1 **Goblet Cell Hyperplasia Increases SARS-CoV-2 Infection in COPD.**

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14 **Summary.** SARS-CoV-2 has become a major problem across the globe, with approximately 50

15 million cases and more than 1 million deaths and currently no approved treatment or vaccine.

16 Chronic obstructive pulmonary disease (COPD) is one of the underlying conditions in adults of

17 any age that place them at risk for developing severe illness associated with COVID-19. We

18 established an airway epithelium model to study SARS-CoV-2 infection in healthy and COPD

19 lung cells. We found that both the entry receptor ACE2 and the co-factor transmembrane

20 protease TMPRSS2 are expressed at higher levels on nonciliated goblet cell, a novel target for

21 SARS-CoV-2 infection. We observed that SARS-CoV-2 infected goblet cells and induced

22 syncytium formation and cell sloughing. We also found that SARS-CoV-2 replication was

23   increased in the COPD airway epithelium likely due to COPD associated goblet cell hyperplasia.

24   Our results reveal goblet cells play a critical role in SARS-CoV-2 infection in the lung.

25

26   **Keywords.** SARS-CoV-2, COVID-19, goblet cells, ciliated cells, COPD, squamous metaplasia,

27   air-liquid interface, syncytium, cell sloughing, goblet cell hyperplasia.

28

29   **Introduction.** Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2, a causative agent

30   of coronavirus disease 2019, COVID-19) that emerged in December 2019 in Wuhan, China.

31   Since then, this pathogen has caused havoc in the healthcare systems worldwide and

32   consequentially ravaged the economy of countries with COVID-19 outbreaks. There is currently

33   no FDA-approved vaccine against SARS-CoV-2. SARS-CoV-2 is a nonsegmented, positive-

34   sense, single-strand RNA virus that causes both upper and lower respiratory tract infections.

35   Most patients exhibit fever and cough, and a subset of patients advance to severe acute

36   respiratory distress syndrome (ARDS) (Guan et al., 2020; Yang et al., 2020). Therefore, patients

37   with underlying chronic obstructive pulmonary disease (COPD) are vulnerable to COVID-19,

38   and in fact, COPD is one of the high-risk factors for severe illness associated with COVID-19

39   (CDC, 2020; Leung et al., 2020; Sin, 2020).

40   Viral infections begin by the attachment of viral particles to entry receptors on the host

41   cell. The tissue expression and distribution of the SARS-CoV-2 entry receptor angiotensin-

42   converting enzyme 2 (ACE2) and its co-factor transmembrane serine protease 2 (TMPRSS2)

43   determine the tropism of virus infection (Hoffmann et al., 2020; Li et al., 2003), and viral

44   infection in human airway epithelium depends on ACE2 expression (Hamming et al., 2004; Jia et

45   al., 2006). For successful entry into cells, SARS-CoV-2 uses the serine protease TMPRSS2 for S

46 protein priming (Hoffmann et al., 2020). ACE2 is highly expressed in the small intestine, testis,

47 kidneys, heart, thyroid, and adipose tissue and is expressed at moderate expression levels in the

48 lung, colon, liver, bladder, and adrenal gland; and lowest in the blood, spleen, bone marrow,

49 brain, blood vessels, and muscle (Hamming et al., 2004; Li et al., 2020). ACE2 expression in the

50 lungs is predominantly observed in alveolar type 2 (AT2) cells (Lukassen et al., 2020; Qi et al.,

51 2020; To and Lo, 2004; Ziegler et al., 2020), but ciliated cells also express ACE2 in the

52 respiratory epithelium (Sims et al., 2005). Recent RNAseq-based studies have suggested that

53 ACE2 is more highly expressed on goblet cells in the nasal airways and on secretory cells in

54 subsegmental bronchial branches of the lung (Lukassen et al., 2020; Sungnak et al., 2020;

55 Ziegler et al., 2020). Although ACE2 and TMPRSS2 expressions are higher in nonciliated goblet

56 cells compared to ciliated cells (Lukassen et al., 2020; Sungnak et al., 2020; Zhang et al., 2020;

57 Ziegler et al., 2020), it appears that goblet cells are underappreciated in the SARS-CoV-2

58 infection studies. The possibility that SARS-CoV-2 infects goblet cells could explain the

59 presence of viral RNA in sputum (Wang et al., 2020) and might explain the efficient

60 transmission of the virus from person to person (Dhand and Li, 2020; Wolfel et al., 2020).

61 Importantly, goblet cell hyperplasia is a characteristic pathological feature of COPD patients,

62 who are vulnerable to severe disease associated with COVID-19 (Lippi and Henry, 2020;

63 Shimura et al., 1996; Zhao et al., 2020). Therefore, it is prudent to determine to what extent

64 SARS-CoV-2 infects goblet cells in the lung.

65 To determine the expression of the SARS-CoV-2 receptor and its preferential cell tropism

66 in the lung, we developed an in vitro airway epithelium model by differentiating primary normal

67 human bronchial (NHBE) cells derived from either a patient with COPD or a healthy adult (non-

68 COPD). The COPD airway epithelium model recapitulates many bronchial characteristics of

69   COPD. We evaluated the expression of ACE2 and TMPRSS2 and studied SARS-CoV-2

70   infection in these in vitro airway epithelium models. We found that SARS-CoV-2 primarily

71   infects nonciliated goblet cells due to high expression of both ACE2 and TMPRSS2 in these

72   cells. Goblet cell hyperplasia increases of SARS-CoV-2 infection in the COPD airway

73   epithelium. Thus, SARS-CoV-2 targeting and replication in goblet cells may explain the

74   development of more severe COVID-19 in COPD patients.

75

76   **Results.**

77   **The airway epithelium model recapitulates the chronic bronchial characteristics of COPD.**

78   We first established an in vitro airway epithelium model by differentiating NHBE cells from

79   either a healthy adult or a COPD patient (deidentified) at the air-liquid interface (ALI). We found

80   that 4 weeks of differentiation provides a fully differentiated pseudostratified mucociliary airway

81   epithelium for both conditions (Figures 1A and B), which allows the use of this model for side-

82   by-side comparisons. NHBE cells from a healthy adult differentiated primarily into a

83   pseudostratified columnar epithelium, whereas the NHBE cells from a COPD patient

84   differentiated into a mix of pseudostratified and stratified columnar epithelium that contained all

85   three main cell types of the respiratory epithelium (Pawlina, 2016; Rayner et al., 2019; Rigden et

86   al., 2016): ciliated cells, goblet cells, and basal cells (Figure 1B). We found that the apical site of

87   the epithelium mainly consists of ciliated and nonciliated goblet cells (Figures 1C and D).

88   Studies have shown that mucin 5AC (MUC5AC) is predominately expressed by airway goblet

89   cells and that mucin 5B (MUC5B) is expressed by goblet cells of submucosal glands (Whitsett,

90   2018). Club cells are the progenitors of goblet cells, which might express both MUC5AC and

91   MUC5B (Kiyokawa and Morimoto, 2020; Okuda et al., 2019). As expected, we found

4

92   heterogenicity in the cell population with differential expression of common goblet cell markers

93   (Figure 1E) (Lukassen et al., 2020). Basal cells are known for their self-renewal property and

94   give rise to multiple types of differentiated airway epithelial cells (Crystal, 2014). For the

95   detection of basal cells, we sectioned the epithelium and stained the sections for the basal cell

96   marker P63 (Persson et al., 2014; Wang et al., 2002). We found that basal cells reside at the basal

97   membrane of both epithelia and that the COPD epithelium has more basal cells than the healthy

98   epithelium as it is known from respiratory epithelium of COPD patients (Figure 1F) (Higham et

99   al., 2019; Polosukhin et al., 2011).

100   COPD is associated with abnormal airway and alveolar responses during exposure to

101   noxious stimuli (Brusselle et al., 2011). Because our COPD airway epithelium model was

102   differentiated from NHBE cells, it should recapitulate the bronchial airway phenotypes instead of

103   the alveolar phenotype as more commonly associated with emphysema (Barnes, 2013). To

104   determine whether our in vitro COPD airway epithelium model recapitulates some of the

105   bronchial pathophysiological characteristics of COPD, we focused on two different aspects:

106   goblet cell hyperplasia and squamous metaplasia. First, we compared the number of MUC5AC$^+$

107   or MUC5B$^+$ goblet cells between healthy and COPD epithelia. We found a higher ratio of goblet

108   cells in the COPD airway epithelium (Figures 1G and H). The higher number of goblet cells in

109   the COPD epithelium suggests a persistent goblet cell differentiation, which results in goblet cell

110   hyperplasia (Kim et al., 2015; Reid et al., 2018; Shaykhiev, 2019). Indeed, we found a patch of

111   higher number of goblet cells with extensive mucus secretion in the COPD epithelium (Figure

112   1I, center). We also found an apparent loss of pseudostratified epithelium accompanied by

113   squamous metaplasia in the COPD epithelium, which is a common pathological phenotype in

114   COPD (Figure 1I, right) (Rigden et al., 2016).

115   To determine the biophysical properties of these respiratory epithelia, we assessed the

116   tissue membrane integrity (transepithelial electrical resistance, TEER) and ciliary function

117   (ciliary beat frequency, CBF) and found no significant difference in these biophysical properties

118   between healthy and COPD epithelia (Figures 1J and K). These results indicated that NHBE

119   cells from patients with COPD produced a mix of pseudostratified and stratified highly

120   differentiated mucociliary epithelium with appropriate biophysical properties.

121

122   **ACE2 and TMPRSS2 are expressed at higher levels in goblet cells.** SARS-CoV-2 infects the

123   human airway epithelium, and virus entry depends on the host cell receptors ACE2 and its co-

124   factor TMPRSS2 (Hoffmann et al., 2020). We quantified the ACE2 transcript levels in a lung

125   epithelial cell line (A549 cells) and primary NHBE cells in a monolayer or in the differentiated

126   airway epithelium by real-time PCR. We did not detect ACE2 transcripts in the A549 cells

127   (Figure S1A), which might indicate low or no expression of ACE2 confirming previous data

128   (Blanco-Melo et al., 2020; Harcourt et al., 2020; Hoffmann et al., 2020; Jia et al., 2005).

129   However, we detected ACE2 transcripts in primary NHBE cells in both the monolayer and

130   differentiated airway epithelium (Figure S1A). Despite their similar biophysical properties

131   (tissue barrier integrity and ciliary function, Figures 1J and K, respectively), ACE2 expression

132   was higher in the COPD epithelium compared to those derived from a healthy donor (Figures

133   S1A-C). We then visualized the expression of ACE2 in the airway epithelium by

134   immunofluorescence imaging. We observed ACE2 expression in both the healthy and COPD

135   airway epithelia and found that ACE2 expression hardly overlapped with cilia on the apical site

136   of the epithelium (Figures 2A and S1D). Nevertheless, low levels of ACE2 expression were

137   observed on ciliated cells in our model (Figure S1E). These results suggest that the SARS-CoV-2

138   entry receptor ACE2 is mainly expressed on non-ciliated cells in the respiratory epithelium.

139   Because ACE2 staining hardly overlapped with acetyl-alpha-tubulin, we tested the

140   expression of ACE2 along with that of the goblet and club cell markers MUC5AC and MUC5B

141   (Lukassen et al., 2020). Indeed, ACE2 overlapped with MUC5AC (Figure 2B) and MUC5B

142   (Figures 2C and S1F). We also compared ACE2 expression with the expression of E-cadherin

143   and Zonula occludens-1 (ZO-1), markers for adherens junction and tight junction proteins,

144   respectively. The results showed that ACE2 expression did not overlap with apical tight

145   junctions or adherent junctions (Figure S2), which suggests that ACE2 expression is primarily

146   located within the cellular boundary and does not impact the tissue barrier integrity of the

147   respiratory epithelium. Overall, ACE2 expression was higher in the COPD than in the healthy

148   epithelium (Figures 2 and S1-2), which is likely due to the presence of goblet cell hyperplasia in

149   the COPD epithelium and thus a higher number of goblet cells.

150

151   TMPRSS2 is an important host co-factor for SARS-CoV-2 entry into target cells

152   (Hoffmann et al., 2020; Lukassen et al., 2020; Shulla et al., 2011). We visualized TMPRSS2 in

153   the apical site of the airway epithelium by staining with anti-TMPRSS2 and found that

154   TMPRSS2 expression hardly overlapped with cilium (Figure 3A). We then tested the expression

155   of TMPRSS2 along with that of the goblet cell markers MUC5AC and MUC5B. Indeed,

156   TMPRSS2 overlapped with MUC5AC (Figure 3B) and MUC5B (Figures 3C). Therefore, it

157   appears that both TMPRSS2 and ACE2 are expressed on the same cell surface (Figures 2 and 3).

158   These results indicate that goblet cells may be a novel target of SARS-CoV-2 infection in the

159   respiratory epithelium.

160

161   **SARS-CoV-2 infects goblet cells.** Although we confirmed that SARS-CoV-2 entry receptors are

162   expressed at higher levels on nonciliated goblet cells, a number of previous studies have

163   suggested that SARS-CoV-2 targets ciliated cells (Hou et al., 2020; Lamers et al., 2020).

164   Therefore, we first examined whether SARS-CoV-2 infects nonciliated goblet cells. We infected

165   the apical side of the airway epithelium with SARS-CoV-2 at a multiplicity of infection of 0.1

166   (MOI = 0.1). At 4 days post infection (DPI), we fixed the cells and stained them for SARS-CoV-

167   2 nucleoprotein (N) and a ciliated cell marker. The results revealed that SARS-CoV-2 infects

168   both healthy and COPD epithelium and causes a substantial cytopathic effect (CPE) (Figures

169   S3A and B). SARS-CoV-2 infection was higher in the COPD epithelium than in the healthy

170   epithelium, as will be addressed later in the manuscript. Although in some cases the virus-

171   induced extensive CPE made it difficult to distinguish SARS-CoV-2 cell tropism, we focused on

172   multiple random areas with less CPE but virus infection. We found that SARS-CoV-2 infects

173   nonciliated cells in both healthy and COPD epithelium (Figure 4A). We also used a second

174   detection method to visualize viral and cellular markers in cross sections of the epithelium.

175   Immunohistochemistry-based staining confirmed the extensive CPE induced by SARS-CoV-2 in

176   both healthy and COPD epithelium. Apparently, SARS-CoV-2 infected both ciliated and

177   nonciliated epithelial cells in the airway epithelium (Figure 4B). Using staining strategies similar

178   to those described before, we found that SARS-CoV-2 infects MUC5B-positive (Figures 4C and

179   D) and MUC5AC-postive (Figure 4E) goblet cells. To determine whether SARS-CoV-2 infects

180   basal cells, we stained the sectioned epithelium for P63 and the SARS-CoV-2 spike (S) protein.

181   We did not observe any overlap between the SARS-CoV-2 S protein and the basal cell marker

182   (Figure S4). These results suggest that SARS-CoV-2 infects nonciliated goblet cells in addition

183   to ciliated cells.

184

185   **SARS-CoV-2 induces syncytia and cell sloughing in the airway epithelium.** To determine

186   whether SARS-CoV-2 infection in the airway epithelium recapitulates the virus-induced

187   pathophysiology in the lung, we examined the infected epithelium under a confocal microscope

188   and found that SARS-CoV-2 infection causes substantial damage to the apical site of the infected

189   epithelium, as confirmed by extensive CPE in both healthy and COPD epithelium (Figure S3).

190   We also found substantial mucus secretion due to SARS-CoV-2 infection. The apical damage of

191   the SARS-CoV-2-infected epithelium included loss of cellular junctions, loss of ciliary damage,

192   substantial mucus production, and the protraction of nuclei, which are all common features

193   observed in SARS-CoV-2-infected lungs (Schaefer et al., 2020). Additionally, we investigated

194   whether SARS-CoV-2-infected cells in the epithelium might form syncytium (multinucleated

195   cell), a hallmark of SARS-CoV-2 infection in the lung (Giacca et al., 2020) that had also been

196   reported for SARS-CoV-1 (Franks et al., 2003). Indeed, we found that SARS-CoV-2-infected

197   cells formed syncytia in both healthy and COPD epithelia (Figure 5A and B). Cell sloughing has

198   been reported from lung autopsy findings of SARS-CoV-2-infected patients (Schaefer et al.,

199   2020). Therefore, we examined whether SARS-CoV-2 infection in our epithelium model

200   recapitulates cell sloughing. Indeed, we found that SARS-CoV-2 induces cell sloughing in both

201   healthy and COPD epithelia as confirmed by two independent methods, immunofluorescence

202   and IHC (Figures 5C and D). These results demonstrate that hallmark pathological features of

203   SARS-CoV-2 are recapitulated in the infected airway epithelium model.

204

205 **SARS-CoV-2 replicates higher and exacerbates pathophysiology in COPD epithelium.** To

206 determine whether SARS-CoV-2 replicates better in the COPD epithelium, we titered SARS-

207 CoV-2 in the apical wash of the infected epithelium and found that SARS-CoV-2 replication was

208 increased by almost a log in the COPD compared to the healthy epithelium (Figure 6A). In

209 addition, we found a squamous metaplasia in SARS-CoV-2-infected COPD epithelium which

210 was rather infrequently found in the SARS-CoV-2-infected healthy epithelium (Figures 1I; 4B,

211 D, and E, and 5B and D) (Borczuk et al., 2020). Squamous metaplasia is known to increase

212 bronchial wall thickening as seen in bronchitis (Randell, 2006; Reid et al., 2018; Rigden et al.,

213 2016). As tracheobronchitis is one of the most common histopathological features in the

214 COVID-19 disease fatalities (Martines et al., 2020), we evaluated whether SARS-CoV-2

215 infection increases height of the epithelium. First, there was a substantial increase in squamous

216 metaplasia in the COPD epithelium due to SARS-CoV-2 infection (Figure S5). Second, the

217 increased metaplasia apparently changed the morphology of the nonciliated goblet cells in the

218 infected COPD epithelium (Figures 6B and C). Whether this change in the goblet cell

219 morphology impacted the mucus hyperplasia remains to be determined. Third, in contrast to the

220 healthy epithelium, SARS-CoV-2 induced higher squamous metaplasia in the infected COPD

221 epithelium and caused a substantial increase in the height of the epithelium (Figure 6D). These

222 results suggest that SARS-CoV-2 replicates better in the COPD epithelium and exacerbates

223 pathophysiology in the infected airway epithelium.

224

225 **Discussion.**

226 Here, we have shown that the COPD epithelium model recapitulates the bronchial

227 biophysical and pathophysiological characteristics of COPD, such as goblet cell hyperplasia and

10

228   squamous metaplasia (Gohy et al., 2019; Kim et al., 2015; Reid et al., 2018; Rigden et al., 2016).

229   A previous report suggested the presence of altered ciliated cells in COPD airway epithelium

230   (Gohy et al., 2019), which was not observed in our model. The duration of cell differentiation

231   may be the reason, as we differentiated NHBE cells for four weeks that may require to see

232   terminal differentiation (including ciliogenesis) (Gohy et al., 2019). We used an air-liquid

233   interface (ALI) culture method to generate multi-cellular diversity and physiologic functioning

234   airway epithelium that resembles the airway surface in vivo (Fulcher et al., 2005; Pawlina, 2016;

235   Rayner et al., 2019). One of the limitations in the ALI culture research is passaging of primary

236   NHBE cells may impact on their ability to differentiate into airway epithelium. We could

237   demonstrate that primary NHBE cells obtained after four passages without using any additional

238   supplements can still be differentiated into human airway epithelium (Rayner et al., 2019). In a

239   separate study, we confirmed that passaging NHBE cells up to four times has insignificant effect

240   on the whole-genome transcriptome by comparing transcriptome profiles of each passage cells

241   (data not shown). The ability to expand primary cells that also form fully differentiated

242   mucociliary epithelium reduces repeat sample collections from patients where samples are

243   difficult and limited, such as infants and deceased patients (Rayner et al., 2019; Wolf et al.,

244   2017). Our results demonstrate primary NHBE cells either from healthy or a COPD patient can

245   be passaged up to four times and that normal epithelial phenotypic features are maintained in

246   passaged primary NHBE cells.

247   One emergent question is why the human-to-human transmission of SARS-CoV-2 is

248   much higher compared to SARS-CoV-1, although both viruses share ACE2 as cell surface

249   receptor and use TMPRSS2 to facilitate their entry into the host cell (Hoffmann et al., 2020;

250   Lukassen et al., 2020). The SARS-CoV-2 spike protein has an additional furin cleavage site that

251 is absent in SARS-CoV-1, and it is hypothesized that furin cleavage facilitates human-to-human

252 transmission (Coutard et al., 2020; Lukassen et al., 2020). We found that SARS-CoV-2 infected

253 both ciliated cells and nonciliated goblet cells, but not basal cells in the airway epithelium.

254 Although SARS-CoV-2 may preferentially infect goblet cells due to the higher expression of

255 ACE2 and TMPRSS2, further studies are needed to confirm temporal and spatial regulations of

256 SARS-CoV-2 infection in the airway epithelium. The major function of goblet cells in the lung

257 epithelium is mucin production to trap pathogens, dust, and particles, which are cleared by a

258 process known as mucociliary clearance (Rogers, 2003). The possibility that SARS-CoV-2

259 infects goblet cells could explain the presence of viral RNA in sputum (Wang et al., 2020) and

260 might explain the easy transmission of the virus from person to person. While we are preparing

261 our manuscript, Hao et al., have shown that goblet cells are permissive to SARS-CoV-2 infection

262 (Hao et al., 2020). As SARS-CoV-1 infection is limited to ciliated cells (Sims et al., 2005), we

263 think that SARS-CoV-2 infection in goblet cells could explain why SARS-CoV-2 is more

264 transmissible than SARS-CoV-1. Influenza A virus infects goblet cells in addition to ciliated

265 cells (Matrosovich et al., 2004), whereas respiratory syncytial virus (RSV) infects only ciliated

266 cells (Zhang et al., 2002). Therefore, virus-specific preferential cell tropism in the lung could

267 explain the difference in respiratory virus pathogenicity and transmissibility.

268 Our airway epithelium model recapitulated some SARS-CoV-2-induced lung

269 histopathological findings, such as prominent nucleoli (Schaefer et al., 2020), cytoplasmic

270 vacuolation, multinucleated giant cells (Falasca et al., 2020), squamous metaplasia (Martines et

271 al., 2020) and epithelial cell sloughing, which is the most common finding (Bradley et al., 2020).

272 SARS-CoV-2 infection in the bronchial epithelium models induced a substantial amount of cell

273 syncytium formation and cell sloughing, which indicates that tracheobronchial cells are highly

274 associated with virus-induced pathogenesis. Therefore, our data suggest that bronchial cells are

275 equally important to alveolar cells in the SARS-CoV-2 pathogenesis (Martines et al., 2020). In

276 patients with COPD, goblet cell hyperplasia is a common clinical manifestation, and an

277 increasing number of goblet cells results in more mucus production (Shaykhiev, 2019). Because

278 we have shown that SARS-CoV-2 replicated at a higher rate in the COPD epithelium, we believe

279 that goblet cell hyperplasia is responsible for this phenomenon and may explain why COPD

280 patients are at higher risk for severe outcome of COVID-19. Interestingly, we found that SARS-

281 CoV-2 infection induced "squamous metaplasia" in the COPD epithelium. Squamous metaplasia

282 in the columnar epithelium corelates with increased severity of airway obstruction in COPD

283 (Araya et al., 2007). Although squamous metaplasia is one of the histopathological findings in

284 the SARS-CoV-2 infected patient fatalities (Martines et al., 2020), our results provide evidence

285 for the first time that SARS-CoV-2 infection increases squamous metaplasia, which may result in

286 bronchitis in the infected COPD patients. In fact, tracheobronchitis is one of the most common

287 histopathological features in the COVID-19 disease fatalities (Martines et al., 2020). No SARS-

288 CoV-2 infectious virion was detected in the basal medium of the infected healthy and COPD

289 epithelia, which might suggest that the initial virus-induced substantial damage was limited at the

290 apical site.

291 Immune responses to viral infection play a critical role in determining the clinical

292 outcomes of SARS-CoV-2 infection (Huang et al., 2020). Unfortunately, our airway epithelium

293 model lacks both resident and infiltrating immune cells (dendritic cells, macrophages, and

294 lymphocytes, etc.) and is devoid of the endothelial layer, and therefore cannot study the impact

295 of immune responses on the SARS-CoV-2 infections. However, we can provide inferences based

296 on the detection of immunoregulatory factors (chemokines and cytokines), but these hypotheses

297　remain to be confirmed. Overall, we believe that the airway epithelium model provides an

298　excellent tool for demystifying some of the SARS-CoV-2 pathophysiological features and

299　identifying and testing novel therapeutics against the virus.

300　　　In conclusion, we developed in vitro lung airway epithelium models from passaged

301　primary NHBE cells of either a healthy or a COPD patient. Our studies revealed SARS-CoV-2

302　infection of goblet cells leading to virus-induced syncytium formation and cell sloughing in the

303　airway epithelium. We found that SARS-CoV-2 replicates better COPD airway epithelium likely

304　due to COPD associated goblet cell hyperplasia. Thus, we postulate that goblet cells play a

305　critical role in SARS-CoV-2 infection of the lung and are responsible for more severe outcome

306　of SARS-CoV-2 infection in COPD patients.

307

308　**Figure legends.**

309　**Figure 1. The airway epithelium model recapitulates the bronchial characteristics of**

310　**COPD. (A).** Airway epithelium derived from differentiated NHBE cells obtained from a healthy

311　adult or COPD patient were fixed, embedded in paraffin, sectioned and stained with hematoxylin

312　and eosin (H&E). The nuclei are stained dark-purple, and the cytoplasmic components are pink.

313　Bar = 50 µm. **(B)**. The sectioned epithelia were stained with Alcian blue. Mucosubstances are

314　stained blue, whereas the cytoplasmic components are pale pink, and the nuclei are pink/red. Bar

315　= 50 µm. **(C).** The apical sites of the airway epithelia were fixed, permeabilized and stained for

316　the goblet cell marker MUC5AC (anti-MUC5AC, green), the ciliated cell marker acetyl-alpha

317　tubulin (anti-acetyl-alpha tubulin, cyan), and F-actin (rhodamine phalloidin, red); the nuclei were

318　also stained with DAPI (blue). Bar = 10 µm. **(D).** The sectioned epithelia were stained for the

319　ciliated cell marker acetyl-alpha tubulin (anti-acetyl-alpha tubulin, cyan), the goblet cell marker

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320   MUC5AC (anti-MUC5AC, green), and F-actin (rhodamine phalloidin, red); the nuclei were also

321   stained with DAPI (blue). Bar = 20 µm. **(E).** The apical sites of the airway epithelia were fixed

322   and stained for two goblet cell markers, MUC5AC and MUC5B [anti-MUC5AC (green) and

323   anti-MUC5B (cyan), respectively] and F-actin (rhodamine phalloidin, red); the nuclei were also

324   stained with DAPI (blue). Bar = 10 µm. **(F).** The sectioned epithelia were stained for the basal

325   cell marker P63 (anti-P63, green), the goblet cell marker MUC5B (anti-MUC5B, cyan), and F-

326   actin (rhodamine phalloidin, red); the nuclei were also stained with DAPI (blue). Bar = 20 µm.

327   **(G).** The MUC5B$^+$ goblet cells were counted from tiled images of the airway epithelium stained

328   for MUC5B (anti-MUC5B, cyan) (described above) obtained with a Leica DMi8 epifluorescence

329   microscope. A total of approximately 3,500 cells were counted from each epithelium to

330   determine the ratio. **(H).** The MUC5AC$^+$ goblet cells were counted from random fields in

331   confocal images of the airway epithelium stained for MUC5AC (anti-MUCAC, cyan) (described

332   above). A total of approximately 1,500 cells were counted from each epithelium to determine the

333   ratio. **(I).** The sectioned epithelia were stained for MUC5B (anti-MUC5B, cyan); the nuclei were

334   stained with DAPI (blue). Bar = 20 µm. **(J).** Transepithelial electrical measurements (TEERs) of

335   the airway epithelia were obtained. The data were obtained by combining three independent

336   Transwell reads, and each Transwell read was an average of three independent reads. The error

337   bars represent the SEMs. **(K).** The ciliary beat frequency (CBF) was measured on the airway

338   epithelia. The data were obtained by combining three independent Transwell reads, and each

339   Transwell read was an average of six random point reads. The error bars represent the SEMs.

340   The statistical significance was determined by unpaired two-tailed t-tests. The results from one

341   independent experiment are shown.

342

15

343 **Figure 2. ACE2 is expressed at higher levels in goblet cells**. The airway epithelia were

344 generated and fixed as described in Figure 1 **(A)** Cells were stained for ACE2 (anti-ACE2,

345 green), cilia (anti-acetyl-alpha tubulin, cyan) F-actin (rhodamine phalloidin, red) and nuclei

346 (DAPI, blue). **(B).** Cells were stained for ACE2 (anti-ACE2, green), MUC5AC+ goblet cells

347 (anti-MUC5AC, cyan) F-actin (rhodamine phalloidin, red) and nuclei (DAPI, blue). **(C).** Cells

348 were stained for ACE2 (anti-ACE2, green), MUC5B+ goblet cells (anti-MUC5B, cyan), F-actin

349 (rhodamine phalloidin, red) and nuclei (DAPI, blue). Bar =10 µm. See also Figures S1 and S2.

350

351 **Figure 3. TMPRSS2 is expressed at higher levels in goblet cells. (A).** The airway epithelia

352 (described in Figure 1) were stained for cilia (anti-acetyl-alpha tubulin, cyan), TMPRSS2 (anti-

353 TMPRSS2, green), and F-actin (rhodamine phalloidin, red); the nuclei were stained with DAPI

354 (blue). **(B)**. The airway epithelia were stained for the identification of MUC5AC$^+$ goblet cells

355 (anti-MUC5AC, cyan), TMPRSS2 (anti-TMPRSS2, green), F-actin (rhodamine phalloidin, red)

356 and nuclei (DAPI, blue). **(C).** The airway epithelia were stained to identify MUC5B$^+$ goblet cells

357 (anti-MUC5B, cyan), TMPRSS2 (anti-TMPRSS2, green), F-actin (rhodamine phalloidin, red)

358 and nuclei (DAPI, blue). Bar =10 µm.

359

360 **Figure 4. SARS-CoV-2 infects goblet cells. (A).** The airway epithelia were mock-infected or

361 infected with SARS-CoV-2 at an MOI of 0.1. At 4 days post infection (DPI), the epithelia were

362 fixed, permeabilized, and stained for the identification of cilia (anti-acetyl-alpha tubulin, cyan),

363 SARS-CoV-2 N (anti-N, green), F-actin (rhodamine phalloidin, red) and nuclei (DAPI, blue).

364 The arrows indicate SARS-CoV-2-infected nonciliated cells. **(B).** At 4 DPI, the epithelia

365 (described in A) were stained for the goblet cell marker MUC5B (anti-MUC5B, cyan), SARS-

16

366   CoV-2 N (anti-N, green), and F-actin (rhodamine phalloidin, red); the nuclei were stained with

367   DAPI (blue). The arrows indicate SARS-CoV-2-infected MUC5B$^+$ goblet cells. **(C).** At 4 DPI,

368   the epithelia (described in A) were embedded in paraffin, sectioned and stained for the detection

369   of cilia (anti-acetyl-alpha tubulin, cyan) and SARS-CoV-2 N (anti-N, green); the nuclei were

370   stained with DAPI (blue). The arrows indicate SARS-CoV-2-infected nonciliated cells. **(D).** At 4

371   DPI, the sectioned epithelia were stained for the goblet cell marker MUC5AC (anti-MUC5AC,

372   cyan) and SARS-CoV-2 N (anti-N, green); the nuclei were also stained with DAPI (blue). The

373   arrows indicate SARS-CoV-2-infected MUC5AC$^+$ goblet cells. **(E).** At 4 DPI, the sectioned

374   epithelia were stained for the goblet cell marker MUC5B (anti-MUC5B, cyan) and SARS-CoV-2

375   N (anti-N, green); the nuclei were also stained with DAPI (blue). The arrows indicate SARS-

376   CoV-2-infected MUC5B$^+$ goblet cells. (A and B) Bar = 5 µm. (C, D and E) Bar = 20 µm. See

377   also Figures S3 and S4.

378

379   **Figure 5. SARS-CoV-2 induces syncytia and cell sloughing. (A).** The airway epithelia (mock

380   or infected; described in Figure 4) were stained for SARS-CoV-2 N (anti-N, green) and F-actin

381   (rhodamine phalloidin, red); the nuclei were also stained with DAPI (blue). The arrows indicate

382   syncytia. Bar = 5 µm. **(B).** At 4 DPI, the sectioned epithelia were stained for the goblet cell

383   marker MUC5AC (anti-MUC5AC, cyan) and SARS-CoV-2 N (anti-N, green), and the nuclei

384   were also stained with DAPI (blue). The arrows indicate syncytia. Bar = 20 µm. **(C).** At 4 DPI,

385   the airway epithelia were stained for SARS-CoV-2 N (anti-N, green) and F-actin (rhodamine

386   phalloidin, red), and the nuclei were stained with DAPI (blue). The arrows indicate cell

387   sloughing. Bar = 5 µm. **(D).** At 4 DPI, the sectioned epithelia were stained for cilia (anti-acetyl-

17

388   alpha tubulin, cyan) and SARS-CoV-2 N (anti-N, green), and the nuclei were stained with DAPI

389   (blue). The arrows indicate cell sloughing. Bar = 20 µm.

390

391   **Figure 6. SARS-CoV-2 replicates at a higher rate and increases squamous metaplasia in the**

392   **COPD epithelium. (A).** The apical wash of the SARS-CoV-2-infected airway epithelia

393   (described in Figure 4) was collected, and SARS-CoV-2 titration was performed based on the

394   tissue culture infective dose 50 ($TCID_{50}$). The results from an independent experiment (n=3) are

395   shown. The error bars show the SEMs. **(B).** The sectioned epithelia (described in Figure 4) were

396   stained with Alcian blue. Bar = 50 µm. **(C).** At 4 DPI, the sectioned epithelia (COPD epithelia

397   after mock or SARS-CoV-2 infection) were stained for the goblet cell marker MUC5B (anti-

398   MUC5B, cyan) and SARS-CoV-2 N (anti-N, green), and the nuclei were stained with DAPI

399   (blue). Bar = 20 µm. **(D).** The height of the airway epithelia (described in C) was measured from

400   the images (n=5), the average of 12 points was plotted. See also Figure S5.

401

402   **Figure S1. ACE2 expression is higher in the COPD airway epithelium. (A)** ACE2 mRNA

403   expression in total RNA extracted from the lung epithelial cell line (A549 cells) and primary

404   NHBE cells from healthy adults and patients with COPD in monolayers or differentiated into

405   airway epithelia was quantified by real-time PCR. The data were plotted as expression levels

406   normalized to those in the NHBE healthy monolayer. The data were obtained by combining the

407   quadruplet technical replicates of each sample. The graph represents the results from two

408   independent real-time PCR runs. **(B).** ACE2 was detected in the airway epithelium (obtained

409   after 4 weeks of differentiation of NHBE cells) by Western blotting. **(C).** The ACE2 signal

410   (shown in B) was quantified (normalized to the alpha-tubulin level) and plotted relative to that in

18

411   the healthy epithelium. **(D).** The apical sites of the airway epithelia were fixed and stained for

412   ACE2 (anti-ACE2, green), cilia (anti-acetyl-alpha tubulin, cyan) and F-actin (rhodamine

413   phalloidin, red). Deconvoluted Z-stack images are presented in the 3D view. **(E).** The apical sites

414   of the airway epithelia were fixed and stained for cilia (anti-acetyl-alpha tubulin, cyan), ACE2

415   (anti-ACE2, green), F-actin (rhodamine phalloidin, red) and nucleus (DAPI, blue). Bar = 15 µm.

416   **(F).** The airway epithelia (described in D) were stained for ACE2 (anti-ACE2, green), MUC5B

417   (anti-MUC5B, cyan) and F-actin (rhodamine phalloidin, red). Deconvoluted Z-stack images are

418   presented in the 3D view.

419

420   **Figure S2. ACE2 expression within the goblet cell boundary**. The airway epithelia (obtained

421   after 4 weeks of differentiation of NHBE cells) were fixed and stained for ACE (anti-ACE2

422   antibody, green) and MUC5B (anti-MUC5B, cyan) or ZO-1 (anti-ZO-1 antibody, cyan) or e-

423   cadherin (anti-e-cadherin antibody, cyan). F-actin (red) and nuclei (blue) were stained with

424   rhodamine phalloidin and DAPI, respectively. The images represent multiple random areas

425   obtained from an independent experiment. Bar = 10 µm.

426

427   **Figure S3. SARS-CoV-2 induces a cytopathic effect in the airway epithelia.** The airway

428   epithelia were mock-infected or infected with SARS-CoV-2 at an MOI of 0.1. At 4 days post

429   infection (DPI), the epithelia were fixed, embedded in paraffin, sectioned, and stained for the

430   basal cell marker P63 (anti-P63, green), SARS-CoV-2 spike (S) (anti-S, cyan), and F-actin

431   (rhodamine phalloidin, red); the nuclei were stained with DAPI (blue). Bar = 20 µm.

432

433 **Figure S4. SARS-CoV-2 does not infect basal cells in the airway epithelia.** The airway

434 epithelia were mock-infected or infected with SARS-CoV-2 at an MOI of 0.1. At 4 days post

435 infection (DPI), the epithelia were fixed, permeabilized, and stained for cilia (anti-acetyl-alpha

436 tubulin, cyan), SARS-CoV-2 N (anti-N, green), and F-actin (rhodamine phalloidin, red); the

437 nuclei were stained with DAPI (blue). The images represent multiple independent random areas

438 from an independent experiment. Bar = 30 µm.

439

440 **Figure S5. SARS-CoV-2 increases squamous metaplasia in the COPD airway epithelia.** The

441 airway epithelia were mock-infected or infected with SARS-CoV-2 at an MOI of 0.1. At 4 days

442 post infection (DPI), the epithelia were fixed, permeabilized, and stained with hematoxylin and

443 eosin (H&E). Bar = 50 µm.

444

445 **Graphical Abstract.**

446 Highlights:

447   1. The airway epithelium model recapitulates many bronchial pathological characteristics of

448   COPD, such as goblet cell hyperplasia and squamous metaplasia

449   2. ACE2 and TMPRSS2 are highly expressed on nonciliated goblet cells

450   3. SARS-CoV-2 infects goblet cells and induces syncytium formation and cell sloughing

451   4. SARS-CoV-2 replicates at a higher rate in the COPD epithelium and increases squamous

452   metaplasia

453 In Brief:

454   Osan et al. showed that SARS-CoV-2 preferentially infects and replicates in nonciliated goblet

455   cells inducing syncytium formation and cell sloughing. Our results suggest that goblet cells play

456   a critical role in SARS-CoV-2-induced pathophysiology in the lung.

457

467

468   **Author contributions.** M.M., J.K.O., and S.N.T. conceived the project and designed the

469   experiments. K.L.B. provided the primary cells as well as training and guidance on primary cell

470   differentiation. M.M., J.K.O., and S.N.T. performed the experiments. S.N.T. generated the

471   confocal images. F.F. and H.F. performed the virus infection experiments in the BSL-4

472   laboratory. J.K.O., B.A.D., and K.J. generated the IHC images, B.A.D. performed the H&E and

473   Alcian Blue staining, and M.M., J.K.O., and S.N.T. wrote the paper. M.M. and H.F. reviewed

474   and edited the paper.

475   **STAR METHOD**

476   **KEY RESOURCE TABLE**

REAGENT or RESOURSE	SOURCE	IDENTIFIER
Antibodies		
Rabbit monoclonal Anti-E-cadherin	Cell Signaling Technology	Cat#3195S; Clone 24E10; RRID:AB_2291471
Rabbit monoclonal Anti-MUC5B	Atlas Antibodies	Cat# HPA008246; RRID:AB_1854203
Mouse polyclonal Anti-MUC5AC	Abnova	Cat# H00004586-A01; RRID:AB_606620
Rabbit monoclonal Anti-MUC5AC	Cell Signaling Technology	Cat#61193; Clone E3O9I; RRID: AB_2799603
Mouse monoclonal Anti-α-tubulin	Sigma-Aldrich	Cat#T6199; RRID:AB_477583
Rabbit polyclonal Anti-ZO-1	Thermo Fisher Scientific	Cat#40-2200; RRID:AB_2533456
Rabbit monoclonal Anti-Acetyl-α-Tubulin	Cell Signaling Technology	Cat#5335; RRID:AB_10544694
Mouse monoclonal Anti-TMPRSS2	Santa Cruz Biotechnology	Cat#sc-515727
Goat polyclonal Anti-ACE2	R and D Systems	Cat# AF933; RRID:AB_355722
Mouse monoclonal Anti-p63	Abcam	Cat# ab735; Clone BC4A4; RRID: AB_305870
Mouse monoclonal Anti-ACE2	R and D Systems	Cat# MAB933; Clone 171606 RRID:AB_2223153
Mouse monoclonal Anti-SARS/SARS-CoV-2 Nucleocapsid protein	Thermo Fisher Scientific	Cat# MA1-7404; Clone B46F RRID:AB_1018422
Rabbit polyclonal Anti-SARS/SARS-CoV-2 Coronavirus Spike Protein	Thermo Fisher Scientific	Cat# PA5-81795; RRID:AB_2788969

Donkey Anti-Goat IgG antibody IRDye 800CW	LI-COR Biosciences	Cat# 926-32214, RRID:AB_621846
Goat Anti-Mouse IgG antibody IRDye 680RD	LI-COR Biosciences	Cat#926-68070;RRID:AB_10956588
Goat anti-Mouse IgG Antibody, Alexa Fluor 488	Thermo Fisher Scientific	Cat# A-11029; RRID:AB_2534088
Goat anti-Rabbit IgG Antibody, Alexa Fluor 647	Thermo Fisher Scientific	Cat# A-21245; RRID:AB_2535813
Critical Commercial Assays		
BCA protein assay kit	Thermo Fisher Scientific	23225
AllPrep DNA/RNA Mini Kit	Qiagen	80204
RNeasy Mini Kit	Qiagen	74104
Virus strains		
SARS CoV-2 WA 1 isolate	Natalie J Thornburg, CDC	GenBank: MN985325.1
Software		
IMARIS 9.5.1	Oxford Instruments	https://imaris.oxinst.com/
Fiji	ImageJ	https://imagej.net/Downloads
Prism 8	GraphPad	https://www.graphpad.com/scientific-software/prism/
Sisson-Ammons Video Analysis (SAVA)	Ammons Engineering	N/A
LASX	Leica Microsystem	N/A
Image Studio 5.2	LI-COR Biosciences	https://www.licor.com/bio/image-studio/
Photoshop 2020	Adobe	N/A
Experimental models:		

Primary cells/ Cell lines		
Normal Bronchial Epithelial Cells (NHBE C16)	Provided by Dr. Kristina Bailey laboratory	
COPD Bronchial Epithelial Cells	Provided by Dr. Kristina Bailey laboratory	
A549 cells	Provided by Dr. Peter Collins laboratory	
Vero cells	Heinz Feldmann, RML	
Chemical reagents, siRNA and Media		
Airway Epithelial Cell Growth Medium	PromoCell	C21060
PneumaCult™-ALI Medium	Stemcell Technologies	05002
F-12 Medium 2-mercaptoethanol	Thermo Fisher Scientific Sigma-Aldrich	11765054 M6250
Penicillin-Streptomycin	Thermo Fisher Scientific	15140122
HyClone™ Fetal Bovine Serum Amphotericin B Goat Serum Blocking Solution	Thermo Fisher Scientific Thermo Fisher Scientific Vector Laboratories	SH3007103 15290026 S-1000-20
PureCol, Bovine Collagen	Cell Systems	5005
NucBlue TM fixed cell stain ReadyProbes	Thermo Fisher Scientific	R37606
ProLong^{TM} Gold anti-fade Mounting	Thermo Fisher Scientific	P36930
Rhodamine Phalloidin (Amanita phalloides)	Cytoskeleton Inc.	PHDR1
16% Formaldehyde (Methanol free)	Polysciences	18814-10
Intercept® (PBS) Blocking Buffer and Diluent Kit	LI-COR Biosciences	927-76003

TrypLE™ Express Enzyme	Thermo Fisher Scientific	12604021
Other		
6.5 mm Transwell® with 0.4 μm Pore Polyester Membrane Insert	Corning	3470
Bolt™ 4-12% Bis-Tris Plus Gels	Thermo Fisher Scientific	NW04120BOX
iBlot™ 2 Transfer Stacks, PVDF	Thermo Fisher Scientific	IB24002
EVOM2 epithelial volt-ohmmeter	World Precision Instruments Inc	177776 AB06J
FV3000 Confocal Laser Scanning Microscope	Olympus Corporation	N/A
Leica DMi8 Inverted Microscope	Leica Microsystem	N/A
Odyssey® CLx Imaging System	LI-COR Biosciences	N/A
Transmission Electron Microscope HT7700	Hitachi	N/A
CFX384 Real-Time PCR System	Bio-Rad	N/A
Epoch microplate spectrophotometer	BioTek	N/A

477

478   **RESOURCE AVIALABILITY**

479   **Lead Contact**

480   Further information and requests for resources and reagents should be directed to and will be

481   fulfilled by the lead contact, Masfique Mehedi (masfique.mehedi@und.edu).

482

483   **Material Availability**

484   The materials and reagents generated in this study will be made available upon installment of a

485   material transfer agreement (MTA).

486   **Competing interest.** The authors declare no competing interests.

487   **EXPERIMENTAL MODEL AND SUBJECT DETAILS**

488   **Cells.** Primary normal human bronchial epithelial (NHBE) cells from either a deidentified

489   healthy adult (female, aged 52 years, never-smoker) or a patient with chronic obstructive

490   pulmonary disease (COPD) were obtained under an approved material transfer agreement

491   (MTA) between the Mehedi Laboratory at University of North Dakota (UND) and Bailey

492   Laboratory at University of Nebraska Medical Center (UNMC), Omaha, NE. A549 cells (a

493   human lung epithelial cell line, ATCC-185) were obtained from Dr. Peter Collins at the National

494   Institutes of Health (NIH), Bethesda, MD, USA. Vero cells used for $TCID_{50}$ assay were a

495   resource of the Feldmann Lab at the Rocky Mountain Laboratories, Hamilton, MT.

496

497   **Virus.** The SARS-CoV-2 isolate nCoV-WA1-2020 (MN985325.1) was kindly provided by CDC

498   as Vero passage 3 (Harcourt et al., 2020). The virus was propagated once in Vero E6 cells in

499   DMEM (Sigma) supplemented with 2% fetal bovine serum (Thermo Fisher Scientific), 1 mM L-

500   glutamine (Thermo Fisher Scientific), 50 U/ml penicillin and 50 μg/ml streptomycin (Thermo

501   Fisher Scientific) (virus isolation medium). The virus stock used in this study was 100%

502   identical to the initially deposited GenBank sequence (MN985325.1); sequencing did not detect

503   any virus stock contaminants.

504

505   **Biosafety Statement.** Work with SARS-CoV-2 was performed in the high biocontainment

506   facilities at the Rocky Mountain Laboratories (RML), NAID, NIH in Hamilton, MT. All

507   infectious work followed standard operating procedures (SOPs) approved by the Institutional

508   Biosafety Committee.

26

509

METHOD DETAILS:

Cell culture. We passaged the primary NHBE cells (passage 1) three times before differentiating them (passage 4) into a pseudostratified epithelium. For each passage, the cells were grown in a 100-mm culture dish (Corning Inc.) precoated with PurCol (Advanced Biometrics). The cells were maintained in airway epithelial cell (AEC) growth medium (PromoCell) containing AEC supplements (PromoCell), 2% penicillin/streptomycin (Thermos Fisher Scientific), and 1% amphotericin B (Thermos Fisher Scientific) (complete AEC medium) at 37°C in an incubator with 5% CO_2. The cells were grown to 90% confluency, and the medium was changed every other day. Confluent monolayers of cells were dissociated with 5 ml of TrypLE (Thermo Fisher Scientific) and pelleted, and one-third of the cells were reseeded in a culture dish containing complete AEC medium for passaging. A549 cells were grown in F-12 medium (Thermo Fisher Scientific) with 10% HyClone fetal bovine serum (GE Healthcare), 2% penicillin/streptomycin, and 1% amphotericin B.

523

Air-liquid interface (ALI) culture. Transwells (6.5 mm) with 0.4-µm-pore polyester membrane inserts (Corning Inc.) were coated with PureCol for 20 minutes before cell seeding. NHBE cells (5×10^4) suspended in 200 µl of complete AEC medium were seeded in the apical part of a Transwell. Subsequently, 500 µl of complete AEC medium was added to the basal part of the Transwell. When the cells formed a confluent layer on the Transwell insert, the AEC medium was removed from the apical part, and PneumaCult-ALI basal medium (Stemcell Technologies) with the required supplements (Stemcell Technologies), 2% penicillin/streptomycin and 1% amphotericin B (complete ALI basal medium) was added to the basal part. The ALI medium in

532 the basal part was changed every other day, and the apical surface was washed with 1x

533 Dulbecco's phosphate-buffered saline (DPBS) (Thermo Fisher Scientific) once per week initially

534 but more frequently when more mucus was observed on later days. However, the thickness of the

535 mucus was not determined. All the cells were differentiated for at least 4 weeks at 37°C in an

536 incubator with 5% CO_2.

537

538 **Transportation of Transwells containing airway epithelia.** The differentiated airway epithelia

539 for 22 days were transported in transportation medium at 4°C via overnight FedEx biological

540 material shipment to the Rocky Mountain Laboratories (RML, Hamilton, MT, USA). The

541 semisolid transportation medium was prepared by adding agarose to the complete AEC medium.

542 Upon receipt, the Transwells containing airway epithelia were immediately transferred into new

543 plates with complete AEC medium in the basal part and maintained before infection as described

544 in the section on ALI culture.

545

546 **Virus infection.** Virus infection was conducted in the high biocontainment facilities at RML,

547 NIAID, NIH. The 4-week-differentiated airway epithelia were washed with 200 µl of 1x PBS to

548 remove mucus and infected on the apical site with SARS-CoV-2 at an MOI of 0.1 in 1x PBS for

549 1 hour (at 37°C with 5% CO_2). For mock infection, the assigned Transwells were similarly

550 incubated with 1x PBS without virus. The viral inoculum was then removed, and the epithelia

551 were washed twice with 200 µl of 1x PBS. Two hundred microliters of apical wash and basal

552 medium were collected for virus titration. Fresh ALI medium (1000 µl) with supplements was

553 added to the basal part of each Transwell, and the apical part was kept empty. Mock-infected and

28

554   SARS-CoV-2-infected Transwells were incubated for 4 days at 37°C in an incubator with 5%

555   CO_2.

556

557   **Tissue culture infective dose 50 (TCID$_{50}$).** Vero cells were seeded on 96-well plates to

558   confluency, and SARS-CoV-2 endpoint titration was performed. The cells were inoculated with

559   10-fold dilutions of the supernatants collected during the experiment in DMEM, 2% FBS, L-

560   glutamine and P/S. The cells were incubated for 7 days, and the cytopathic effect (CPE) was

561   scored under a microscope. The TCID$_{50}$ was calculated using the Reed-Muench formula (Reed

562   and Muench, 1938).

563

564   **Virus-infected sample collection.** At 4 DPI, 200 µl of 1x PBS was added to the apical site of

565   the Transwell and incubated for several minutes, and the apical wash was collected for virus

566   titration. Similarly, basal medium was collected for virus titration. All the samples were stored at

567   -80°C until titration. For PFA fixation, 200 µl of 4% PFA was added at the apical site of the

568   Transwells and incubated for 30 minutes, and the Transwells were further maintained overnight

569   in 4% PFA before being transported outside of the BSL4 lab according to the standard protocol

570   of RML. For protein and RNA sample collections, 200 µl of 1x PBS was added to the apical side

571   of each Transwell, and cells were scrapped with a mini scrapper, collected into a cryovial, and

572   pelleted by being spun down with a table-top bench centrifuge. For the protein samples, 100 µl

573   of 2x SDS buffer was added to the cryovials containing the cell pellet, and the tube was boiled

574   for 10 minutes. After a quick spin, the samples were transferred into new tubes and removed

575   from the BSL4 laboratory using a standard RML protocol. For the RNA samples, the cell pellets

576   were resuspended in 600 µl of RLT buffer (Qiagen) and transported out of the laboratory based

577   on the RML protocol with 600 µl of ethanol. RNA was extracted using a Qiagen RNeasy mini

578   kit (QIAGEN) and eluted in 50 µl of RNase-free water. All the samples were shipped to Mehedi

579   Lab at UND using an appropriate overnight biological shipment procedure.

580

581   **Hematoxylin and eosin (H&E) staining.** Four days post infection, NHBE-ALI and COPD-ALI

582   mock and infected Transwells were fixed with 4% PFA for 24 hours. The membrane was cut off

583   using a scalpel and embedded in paraffin mold. Five-micrometer sections were cut using a

584   microtome. H&E staining was performed by two 5-minute Histo-Clear incubations followed by

585   three 5-minute incubations with 100% ethanol, one 5-minute incubation with 95% ethanol and

586   one 5-minute incubation with 70% ethanol. Subsequently, the samples were washed with tap

587   water for 2 minutes, incubated with 4% acidified Harries Hematoxylin for 2 minutes, washed

588   with tap water for 2 minutes, and incubated with 0.5% lithium carbonate for 20 seconds. The

589   samples were subsequently washed with tap water for 5 minutes, incubated with Eosin Y

590   Solution with Pholoxine for 2 minutes and then subjected to a series of ethanol incubations:

591   incubation with 70% ethanol for 5 minutes, incubation with 95% ethanol for 5 minutes and three

592   5-minutes incubation with 100% ethanol. Finally, two 5-minute incubations with Histo-Clear

593   were performed, and coverslips were then placed using permanent mounting medium.

594

595   **Alcian blue staining.** The 5-µm sectioned ALI-NHBE and COPD mock and infected samples

596   were also subjected to Alcian blue staining. The sections were deparaffinized as described in the

597   section on H&E staining using Histo-Clear and ethanol. The cells were then washed with tap

598   water for 2 minutes, incubated in 3% acetic acid for 3 minutes and stained with Alcian blue for

599   30 minutes. The sections were subsequently rinsed with 3% acetic acid for 10 seconds to help

600   prevent nonspecific staining. The sections were washed under tap water for 10 minutes and then

601   rinsed for 2 minutes in Milli-Q water. The sections were counterstained with nuclear fast red

602   solution for 5 minutes and washed under tap water for 5 minutes. The sections were subsequently

603   dehydrated and cleared with a gradient of alcohol and Histo-Clear as in the protocol used for

604   H&E staining. The slides were mounted with permanent mounting medium and coverslipped.

605

606   **Fluorescence imaging.** The apical site of the airway epithelium was washed with PBS, and both

607   the apical and basal parts were fixed with 4% paraformaldehyde (PFA) (Polysciences, Inc.), and

608   blocked with 10% goat serum (Vector Laboratories) solution in immunofluorescence (IF)

609   washing buffer (130 mM $NaCl_2$, 7 mM Na_2HPO_4, 3.5 mM NaH_2PO_4, 7.7 mM NaN_3, 0.1% BSA,

610   0.2% Triton-X 100 and 0.05% Tween-20) for 1 hour. The Transwell inserts were then incubated

611   with the following primary antibodies (Abs) (alone or in combination) in IF washing buffer

612   overnight at 4°C: anti-ZO-1 rabbit polyclonal (1:200) (Thermo Fisher Scientific), anti-E-

613   cadherin rabbit monoclonal (1:200) (Cell Signaling Technologies), anti-MUC5B rabbit

614   monoclonal (1:500) (Atlas Antibodies), anti-MUC5AC mouse polyclonal (1:100) (Abnova),

615   anti-MUC5AC rabbit monoclonal (1:200) (Cell Signaling Technology), anti-acetyl-alpha-tubulin

616   rabbit monoclonal (1:500) (Cell Signaling Technologies), anti-TMPRSS2 mouse monoclonal

617   (1:50) (Santa Cruz Biotechnology), anti-SARS CoV-2 nucleocapsid mouse monoclonal (1:10)

618   (Thermo Fisher Scientific) and anti-ACE2 mouse monoclonal (1:20) (R&D Systems). The next

619   day, the inserts were washed with the IF washing buffer and incubated with an anti-mouse

620   AlexaFlour 488-conjugated Ab (1:200) (Thermo Fisher Scientific) and/or an anti-rabbit

621   AlexaFlour 647 (1:200) (Thermo Fisher Scientific) in IF washing buffer for 3 hours in the dark at

622   4°C. The cells were washed twice with PBS and incubated with rhodamine phalloidin (1:500)

623   (Cytoskeleton Inc.) in IF washing buffer for 30 minutes at room temperature in the dark. After

624   two washes with PBS, the cell nuclei were stained with NucBlue Fixed Cell Stain ReadyProbes

625   (Thermo Fisher Scientific) for 30 minutes in the dark at room temperature. The epithelium was

626   mounted on Tech-Med microscope slides (Thomas Scientific) using ProLong-Gold anti-fade

627   mounting medium (Thermo Fisher Scientific). Images were captured using a confocal laser-

628   scanning microscope (Olympus FV3000) enabled with a 60X objective. The 405-nm laser was

629   used to excite the DAPI signal for nucleus detection, the 488-nm laser was used to excite Alexa

630   Flour 488 for MUC5AC, ACE2, TMPRSS2 or SARS CoV-2 nucleocapsid protein detection, the

631   561-nm laser was used to excite rhodamine phalloidin for F-actin detection, and the 640-nm laser

632   was used to excite Alexa Flour 647 for MUC5B, acetyl-alpha-tubulin, E-cadherin, ZO-1 or

633   MUC5AC detection. At least two random fields were selected per sample and imaged. The

634   images were processed with Imaris software version 9.5.1 (Oxford Instruments Group) and used

635   for the conversion of Z-stack images (.oir format) to .tiff format and for additional image

636   postprocessing. Separately, the tiling of Z-stack (3x3) images was captured using a Leica DMi8

637   microscope followed by image processing using a 3D deconvolution image processing module in

638   the LASX software (Leica Microsystem) associated with the microscope. We used nine

639   independent images to quantify the total cell number based on F-actin (Texas Red channel) and

640   goblet cells using the Alexa Flour 647 channel (anti-MUC5B) with the Fiji multipointer option.

641   Similarly, we quantified the MUC5AC$^+$ cells using at least two confocal images.

642

643   **Immunohistochemistry:** Healthy and COPD airway epithelia (mock or infected) were sectioned

644   into 5-µm sections as described previously for immunohistochemistry. Before staining, the

645   antigen retrieval process was performed using R-buffer A in a Retriever 2100. The slides were

646   allowed to cool in the buffer overnight and washed three times in PBST (0.05% Tween-20) for

647   10 minutes. Using a Pap pen, a hydrophobic barrier was drawn around the tissue. Once the

648   hydrophobic barrier was dry, the tissue was incubated with blocking buffer (10% goat serum in

649   PBS) for 2 hours in a humidified, light-protected chamber. After incubation with blocking buffer,

650   the tissue was immediately incubated with the following primary antibodies overnight at 4°C in a

651   humidified, light-protected chamber: SARS-CoV-2 N (1:50) (Thermo Fischer Scientific) for the

652   detection of SARS-CoV-2 nucleoprotein (N), SARS-CoV-2 spike (S) protein (1:100) (Thermo

653   Fischer Scientific) for the detection of SARS-CoV2 S protein, acetylated-alpha-tubulin (1:500)

654   (Cell Signaling Technologies) for the staining of ciliated cells, MUC5AC (1:500) (Cell Signaling

655   Technologies) and MUC5B (1:500) (Atlas Antibodies) for the staining of goblet cells, and P63

656   (1:100) (Abcam Inc.) for the staining of basal cells. The next day, the slides were washed three

657   times with PBST and then incubated for 45 minutes with anti-mouse Alexa Fluor 488 (Thermo

658   Fisher Scientific) and anti-rabbit Alexa Fluor 647 (Thermo Fisher Scientific) secondary

659   antibodies in a humidified, light-protected chamber. The slides were subsequently washed three

660   times with PBST, incubated with rhodamine phalloidin (1:100) (Cytoskeleton Inc.) for 30

661   minutes at room temperature in a humidified, light-protected chamber, and washed three times

662   with PBST. The nuclei were then stained by incubation with NucBlue Fixed Cell Stain

663   ReadyProbes (Thermo Fisher Scientific) for 5 minutes at room temperature in a humidified,

664   light-protected chamber. After incubation with the nuclear dye, the slides were washed once with

665   PBST and twice with DI water, and coverglass was then placed on the tissue section using

666   ProLong-Gold anti-fade mounting medium (Thermo Fisher Scientific). The slides were scanned

667   using a Leica DMi8 inverted fluorescence microscope with a 63X oil objective, and the images

668   were further processed using a 3D deconvolution image processing module in the LASX

669   software (Leica Microsystem) associated with the microscope.

670

671   **Ciliary beat frequency (CBF).** Cilia on the apical surface of the cells in the differentiated

672   epithelial layer (after 4 weeks of differentiation) were visualized in the phase-contrast mode with

673   a Leica DMi8 microscope with a 20X objective and an attached environment control chamber

674   (37°C with 5% CO_2) (Leica Microsystems). For each Transwell, six different random fields were

675   recorded for approximately 2.1 seconds at 120 frames per second. The images were captured at

676   37°C and analyzed using the Sisson-Ammons Video Analysis (SAVA) system V.2.1.15 to

677   determine the CBF (Hz) (Ammons Engineering).

678

679   **Transepithelial electrical resistance (TEER).** The permeability of the differentiated epithelial

680   layer (after 4 weeks of differentiation) was determined by measuring the TEER using an

681   epithelial volt-ohm meter (EVOM2, World Precision Instruments, Inc.). The EVOM2 was

682   calibrated according to the manufacturer's instructions, and the STX2 electrode was sterilized

683   with 70% ethanol before use. The internal electrode (smaller in size) was placed in the apical

684   part of each Transwell (PBS was added during the TEER reading), and the external electrode

685   (larger in size) was placed in the basal part of the Transwell, which contained ALI basal medium,

686   to measure the membrane voltage and resistance of the epithelial layer. An empty Transwell

687   insert (filled with PBS) containing no NHBE cells was used to correct for the background

688   resistance. Three readings were taken for each Transwell. The TEER value of each sample was

689   calculated by subtracting the background value.

690

691 **Quantitative real-time PCR.** Airway epithelia cultured on 6.5-mm Transwell membranes were

692 washed and treated for 1 minute at RT with RLT buffer (Qiagen) with 1% β-mercaptoethanol

693 (Sigma-Aldrich). The cells were scraped using a cell scraper, collected into a QIAshredder tube

694 and centrifuged at 15,000 rpm and 4°C for 3 minutes. The eluate was used for the extraction of

695 total RNA using a Total DNA/RNA Extraction Kit (Qiagen), and DNase I treatment was

696 performed to remove DNA from the samples according to the manufacturer's instructions. We

697 also followed a similar approach for the extraction of RNA from A549 cells. The RNA

698 concentration was determined with an Epoch microplate spectrophotometer (BioTek). Five

699 hundred nanograms of RNA was used for first-strand cDNA synthesis (Thermo Fisher Scientific)

700 using Oligo(dT) primers (Thermo Fisher Scientific). qRT-PCR was performed using TaqMan

701 assays (ACE2: Hs1085333_m1 and ACTB: Hs99999903_m1, for calibration) (Thermo Fisher

702 Scientific) with the CFX384 Real-Time PCR System (Bio-Rad), and fold changes were

703 calculated to determine the relative expression levels.

704

705 **Western blotting.** The airway epithelium cultured on 6.5-mm Transwell membranes was

706 washed with PBS, scraped out of all the cells, and pelleted by centrifugation at 10,000 rpm for 5

707 minutes. The cell pellet was incubated with 1x LDS loading buffer (Thermo Fisher Scientific)

708 with proteinase inhibitor (Roche), transferred into a QIAshredder microcentrifuge tube, and

709 centrifuged for 3 minutes at 15,000 rpm in a tabletop centrifuge. The elusion from the

710 QIAshredder was collected and stored in a freezer. The protein concentration was measured

711 using a BCA protein assay kit (Thermo Fisher Scientific). For the detection of ACE2, total

712 protein (30 µg) was separated on 4-12% Bis-tris SDS polyacrylamide gels (reducing) and then

713 subjected to dry blot transfer onto PVDF membranes according to the manufacturer's instructions

714   (Life Technologies). The PVDF membrane was imaged using an Odyssey CLX system (Li-Cor

715   Biosciences). ACE2 was detected by Western blotting using anti-ACE2 goat polyclonal antibody

716   (R&D Systems) and corresponding donkey anti-goat IRDye 800 secondary antibodies (Li-Cor

717   Biosciences). For the loading control, alpha-tubulin was detected by anti-alpha-tubulin mouse

718   monoclonal antibody (Sigma-Aldrich) and the corresponding goat anti-mouse IRDye 680

719   secondary antibody (Li-Cor Biosciences). Image Studio 5.2 (LI-COR Biosciences) was used to

720   quantify the protein signal.

721

722   **Epithelial height measurement.** Microscopic images of the IHC (described above) were used to

723   quantify epithelial height by using the scale feature in LASX software of Leica DMi8

724   microscope. We used at least 4 independent slides for the measurement, at least three

725   independent reads per slide. The plastic membrane of the Transwell was not included in the

726   height measurement.

727

728   **QUANTIFICATION AND STATISTICAL ANALSYSIS**

729

730   **Statistical analysis:** Parameters such as the number of replicates, number of independent

731   experiments, mean +/- SEM, and statistical significance are reported in the figures and figure

732   legends. A p-value less than 0.05 was considered to indicate significance. Where appropriate, the

733   statistical tests and post hoc statistical analysis methods are noted in the figure legends or

734   Methods section.

735

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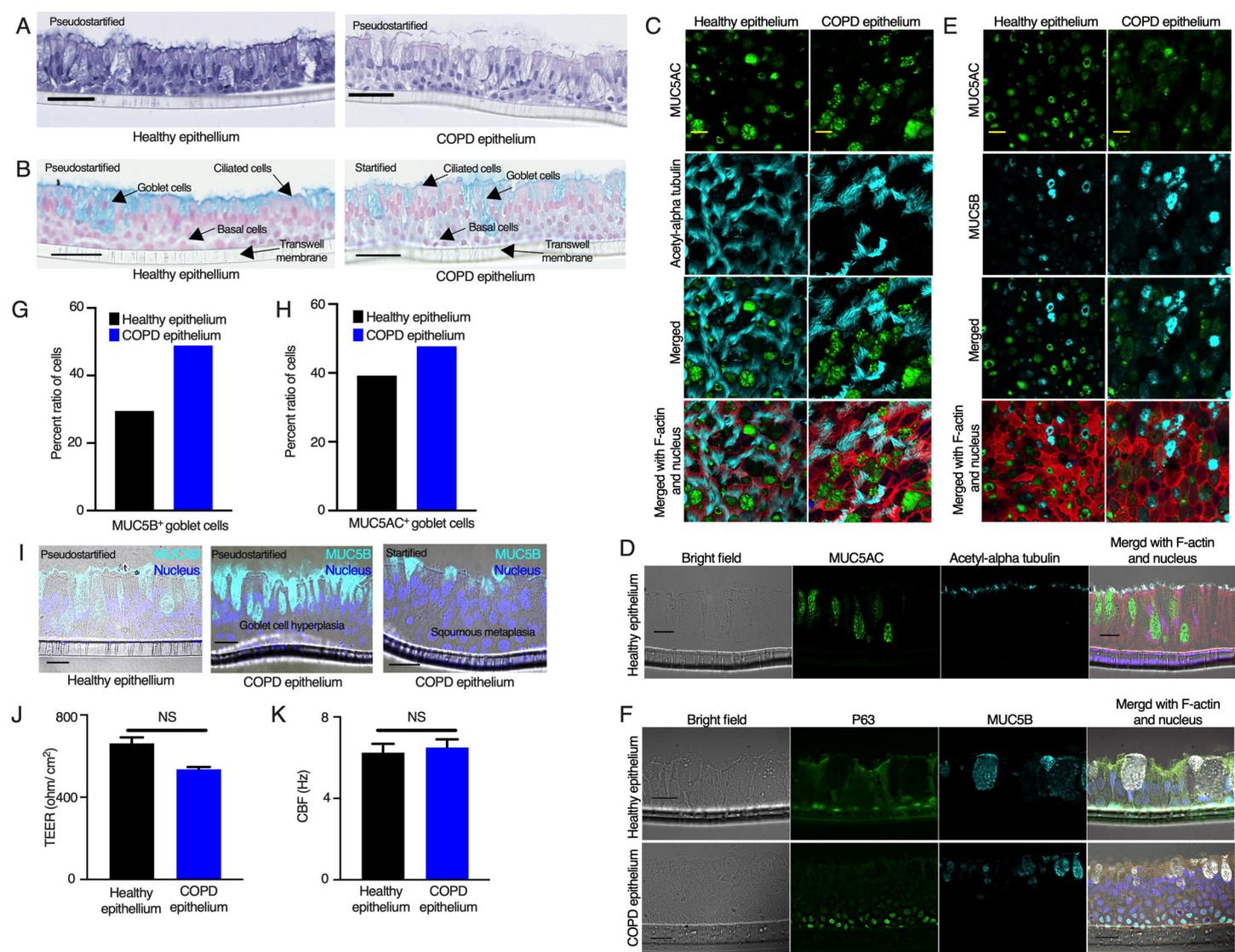
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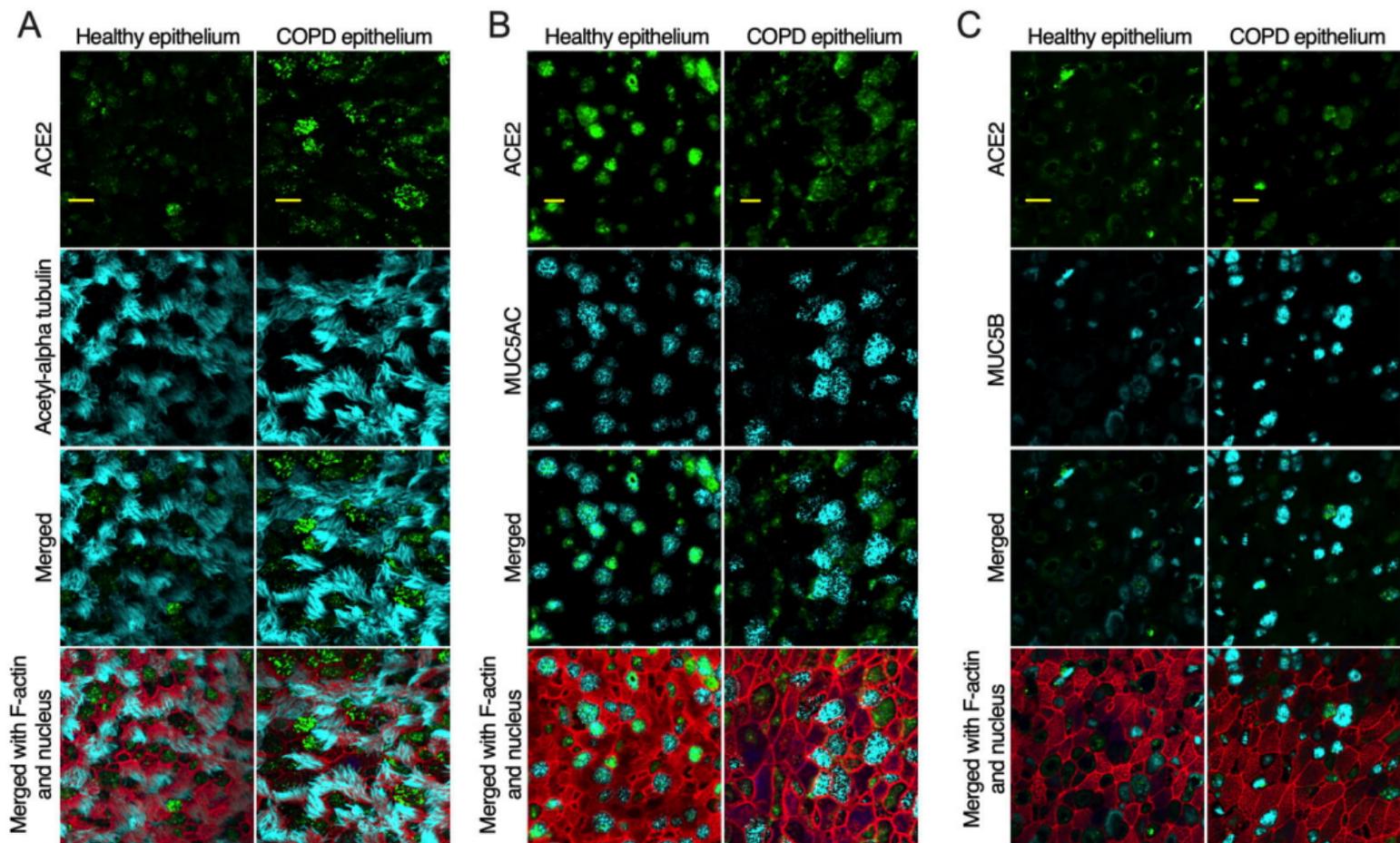
Figure 2

Figure 3

Figure 4

A

SARS-CoV-2 infected healthy epithelium, 4 DPI   SARS-CoV-2 infected COPD epithelium, 4DPI

F-actin
Nucleus
SARS-CoV-2 N

C

SARS-CoV-2 infected healthy epithelium, 4 DPI   SARS-CoV-2 infected COPD epithelium, 4DPI

F-actin
Nucleus
SARS-CoV-2 N

B

| | Bright field | Nucleus | MUC5AC | SARS-CoV-2 N | Merged with F-actin | All merged |

SARS-CoV-2 + healthy epithelium

SARS-CoV-2 + COPD epithelium

D

| | Bright field | Nucleus | Acetyl-alpha tubulin | SARS-CoV-2 N | Merged with F-actin | All merged |

SARS-CoV-2 + healthy epithelium

SARS-CoV-2 + COPD epithelium

Figure 5

Figure 6

Figure S1

Figure S2

A

	Acetyl-alpha tubulin	SARS-CoV-2 N	F-actin	Nucleus	Merged
Mock healthy epithelium					
SARS-CoV-2 + healthy epithelium					
Mock COPD epithelium					
SARS-CoV-2 + COPD epithelium					

B

	MUC5B	SARS-CoV-2 N	F-actin	Nucleus	Merged
Mock healthy epithelium					
SARS-CoV-2 + healthy epithelium					
Mock COPD epithelium					
SARS-CoV-2 + COPD epithelium					

Figure S3

Figure S4

	Mock-infected	SARS-CoV-2 infected, 4 DPI
Healthy epithelium	Pseudostartified	Pseudostartified
Healthy epithelium	Pseudostartified	Pseudostartified
COPD epithelium	Startified / Squamous metaplasia	Startified / Increased squamous metaplasia
COPD epithelium	Pseudostartified / Squamous metaplasia	Startified / Increased squamous metaplasia

Figure S5

Healthy Epithelium

COPD Epithelium

TMPRSS2

ACE2

Cell Sloughing

Syncytium