytic dysfunction in the olfactory mucosa

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297   The tropism of SARS-CoV-2 in olfactory epithelium indicates the capacity of local immune

298   system against viral infection could involve in the pathogenesis of COVID-19. It has been

299   reported that reduced innate antiviral defenses including type I and type III interferons coupled

300   with a hyperinflammatory response is the major cause of disease severity and death in COVID-

301   19 patients[39,40]. Corresponding to the high viral load in olfactory epithelium, our qPCR analysis

302   revealed an extensive upregulation of the anti-viral gene Ifng (type II interferon) in the nasal

303   turbinate tissue post infection (Extended Data Fig. 7a), suggesting activated local immune

304   defense. We next studied the potential age-related alternation of olfactory immune response to

305   SARS-CoV-2 infection.

306   Because of the limited cross-reactivity of CD45 antibodies with hamster tissue, we took

307   advantage of the mouse adapted SARS-CoV-2 (maSARS) infection model in C57BL/6J

308   wildtype mice[41]. Normally, a low number of CD45$^+$ immune cells and Iba1$^+$

309   macrophages/dendritic cells reside in the mouse olfactory mucosa. In maSARS infected group,

310   we observed striking CD45$^+$ immune cell infiltration into the lamina propria, crossing the basal

311   cell layer and migrating into the neuroepithelium, suggesting a nasal immune defense in response

312   to viral infection (Fig. 6a). On 6 dpi, approximately 48.3% of CD45$^+$ immune cells in olfactory

313   mucosa were Iba1$^+$ macrophages/dendritic cells, which is similar to single-cell RNA sequencing

314   data of BALF samples from critical COVID 19 patients[42,43].

315   In hamsters, intranasal inoculation of SARS-CoV-2 induced massive shedding of NP$^+$ infected

316   cells into the nasal lumen at 4 dpi (Fig. 6b-d), consistent with our findings in infected human

317   olfactory biopsies. Iba1$^+$ macrophages/dendritic cells were widely distributed in the olfactory

318   mucosa and the detached cells in the lumen (Fig. 6b,c). Co-staining analysis showed that the

319   Iba1$^+$ macrophages are the major population producing CXCL10 (Extended Data Fig. 7b), a

320   chemokine that has been reported in macrophages from COVID-19 patients' BALF sample.

321   Notably, 72% of Iba1$^+$ cells were also positive for viral NP antigen at 4dpi (Fig. 6b), indicating

322   uptake of infected cell debris. In addition, some of the apoptotic cells sloughed into the nasal

323   lumen were Iba1$^+$/Caspase-3$^+$, suggesting the viral clearance by macrophages (Fig. 6c).

324   Compared to the young hamsters, the number of Iba1$^+$ cells in the nasal lumen significantly

325   increased in the older group at 6dpi (Fig. 6d,e). In parallel to the increased macrophages, we

326   observed the number of remaining NP+ cells/debris in the serial sections of older hamster nasal

327   cavities was increased 3.7-fold when compared to young hamsters (Fig. 6d,f), in line with the

328   reported prolonged virus load/delayed viral clearance in older COVID patients[44]. The delayed

329   viral clearance could be a consequence of impaired phagocytic function in aging macrophages,

330   as reported in an influenza infection model[45].

331   By analyzing a previously published single cell RNA sequencing (scRNA-seq) dataset[46]

332   derived from mouse lung CD45$^+$ inflammatory cells, we noted significant reduction of

333   phagocytosis related genes[47] including Clec4n (Dectin2), Fabp5, Fpr2, and Cd9 in old

334   macrophage/dendritic lineages compared to young mice (Fig. 6g). We further verified that the

335   expression of Dectin2 was dominantly located in Iba1$^+$ macrophages/dendritic cells in olfactory

336 mucosa of young mice and dramatically declined with age (Fig. 6h). Collectively, our data

337 support that the macrophages are the critical population involved in SARS-CoV-2 defense, and

338 their impaired viral clearance capacity could involve in the prolonged virus retention in the

339 olfactory mucosa of the aged population.

340 **Regenerated olfactory epithelium regains ACE2 expression**

341 Given the robust reparative capacity of the olfactory mucosa[48] and the rapid reconstitution

342 post SARS-CoV2 infection[31,49], we next systematically examined post-viral stem cell-mediated

343 regeneration using an adult hamster model (2-month old). As a consequence of viral infection,

344 nearly complete loss of neuroepithelium was observed at 4dpi, and ACE2 was not detectable in

345 newly regenerated epithelium (Fig. 7a). Compared to the single layer of Krt5-expressing

346 olfactory stem cells in mock control, SARS-CoV-2 induced widespread epithelial damage and

347 activated robust basal cell proliferation simultaneously (Extended Data Fig. 7c). qPCR analysis

348 revealed that the increased expression of Sox2 (basal cell /sustentacular cell marker), Lgr5

349 (globose basal cell marker), and Tubb3 (immature neuron marker) was coincident with gradual

350 re-expression of ACE2 as olfactory epithelium regeneration proceeded (Fig. 7b, Extended Data

351 Fig. 7d). The expression of ACE2 and the olfactory sensory neuron marker, OMP, recovered to

352 78% and 56% of mock on 28 dpi, respectively (Fig. 7b,c). The incomplete recovery of OMP on

353 28 dpi partially may explain the slow return of olfactory function in human cases with severe

354 damage.

355 Coincident with epithelial repair, production of CXCL10 in Iba1+ macrophages vanished in

356 both the young and old groups on 6dpi (Extended Data Fig. 7b). Compared to the old group, the

357 newly regenerated olfactory epithelium in young hamsters is significantly thicker at 6dpi (Fig.

358 7d,e), suggesting age-related delay in regeneration post infection. Furthermore, recovery of

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359   ACE2 protein could be detected in hamsters at 28 dpi, and ACE2 expression was also observed

360   in a COVID-19 patient who had lost the sense of smell (Fig. 7f,g).

361   **Discussion**

362   Understanding the cellular tropism and properties of SARS-CoV-2 infection of the upper

363   airway could provide valuable insights for predicting the pathogenicity of new variants.

364   Consistent with the enrichment of ACE2 in human olfactory sustentacular cells[8], we herein

365   present greatly enhanced infection efficiency in human and hamster olfactory epithelium,

366   suggesting that this site is potentially critical for initial SARS-CoV-2 infection and replication,

367   especially for the WA1 and Delta strains. The tropism transition from olfactory to respiratory

368   observed in the Omicron variant may explain the low prevalence of anosmia, while the extended

369   duration that Omicron resides in the sinonasal respiratory epithelium may contribute to increased

370   transmission. Our observations, together with the clinical findings of high viral loads in the nasal

371   passages of COVID-19 patients[1,2], suggests that the nasal cavity is an important site of SARS-

372   CoV-2 infection, cell damage, and host immune reaction in nasal cavity.

373   The mechanisms underlying olfactory loss in SARS-CoV-2 infection are difficult to

374   disentangle from a number of pathological processes at multiple anatomic levels[24].

375   Quantification of SARS-CoV-2 in nasal and throat swabs reveals a gradual decrease in viral load

376   soon after symptom onset [2,50], suggesting a short pathological process in the nose. Together with

377   these findings, the rapid detachment of infected olfactory epithelium presented here may explain

378   variation in viral loads detected on nasal swabs[2]. The subsequent neuroepithelial structural

379   damage upon viral targeting of supporting sustentacular cells and olfactory neurons plausibly

380   underlies the high incidence of olfactory dysfunction in COVID-19 patients. Importantly, the

381   detached olfactory epithelium likely carries a large amount of virus, and shedding of these

16

382   infected cells has the potential for aerosolization, exacerbating lung infection, and facilitating

383   transmission between individuals. Other factors include the disrupted nuclear architecture,

384   downregulated olfactory receptor expression[51] in mild infection, as well as the infection of

385   Bowman's glands[3] may also account for the olfactory dysfunction. However, the contribution of

386   the small proportion of olfactory neurons that are become infected based on our observations is

387   likely very limited.

388   Whether and how SARS-CoV-2 gains access to the brain has been investigated intensively and

389   debated widely[24]. Unlike the obvious infection of the brain in hACE2-expressing mice after

390   SARS-CoV-2 inoculation[52-54], viral antigen in hamster brain was not detectable[28,55,56] while one

391   study recovered SARS-CoV-2 from brain tissue[55]. A recent study in a hamster model showed

392   limited viral antigen located in nasal OMP^+ olfactory axons[29]. The presence of SARS-CoV-2

393   RNA or viral antigen in human postmortem brain tissue reveals that the virus may access the

394   brain even though neuronal infection is rare[25-27]. To avoid the tissue autolysis associated with

395   long postmortem intervals, a bedside endoscopic tissue harvest procedure was developed by

396   Khan et al[3]. In 85 postmortem samples analyzed from COVID-19 cases, even though a uniform

397   sustentacular cell infection was visualized in the olfactory mucosa of a patient within 4 days of

398   diagnosis, no infection in olfactory sensory neurons was identified. It should be noted that the

399   samples in the study by Khan et al were limited to relatively aged (>62 years) patients. Although

400   most children and adolescents are spared from severe COVID-19, it is reported that 22%

401   experience neurologic involvement and 12% develop life-threatening neurologic sequelae[57].

402   Abnormal neuroimaging manifestations, including acute disseminated encephalomyelitis-like

403   changes, were also reported in children with COVID-19[58]. Based on infection of young and old

404   hamsters, our observations provide strong evidence that SARS-CoV-2 WA1 targets a subset of

405   mature and immature olfactory neurons, and gains access to the brain through axon transport in

406   an age-dependent manner. The higher proportion of Nrp1^{+} olfactory neuron in the young

407   population may be associated with the increased neuronal infection. It should be noted that a role

408   for other SARS-CoV-2 entry molecules besides Nrp1[59] for the invasion process cannot be

409   excluded from our data.

410   The unique targeting of SARS-CoV-2 (WA1 and Delta strains) to a small neuronal population

411   may have impeded discovery to date. As previously mentioned, the absence of evidence for

412   olfactory sensory neuron infection in postmortem samples could be attributed to the older age of

413   the cohort studied[3]. The enhanced olfactory bulb viral transport and subsequent greater level of

414   microglial infiltration in younger hosts may call for a reassessment of neurological impairment in

415   children. Indeed, recent clinical evidence indicates a recurring pattern of disease with SARS-

416   CoV2-related abnormal CNS neuroimaging in infected children without pre-existing conditions[58].

417   Therefore, the long-term consequences of brain infection require further investigation.

418   In line with previous observations of aging-related deficits of macrophage phagocytosis in

419   influenza infection models[45], the delayed SARS-CoV-2 clearance in older hamsters' olfactory

420   mucosa and in COVID-19 patients may represent a compromised phagocytic function of aged

421   macrophages. The prolonged viral retention may correlate with disease severity in aged COVID-

422   19 patients or with increased risk of transmission. Therefore, the local immune defense in nasal

423   olfactory and respiratory mucosa represents a potential target for early intervention and

424   prevention.

425   Robust olfactory basal cell activation efficiently regenerates sustentacular cells and restores

426   ACE2 expression. The continued ACE2 expression in the olfactory epithelium may be important,

427   given that anti-SARS-CoV-2 antibodies decay after approximately 6 months from the onset of

428 symptoms, especially in individuals with mild COVID-19 disease[60]. The rapid restoration of

429 ACE2 expression in olfactory epithelium may provide an avenue for re-infection in recovered

430 COVID-19 patients. Taken together, our study identifies the tropism of SARS-CoV-2 WA1 and

431 Delta in olfactory epithelium and the transport of virus to the brain through olfactory neuron

432 axons, especially in younger hosts. In addition, the longer duration of Omicron infection in

433 sinonasal epithelium raises the possibility that early topical intranasal treatment may accelerate

434 viral clearance and reduce transmission.

435   It should be noted that the observed viral tropism in this study only represents characteristics

436 of infection in the nasal cavity. While our observations demonstrate a high olfactory tropism of

437 WA1 and Delta, the infection is not limited to the olfactory epithelium, and recent RNAseq[61] and

438 RNAscope or immunohistochemistry[3] evidence using COVID-19 patient samples suggests the

439 presence of nasal respiratory epithelial infection as well. The extent to which nasal viral load

440 affects lower respiratory infection is not known. In addition, the relatively low amount of virus

441 transported into the olfactory bulb reported here unlikely causes significant neurologic change

442 other than microglial activation and inflammation. Even though the specific cellular tropism in

443 the nasal cavity for each SARS-CoV-2 strain was identified here, it remains to be determined

444 which group of mutations in Spike S protein is associated with altered tropism. Given the

445 predominance of respiratory epithelium by area in the human nasal cavity, the enhanced

446 respiratory infection and the extended viral retention in sinus epithelium may contribute to the

447 increased transmissibility of Omicron, and calls for a reassessment of early local intervention.

448

449

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588

589

590   **Figure Legends**

591 **Fig. 1. SARS-Cov-2 WA1 selectively targets human olfactory neuroepithelium**

592 **a,b**, Confocal images of SARS-CoV-2 viral antigen NP (red, Novus, NB100-56576) and

593 olfactory neuronal marker β-III Tubulin (Tuj1, green) in superior turbinate biopsies from 2

594 separate patients. Images were obtained under tile scan mode, which covered olfactory and

595 adjacent respiratory epithelium in the same piece of tissue. Boxed area in (**b**) was highlighted in

596 **Extended Data Fig. 1c, d.**

597 **c**, Co-staining of NP and Tuj1 in human biopsy collected from the olfactory cleft.

598 **d**, Representative image of NP overlapped with Tuj1-negative ciliated cell (brightfield).

599 Confocal image was obtained from a biopsy which contains only respiratory epithelium.

600 **e**, Quantification of NP^+ cells per mm tissue. 24 independent specimens have exclusively

601 respiratory epithelium (RE), while 7 specimens contained both respiratory and olfactory

602 epithelium (OE).

603 Arrowheads (**a-c**) indicate the detachment of infected cells. Data in (**e**) are represented as mean ±

604 S.D. *p* value was calculated by one-way ANOVA. Scale bars, 50 µm (**a** and **b**); 20 µm (**c,d**).

605

606 **Fig. 2. Omicron variant shows tropism transition from olfactory to respiratory epithelium**

607 **a**, Scheme of the tissue section. To avoid variability across different animals, frozen sections

608 were collected and examined at three consistent levels (L1–3) representing the anterior (mainly

609 respiratory epithelium), middle (respiratory + Olfactory epithelium), and posterior (mainly

610 olfactory epithelium).

611 **b**, Confocal images of NP and Tuj1-labeled hamster nasal sections at L2. WA1, Delta, and

612 Omicron infected hamsters were examined on 4 dpi. Boxed areas are highlighted at bottom.

613 Scale bars = 500 µm.

614   **c**, Percentage of the infected olfactory epithelium. The total length of $Tuj1^+$ or $NP^+/Tuj1^+$

615   epithelium in each section at L1-3 were quantified using Image J.

616   **d**, Quantification of NP^+ cells in nasal respiratory epithelium. The total NP^+ cells in $Tuj1^-$

617   respiratory epithelium including paranasal sinuses of each section were counted.

618   **e**, qPCR analysis of ACE2 expression in mouse nasal respiratory or olfactory epithelium at age

619   of 2 weeks, 2months, and 19months. The entire nasal respiratory or olfactory epithelium from

620   the same animal were isolated separately.

621   Data are represented as mean ± S.D. Statistical significance was determined by unpaired two-

622   tailed *t*-test. Each data point represents an individual animal.

623

624   **Fig. 3. Delta variant infects cells in submucosa of the nose**

625   **a**, Representative image shows $NP^+/Pan\text{-}Keratin^+$ Bowman's glands in Delta treated hamsters.

626   **b**, Quantification of infected Bowman's glands. The average number of NP^+ Bowman's glands

627   in one 14 µm section was calculated. 3 sections per animal were counted.

628   **c**, Confocal image shows $NP^+/\alpha\text{-}SMA^+$ myofibroblasts. Hamsters infected with Delta variant on

629   4dpi were examined.

630   **d**, Co-staining of Tuj1 and NP in nasal sections at 7dpi. Whole nasal cavity images were

631   captured using a tile scan and z stack mode on a 14 µm section. Boxed area in Omicron infected

632   hamster is highlighted on the right. Scale bars = 500 µm.

633   **e**, Quantification of NP^+ respiratory epithelial cells in paranasal sinuses. 3 sections per animal

634   was counted.

635   Data are represented as mean ± S.D. Statistical significance was determined by unpaired two-

636   tailed *t*-test. Each data point represents an individual animal.

637

Fig. 4. Age associated SARS-Cov-2 infection in olfactory sensory neurons

a-c, Confocal image showing WA1-infected hamster olfactory epithelium at 4dpi. Insert in (**a**) highlighting an NP stained axon bundle (horizontal section). Arrowheads indicate virus infected Tuj1$^+$ immature (**b**) or OMP$^+$ mature (**c**) sensory neurons (coronal sections). White line indicated the basal layer of epithelium.

d, **e**, NP$^+$ axon travel from neuroepithelium to laminar propria and merge into Tuj1$^+$ axon bundle.

f-h, Quantification of NP$^+$ axons in young and old hamsters at 6dpi. Representative images show horizontal (**f**) or coronal sections (**g**). NP$^+$ axons were quantified per µm of the diameter of axon bundle.

i,j, Representative images showing NP located in Tuj1$^+$ human olfactory neurons (**j**) and the percentage of NP$^+$ cells in Tuj1$^+$ population (**i**). Dotted line in (**j**) indicates virus infected NP$^+$ axon. Arrowheads denote NP$^+$/Tuj1$^+$ neurons compared to uninfected cells (empty arrowhead). Infected biopsies from 3 young donors (age 25-33 years) and 5 biopsies from older donors (age 54-72 years) were quantified for Tuj1$^+$ neuronal infection.

k,l, Representative images of Nrp1 expression in human olfactory epithelium (**k**) and quantification of Nrp1$^+$ cells in Tuj1$^+$ population (**l**). 3 biopsies from young (age 20-30 years) and 4 biopsies from older donors (age 68-79 years) were examined for Nrp1 expression. Images in (**f**) were captured with 3 µm Z-stack and exported by maximum intensity projections. Each data point represents an individual sample from hamster (**h**), or human (**i** and **l**). Details of human biopsies can be found in Supplementary Table 1. Data are represented as mean ± S.D. Statistical significance was determined by unpaired two-tailed t-test. Scale bars, 20 µm.

659

25

660   **Fig. 5. Increased olfactory bulb transport of SARS-CoV-2 in young hamsters**

661   **a-c**, Confocal images of Iba1 and NP co-staining in hamster olfactory bulbs. Arrowheads

662   indicate infected axon.

663   **d,** Co-staining of NP and Tuj1 in a serial section next to panel (**b**).

664   **e**, Quantification of Iba1$^+$microglials in hamster olfactory bulb. Each data point in (**d**) represents

665   an individual hamster sample. Data are represented as mean ± S.D. Statistical significance was

666   determined by unpaired two-tailed t-test.

667   **f**, Confocal image of cleaved caspase-3$^+$/NeuN$^-$ apoptotic cells (arrowheads) in the glomerular

668   layer at 4dpi.

669   Images were captured with 3 µm (**a-d**) or 4 µm (**f**) Z-stack and exported by maximum intensity

670   projections. Olfactory bulb tissues were collected from young and old hamsters on 6dpi (**a-d**) or

671   from mock control. Scale bars, 50 µm, (**a-d**); 20 µm, (**f**). ONL, olfactory nerve layer; GL,

672   glomerular layer; EPL, external plexiform layer; MCL, mitral cell layer. Boxed areas are

673   highlighted at bottom. Dotted circles indicate glomeruli.

674

675   **Fig. 6. Age-associated delay in viral clearance in olfactory mucosa**

676   **a**, Representative images showing CD45 and Iba1 co-staining in olfactory mucosa. Mock or

677   maSARS-Cov2 infected wildtype mice were examined at 6dpi.

678   **b**, Co-immunostaining shows Iba1$^+$ macrophages engulfing NP$^+$ debris in hamster olfactory

679   mucosa at 4dpi.

680   **c**, Representative image of Iba1 and cleaved caspase-3 co-staining in hamster at 4dpi.

681   Arrowheads highlight the Iba1$^+$ macrophages undergoing apoptosis.

682   **d**, Representative images showing Iba1 or NP staining in serial sections. Each panel combines 6

683   40x images acquired under tile scan mode. Young or old hamsters' olfactory tissues were

684   examined at 6dpi.

685   **e,f**, Quantification of Iba1$^+$ (**e**) or NP$^+$ (**f**) cells in hamster nasal olfactory lumen at 6dpi. Serial

686   sections (**d**) from 4 different levels were quantified.

687   **g**, Violin plots showing the differentially expressed Clec4n (Dectin2) or Fpr2 in young and old

688   macrophage/dendritic lineage.

689   **h**, Confocal images of Iba1 and Dectin2 co-staining in mouse olfactory mucosa.

690   Each data point represents an individual hamster sample. Statistical significance was determined

691   by unpaired two-tailed t-test. The white dotted line in (**a-c**) indicates the basement membrane.

692   Scale bars, 20 µm (**a-c**, **h**)**;** 50 µm (**d**)**.**

693

694   **Fig. 7. Regeneration of olfactory epithelium and re-expression of ACE2**

695   **a**, Confocal images showing ACE2 (red) and Krt5$^+$ horizontal basal cells (green) in olfactory

696   epithelium of mock or SARS-CoV-2 infected hamster at 4 dpi.

697   **b,c**, qPCR analysis of ACE2 (**b**) or OMP (**c**) mRNA expression in SARS-CoV-2 infected

698   hamster turbinate lysate at indicated time points.

699   **d,e**, Representative images of Krt5$^+$ cells in newly regenerated olfactory epithelium (**d**) on 6dpi,

700   and quantification of epithelium thickness (**e**). The thickness of septal olfactory epithelium was

701   measured using Zen lite "line" function. For each section, 8 spots were measured randomly.

702   **f**, Confocal image showing regenerated hamster olfactory epithelium expression of ACE2 at

703   28dpi.

704 **g**, Representative image shows ACE2 and Tuj1$^+$olfactory neurons in an olfactory biopsy from a

705 COVID-19 patient on day 12 post diagnosis.

706 Dots in graph represent independent animal. Data are represented as mean ± S.D. *p* value was

707 calculated by unpaired two-tailed Student's *t* test. Scale bars, 20 µm.

708

709 **Extended Data Fig. 1 Detection of SARS-Cov-2 in the olfactory neuroepithelium.**

710 **a**, SARS-Cov-2 antibody testing. 1 anti-spike S and 3 different anti-NP were verified to be

711 reliable for frozen section immunohistochemistry. Hamster olfactory tissue was examined at 4dpi.

712 All 4 antibodies stained in the same pattern showing intensive viral load mainly located in the

713 apical sustentacular cell layer. No signal could be detected in mock control. Catalog number for

714 each antibody is presented accordingly.

715 **b,** RNAscope analysis showing SARS-Cov-2 viral RNA on 4dpi in hamster olfactory epithelium.

716 **c,d**, Co-staining of NP and sustentacular cell marker Krt18. Image was captured from the boxed

717 area in panel (**b**) of Figure 1.

718 **e**, Confocal image of NP and Tuj1 staining in mock control. Scale bars, 20 µm.

719

720 **Extended Data Fig. 2 Decreased Omicron variant infection in hamster olfactory epithelium.**

721 **a**, Co-staining of neuronal marker Tuj1 and respiratory epithelium marker Foxj1 in mouse nasal

722 cavity. Scale bar, 200 µm.

723 **b**, Representative image shows OMP and rabbit anti ACE2 co-staining in hamster turbinate

724 horizontal section. Intense ACE2 expression is seen in OMP$^+$ olfactory epithelium. The green

725 arrows show the respiratory-olfactory transition area with lower ACE2 expression. Scale bar,

726 100 µm.

727 **c**, Confocal images show the distribution of NP and Tuj1 in a coronal section at L1 of the nasal

728 cavity. Tissues were examined on 4dpi, boxed areas were highlighted at bottom. Note that NP

729 was dramatically declined from Tuj1 negative respiratory epithelium (RE) in hamsters infected

730 with WA1 or Delta. The respiratory infection in Omicron group was markedly increased. Scale

731 bars = 500 µm.

732

733 **Extended Data Fig. 3 Tropism of SARS-CoV-2 variants in the posterior nasal mucosa.**

734 **a**, Confocal images showing the distribution of NP and Tuj1 in posterior nasal cavity sections at

735 4dpi. Coronal sections at L3 were examined, where the proportion of olfactory epithelium is

736 predominant. The olfactory epithelium infection in the Omicron group was decreased remarkably.

737 Scale bar = 500 µm.

738 **b**, Co-staining of NP and Iba1 (macrophage marker) or NP and Vimentin (mesenchymal cell and

739 olfactory ensheathing cell marker) in Delta infected hamster. Scale bars = 20 µm.

740   The white dotted line in (**b**) indicates the basement membrane.

741

742 **Extended Data Fig. 4 SARS-Cov-2 WA1 and Delta variants infect olfactory sensory**

743 **neurons.**

744 **a**, Co-staining of NP and OMP in olfactory epithelium. 5 weeks-old hamsters were infected with

745 SARS-CoV-2-Delta variant (1×10^7 TCID50) and were examined on 4dpi. Arrowhead indicates

746 an infected neuron.

747 **b,c**, Confocal images show co-localization of NP with Tuj1$^+$ or OMP$^+$ axon. (**b**) shows a larger

748 view of Figure 2f. Boxed areas in (b) were highlighted at bottom. 1m (**b**) or 7-8 weeks-old (**c**)

749 hamsters were infected with WA1.

750   **d**, Representative image shows NP signal does not colocalize with Vimentin in axon bundles.

751   **e,f**, Co-staining of NP and OMP in olfactory epithelium.

752   **g**, Confocal image shows Tuj1$^+$ immature olfactory neurons in mock group.

753   7-8 week-old hamsters were infected WA1 (**d, e**) or Delta variant (**f**) at 1×10^5 TCID50 and were

754   examined at 4dpi. Scale bars, 20 µm.

755

756   **Extended Data Fig. 5 Expression of Nrp1 in mouse olfactory epithelium and bulb**

757   **a**, qPCR analysis of Nrp1 expression in mouse olfactory mucosa at ages 2 weeks, 2 months, and

758   19 months. Each data point represents an individual mouse (n=3).

759   **b,c**, Immunostaining analysis shows the expression of Nrp1 in the Tuj1$^+$ immature olfactory

760   neurons and axon bundles. Confocal images were acquired from horizontal section of young (1

761   month) and old (8 month) mice. Boxed areas were highlighted on right. In young mice, a few

762   mature neurons above the Tuj1$^+$ cells also express a low level of Nrp1.

763   **d**, Percentage of Nrp1 expressing cells in Tuj1$^+$ immature neurons. Olfactory tissues from

764   wildtype mice were examined at the indicated age groups.

765   **e**, Confocal images show the expression of Nrp1 in young and old mouse olfactory bulb. Data

766   are represented as mean ± S.D. Statistical significance was determined by unpaired two-tailed *t*-

767   test. Arrowheads highlight Nrp1$^+$ cells in glomerular layer. Scale bars, 20 µm (**b,c**); 50 µm (**e**).

768

769   **Extended Data Fig. 6 Increased brain transport of SARS-CoV-2 in young hamsters**

770   **a-f,** Confocal image capturing a cross section of olfactory epithelium and olfactory bulb. SARS-

771   CoV-2-infected young or old hamsters were examined at 6 dpi. Boxed areas highlight the

772   infected lateral olfactory axons crossing the cribriform plate and projecting to the olfactory bulb.

773   Images were captured with 4 µm Z-stack and exported by maximum intensity projections. OE,

774   olfactory epithelium; OB, olfactory bulb.

775   **g**, RNAscope analysis shows viral RNA in SARS-Cov-2 infected hamster OB glomeruli at 4dpi.

776   **h,** Co-staining of Caspase-3 and Iba1 in olfactory bulb at 4dpi.

777   **h,i**, Confocal image shows co-staining of endothelial cell marker CD31 and ACE2 in mouse (**h**)

778   or hamster (**i,** ACE2 only) olfactory bulb.

779   **j**, Immunostaining of CD45 and microglia marker Iba1 in the olfactory bulb of hACE2 mice at 6

780   dpi. Arrowheads indicate Iba1 negative immune cells. In the hACE2 strain, human ACE2

781   overexpression was driven by mouse Krt18 promoter. Scale bars, 100 µm (**a**,**d**); 50 µm (**h-j**).

782

783   **Extended Data Fig. 7 Regeneration of the olfactory epithelium**

784   **a**, qPCR analysis of Ifng expression in turbinate tissues. SARS-CoV-2 infected hamsters were

785   examined at indicated time points.

786   **b**, Dynamic of Iba1^{+} macrophages infiltration and CXCL10 expression in hamster olfactory

787   epithelium.

788   **c**, Representative images show Krt5^{+} basal cells expressing proliferation marker Ki67 on 4dpi in

789   hamster olfactory epithelium.

790   **d**, qPCR analysis of Sox2, Lgr5, and Tubb3 expression in turbinate samples at indicated time

791   points.

792   Data are represented as mean ± S.D. Statistical significance was determined by unpaired two-

793   tailed *t*-test. Each data point represents an individual mouse. Scale bars, 20 µm.

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811 **Methods**

812 **Human nasal explant *in vitro* infection.** The research protocol involving human specimens was

813 approved by the Johns Hopkins institutional review board, and all subjects provided signed

814 informed consent. Nasal biopsies included olfactory epithelial and/or respiratory epithelial

815 samples were collected from chronic rhinosinusitis (CRS) patients and control subjects

816 undergoing endonasal surgical approaches for non-CRS disease processes[62]. All patients were

817 tested negative for COVID-19 before surgery. In this study, the majority of biopsies were taken

818 from superior turbinates. The human olfactory mucosa is predominantly distributed on the dorsal

819   aspect of the nasal vault[63]. The superomedial portion of superior turbinate that comprises part of

820   the olfactory cleft contains olfactory epithelium, while the inferior and lateral side is entirely

821   respiratory epithelium. Therefore, the coronal sections of superior turbinate samples in this study

822   include both olfactory and respiratory epithelium, with a much smaller proportion of olfactory

823   relative to respiratory. Notably, over 60% of the superior turbinate biopsies contained solely

824   respiratory epithelium. Other specimens were obtained from the olfactory cleft septal mucosa.

825   More details about the clinical specimens are listed in Supplementary Table 1.

826   Human biopsies were placed in PneumaCult medium (Stemcell Technologies) and sent for

827   infection immediately. SARS-CoV-2 infection experiments were conducted in a biosafety level 3

828   facility at the Bloomberg School of Public Health, Johns Hopkins University. After 2 hours

829   incubation with SARS-CoV-2/USA/WA1/2020 (BEI Resources) at 5×10^5 TCID$_{50}$ per mL, the

830   tissues were washed in PBS and transfer into fresh medium at 37°C. Mock controls were

831   maintained in medium without virus. Tissues were fixed at 9 hours post infection in 4% PFA at

832   4°C for 24 hours. 6 control (2 females and 4 males ranged from 45 to 63 years old) and 27 CRS

833   biopsies (11 females and 16 males ranged from 25 to 76 years old) were used for detailed

834   immunohistochemistry analysis.

835   Human biopsies for Nrp1 staining were collected from 3 young (20-30 years) and 4 older (68-

836   79years) subjects. Tissues were fixed in 4% PFA at 4°C overnight, and the olfactory

837   neuroepithelium identity was verified by Tuj1 staining.

838   **Animal *in vivo* infection**

839   Animal infection experiments were carried out in a biosafety level 3 facility at Johns Hopkins

840   Research Animal Resources (RAR) in compliance with the established ethical guidelines.

841   Animal experimental procedures were approved by the Animal Care and Use Committee at the

842   Johns Hopkins University. Animal infection experiments were conducted using wildtype

843   C57BL/6J mice, Syrian golden hamsters (HsdHan®: AURA, Envigo, Haslett MI), and hACE2

844   mice (B6.Cg-Tg(K18-ACE2)2Prlmn/J, JAX, Bar Harbor, Maine). In hACE2 strain, the human

845   ACE2 was driven by the mouse Krt18 promoter. 1×10^7 TCID50/ml of SARS-CoV-

846   2/USA/WA1/2020 or 2.4×10^7 TCID50/ml of Delta variant (SARS-CoV-2/USA/MD-

847   HP05660/2021 B.1.617.2) in 100 µL Dulbecco's modified Eagle medium (DMEM) was

848   intranasally inoculated to hamsters (50 µl per nare). 6.3×10^5 PFU in 20 µL (10 µL per nare)

849   was administered intranasally to hACE2 mice. The mouse-adapted SARS-CoV-2 (courtesy of

850   Michael Schotsaert, Icahn School of Medicine at Mt. Sinai) infection[41] was performed as 10 µL

851   per nare, $2.5 \square 10^8$ PFU. Mock control animals received equivalent volume of DMEM alone.

Tissue processing

853   Animals were euthanized in biosafety level 3 facility at indicated time points. After anesthetized

854   with avertin and transcardially perfused with PBS followed by 4% PFA, the skull bone was

855   removed, and the head was postfixed in 4% PFA at 4°C for 3 days. After decalcification in

856   TBD2 solution (6764003, Thermo) overnight and washing in PBS, tissues were equilibrated

857   sequentially in 15% and 30% sucrose, then embedded in Optimum Cutting Temperature (OCT,

858   Tissue-Tek) for sectioning Fixed human biopsies were processed similarly and embedded in

859   OCT without TBD2 treatment. Frozen sections were processed at 12 µm using MICROM

860   HM560 cryostat (Thermo).

Immunohistochemistry

862   The immunostaining process was carried out on cryosections after an antigen retrieval step.

863   Briefly, sections were washed in PBS and then blocked in 2% BSA containing 0.2% Triton X-

864   100 at 4 °C overnight, followed by incubation with primary antibodies at 4 °C overnight. The

865   following primary antibodies was used: Rabbit anti-SARS-CoV-2 Nucleoprotein (1:200, Novus,

866   NB100-56576), Rabbit anti- SARS-CoV-2 Nucleoprotein (1:500, GeneTex, GTX135357),

867   Rabbit anti- SARS-CoV Nucleoprotein (1:1000, Rockland, 200-401-A50), Rabbit anti- SARS-

868   CoV-2 Spike S (1:200, Sino Biological, 40150-R007), Goat anti-ACE2 (1:100, R&D, AF933, for

869   human samples), Rabbit anti-ACE2 (1:100, Thermo, MA5-32307, for hamster samples), Goat

870   anti-Neuropilin-1 (1:200, R&D, AF566) Mouse anti-Keratin 18 (1:500, Novus, NB500-306),

871   Goat anti OMP (1:500, Wako, 544-10001), Mouse anti-aSMA (1:100, R&D MAB1420);

872   Chicken anti-Vimentin (Novus NB300-223); Goat anti-Foxj1 (1:200, R&D AF3619); Mouse

873   anti-NeuN (1:1000, BioLegend, 834502); Rat anti-CD45 (1:300, Ebioscience, 14-0451-81), Rat

874   anti-CD31(1:50, BD, 550274), Rabbit anti-Krt5 (1:500, Covance, PRB-160P), Chicken anti-Krt5

875   (1:500, BioLegend, 905904), Mouse anti Tuj1 (1:300, BioLegend, 801203), Rabbit anti Iba1

876   (1:500, Wako, 019-19741), Rabbit anti-Cleaved Caspase-3 (1:300, Cell signaling, 9664), Rat anti

877   Dectin2 (1:200, Bio-Rad, MCA2415T), and Goat anti-CXCL10(1:100, R&D, AF-466-NA).

878   After washing in PBS three times, the tissue sections were incubated with Alexa Fluor

879   conjugated, highly cross-adsorbed, secondary antibodies along with DAPI for nuclear

880   counterstaining. The donkey-derived Alexa Fluor-conjugated secondary antibodies included anti-

881   mouse 488 (A21202, Invitrogen); anti-Rat 488 (A21208, Invitrogen); anti-Rabbit 488 (A21206,

882   Invitrogen); anti-Rabbit 546 (A10040, Invitrogen); anti-Goat 488 (A32814, Invitrogen); anti-

883   Goat 546 (A11056, Invitrogen); anti-Chicken 488 (SAB4600031, Sigma).

884   **In situ hybridization**

885   To detect SARS-CoV-2 RNA, in situ hybridization was performed on 12 µm-thick sections of 4%

886   PFA-fixed OCT mounted on charged glass slides using the Leica Bond RX automated system

887   (Leica Biosystems, Richmond, IL). Heat-induced epitope retrieval (HIER) was conducted by

888   heating slides to 95°C for 15 minutes in EDTA-based ER2 buffer (Leica Biosystems, Richmond,

889   IL). Slides were treated in protease (Advanced Cell Diagnostics, Newark, CA) for 15 minutes

890   and probes hybridized to RNA for 1 minute. The SARS-CoV-2 probe (#848568, Advanced Cell

891   Diagnostics, Newark, CA) was detected using the Leica RNAScope 2.5 LS Assay-RED kit with

892   a hematoxylin counterstain (#322150, Leica Biosystems, Richmond, IL). An RNApol2 probe

893   served as a host gene control to evaluate RNA quality; a probe for the bacterial dap2 gene served

894   as a negative control ISH probe.

895   **Confocal Imaging and Quantification**

896   Immunostaining images were obtained using a Zeiss LSM 780 confocal microscope equipped

897   with a 40x, numerical aperture 1.1 water objective. The following laser lines were used DPSS

898   561nm (detection range 560-612nm) for Alexa Fluor 546; Diode 405nm (detection range 410-

899   480nm) for DAPI; and Argon 488nm (detection range 490-550nm) for Alexa Fluor 488. Images

900   for the same primary antibody across different samples were acquired and exported under the

901   same settings. Before exporting, contrast adjustment was applied as necessary for individual

902   channels using Zen lite (Zeiss) under the "Display" option. Images were cut by Photoshop and

903   assembled by Illustrator.

904   For quantification, at least 5 images were collected from each specimen using 40x objectives

905   under the tile scan and z stack mode at same depth. Positive cells were identified according to the

906   subcellular staining pattern and were counted manually using "Events" function of Zen lite

907   (Zeiss). Cells in olfactory or respiratory mucosa were quantified per mm of surface epithelium.

908   By measuring the whole length of Tuj1$^+$ epithelium, The SARS-CoV2 infected axons were

909   quantified per µm diameter of axon bundle. Microglia in the olfactory bulb or shedding cells in

910   nasal cavity were quantified per mm^2 tissue.

RNA isolation, cDNA synthesis and qPCR

Total RNA was isolated from hamster olfactory tissue lysate using a Direct-zol RNA Kits

(Zymo). Equal amounts of RNA were transcribed into cDNA by High-Capacity cDNA Reverse

Transcription Kit (Applied Biosystems). On-Column DNase I digestion was conducted to

remove genomic DNA contamination. Ten nanograms of cDNA was added to a 20-μL PCR

reaction using SYBR Green PCR Master Mix or TaqMan Fast Universal PCR Master Mix

(Applied Biosystems) on StepOne Plus System (Applied Biosystems). For SYBR Green PCR,

post-amplification melting curve analysis was performed to monitor unspecific products. Fold

change in mRNA expression was calculated using the comparative cycle method ($2^{-\Delta\Delta Ct}$). SYBR

Green PCR primer sequences of hamster genes are: ACE2: Forward,

TGGTGGGAGATGAAGCGAGA, and Reverse, GAACAGAGCTGCAGGGTCAC; OMP:

Forward, CAGAAGCTGCAGTTCGACCG, and Reverse, CAGAAGATTGCGGCAGGGTC;

Ifng: Forward, TAATGCACACCACACGTTGC, and Reverse,

AAGACGAGGTCCCCTCCATT. GAPDH: Forward, GTGGAGCCAAGAGGGTCATC, and

Reverse, GGTTCACACCCATCACAAACAT. Mouse genes are: Nrp1: Forward,

CAGTGGCACAGGTGATGACT, and Reverse, ACCGTATGTCGGGAACTCTGAT; ACE2:

Forward, CCATTGGTCTTCTGCCATCCG, and Reverse, CCAACGATCTCCCGCTTCATC;

GAPDH: Forward, TCAATGAAGGGGTCGTTGAT, and Reverse,

CGTCCCGTAGACAAAATGGT.

Single cell RNA-seq analysis

Sc-RNA-seq dataset was retrieved from published study (GSE155006) by Mogilenko, et al[46].

This dataset was generated from sorted lung CD45$^+$ immune cells from 3 or 17-month-old mice.

The Seurat R package was used for subsequent analysis. Quantity control was conducted

934   according to the standard pre-processing workflow. Cells in young and old datasets express 500-

935   2500 genes, mitochondrial genes less than 5% were selected and normalized using a scaling

936   factor 10,000. The highly variable genes in each dataset were selected using the

937   *FindVariableFeatures* function, and combined (10,228 cells in total) for Seurat integration

938   procedure and linear dimensionality reduction. The top 2000 most variable genes per dataset

939   were used for downstream principal component analysis and clustered using the *FindClusters*

940   function. The datasets include 16 clusters were then projected as UMAP plots. According to the

941   expression levels of canonical marker genes, we matched the clusters to known immune cell

942   types. We applied *FindMarkers* function to identify differentially expressed genes in

943   macrophages/dendritic cell lineage between young and old conditions. Average Log_2 fold

944   changes of gene expression and the percentage of cells expressing certain genes in each

945   condition were calculated.

946   **Statistical analyses**

947   Data are expressed as mean ± SD. as indicated. Data analyses were carried out using GraphPad

948   Prism. For experiments with two groups, *P* values were calculated using the unpaired two-tailed

949   Student's t-test. Differences were considered significant when $P < 0.05$.

950   **Reporting Summary.** Further information on research design is available in the

951   Nature Research Reporting Summary linked to this article.

952   **Data and materials availability:** Data, code, and materials will be made available upon request.

953   **Acknowledgements**

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959   **Author contributions:** M.C., A.P.L., and A.P. designed research; M.C. and W.S. performed

960   experiments; M.C. performed the bioinformatic analysis of the single cell RNA-seq dataset;

961   A.P., R.Z. performed *in vitro* infection experiments; N.R.R., A.P.L., M.R., H.K., and Z.L.

962   collected biopsies; J. V., S. B., and J. M. performed animal infection experiments; M.C., A.P., J.

963   M., K. W. and A.P.L. analyzed data; M.C., A.P., and A.P.L. wrote the paper.

964   **Competing interests:** The authors declare no competing interests.
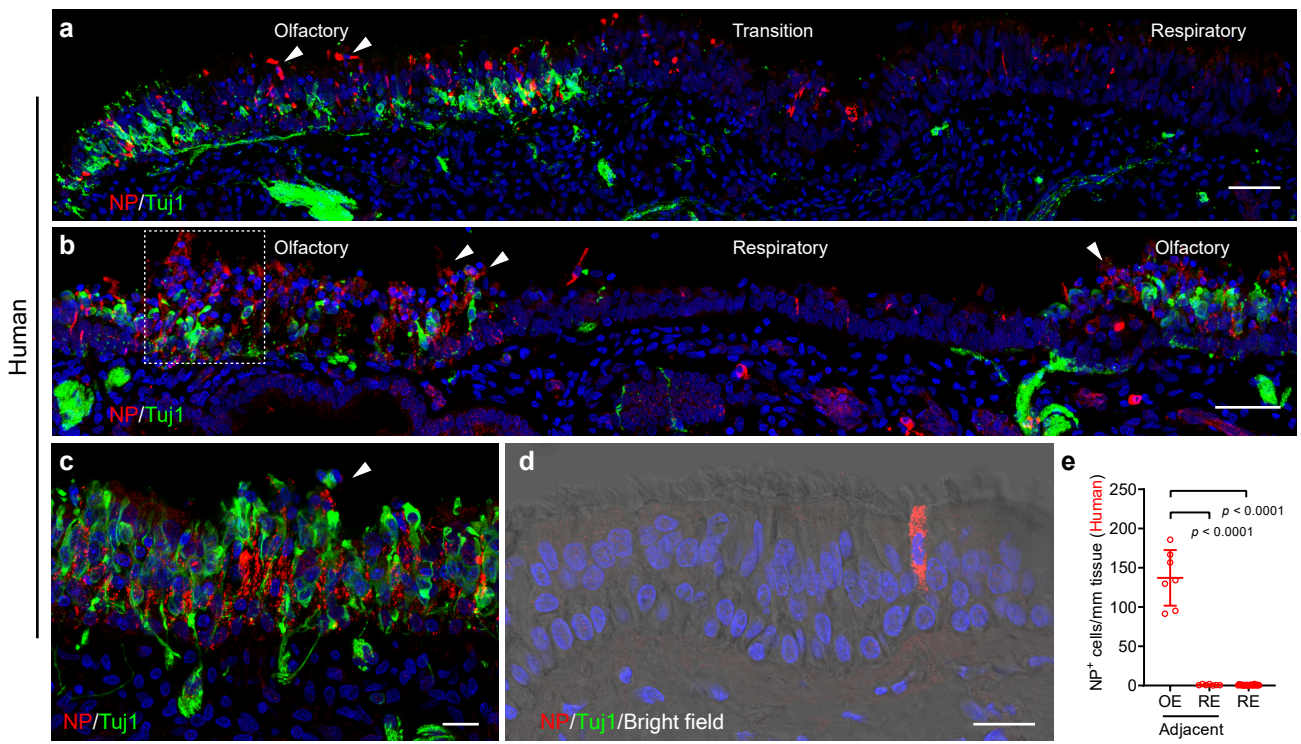
Figure 1   Chen and Lane et al.

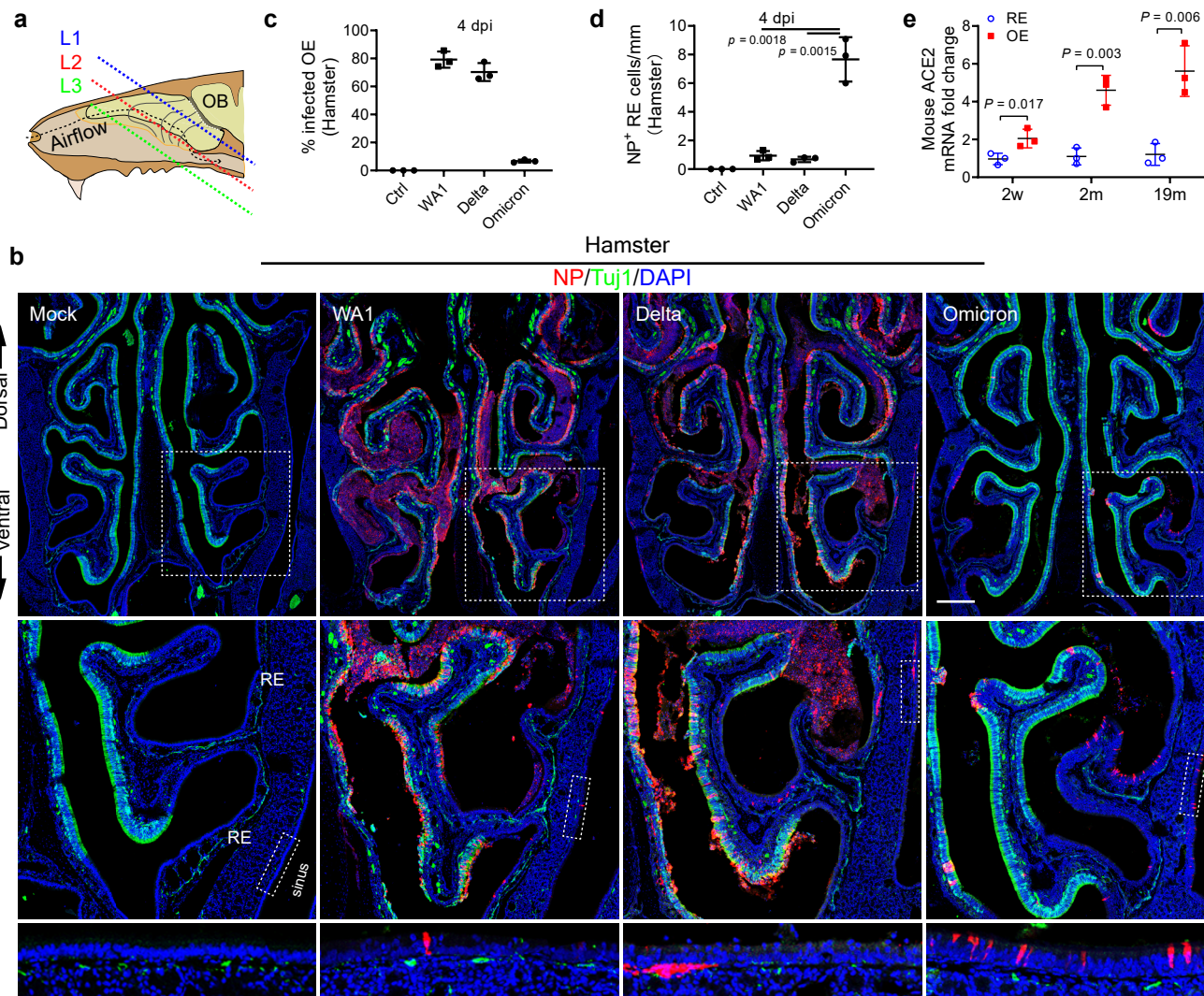**Human**

a — Olfactory / Transition / Respiratory — NP/Tuj1

b — Olfactory / Respiratory / Olfactory — NP/Tuj1

c — NP/Tuj1

d — NP/Tuj1/Bright field

e — NP+ cells/mm tissue (Human) — OE / RE / RE — Adjacent — $p < 0.0001$ / $p < 0.0001$
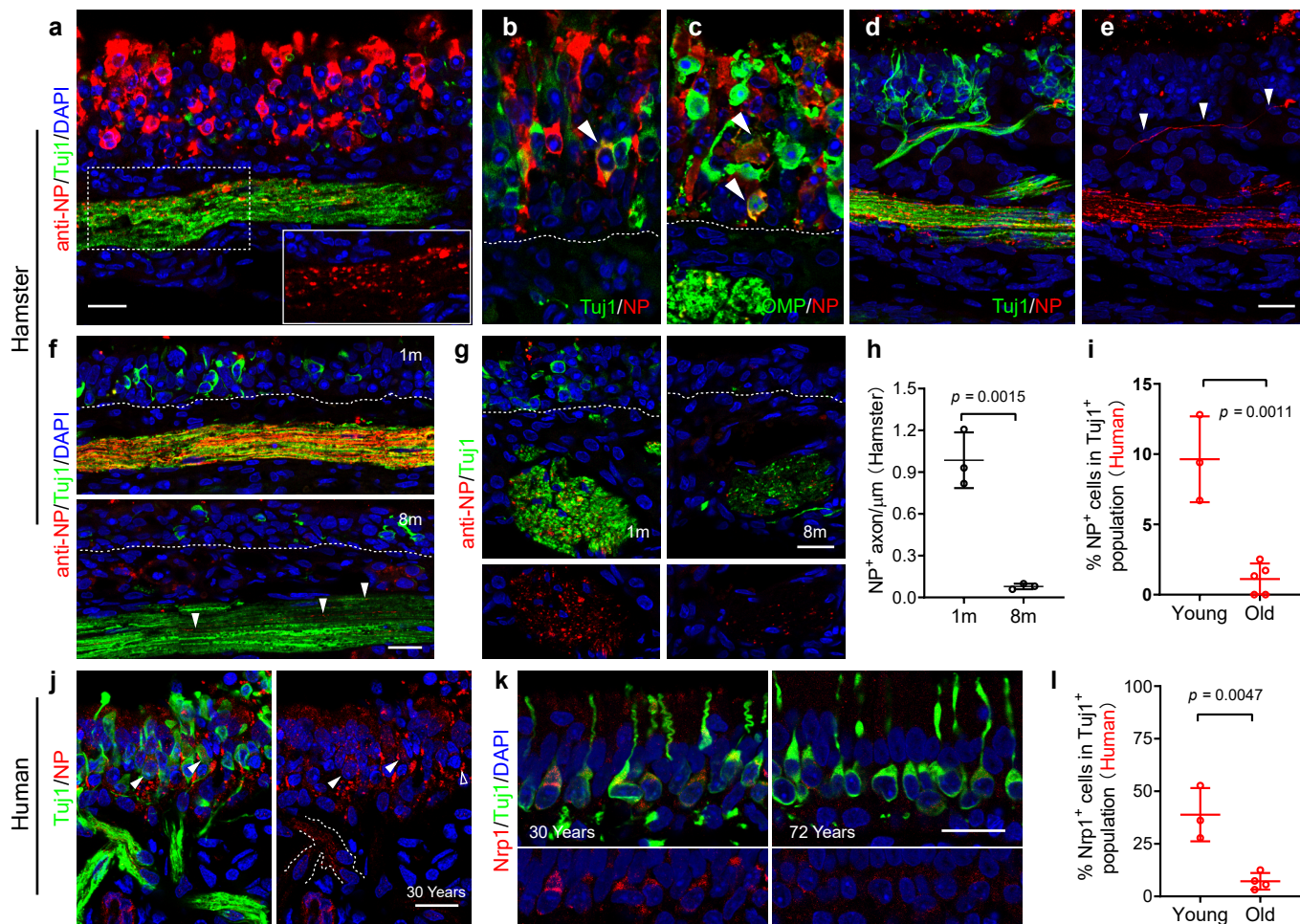
Figure 2   Chen and Lane et al.

a L1 L2 L3 OB Airflow

c % infected OE (Hamster) — 4 dpi — Ctrl, WA1, Delta, Omicron

d NP$^+$ RE cells/mm (Hamster) — 4 dpi — $p = 0.0018$, $p = 0.0015$ — Ctrl, WA1, Delta, Omicron

e Mouse ACE2 mRNA fold change — RE, OE — $P = 0.017$, $P = 0.003$, $P = 0.006$ — 2w, 2m, 19m

b Hamster

NP/Tuj1/DAPI

Dorsal / Ventral

Mock, WA1, Delta, Omicron

RE, RE, sinus
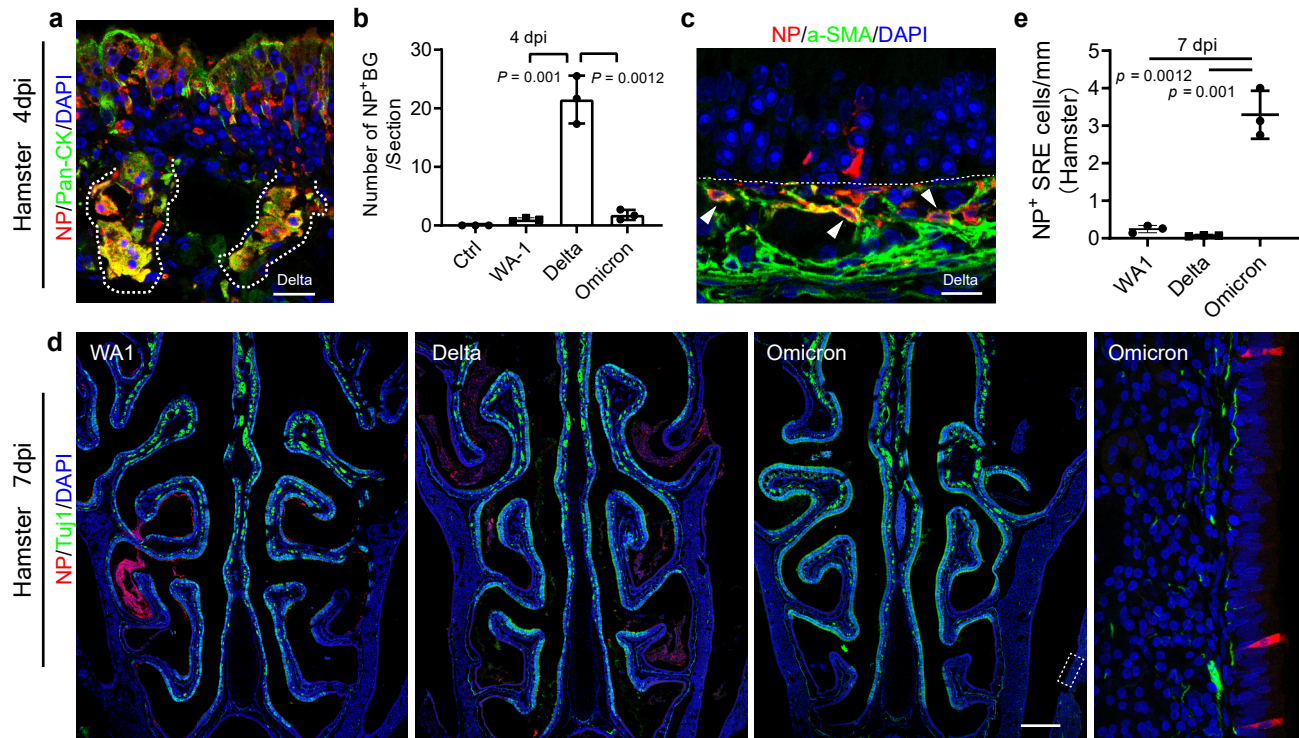
Figure 3

Figure 4   Chen and Lane et al.
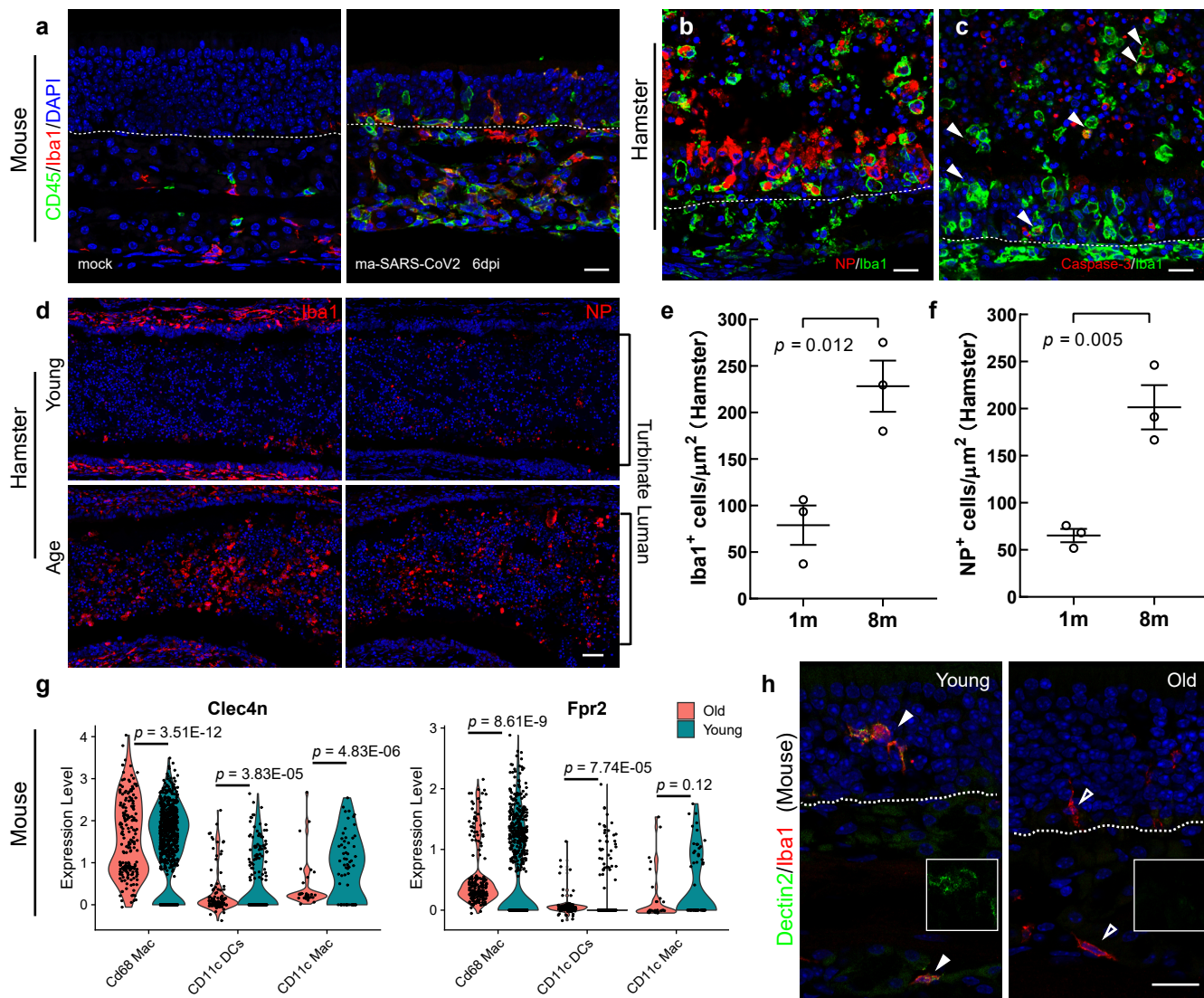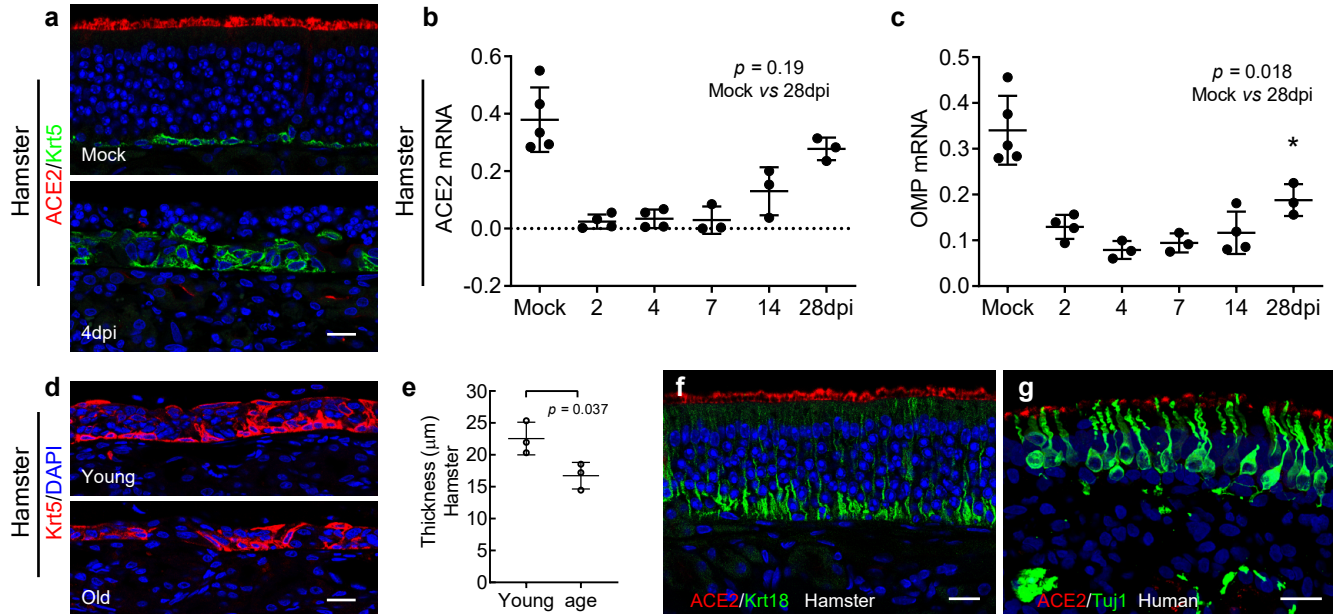
Figure 5

Chen and Lane et al.

a
ONL
GL
EPL
MCL
Np/Iba1/DAPI
Mock

b
1m

c
8m

d
Np/TuJ1/DAPI
1m

e
p = 0.004 p = 0.036
Iba1+ cells/μm^2
Mock   1m   8m

f
Casp3/NeuN

Fig 6   Chen and Lane et al.

Figure 7

a

anti-Spike S/DAPI
Sino Biological 40150 R007

anti-NP/DAPI

Novus NB100-56576

Rockland 200-401-A50

GeneTex GTX135357

Hamster

Mock

4dpi

b

Hamster

Control

V-nCoV2019-S

Human

c NP/Krt18

d

e Mock/Tuj1/NP

a

Nrp1 mRNA fold change (Mouse)

P = 0.002

P = 0.008

2w 2m 19m

b

Nrp1/Tuj1/DAPI

1 month

c

8 month

d

% Nrp1⁺ in Tuj1⁺ immature neurons (Mouse)

p = 0.0042

1m 8m

e

Nrp1/Tuj1/DAPI

1 month

8 month

Mouse Wildtype Control

a

b

c

d