

1 **Title:** Unconventional secretion of unglycosylated ORF8 is critical for the cytokine  
2 storm during SARS-CoV-2 infection

3 **Running Title:** Life Finds a Way: the unconventional secretion of ORF8

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## 23    **Abstract**

24    Coronavirus disease 2019 is a respiratory infectious disease caused by the severe  
25    acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Evidence on the  
26    pathogenesis of SARS-CoV-2 is accumulating rapidly. In addition to structural  
27    proteins such as Spike and Envelope, the functional roles of non-structural and  
28    accessory proteins in regulating viral life cycle and host immune responses remain to  
29    be understood. Here, we show that open reading frame 8 (ORF8) acts as messenger  
30    for inter-cellular communication between alveolar epithelial cells and macrophages  
31    during SARS-CoV-2 infection. Mechanistically, ORF8 is a secretory protein that can  
32    be secreted by infected epithelial cells via both conventional and unconventional  
33    secretory pathways. The unconventionally secreted ORF8 recognizes the IL17RA  
34    receptor of macrophages and induces cytokine release. However, conventionally  
35    secreted ORF8 cannot bind to IL17RA due to N-linked glycosylation. Furthermore,  
36    we found that Yip1 interacting factor homolog B (YIF1B) is a channel protein that  
37    translocates unglycosylated ORF8 into vesicles for unconventional secretion.  
38    Blocking the unconventional secretion of ORF8 via a YIF1B knockout in hACE2  
39    mice attenuates inflammation and yields delayed mortality following SARS-CoV-2  
40    challenge.

41

42    **Keywords:** SARS-CoV-2, Secretory protein, Unconventional secretory pathway,  
43    Glycosylation, YIF1B

44

## 45     **Introduction**

46     Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome  
 47     coronavirus 2 (SARS-CoV-2), is continuing to spread around the world with nearly  
 48     256 million confirmed cases and more than 5.1 million deaths. According to clinical  
 49     case reports, critically ill patients with COVID-19 experience a cytokine storm,  
 50     resulting in acute respiratory distress syndrome and multiple organ failure(1, 2).  
 51     Currently, our understanding of the mechanisms behind the cytokine storm and how  
 52     SARS-CoV-2 affects cytokine release is still limited.

53  
 54     Open reading frame 8 (ORF8) is an accessory protein of SARS-CoV-2 and it is one of  
 55     the most rapidly evolving  $\beta$ -coronaviruses proteins(3). A 29 nucleotide deletion in  
 56     ORF8 is the most obvious genetic change in severe acute respiratory syndrome  
 57     coronavirus (SARS or SARS-CoV-1) during its host-jump from bats to humans(4).  
 58     The  $\Delta$ 382 variant of SARS-CoV-2, which eliminates ORF8 transcription, seems to be  
 59     associated with milder infection and less systemic release of pro-inflammatory  
 60     cytokines(5). In a previous study, we demonstrated that ORF8 contributes to the  
 61     cytokine storm during SARS-CoV-2 infection(6). Specifically, we found ORF8 to  
 62     interact with the IL17RA receptor, leading to excessive activation of IL-17 signaling  
 63     and downstream NF- $\kappa$ B pathway. However, it remains unclear how the virus exposes  
 64     ORF8 to enable access to the extracellular domain of IL17RA.

65

66     In eukaryotes, secretory proteins usually contain a signal peptide that triggers

translocation into the endoplasmic reticulum (ER)(7). Following this translocation to the ER, cargoes will be exported through ER-Golgi trafficking for further processing and modification(8, 9). This process is termed conventional secretion. Besides, many cytosolic proteins without signal peptides, such as fibroblast growth factor 2 and yeast Acb1, can be released through an unconventional protein secretion pathway(10, 11). Recently, Zhang et al. identified TMED10 as a protein channel for vesicle entry and secretion of interleukin 1 family members(12). Evidently, viral proteins can hijack this secretion pathway to become secreted. For example, the HIV-1 Nef protein can be released from infected cells via an exosomal pathway(13, 14). However, very little is known about whether and how SARS-CoV-2 encoded proteins are secreted during infection.

Glycosylation is a common posttranslational modification, it involves the addition of glycans to macromolecules and is considered essential for the correct folding and functional performance of proteins(15-17). It is also not uncommon for proteins from pathogens to be glycosylated by the host. The co-evolution of N-linked glycosylation sites in influenza viruses affects the host specificity(18). The glycosylation of viral envelope proteins has a wide range of functions, including regulating cell tropism, protein stability and immune evasion(19-21). Recent studies have shown that SARS-CoV-2 Spike protein has 22 N-linked glycosylation sites and 17 O-linked glycosylation sites, which may influence viral infectivity and pathogenicity(22-24). In contrast to the situation for the Spike protein, glycosylation and its functional role in

89 accessory proteins of SARS-CoV-2 has not yet been reported.

90

91 Here, we identified SARS-CoV-2 ORF8 as a secretory protein that can be secreted via

92 conventional and unconventional secretory pathways at the same time. We found that

93 unglycosylated ORF8 secreted via an unconventional pathway is responsible for the

94 release of pro-inflammatory cytokines by binding the IL17RA receptor. By contrast,

95 conventionally secreted ORF8 is incapable of binding to IL17RA due to the N-linked

96 glycosylation at Asn78 site. Further, we identified Yip1 interacting factor homolog B

97 (YIF1B) as a channel protein that recognizes and translocates unglycosylated ORF8

98 into vesicles, thus enabling unconventional secretion. Our findings present an

99 important contribution to the understanding of how SARS-CoV-2 promotes the onset

100 of cytokine storm, and provide a promising strategy for the development of

101 COVID-19 therapeutics.

102

103

## 104 **Results**

### 105 **SARS-CoV-2 ORF8 is a secretory protein that is associated with cytokine release**

106 In order to investigate the secretion of ORF8 protein, we infected Calu-3 human lung

107 epithelial cells with SARS-CoV-2 that was generated using a reverse genetic system

108 (25-27)(Fig. 1A). Cell culture supernatant was collected and presence of ORF8 was

109 determined by ELISA and western blotting (Fig. 1B). We found that ORF8 protein

110 can be secreted into cell culture medium (Fig. 1C). To further validate these results,

111 we used the non-secretory SARS-CoV-2 main proteinase (M pro, also known as 3CL  
112 pro) non-secreted control, and the well-known Nef secretory protein of HIV-1 (13, 14)  
113 as a secreted protein control. We found that Jurkat cells secreted Nef protein  
114 following HIV-1 infection, and Calu-3 cells secreted ORF8 protein after being  
115 infected with SARS-CoV-2 (Fig. 1D). By contrast, the structural protein 3CL pro was  
116 not secreted (Fig. 1D). Next, we tested the time-dependency of ORF8 secretion in  
117 Calu-3 epithelial cells. After 12 hours of SARS-CoV-2 infection, the cell culture  
118 medium was replaced and ORF8 protein in the supernatant was detected every 2  
119 hours. Using 3CL pro as a negative control, we found that the secretion of ORF8  
120 continued for at least 12 hours after replacing the culture medium (Fig. 1E). These  
121 results indicated that SARS-CoV-2 ORF8 is a secretory protein.

122

123 Our previous study has shown that ORF8 protein contributes to the cytokine storm  
124 during SARS-CoV-2 infection(6). SARS-CoV-2 mainly invades alveolar epithelial  
125 cells through binding to ACE2 receptors, however monocytes/macrophages play a  
126 critical role in the secretion and regulation of cytokines. Next, we generated a  
127 SARS-CoV-2 variant with an ORF8 deletion (Fig. 1F), and constructed an epithelial  
128 cell-macrophage co-culture system using a Transwell setup (Fig. 1F), to answer the  
129 question that whether ORF8 is a key factor in modulating the transmission process of  
130 infection signals from epithelial cells to monocytes/macrophages. In this system, the  
131 release of pro-inflammatory factors in macrophages infected with either wild-type  
132 SARS-CoV-2 or ORF8-deletion variant was increased, probably due to a small

133 amount of ACE2 receptor expressed on the surface of macrophages (Fig. 1G, H).

134 Interestingly, we found that the amount of pro-inflammatory factors in the co-culture

135 system was much higher than that measured in individual epithelial cells or

136 macrophages infected with wild-type SARS-CoV-2 (Fig. 1G). However, this

137 synergistic pro-inflammatory effect between epithelial cells and macrophages was not

138 observed in the ORF8-deletion virus infected group (Fig. 1H). Considering that ACE2

139 is responsible for virus entry(28, 29), and IL17RA is the receptor of ORF8(6), we

140 generated ACE2-deficient epithelial cells (Calu-3 *Ace2*<sup>-/-</sup>) based on Calu-3 cell line,

141 and IL17RA-deficient macrophages [THP-1-derived macrophages (THP-1 DM)

142 *Il17ra*<sup>-/-</sup>] based on THP-1 cell line (Fig. S1A). By using these two ways to disrupt

143 cellular communication between epithelial cells and macrophages, preventing

144 SARS-CoV-2 entry by ACE2 deletion in Calu-3 *Ace2*<sup>-/-</sup> cells, or interrupting ORF8

145 reception by IL17RA deletion in THP-1 DM *Il17ra*<sup>-/-</sup> cells, a significant

146 downregulation of pro-inflammatory factors was observed in the co-culture system

147 (Fig. 1H). These results implied that inter-cellular communication between epithelial

148 cells and monocytes/macrophages is important for the cytokine release during

149 SARS-CoV-2 infection.

150

151 It is known that secretory proteins, such as Nef, can be secreted in absence of viral

152 infection in an *in vitro* system(13). We therefore tested whether ORF8 protein can be

153 secreted by human embryonic kidney (HEK-293FT) cells transfected with an

154 ORF8-Flag plasmid. The results showed that Flag-tagged ORF8 was secreted in

155 absence of SARS-CoV-2 infection (Fig. S1B, C). Furthermore, the supernatant of  
156 cells transfected with ORF8-Flag was collected and added to the culture medium of  
157 THP-1 DM cells for stimulation. In this setup, the release of pro-inflammatory factors  
158 in THP-1 DM cells stimulated with ORF8-Flag transfection supernatant was  
159 significantly increased compared to cells treated with control supernatant (Fig. S1D).  
160 In summary, we demonstrated that SARS-CoV-2 ORF8 is a secretory protein, and that  
161 extracellular ORF8 protein is of greatly promoting cytokine release.

162

### 163 **SARS-CoV-2 ORF8 has an unconventional secretory pathway**

164 In order to understand the secretion pattern of ORF8, we used an online tool, Simple  
165 Modular Architecture Research Tool (SMART, <http://smart.embl-heidelberg.de>), to  
166 analyze structural domains of the ORF8 protein. We found a hydrophobic central  
167 domain (similar to the conserved signal peptide of eukaryotes) to be located at its  
168 N-terminus. We defined this domain as the signal peptide of ORF8 protein. Signal  
169 peptide-deficient mutant ( $\Delta$ Signal-SARS-CoV-2 ORF8) was constructed and  
170 transfected into Calu-3 epithelial cells and HEK-293FT cells, respectively (Fig. 2A).  
171 The result showed that the secretion ability of ORF8 was substantially impaired by  
172 signal peptide deletion (Fig. 2B, C). Interestingly, an appreciable quantity of signal  
173 peptide-deficient ORF8 was observed in both the supernatant of Calu-3 and  
174 HEK-293FT cells. This result strongly suggested that the secretion of ORF8 is not  
175 completely blocked by the signal peptide deletion (Fig. 2B, C). This data implied that  
176 secretion of ORF8 might not completely depend on the presence of the signal peptide.



177

178 Proteins secreted through the conventional secretory pathway contain an N-terminal  
179 signal peptide, which is recognized by the signal recognition particles and transported  
180 into the ER, followed by signal peptide cleavage and trafficking to the Golgi  
181 apparatus and the subsequent endomembrane system(7). To verify the existence of an  
182 unconventional secretory pathway for ORF8, Brefeldin A(30) or Monensin(31) was  
183 used to inhibit the Golgi-related vesicle transportation. Consistent with our previous  
184 result, secretion of ORF8 was still observed when the conventional secretory pathway  
185 was blocked (Fig. 2D, E).

186

187 To understand whether the secretion pattern of ORF8 is evolutionary conserved, we  
188 analyzed the homologous ORF8 of SARS and found the SARS ORF8a isoform to  
189 contain an N-terminal signal peptide. We then constructed a  $\Delta$ Signal-SARS ORF8a  
190 mutant and transfected it into epithelial cells (Fig. 2F). While the intact SARS ORF8a  
191 was secreted normally (Fig. 2G), the  $\Delta$ Signal-SARS ORF8a lost the secretory ability  
192 completely (Fig. 2G). This finding stands in interesting contrast to our results showing  
193 that SARS-CoV-2 ORF8 is secreted in absence of the signal peptide (Fig. 2G). This  
194 finding was further supported by inhibition of the Golgi-related vesicle transport  
195 using Brefeldin A or Monensin in Calu-3 (Fig. 2H) and HEK-293FT cells (Fig. 2I).  
196 The results obtained here show that SARS ORF8a can only be secreted under the  
197 guidance of signal peptide through the conventional secretory pathway.

198

199 Considering that the signal peptides of these two different ORF8 proteins share  
200 distinct sequences, we asked whether the different secretion patterns of ORF8 is due  
201 to the different types of signal peptide. To examine this hypothesis, we exchanged the  
202 ORF8 signal peptides between SARS and SARS-CoV-2 (Fig. 2J). As a result,  
203 exchange of signal peptides did not change the secretory patterns observed for the two  
204 viruses (Fig. 2K). Specifically, SARS-CoV-2 ORF8 carrying the SARS ORF8a signal  
205 peptide was still secreted when the Golgi-dependent secretory pathway was blocked  
206 (Fig. 2K). Taken together, these results indicated that SARS-CoV-2 ORF8 is likely to  
207 have an unconventional secretion pattern that does not depend on the presence of a  
208 signal peptide.

209

# **210 Unconventional secretion of ORF8 is required for cytokine storm**

211 Next, we studied whether the different secretion patterns of ORF8 are associated with  
212 the release of pro-inflammatory factors. Calu-3 epithelial cells were pretreated with  
213 Brefeldin A or Monensin to block the conventional secretory pathway, then cells were  
214 infected with SARS-CoV-2 and culture supernatant was collected to stimulate THP-1  
215 DM cells. After 12 hours, pro-inflammatory factors secreted by macrophages were  
216 examined by ELISA (Fig. 3A). Surprisingly, although the amount of secreted ORF8  
217 was significantly decreased (Fig. 3B), the release of pro-inflammatory factors was  
218 barely affected by Brefeldin A or Monensin treatment (Fig. 3C). In order to rule out  
219 the possibility of mutual influence between macrophages themselves, we tested  
220 pro-inflammatory factors released by THP-1 DM cells stimulated with supernatant at

different time intervals. The result showed that there were no significant differences in the secretion of pro-inflammatory factors by macrophages within 0-12 h (Fig. 3C, Fig. S2A). Further, we used exogenously expressed Flag-tagged ORF8 (Fig. 3A), instead of SARS-CoV-2 virus, to validate this finding. Consistent with previous results, we observed almost equal expression levels of pro-inflammatory factors induced by ORF8-Flag regardless of the Golgi-dependent secretory pathway blockade (Fig. 3D, Fig. S2B). These results implied that the unconventionally, instead of the conventionally secreted ORF8 is responsible for the cytokines release.

The current data point to a potential possibility that ORF8 secreted via different pathways might undertake different responsibilities. Interestingly, in our western blots, a smear band shifted to a higher molecular mass compared to ORF8 was regularly observed, this smear however disappeared when the conventional secretory pathway was inhibited by signal peptide deletion or Golgi apparatus damage (Fig. 2B, D, E). Increasing the acrylamide concentration of the SDS-PAGE gel and prolonging the electrophoresis time, we were finally able to distinguish a second band of secreted SARS-CoV-2 ORF8 (Fig. 3E). The Golgi apparatus is known to be the workshop for protein trafficking and processing(32), the most common form of protein processing in Golgi apparatus is glycosylation(16). With this in mind, we asked whether ORF8 is glycosylated during the conventional secretory pathway, which might be responsible for the band shift. Using PNGase F or O-Glycosidase +  $\alpha$ 2-3, 6, 8, 9 Neuraminidase A, we tested the glycosylation status of ORF8 protein secreted from Calu-3 cells infected

243 with SARS-CoV-2. We found that PNGase F treatment, which hydrolyzes most of the  
244 N-linked glycans(33, 34), was leading to the formation of a single ORF8 protein band  
245 (Fig. 3E). Digesting SARS-CoV-2 ORF8 with O-linked glycan hydrolase  
246 O-Glycosidase and  $\alpha$ 2-3, 6, 8, 9 Neuraminidase A did not change the type of bands  
247 compared to untreated samples (Fig. 3E). These data suggested that part of the  
248 secreted SARS-CoV-2 ORF8 protein was N-glycosylated, and that this is likely a  
249 result of conventional secretion through the Golgi apparatus. By contrast, SARS  
250 ORF8a did not respond to glycoside hydrolases at all (Fig. S2C), which means that  
251 conventional pathway secreted SARS ORF8a is not glycosylated.

252

253 Next, we asked whether the glycosylation status of ORF8 is associated with the  
254 release of pro-inflammatory factors. Calu-3 epithelial cells were infected with  
255 SARS-CoV-2, and tunicamycin(35) or PNGase F was used to oppose the N-linked  
256 glycosylation, respectively. Then purified ORF8 protein was added into the culture  
257 medium of THP-1 DM cells for stimulation. The results showed that macrophages  
258 stimulated with non-glycosylated ORF8 showed an elevated level of cytokine  
259 secretion (Fig. 3F). Furthermore, we used plasmid transfection instead of  
260 SARS-CoV-2 infection to validate this result. In line with previous results, a similar  
261 upregulation in cytokine release was observed in the tunicamycin and PNGase F  
262 treatment groups (Fig. 3G). In contrast, the pro-inflammatory factor expression of  
263 Calu-3 cells transfected with SARS ORF8a-Flag plasmid did not change upon  
264 tunicamycin or PNGase F treatment (Fig. S2D). Taken together, these results

265 suggested that glycosylation state of ORF8 is closely associated with the release of  
266 pro-inflammatory factors.

267

# **268 N-linked glycosylation at Asn78 impedes ORF8 binding to IL17RA**

269 In order to determine the specific glycosylation site of SARS-CoV-2 ORF8, we  
270 collected the supernatant of SARS-CoV-2 infected Calu-3 epithelial cells and  
271 performed high performance liquid chromatography-tandem mass spectrometry  
272 (HPLC-MS/MS) for N-glycosylation site mapping (Fig. 4A). According to the  
273 identification by MS, we found the Asparagine 78 (Asn78 or N78) of ORF8 protein to  
274 be glycosylated (Fig. 4B). We further investigated the glycosylation site by creating a  
275 SARS-CoV-2 variant carrying the ORF8 N78Q mutation. Following infection with a  
276 SARS-CoV-2 ORF8-N78Q mutant, Calu-3 epithelial cells secreted a single form of  
277 unglycosylated ORF8 (Fig. 4C). This finding was also validated by transfection of  
278 exogenously expressed Flag-tagged N78Q ORF8 plasmid (Fig. 4C). Further, we  
279 treated THP-1 DM cells with supernatant enriched from Calu-3 cells infected with  
280 wild-type or the SARS-CoV-2 ORF8-N78Q mutant, respectively. In this context,  
281 more pronounced cytokine release was observed in macrophages stimulated with the  
282 ORF8-N78Q mutant protein (Fig. 4D). This result was further validated by exogenous  
283 expression of wild-type ORF8 and N78Q mutants. Consistent with previous results,  
284 transfection of N78Q mutant led to the excessive expression of pro-inflammatory  
285 factors (Fig. 4E). These data show that unglycosylated ORF8, instead of glycosylated  
286 ORF8, is involved in the inflammation response upon SARS-CoV-2 infection.

287

288 In our previous studies, we found that the interaction between ORF8 and host IL17RA  
289 contributes to the formation of a cytokine storm(6). Here, we tested the effect of  
290 ORF8 glycosylation on the activation of the IL-17 pathway. We found that ORF8  
291 protein secreted from the SARS-CoV-2 ORF8-N78Q variant or Flag-tagged  
292 ORF8-N78Q mutant, which could not be N-glycosylated at N78, exhibited stronger  
293 binding to the IL17RA receptor compared to a wild-type control (Fig. 4F). Further,  
294 the interaction between SARS-CoV-2 ORF8 and IL17RA was significantly increased  
295 when PNGase F was used to remove glycosylation (Fig. 4G). However, ORF8-N78Q  
296 showed consistently strong binding to IL17RA regardless of PNGase F treatment (Fig.  
297 4G). We also tested the activation of NF- $\kappa$ B signaling downstream of the IL-17  
298 pathway(36). Consistently, the activation of NF- $\kappa$ B signaling was positively  
299 correlated with the binding affinity between ORF8 and IL17RA (Fig. S3A, B). Data  
300 collected from plasmid transfection of Flag-tagged ORF8 mutant further validated this  
301 result (Fig. 4H, S3C). To further confirm that the N-linked glycosylated ORF8 was  
302 secreted via a conventional pathway, Brefeldin A or Monensin was used to pretreat  
303 Calu-3 epithelial cells to block conventional secretory transport. As a result, inhibition  
304 of Golgi-dependent vesicle transport decreased the interaction between ORF8 and  
305 IL17RA in N78Q groups, because less ORF8 was secreted when ER-Golgi trafficking  
306 was blocked (Fig. S3D, E); however, this inhibition did not affect the interaction  
307 between ORF8 and IL17RA in control groups, mainly due to conventionally secreted  
308 ORF8 was glycosylated (Fig. 4I, J, S3F, G).

309

310 Further, we prepared N-glycosylated ORF8 protein (ORF8-N-Glyc) *in vitro* and  
 311 stimulated THP-1 DM cells directly. The results showed that glycosylated ORF8  
 312 could not bind the IL17RA receptor (Fig. 5A), and that secretion of pro-inflammatory  
 313 factors was significantly reduced (Fig. 5B). These data indicated that  
 314 glycosylation-deficient ORF8 is capable of binding to IL17RA and subsequent  
 315 activation of the IL-17 pathway, thus promoting the cytokine storm. In order to further  
 316 verify the contribution of ORF8 glycosylation to cytokine release during  
 317 SARS-CoV-2 infection *in vivo*, we treated humanized ACE2 (hACE2) mice with  
 318 aerosols of synthetic ORF8 or ORF8-N-Glyc. Compared with control group, the  
 319 survival time of mice exposed to ORF8-N-Glyc was significantly prolonged (Fig. 5C).  
 320 We also observed very little inflammation in the lungs from mice treated with  
 321 ORF8-N-Glyc, while the lung lesions in unglycosylated ORF8-exposed mice were  
 322 much more severe (Fig. 5D). Additionally, mice treated with ORF8-N-Glyc secreted  
 323 decreased levels of cytokines and chemokines in lungs and livers (Fig. 5E, F). Taken  
 324 together, these data indicated that the N78 glycosylation of ORF8 participates in the  
 325 regulation of inflammatory response during SARS-CoV-2 infection.

326

### 327 **YIF1B is essential for the unconventional secretion of ORF8**

328 A substantial number of secreted eukaryotic proteins lacking classical signal peptides  
 329 (called leaderless cargoes) are released through unconventional secretion(37, 38). It  
 330 has been reported that channel proteins located on the ER-Golgi intermediate

331 compartment (ERGIC) might mediate translocation of leaderless cargoes into  
332 transport vesicles(39). However, the driving factors of initial vesicle formation have  
333 not yet been fully elucidated. Recent evidence suggests that autophagy contributes to  
334 the formation of unconventional secretory vesicles(39-41). Therefore, we next  
335 examined whether the unconventional secretion of ORF8 is regulated by autophagy.  
336 Calu-3 epithelial cells were pretreated with Brefeldin A to block the conventional  
337 secretory pathway, and then infected with SARS-CoV-2. Under the stimulation of  
338 starvation or Rapamycin (an autophagy activator), the secretion of ORF8 increased  
339 significantly (Fig. S4A). Autophagy inhibitor 3- Methyladenine (3-MA) or  
340 Wortmannin (Wtm) could counteract the starvation-induced ORF8 secretion (Fig.  
341 S4B). Consistently, knockdown of autophagy-related genes, such as *Atg5*, *Atg2a* or  
342 *Atg2b*, also inhibited the release of ORF8 (Fig. S4C). Additionally, we observed  
343 co-localization of ORF8 and ERGIC-53, indicating the possibility of ORF8  
344 translocation into ERGIC (Fig. S4D). The proteinase K protection test also provided  
345 evidence of vesicle-mediated ORF8 transportation (Fig. S4E). These data suggested  
346 that SARS-CoV-2 ORF8 likely features an unconventional secretion pattern similar to  
347 eukaryotic leaderless cargoes.

348

349 To further determine the channel protein that guides the translocation of ORF8 into an  
350 unconventional secretion pattern, we performed mass spectrometry analysis to  
351 identify proteins that interact with unglycosylated ORF8. To enrich as many potential  
352 channel proteins on ORF8 transport vesicles as possible, dihydrofolate reductase



(DHFR)-tagged ORF8 was transfected into HEK-293FT cells pretreated with aminopterin and Brefeldin A/Monensin as previously described (12)(Fig. S4F). Of the 156 potential interacting proteins, seven channel proteins appeared to be related to cargo transport (Fig. S4G). We then screened the seven candidates using knockdown strategy by siRNAs, and found that YIF1B was associated with the unconventional secretion of ORF8 (Fig. S4G).

Next, we verified the interaction between ORF8 and YIF1B by immunoprecipitation. Flag-tagged ORF8 was transfected into HEK-293FT cells and an interaction between ORF8 and YIF1B was observed (Fig. 6A). The co-localization of exogenously expressed HA-tagged YIF1B and Flag-tagged ORF8 was detected in HEK-293FT cells by immunoprecipitation (Fig. 6B) and the Duolink proximity ligation assay (Fig. 6C). Further, endogenous YIF1B and virus-derived ORF8 were able to form a complex in SARS-CoV-2 infected Calu-3 epithelial cells (Fig. 6D).

Then we generated YIF1B-deficient cells (YIF1B-KO) based on the Calu-3 cell line, and found that unconventional secretion of ORF8 disappeared in absence of YIF1B; however, exogenous supplementation of YIF1B by plasmid transfection rescued the secretion of ORF8 (Fig. 6E, F). Moreover, cytokine release assays further proved that YIF1B overexpression reconstituted the function of ORF8 protein in YIF1B-KO cells (Fig. 6G). Likewise, ORF8 secretion and cytokine release data collected from ORF8-Flag plasmid transfection further confirmed above findings (Fig. 6H-J). These

375 results indicated that the unconventional secretion pattern of SARS-CoV-2 ORF8  
376 requires host YIF1B.

377

### 378 **The $\alpha 4$ helix of YIF1B recognizes the $\beta 8$ sheet of ORF8 for interaction**

379 The structural domain of ORF8 protein, which is eight  $\beta$ -pleated sheets consisted by  
380 165 amino acid chain, has been reported(42) (Fig. 7A). We constructed ORF8  
381  $\Delta\beta 1$ - $\Delta\beta 8$  deletion mutants, respectively. The results of co-immunoprecipitation  
382 showed that an ORF8 mutant lacking the  $\beta 8$  sheet could not bind to YIF1B (Fig. 7B).  
383 Similarly, we observed that  $\beta 8$  sheet-deleted ORF8 could not be secreted when the  
384 conventional secretion pathway was blocked by Brefeldin A pretreatment (Fig. 7C, D).  
385 Likewise, the cytokine release by THP-1 DM cells stimulated with supernatant proved  
386 that  $\beta 8$  sheet domain was closely associated with the unconventional secretory  
387 pathway of ORF8 protein (Fig. 7E).

388

389 In order to understand how YIF1B mediates the recognition and secretion of leadless  
390 cargoes, we constructed deletion mutants of five  $\alpha$  helices located on the  
391 transmembrane domains of YIF1B according to the structural predication  
392 (<https://alphafold.ebi.ac.uk/entry/Q5BJH7>) (Fig. 7F). Co-immunoprecipitation results  
393 showed that a  $\alpha 4$  helix deletion impaired the interaction between YIF1B and ORF8  
394 (Fig. 7G). Next, we transfected exogenous YIF1B mutants into wild-type epithelial  
395 Calu-3 cells. We found that the unconventional secretion of ORF8 increased  
396 significantly in YIF1B mutants groups; this however was not observed in the  $\Delta\alpha 4$

mutant group (Fig. 7H-J). Further, after transfection of YIF1B mutants into YIF1B-KO cells, the unconventional secretion of ORF8 was rescued by all YIF1B mutants, except the  $\Delta\alpha4$  mutant (Fig. 7K-M). These data implied that the unconventional secretion of ORF8 relies on the  $\alpha4$  helix of YIF1B and depends on its presence in a dose-dependent manner (Fig. 7H-J).

Next, we asked whether YIF1B-guided unconventional secretion of leaderless ORF8 is driven by direct recognition of the  $\beta8$  sheet. To answer this question, we constructed a 3CL pro mutant with a  $\beta8$  sheet fused to the C-terminus. Interestingly, an interaction between 3CL pro- $\beta8$  fusion and YIF1B was observed (Fig. 7N), and secreted 3CL pro could be collected from culture supernatant (Fig. 7O). Collectively, these evidences indicated that unconventional secretion of ORF8 is mediated by host YIF1B through recognition and interaction between the  $\beta8$  sheet and  $\alpha4$  helix domain.

# **YIF1B directly promotes the translocation of ORF8**

Since the interaction between ORF8 and YIF1B is important for the unconventional secretion pattern of ORF8, we first looked for intracellular co-localization of ORF8 and YIF1B during SARS-CoV-2 infection. Immunofluorescence results showed that full-length ORF8 and YIF1B did co-localized (Fig. 8A). However, neither an ORF8  $\beta8$  sheet-deleted mutant nor a YIF1B  $\alpha4$  helix-deficient mutant co-localized with their respective partner (Fig. 8B, C). Next, a proteinase K protection test was performed to examine whether ORF8 transportation into vesicles relies on ORF8-YIF1B interaction.

419 Full-length ORF8-Flag or YIF1B truncations were transfected into YIF1B-KO cells as  
 420 indicated, the membrane pellets were collected by centrifugation and treated with  
 421 proteinase K or Triton X-100. The results showed that in YIF1B-deficient cells, ORF8  
 422 was not transported into vesicles, leading to its degradation by proteinase K (Fig. 8D).  
 423 On the contrary, ORF8 was able to resist the degradation by proteinase K when  
 424 YIF1B truncation was counteracted by transfection of YIF1B plasmids, except in case  
 425 of the  $\Delta\alpha 4$  mutant (Fig. 8D).  
 426  
 427 Furthermore, we constructed an *in vitro* ORF8 transport system as previously  
 428 described(12). In this system, synthesized ORF8 was mixed with assembled  
 429 proteoliposomes, and HEK-293FT cell lysate without endomembrane was used as the  
 430 reaction buffer to promote the transport of ORF8 (Fig. 8E). A proteinase K protection  
 431 test showed that YIF1B mediated the transport of ORF8 into proteoliposomes in a  
 432 dose-dependent manner (Fig. 8F).  
 433  
 434 We also established a GFP fluorescence complementation system(43) to validate if  
 435 ORF8 resides in the same vesicle that also harbors YIF1B. ORF8 fused with GFP(11)  
 436 and YIF1B containing GFP(1-10) were co-transfected into HEK-293FT cells and flow  
 437 cytometry was used to measure the fluorescence emitted by the combination of ORF8  
 438 and YIF1B (Fig. 8G). When the amount of transfected ORF8 or YIF1B increased, an  
 439 elevation in fluorescence intensity was observed, suggesting that the binding rate of  
 440 ORF8-YIF1B complex increased in a concentration depended manner(Fig. 8H, I).

Collectively, these data clarified that YIF1B directly promotes the translocation of ORF8 into vesicles for unconventional secretion.

# **YIF1B regulates cytokine storm upon SARS-CoV-2 infection**

YIF1B regulates the translocation of SARS-CoV-2 ORF8 into vesicles, and thereby promotes the unconventional secretion of unglycosylated ORF8 protein, which is required for the downstream IL-17 signaling activation and release of pro-inflammatory. To ascertain the biologic significance of our findings, we generated YIF1B-deficient (*Yif1b*<sup>-/-</sup>) mice in a hACE2 background (Fig. S5A-C). After SARS-CoV-2 infection, the interaction between ORF8 and YIF1B disappeared (Fig. 9A), and ORF8 secretion was significantly decreased in alveolar epithelial cells collected from these mice (Fig. 9B). In line with our expectations, supplementation of exogenous YIF1B restored the secretion of ORF8 (Fig. 9B). Further, epithelial cells obtained from *Yif1b*<sup>-/-</sup> mice only secreted N-glycosylated ORF8, indicating a deficiency of the unconventional secretory pathway (Fig. 9C). Consistently, ORF8 secreted from *Yif1b*<sup>-/-</sup> epithelial cells neither bound the IL17RA receptor nor did it trigger the downstream NF-κB pathway (Fig. 9D, E). These data again illustrated that the unconventional secretory pathway mediated by YIF1B allows ORF8 to escape host cell glycosylation, thus enabling activation of the IL-17 signaling pathway.

To further characterize the functional role of host YIF1B in the development of a cytokine storm during SARS-CoV-2 infection, *Yif1b*<sup>-/-</sup> hACE2 mice were intranasally

infected with  $4 \times 10^5$  PFU plaque-forming units (PFU) of SARS-CoV-2. Compared to the *Yif1b*<sup>+/+</sup> littermates, the survival time of *Yif1b*<sup>-/-</sup> mice infected with SARS-CoV-2 was significantly prolonged (Fig. 9F). The lungs of *Yif1b*<sup>-/-</sup> mice showed only mild inflammation compared to the extensive lung lesions observed in *Yif1b*<sup>+/+</sup> mice (Fig. 9G). Further, we examined the viral loads and cytokine release in the spleens and livers of *Yif1b*<sup>-/-</sup> mice and their *Yif1b*<sup>+/+</sup> littermates. In this assay, although the viral loads showed no significant difference in the two groups (Fig. 9H), the cytokine release in spleens and livers of *Yif1b*<sup>-/-</sup> mice was strongly alleviated compared to the control group (Fig. 9I, J). These results emphasize both the functional role of YIF1B in the unconventional secretion of ORF8 and the mechanistic role of unconventional ORF8 protein secretion in the development of a cytokine storm during SARS-CoV-2 infection.

Overall, we found that after invading host epithelial cells, SARS-CoV-2 ORF8 can be secreted through both conventional and unconventional secretory pathways. In the conventional secretory pathway, ORF8 is N-glycosylated during the ER-Golgi trafficking, consequently, extracellular ORF8 lost the ability to recognize the IL17RA receptor of macrophages, likely due to steric hindrances imposed by N-glycosylation at the Asn78 site. By contrast, ORF8 is recognized and then translocated into vesicles directly by host YIF1B in an unconventional secretory pathway. Without experiencing the conventional ER-Golgi trafficking, ORF8 protein does not become glycosylated. Hence, extracellular ORF8 can be distributed through body fluid circulation and to get

485 in contact with macrophages, where unglycosylated ORF8 binds the IL17RA receptor  
486 and activates the IL17 pathway and downstream NF-κB signaling facilitating the  
487 onset of a cytokine storm (Fig. 10).

488

489

## 490 **Discussion**

491 Understanding the specific functions of SARS-CoV-2 proteins is pivotal for us to  
492 perceive the mechanisms which contribute to its high infectivity, fitness, and  
493 virulence. Numerous studies unravelling the differential functions of structural  
494 proteins have appeared recently(28, 44). However, it is worth noting that  
495 non-structural and accessory proteins encoded by SARS-CoV-2 likewise play  
496 significant roles in the regulation of the viral life cycle and also affect the immune  
497 response of the host. For example, ORF3a has been reported to induce apoptosis and  
498 promote lysosomal exocytosis-mediated viral egress(45, 46), while ORF6 protein of  
499 SARS-CoV-2 hampers the induction of host interferon signaling(47).

500

501 ORF8, a non-conserved accessory protein, is likely to be associated with the unique  
502 characteristics of SARS-CoV-2. According to clinical reports, ORF8 is highly  
503 immunogenic, anti-ORF8 antibodies are formed in the early stage of infection(48) and  
504 a significant T-cell response to ORF8 is observed in recovered patients(49). It is  
505 further reported that an ORF8-deficient SARS-CoV-2 strain (Δ382) in Singapore  
506 displays a significant reduction in virulence(5). In a recent study, Zhang et al. reported

507 that SARS-CoV-2 ORF8 interacts with MHC-I, a marker protein located on the cell  
508 surface, and activates the lysosomal degradation pathway, thus achieving escape from  
509 immune surveillance by decreasing the expression of MHC I(50). These data indicate  
510 the specific role of ORF8 in infectivity and pathogenicity of SARS-CoV-2.

511

512 Our previous work showed that SARS-CoV-2 ORF8 could interact with the IL17RA  
513 receptor, thereby leading to IL-17 pathway activation and an increased secretion of  
514 pro-inflammatory factors(6). Considering that IL17RA is a transmembrane protein  
515 and ORF8 binds to the extracellular domain of IL17RA, as well as combining with  
516 the existing evidences, we proposed that ORF8 might be a secretory protein that is  
517 secreted into extracellular compartments. In this study, we show that SARS-CoV-2  
518 ORF8 protein can be secreted by infected epithelial cells, which is supporting the role  
519 of ORF8 as a cellular messenger between alveolar epithelial cells and macrophages in  
520 the occurrence and development of a cytokine storm. Interestingly, we discovered that  
521 ORF8 can be secreted through both a conventional and an unconventional secretory  
522 pathway, and that ORF8 secreted via different pathways has different mechanistic  
523 functions in SARS-CoV-2 infection. Specifically, conventionally secreted ORF8 is  
524 N-glycosylated and loses the ability of binding the IL17RA receptor.

525 Unconventionally secreted ORF8 is undergoing the conventional ER-Golgi trafficking  
526 pathway and consequently does not become glycosylated. Therefore, it is able to bind  
527 the IL17RA receptor and activate IL-17 signaling induce the expression of  
528 pro-inflammatory factors. The existence of this unique indirect cellular



529 communication mechanism, instead of virion release, during the course of

530 SARS-CoV-2 infection can at least partly explain why viral loads in patients are not

531 directly proportional to the severity of disease symptoms in COVID-19(51, 52).

532

533 In this study, we found that SARS ORF8a is also a secretory protein. Unlike

534 SARS-CoV-2 ORF8, SARS ORF8a could only be secreted through the conventional

535 secretory pathway. Notably, the conventionally secreted SARS ORF8a is not

536 glycosylated and is able to induce the release of pro-inflammatory factors. These

537 differences might be due to the fact that ORF8 is the only SARS-CoV-2 protein with

538 as low as approximate 20% homology to SARS(42). In this context, the very different

539 secretory pattern and functional role of ORF8 in SARS-CoV-2 can partly explain why

540 the disease spectrum of COVID-19 is different from that of SARS. Based on our data,

541 we propose a potential competition between host and guest regarding the ORF8

542 protein: Primitive SARS invades host cells and secretes ORF8 through the

543 conventional ER-Golgi trafficking pathway. ORF8 is not glycosylated in the Golgi

544 apparatus and can induce the release of pro-inflammatory factors. However, the host

545 does not just surrender to viral infection. During the evolutionary process, the host

546 blocks the induction of IL-17 signaling though ORF8 glycosylation to maintain

547 homeostasis. However, life finds a way; an evolved coronavirus develops a new

548 pathway to bypass the glycosylation modification and secrete unglycosylated ORF8

549 protein, thus regaining an advantageous position in this competition for the moment.

550 Nevertheless, this storyline needs to be validated by further studies.

551

552 In the current work, we identified YIF1B, a five-transmembrane protein, as a key  
 553 molecule that mediates transport of cargo into vesicles to facilitate the unconventional  
 554 secretion pattern of ORF8. Knockout of YIF1B in epithelial cells prevents the  
 555 unconventional secretion of ORF8, and exogenous supplementation of YIF1B rescues  
 556 the secretion of ORF8 even when Golgi apparatus is destructed by Brefeldin A. The *in*  
 557 *vitro* translocation assay proved that YIF1B promotes the translocation of ORF8 into  
 558 vesicles directly. Generally, the translocation process of leaderless cargoes into  
 559 vesicles depends on chaperones(53). For example, leaderless cargoes bind to  
 560 chaperone HSP90A that likely recognizes and unfolds the cargoes in the  
 561 TMED10-channeled unconventional protein secretion pathway(40). Whether the  
 562 translocation of leaderless ORF8 into vesicles needs chaperones in addition to the  
 563 YIF1B channel is not yet known. Notably, the proteinase K protection assays shows  
 564 that ORF8 cannot be translocated into vesicles without the supplementation of cell  
 565 lysates containing non-inner membranes. This result does suggest the necessity of  
 566 chaperones in the translocation of leaderless ORF8. It would be interesting to further  
 567 investigate the specific role of chaperones in the unconventional secretion of  
 568 SARS-CoV-2 ORF8.

569

570

571

572 **Materials and Methods**

## 573 **Ethic statements**

574 This study was carried out in strict accordance with the Guidelines for the Care and  
575 Use of Animals of Chongqing University. All animal experimental procedures were  
576 approved by the Animal Ethics Committees of the School of Life Sciences,  
577 Chongqing University.

578

## 579 **Mice**

580 B6.Cg-Tg(K18-ACE2)2PrImn/J mice (hACE2) were obtained from The Jackson  
581 Laboratory. To generate YIF1B-deficient (*Yif1b*<sup>-/-</sup>) mice based on K18-ACE2  
582 transgenic background, we designed sgRNAs targeting the Exon 3-5 of *Yif1b*. Cas9  
583 and sgRNAs (sgRNA1: CAGCTAACACGGTGGGTTGT, sgRNA2:  
584 GAGGCCAAGAGCCATCAGAG) were co-injected into fertilized eggs of hACE2  
585 mice. PCR followed by sequencing analysis and western blotting were used for  
586 validation. All animal study protocols were reviewed and approved by Chongqing  
587 University School of Life Sciences review boards for animal studies.

588

## 589 **Cell lines and coronavirus**

590 Calu-3 epithelial cells (HTB-55), Jurkat cells (Clone E6-1, TIB-152), THP-1 cells  
591 (TIB-202), Vero E6 cells (CRL-1586) and Sf9 cells (CRL-1711) were purchased from  
592 ATCC. HEK-293FT cells (R70007) were purchased from Thermo Fisher Scientific.  
593 Differentiation of THP-1 monocytes to macrophages was induced by 15 ng/mL  
594 phorbol 12-myristate 13-acetate (PMA) as previously described(54). *Ace2*<sup>-/-</sup> Calu-3

595 epithelial cell line, *Il17ra*<sup>-/-</sup> THP-1-derived macrophage cell line and YIF1B-KO  
596 Calu-3 epithelial cell line were generated using CRISPR-Cas9 system with short  
597 guide RNA sequences (*Ace2*<sup>-/-</sup> cell line: ACAGTTTAGACTACAATGAG; *Il17ra*<sup>-/-</sup>  
598 cell line: TGTCCATTCGATGTGAGCCA; YIF1B-KO cell line:  
599 GAGAGGCTGCAGGATAACTC). Murine alveolar epithelial cells (AECs) were  
600 isolated using the method developed by Corti and colleagues with modifications(55,  
601 56). Plasmid and siRNA transfections were performed using a LONZA  
602 4D-Nucleofector system according to the manufacturer's instruction. The  
603 SARS-CoV-2 virus was generated by using a reverse genetic method as previously  
604 described(25-27). In brief, seven different DNA fragments spanning the entire  
605 genome of SARS-CoV-2 (USA\_WA1/2020 SARS-CoV-2 sequence, GenBank  
606 accession No. MT020880) were synthesized by Beijing Genomics Institute (BGI,  
607 Shanghai, China) and cloned into the pUC57 or pCC1 (kindly provided by Dr.  
608 Yonghui Zheng) plasmid by standard molecular cloning methods. The sequences of  
609 the F1~F7 fragments and the restriction enzymes used for digestion and ligation were  
610 shown in our previous study(25). Full-length cDNA assembly and recombinant  
611 SARS-CoV-2 virus recovery were performed as previously described(25-27). Virus  
612 titer was determined using a standard TCID<sub>50</sub> assay. For the generation of  
613 SARS-CoV-2 ORF8-N78Q variant, AAT→CAA nucleotide substitutions were  
614 introduced into a subclone of pUC57-F7 containing the ORF8 gene of the  
615 SARS-CoV-2 wild-type infectious clone by overlap-extension PCR. Primers are as  
616 follows: F- TCAGTACATCGATATCGGTCAATAT, R-

617 GTAAACAGGAACTGTATATTGACC.

618

# 619 **Viral infection**

620 Specific-pathogen-free, ten-week-old male mice were inoculated intranasally with  
621 SARS-CoV-2 virus with  $4 \times 10^5$  plaque-forming units (PFU). Cells were infected with  
622 SARS-CoV-2 virus at a dosage of  $10^5$  TCID<sub>50</sub>/mL within the indicated time. Cell  
623 culture supernatant obtained from infected epithelial cells was filtered by Ultipor VF  
624 Grade UDV20 Virus Removal Filter Cartridges (PALL) to remove virion, and used  
625 for macrophage stimulation. All experiments with infectious virus were performed  
626 under biosafety level 3 (BSL3+) conditions.

627

# 628 **Collection of secretory proteins and immunoblot**

629 Cell culture medium was collected and centrifuged twice to remove cell debris. The  
630 supernatant was concentrated through a 10 kDa Amicon-Ultra centrifugal tube  
631 (Millipore) and prepared for immunoblot. GAPDH was used to indicate the absence  
632 of cell lysates. Immunoblot analysis was performed as previously described(57). Blots  
633 were probed with the indicated antibodies: anti-ORF8 (NBP3-05720), anti-GAPDH  
634 (NBP2-27103) (Novus Biologicals), anti-3CL pro (GTX135470 ) (GeneTex),  
635 anti-NEF (MA1-71507), anti-IL17RA (PA5-47199) (Thermo Fisher Scientific),  
636 anti-GFP (ab6556), anti-YIF1B (ab188127), anti-SEC22B (ab241585), anti-V5  
637 (ab9137) (Abcam), anti-PDI (2446) (Cell Signaling Technology), anti-Flag (AF0036),  
638 anti-His (AF5060), anti-GST (AF5063), anti-HA (AF0039) (Beyotime

639 Biotechnology).

640

#### 641 **Enzyme linked immunosorbent assay**

642 Cell culture supernatant was purified by centrifugation, and assayed by enzyme linked

643 immunosorbent assays (ELISA). ORF8 antibody (NBP3-05720, Novus Biologicals)

644 was coated with blank ELISA plates in carbonate buffer to prepare ELISA kit.

645 Cytokine and chemokine ELISA kits were purchased from eBiosciences (Thermo

646 Fisher Scientific).

647

#### 648 **Block of conventional secretory pathway**

649 Brefeldin A (00-4506-51, eBioscience, 3 µg/mL) or Monensin (00-4505-51,

650 eBioscience, 2 µM) was added in the culture medium of epithelial cells for 2 hours to

651 inhibit the ER-Golgi trafficking and block the ER-Golgi conventional secretion

652 pathway.

653

#### 654 **N-linked glycosylation identification by HPLC-MS/MS**

655 N-linked glycosylation identification of SARS-CoV-2 ORF8 protein was performed

656 as previously described with slight modifications(58). Firstly, Calu-3 epithelial cells

657 were infected by SARS-CoV-2 with/without Brefeldin A or Monensin pretreatment.

658 The supernatant was collected and concentrated through a 10 kDa Amicon-Ultra

659 centrifugal tube (Millipore). Then, the N-glycopeptides were enriched with

660 Zic-HILIC (Fresh Bioscience), eluted and dried for deglycosylation. Enriched

661 N-glycopeptides were digested using PNGase F dissolved in 50 mM  $\text{NH}_4\text{HCO}_3$  for 2  
662 hours at 37 °C to remove N-linked glycosylation. Finally, deglycosylated peptides  
663 were dissolved in 0.1% FA for tandem mass spectrum analysis. MS1 was analyzed at  
664 an Orbitrap resolution of 120,000 using a scan range (m/z) of 800 to 2000  
665 (N-glycopeptides before and after enrichment), or 350 to 1550 (deglycosylated  
666 peptides). The RF lens, AGC target, maximum injection time, and exclusion duration  
667 were 30%,  $2.0 \times 10^4$ , 100 ms and 15 s, respectively. MS2 was analyzed with an isolation  
668 window (m/z) of 2 at an Orbitrap resolution of 15,000. The AGC target, maximum  
669 injection time, and the HCD type were standard, 250 ms, and 30%, respectively.

670

#### 671 **Deglycosylation assays**

672 Tunicamycin (12819, Cell Signaling Technology, 2µg/mL) was added into epithelial  
673 cells to prevent N-linked glycosylation. PNGase F (P0704, NEB, 1,000 units/µg  
674 protein) was used to remove the N-linked glycosylation in purified ORF8 protein. 1  
675 µg glycoprotein, 2 µL GlycoBuffer 2 (10×), 2 µL PNGase F and  $\text{H}_2\text{O}$  were mixed to  
676 form a 20 µL mixture. The mixture was incubated at 37°C for 8 hours, followed by  
677 western blotting analysis. To remove O-linked glycosylation, purified proteins were  
678 directly digested by O-glycosidase (P0733, NEB, 4,000 units/µg protein) and  $\alpha$ 2-3, 6,  
679 8, 9 Neuraminidase A (P0722, NEB, 4 units/µg protein). 10 µg glycoprotein, 1 µL  
680 10× Glycoprotein Denaturing Buffer and  $\text{H}_2\text{O}$  were combined to make a 10 µL  
681 mixture. The glycoprotein was denatured by heating the mixture at 100°C for 10 min.  
682 Then, 2 µL 10× GlycoBuffer 2, 2 µL 10% NP40, 2 µL  $\alpha$ 2-3, 6, 8, 9 Neuraminidase A,

683 1  $\mu$ L O-Glycosidase and H<sub>2</sub>O were mixed to form a total volume of 20  $\mu$ L and

684 incubated at 37°C for 4 hours, followed by western blotting analysis.

685

# 686 **Peptide synthesis and artificial glycosylation modification**

687 SARS-CoV-2 ORF8 peptide was synthesized according to the NCBI published

688 sequence (accession number: YP\_009724396.1). Fmoc-L-Asn

689 ((Ac)3- $\beta$ -D-GlcNAc)-OH modification was performed by Shanghai Science Peptide

690 Biological Technology Co., Ltd. ORF8 peptides with or without N-glycosylation were

691 dissolved in 40  $\mu$ L DMSO and diluted with PBS buffer for cell stimulation (1  $\mu$ g/mL)

692 or mice aerosol infection (10  $\mu$ g/g).

693

# 694 **Identification of the interaction channel proteins of ORF8**

695 DHFR assay followed by HPLC-MS/MS was used to screen the channel proteins

696 mediating ORF8 translocation in an unconventional secretion pathway as previously

697 described(12). Calu-3 cells expressing ORF8-Flag-DHFR were treated with Brefeldin

698 A (3  $\mu$ g/mL) or Monensin (2  $\mu$ M) to inhibit conventional secretion pathways.

699 Aminopterin (15  $\mu$ M) was added to inhibit DHFR unfolding. After crosslinking,

700 membrane fractions were collected, lysed and immunoprecipitated with anti-Flag

701 beads for HPLC-MS/MS analysis.

702

# 703 **Duolink PLA assay**

704 Duolink PLA assay was performed according to the kit manual (Duolink In Situ



705 Detection Reagents Red, DUO92008, Sigma). In brief, Flag-tagged ORF8 and  
706 HA-tagged YIF1B were co-transfected into HEK-293FT cells. After 12 hours, cells  
707 were fixed with 4% paraformaldehyde (PFA) for 20 min and permeabilized with 0.2%  
708 Triton X-100. Next, the fixed cells were blocked, incubated with antibodies and PLA  
709 probes according to the protocol. Images were captured followed by quantification  
710 using Image J software.

711

# ***In vitro* translocation assay**

713 Purified YIF1B proteins (NCBI accession number: NP\_001034761.1) was  
714 synthesized by MembraneMax<sup>TM</sup> cell-free protein synthesis system according to the  
715 manufacturers' protocol (A10633, Thermo Fisher Scientific). Purified ORF8 protein  
716 was expressed in baculovirus expression vector system (Thermo Fisher Scientific). In  
717 brief, ORF8 protein-coding sequence without signal peptide was optimized according  
718 to the insect cell codon preference, and synthesized by Sangon Biotech. The  
719 Asparagine 78 was mutated to Glutamine to prevent glycosylation. Then, synthesized  
720 ORF8 sequence was amplified by PCR and inserted into a pFastBac HT A plasmid  
721 (Thermo Fisher Scientific). Recombinant pFastBac-ORF8 plasmid was transformed  
722 into DH10Bac-competent cells to obtain Bacmid-ORF8, which was transfected into  
723 Sf9 cells to produce ORF8 protein. Total lipids were extracted from HEK-293FT cells  
724 as previously described(12). Purified YIF1B and lipids were reconstituted into  
725 proteoliposomes. To be specific, lipids were repeatedly frozen and thawed in a 42°C  
726 water bath for 10 times. Triton X-100 was used to dilute lipids to a final concentration

of 0.05%, and the lipid solution was rotated at 4°C for 30 min. Then, the purified YIF1B protein was added into the lipid solution and rotated for 1 hour, followed by incubation with Biobeads SM2 (Bio-Rad Laboratories) to absorb the detergent. After centrifugation to remove the beads, a membrane flotation procedure was performed to collect top fractions containing proteolioposomes. Next, proteolioposomes were mixed with ORF8 protein in HEK-293FT lysates (whole cell lysates were centrifuged at 1,000×g for 10 min and the supernatant was ultra-centrifuged at 100,000×g for 40 min to remove endomembrane) for 1 hour. The mixture was centrifuged, aliquoted into three parts, and collected for proteinase K protection test.

736

### 737 **Proteinase K protection test**

Proteinase K protection test was performed as previously described(40). Briefly, cells were harvested and lysed in HB1 buffer (20 mM HEPES-KOH, pH 7.2, 400 mM sucrose, and 1 mM EDTA) with 0.3 mM DTT and protease inhibitors. After centrifugation, supernatant was ultra-centrifuged at 100,000×g for 40 min to collect the total membrane pellet, followed by membrane flotation assay. The membrane fraction floating on the top was collected and divided into three parts (without proteinase K, with proteinase K, or with proteinase K and 0.5% Triton X-100). The reactions were performed on ice and stopped by adding PMSF and SDS loading buffer. The samples were immediately heated at 98°C for 5 min, followed by SDS-PAGE. Protein disulfide isomerase (PDI) and a vesicle trafficking protein, SEC22 Homolog B (SEC22B), were used as the positive and negative control,

749 respectively.

750

## 751 **H&E staining**

752 Mice were anaesthetized with isoflurane, and lung lobes were harvested at indicated

753 time points. Tissues were fixed with 10% PFA for more than 24 hours and embedded

754 in paraffin. The paraffin blocks were cut into 2 µm-thick sections and stained using a

755 standard Hematoxylin and eosin (H&E) procedure.

756

## 757 **Statistical Analysis**

758 Sample size was based on empirical data from pilot experiments. The investigators

759 were blinded during data collection and analysis. A value of  $P < 0.05$  was considered

760 significant.

761

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772

## 773 **Author contributions**

774 H. Wu, X. Lin and B. Fu conceived and designed the study. H. Wu, X. Lin, B. Fu, Y.

775 Xiong, N. Xing, W. Xue, D. Guo, M. Y. Zaky, K. C. Pavani, D. Kunec and J. Trimpert

776 performed the experiments. H. Wu, X. Lin and B. Fu analyzed the data. H. Wu, X. Lin,

777 B. Fu and J. Trimpert wrote the manuscript. All authors read and approved the final

778 manuscript.

779

## 780 **Conflict of Interest**

781 The authors declare that no conflict of interest exists.

782

783

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908

## 909 **Figure legends**

### 910 **Figure 1 SARS-CoV-2 ORF8 can be secreted by epithelial cells**

911 (A) Schematic diagram of SARS-CoV-2 infectious cDNA clone generated by a  
912 reverse genetic system. The cDNA fragments F1-F7 were synthesized and assembled  
913 into full-length SARS-CoV-2 cDNA, and RNA transcription, electroporation, and  
914 virus production were carried out in Vero E6 cells.

915 (B) Schematic diagram of SARS-CoV-2 infection model. Calu-3 epithelial cells were  
916 infected with SARS-CoV-2 for 12 hours at a dosage of  $10^5$  TCID<sub>50</sub>/mL. Cell culture  
917 supernatant was centrifuged and divided into two parts for western blotting and  
918 ELISA, respectively.

919 (C) The secretion of ORF8 in (B) was detected by western blotting and ELISA.

920 Representative images from n = 3 biological replicates are shown. Data in histogram

921 are shown as the mean  $\pm$  s.e.m. of  $n = 3$  biological replicates.

922 (D) Jurkat cells and Calu-3 epithelial cells were infected with HIV-1 and

923 SARS-CoV-2 for 12 hours at a dosage of  $10^5$  TCID<sub>50</sub>/mL. The secretion of ORF8, Nef

924 and 3CL pro was detected by western blotting. Representative images from  $n = 3$

925 biological replicates are shown.

926 (E) Schematic diagram of time-dependent ORF8 secretion upon SARS-CoV-2

927 infection. After 12 hours of SARS-CoV-2 infection, cell culture medium was replaced,

928 and the amount of ORF8 protein in the supernatant was detected by ELISA every 2

929 hours. Data are shown as the mean  $\pm$  s.e.m. of  $n = 3$  biological replicates.

930 (F-H) Schematic diagram of ORF8-deleted SARS-CoV-2 variant (F). ORF8 coding

931 sequence was deleted from the cDNA of F7 fragment. SARS-CoV-2  $\Delta$ ORF8 variant

932 was used to infect Calu-3 cells, THP-1 DM cells, and the co-culture system at a

933 dosage of  $10^5$  TCID<sub>50</sub>/mL. The secretion of cytokines and chemokines related to

934 cytokine storm was detected by ELISA (G, H). Data are shown as the mean  $\pm$  s.e.m.

935 of  $n = 3$  biological replicates (G, H).

936 (H) Calu-3 *Ace2*<sup>+/+</sup>, Calu-3 *Ace2*<sup>-/-</sup>, THP-1 DM *Il17ra*<sup>+/+</sup> and THP-1 DM *Il17ra*<sup>-/-</sup> cells

937 were used to form four kinds of culture systems. The secretion of cytokines and

938 chemokines in different cell culture systems was detected by ELISA. Data are shown

939 as the mean  $\pm$  s.e.m. of  $n = 3$  biological replicates.

940 Unpaired two-tailed Student *t* test (C) and one-way ANOVA followed by Bonferroni

941 post *hoc* test (G, I) were used for data analysis. \*,  $p < 0.05$ , \*\*,  $p < 0.01$ .

942



943 **Figure 2 SARS-CoV-2 ORF8, rather than SARS ORF8, has an unconventional**  
 944 **secretion pattern**

945 (A) Structures of SARS-CoV-2 ORF8 and its signal peptide-deleted mutant.

946 (B, C) Full-length SARS-CoV-2 ORF8 or its signal peptide-deleted mutant were  
 947 transfected into Calu-3 cells or HEK-293FT cells. The secretion of ORF8 was  
 948 detected by western blotting (B) and ELISA (C). Representative images from n = 3  
 949 biological replicates are shown (B). Data are shown as the mean  $\pm$  s.e.m. of n = 3  
 950 biological replicates (C).

951 (D, E) Brefeldin A (3  $\mu$ g/mL) or Monensin (2  $\mu$ M) was used to pretreat Calu-3 cells  
 952 (D) or HEK-293FT cells (E) for 2 hours. Full-length SARS-CoV-2 ORF8 was  
 953 transfected into pretreated cells. After 12 hours, the secretion of ORF8 was detected  
 954 by western blotting. Representative images from n = 3 biological replicates are  
 955 shown.

956 (F) Structures of SARS ORF8a and its signal peptide-deleted mutant.

957 (G) Full-length SARS ORF8a or its signal peptide-deleted mutant were transfected  
 958 into Calu-3 cells or HEK-293FT cells. The secretion of ORF8a was detected by  
 959 western blotting. Representative images from n = 3 biological replicates are shown.

960 (H, I) Brefeldin A (3  $\mu$ g/mL) or Monensin (2  $\mu$ M) was used to pretreat Calu-3 cells (H)  
 961 or HEK-293FT cells (I) for 2 hours. Full-length SARS ORF8a was transfected into  
 962 pretreated cells. After 12 hours, the secretion of ORF8a was detected by western  
 963 blotting. Representative images from n = 3 biological replicates are shown.

964 (J) Structures of SARS-CoV-2 ORF8 mutant with signal peptide from SARS ORF8a

(with SARS-Signal), and SARS ORF8a mutant with signal peptide from SARS-CoV-2 ORF8 (with SARS-CoV-2-Signal). (K) Brefeldin A (3  $\mu$ g/mL) or Monensin (2  $\mu$ M) was used to pretreat Calu-3 cells for 2 hours. SARS-CoV-2 ORF8 with SARS-Signal and SARS ORF8a with SARS-CoV-2-Signal were transfected into pretreated Calu-3 cells. After 12 hours, the secretion of ORF8 was detected by western blotting. Representative images from n = 3 biological replicates are shown. Unpaired two-tailed Student *t* test (C) was used for data analysis. \*,  $p < 0.05$ , \*\*,  $p < 0.01$ .

### Figure 3 **Unconventional secretion of ORF8 induces cytokine storm**

(A) Schematic diagram of unconventional secretion model upon SARS-CoV-2 infection. Brefeldin A (3  $\mu$ g/mL) or Monensin (2  $\mu$ M) was used to pretreat Calu-3 cells for 2 hours, followed by SARS-CoV-2 infection for 12 hours at a dosage of  $10^5$  TCID<sub>50</sub>/mL, or Flag-tagged ORF8 plasmid transfection for 12 hours. The supernatant was collected to stimulate THP-1 DM cells. (B) The secretion of ORF8 in (A) was detected by ELISA. Data are shown as the mean  $\pm$  s.e.m. of n = 3 biological replicates. (C, D) The release of cytokines and chemokines in (A) was detected by ELISA. Data are shown as the mean  $\pm$  s.e.m. of n = 3 biological replicates. (E) PNGase F (1,000 units/ $\mu$ g protein), O-Glycosidase (4,000 units/ $\mu$ g protein) or  $\alpha$ 2-3, 6, 8, 9 Neuraminidase A (4 units/ $\mu$ g glycoprotein) was added into the purified

987 SARS-CoV-2 ORF8 protein to release glycans. Western blotting was used to detect  
988 the glycosylation of ORF8 protein. Representative images from n = 3 biological  
989 replicates are shown.

990 (F, G) Calu-3 cells were infected with SARS-CoV-2 (F) or transfected with ORF-Flag  
991 plasmids (G). Tunicamycin (2µg/mL) was added into Calu-3 cells for 2 hours to  
992 prevent N-linked glycosylation; PNGase F (1,000 units/µg protein) was used to  
993 remove the N-linked glycosylation in purified ORF8 protein. After deglycosylation  
994 assays, the cell culture supernatant or purified ORF8 protein was used to stimulate  
995 THP-1 DM cells. After 12 hours, the release of cytokines and chemokines was  
996 detected by ELISA. Data are shown as the mean ± s.e.m. of n = 3 biological  
997 replicates.

998 One-way ANOVA followed by Bonferroni post *hoc* test (B-D, F, G) was used for data  
999 analysis. \*, p < 0.05, \*\*, p < 0.01. Abbreviations: n.s., not significant.

1000

#### 1001 **Figure 4 ORF8 N78 glycosylation blocks its interaction with IL17RA**

1002 (A) Schematic diagram of glycosylation identification based on HLPC-MS/MS.  
1003 DMSO (control), Brefeldin A (3 µg/mL) or Monensin (2 µM) was used to pretreat  
1004 Calu-3 cells for 2 hours. The supernatant was collected for HLPC-MS/MS analysis.

1005 (B) SARS-CoV-2 ORF8 secreted through conventional pattern has N78 glycosylation.  
1006 An increase of 0.002989 Da of Asn residue was used to determine N-linked  
1007 glycosylation.

1008 (C-E) Calu-3 cells were infected with SARS-CoV-2 variant carrying ORF8 N78Q

mutant (C, D), or transfected with ORF8 N78Q plasmids (C, E). After 12 hours, the supernatant was collected and divided into two parts. One part was used to purify ORF8 protein, followed by PNGase F digestion and western blotting (C); the other part was used to stimulate THP-1 DM cells for 12 hours, followed by detection of cytokines and chemokines by ELISA (D, E). Representative images from n = 3 biological replicates are shown (C). Data are shown as the mean  $\pm$  s.e.m. of n = 3 biological replicates (D, E).

(F) Calu-3 cells were infected with SARS-CoV-2 ORF8-N78Q variant, or transfected with ORF8-N78Q plasmids. Twelve hours later, the supernatant was used to stimulate THP-1 DM cells for another 12 hours. The interaction of ORF8 and IL17RA was detected by co-immunoprecipitation. Representative images from n = 3 biological replicates are shown.

(G, H) Calu-3 cells were infected with SARS-CoV-2 ORF8-N78Q variant (G), or transfected with ORF8-N78Q plasmids (H). After 12 hours, the supernatant was collected to purify ORF8 protein. After PNGase F digestion, the ORF8 protein was used to stimulate THP-1 DM cells. After 12 hours, the interaction of ORF8 and IL17RA was detected by co-immunoprecipitation. Representative images from n = 3 biological replicates are shown.

(I, J) Brefeldin A (3  $\mu$ g/mL) or Monensin (2  $\mu$ M) was used to pretreat Calu-3 cells for 2 hours, followed by infection with SARS-CoV-2 ORF8-N78Q variant (I), or transfection with ORF8-N78Q plasmids (J). Twelve hours later, the supernatant was used to stimulate THP-1 DM cells for another 12 hours. The interaction of ORF8 and

1031 IL17RA was detected by co-immunoprecipitation. Representative images from n = 3

1032 biological replicates are shown.

1033 One-way ANOVA followed by Bonferroni post *hoc* test (D, E) was used for data

1034 analysis. \*,  $p < 0.05$ , \*\*,  $p < 0.01$ .

1035

1036 **Figure 5 N-linked glycosylation of ORF8 protects mice from cytokine storm**

1037 (A, B) Synthetic N-linked-glycosylated ORF8 (ORF8-N-Glyc) (1  $\mu\text{g/mL}$ ) was added

1038 into the cell culture medium of THP-1 DM cells to stimulate IL-17 pathway. The

1039 interaction of ORF8 and IL17RA was detected by co-immunoprecipitation (A). The

1040 release of cytokines and chemokines was detected by ELISA (B). Representative

1041 images from n = 3 biological replicates are shown (A). Data are shown as the mean  $\pm$

1042 s.e.m. of n = 3 biological replicates (B).

1043 (C) Survival of K18-hACE2 mice infected with PBS (n = 11), unglycosylated ORF8

1044 (n = 18) or synthetic N-linked-glycosylated ORF8 proteins (n = 17) (200  $\mu\text{g}/\text{mouse}$ ).

1045 Data are shown as Kaplan-Meier curves.

1046 (D) Lung lesions of K18-hACE2 mice infected with unglycosylated ORF8 or

1047 synthetic N-linked-glycosylated ORF8 proteins (200  $\mu\text{g}/\text{mouse}$ ) were detected by

1048 H&E staining at day 7 post infection (dpi). Representative images from n = 6

1049 biological replicates are shown. Scale bar = 500  $\mu\text{m}$ .

1050 (E, F) The release of cytokines and chemokines in lungs (E) and livers (F), which

1051 were obtained from K18-hACE2 mice intranasally infected with unglycosylated

1052 ORF8 or synthetic N-linked-glycosylated ORF8 proteins (200  $\mu\text{g}/\text{mouse}$ ), was

1053 detected by ELISA at 7 dpi. Data are shown as the mean  $\pm$  s.e.m. of n = 6 biological  
1054 replicates.

1055 Log-rank (Mantel-Cox) test (C) and one-way ANOVA followed by Bonferroni post  
1056 *hoc* test (B, E, F) were used for data analysis. \*,  $p < 0.05$ , \*\*,  $p < 0.01$ .

1057

# 1058 **Figure 6 YIF1B interacts with unconventionally secreted ORF8**

1059 (A) Flag-tagged ORF8 was transfected into HEK-293FT cells. After 12 hours, the  
1060 interaction of ORF8 and endogenic YIF1B was detected by co-immunoprecipitation.  
1061 Representative images from n = 3 biological replicates are shown.

1062 (B, C) Flag-tagged ORF8 and HA-tagged YIF1B were co-transfected into  
1063 HEK-293FT cells. After 12 hours, the interaction of ORF8 and YIF1B was detected  
1064 by co-immunoprecipitation (B) and Duolink PLA assay (C). Representative images  
1065 from n = 3 biological replicates are shown.

1066 (D) Calu-3 cells were infected with SARS-CoV-2 for 12 hours at a dosage of  $10^5$   
1067 TCID<sub>50</sub>/mL. The interaction of ORF8 and YIF1B was detected by  
1068 co-immunoprecipitation. Representative images from n = 3 biological replicates are  
1069 shown. Scale bar = 10  $\mu$ m.

1070 (E, F) Wild-type and YIF1B-KO cells were transfected with HA-tagged YIF1B,  
1071 followed by infection with SARS-CoV-2 for 12 hours at a dosage of  $10^5$  TCID<sub>50</sub>/mL.  
1072 The secretion of ORF8 was detected by western blotting (E) and ELISA (F). Brefeldin  
1073 A (3  $\mu$ g/mL) was used to block conventional secretion of ORF8. Representative  
1074 images from n = 3 biological replicates are shown (E). Data are shown as the mean  $\pm$

1075 s.e.m. of  $n = 3$  biological replicates (F).

1076 (G) Wild-type and YIF1B-KO cells were transfected with HA-tagged YIF1B, and

1077 were infected with SARS-CoV-2 for 12 hours at a dosage of  $10^5$  TCID<sub>50</sub>/mL. The cell

1078 culture supernatant was collected to stimulate THP-1 DM cells for 12 hours. The

1079 release of cytokines and chemokines was detected by ELISA. Data are shown as the

1080 mean  $\pm$  s.e.m. of  $n = 3$  biological replicates.

1081 (H, I) Wild-type and YIF1B-KO cells were co-transfected with Flag-tagged ORF8 and

1082 HA-tagged YIF1B. After 12 hours, the secretion of ORF8 was detected by western

1083 blotting (H) and ELISA (I). Brefeldin A ( $3 \mu\text{g/mL}$ ) was used to block conventional

1084 secretion of ORF8. Representative images from  $n = 3$  biological replicates are shown

1085 (H). Data are shown as the mean  $\pm$  s.e.m. of  $n = 3$  biological replicates (I).

1086 (J) Wild-type and YIF1B-KO cells were co-transfected with Flag-tagged ORF8 and

1087 HA-tagged YIF1B. Twelve hours later, the cell culture supernatant was collected to

1088 stimulate THP-1 DM cells for another 12 hours. The release of cytokines and

1089 chemokines was detected by ELISA. Data are shown as the mean  $\pm$  s.e.m. of  $n = 3$

1090 biological replicates.

1091 Unpaired two-tailed Student  $t$  test (F, I) and one-way ANOVA followed by Bonferroni

1092 post *hoc* test (G, J) were used for data analysis. \*,  $p < 0.05$ , \*\*,  $p < 0.01$ .

1093

1094

1095 **Figure 7  $\beta$ 8 sheet of ORF8 and  $\alpha$ 4 helix of YIF1B are essential for the interaction**

1096 (A) Structure of  $\beta$ -pleated sheet in SARS-CoV-2 ORF8 protein.

1097 (B) ORF8  $\beta$  sheet mutants ( $\Delta\beta 1$ - $\Delta\beta 8$ ) were transfected into Calu-3 cells. After 12  
1098 hours, the interaction of ORF8 and YIF1B was detected by co-immunoprecipitation.  
1099 Representative images from  $n = 3$  biological replicates are shown.

1100 (C, D) ORF8  $\beta$  sheet mutants ( $\Delta\beta 1$ - $\Delta\beta 8$ ) were transfected into Calu-3 cells. After 12  
1101 hours, the secretion of ORF8 was detected by western blotting (C) and ELISA (D).  
1102 Brefeldin A ( $3 \mu\text{g/mL}$ ) was used to block conventional secretion of ORF8.  
1103 Representative images from  $n = 3$  biological replicates are shown (C). Data are shown  
1104 as the mean  $\pm$  s.e.m. of  $n = 3$  biological replicates (D).

1105 (E) ORF8  $\Delta\beta 8$  mutant was transfected into Calu-3 cells. Twelve hours later, the cell  
1106 culture supernatant was collected to stimulate THP-1 DM cells for another 12 hours.  
1107 The release of cytokines and chemokines was detected by ELISA. Brefeldin A ( $3$   
1108  $\mu\text{g/mL}$ ) was used to block conventional secretion of ORF8. Data are shown as the  
1109 mean  $\pm$  s.e.m. of  $n = 3$  biological replicates.

1110 (F) Structure of  $\alpha$  helix and corresponding deletion mutants of YIF1B protein.

1111 (G) YIF1B  $\alpha$  helix mutants ( $\Delta\alpha 1$ - $\Delta\alpha 5$ ) were transfected into Calu-3 cells. After 12  
1112 hours, the interaction of ORF8 and YIF1B was detected by co-immunoprecipitation.  
1113 Representative images from  $n = 3$  biological replicates are shown.

1114 (H, I) YIF1B  $\alpha$ -helix mutants ( $\Delta\alpha 1$ - $\Delta\alpha 5$ ) were transfected into wild-type Calu-3 cells.  
1115 After 12 hours, the secretion of ORF8 was detected by western blotting (H) and  
1116 ELISA (I). Brefeldin A ( $3 \mu\text{g/mL}$ ) was used to block conventional secretion of ORF8.  
1117 Representative images from  $n = 3$  biological replicates are shown (H). Data are shown  
1118 as the mean  $\pm$  s.e.m. of  $n = 3$  biological replicates (I).



(J) YIF1B  $\Delta\alpha4$  mutant was transfected into wild-type Calu-3 cells. Twelve hours later, the cell culture supernatant was collected to stimulate THP-1 DM cells for another 12 hours. The release of cytokines and chemokines was detected by ELISA. Brefeldin A (3  $\mu\text{g/mL}$ ) was used to block conventional secretion of ORF8. Data are shown as the mean  $\pm$  s.e.m. of  $n = 3$  biological replicates.

(K, L) YIF1B  $\alpha$ -helix mutants ( $\Delta\alpha1$ - $\Delta\alpha5$ ) were transfected into YIF1B-KO Calu-3 cells. After 12 hours, the secretion of ORF8 was detected by western blotting (K) and ELISA (L). Brefeldin A (3  $\mu\text{g/mL}$ ) was used to block conventional secretion of ORF8. Representative images from  $n = 3$  biological replicates are shown (K). Data are shown as the mean  $\pm$  s.e.m. of  $n = 3$  biological replicates (L).

(M) YIF1B  $\Delta\alpha4$  mutant was transfected into YIF1B-KO Calu-3 cells. The cell culture supernatant was collected to stimulate THP-1 DM cells for 12 hours. The release of cytokines and chemokines was detected by ELISA. Brefeldin A (3  $\mu\text{g/mL}$ ) was used to block conventional secretion of ORF8. Data are shown as the mean  $\pm$  s.e.m. of  $n = 3$  biological replicates.

(N, O) HA-tagged YIF1B, V5-tagged 3CL pro and V5-tagged 3CL pro containing  $\beta8$  sheet were transfected into YIF1B-KO Calu-3 cells. After 12 hours later, the interaction of YIF1B and 3CL pro was detected by co-immunoprecipitation (N). The secretion of 3CL pro was detected by western blotting (O). Representative images from  $n = 3$  biological replicates are shown.

One-way ANOVA followed by Bonferroni post *hoc* test (E, I, J, L, M) was used for data analysis. \*,  $p < 0.05$ , \*\*,  $p < 0.01$ . Abbreviations: n.s., not significant.

1141

1142

1143 **Figure 8 YIF1B promotes the transport of ORF8**

1144 (A) Calu-3 cells were infected with SARS-CoV-2, or transfected with ORF8-Flag.

1145 After 12 hours, the co-localization of ORF8 and YIF1B was observed by

1146 immunofluorescence. Representative images from n = 3 biological replicates are

1147 shown. Scale bar = 10  $\mu$ m.

1148 (B) Calu-3 cells were transfected with ORF8- $\Delta\beta 8$  mutant. After 12 hours, the

1149 co-localization of ORF8 and YIF1B was observed by immunofluorescence.

1150 Representative images from n = 3 biological replicates are shown. Scale bar = 10  $\mu$ m.

1151 (C) Calu-3 cells were infected with SARS-CoV-2, or transfected with ORF8-Flag.

1152 YIF1B- $\Delta\alpha 4$  mutant was also transfected into Calu-3 cells. After 12 hours, the

1153 co-localization of ORF8 and YIF1B was observed by immunofluorescence.

1154 Representative images from n = 3 biological replicates are shown. Scale bar = 10  $\mu$ m.

1155 (D) ORF8 and YIF1B  $\alpha$ -helix mutants ( $\Delta\alpha 1$ - $\Delta\alpha 5$ ) were transfected into YIF1B-KO

1156 Calu-3 cells. Whole cell lysates were collected for Proteinase K protection assay. PDI

1157 and SEC22B were used as positive control and negative control, respectively.

1158 Representative images from n = 3 biological replicates are shown.

1159 (E, F) Schematic diagram of *in vitro* translocation assay (E). Different doses of YIF1B

1160 protein and lipids were assembled to proteoliposomes. ORF8 protein and synthetic

1161 proteoliposomes were mixed in the lysate of HEK-293FT cells (without

1162 endomembrane). Proteoliposomes containing ORF8 were collected and aliquoted into

1163 three parts for Proteinase K protection assay (F). Representative images from n = 3  
1164 biological replicates are shown (F).  
1165 (G) Schematic diagram of GFP fluorescence complementation system. ORF8 fused  
1166 with GFP (11) and YIF1B containing GFP (1-10), were co-transfected into  
1167 HEK-293FT cells, and flow cytometry was used to measure GFP fluorescence  
1168 intensity.  
1169 (H, I) Different doses of ORF8-GFP(11) were co-transfected with YIF1B-GFP(1-10)  
1170 (H), or different doses of YIF1B-GFP(1-10) (I) were co-transfected with  
1171 ORF8-GFP(11) into HEK-293FT cells, and flow cytometry was used to measure GFP  
1172 fluorescence intensity. Representative images from n = 3 biological replicates are  
1173 shown. Data are shown as the mean  $\pm$  s.e.m. of n = 3 biological replicates.  
1174 One-way ANOVA followed by Bonferroni post *hoc* test (H, I) was used for data  
1175 analysis. \*, p < 0.05. Abbreviations: n.s., not significant.

1176

1177

# 1178 Figure 9 **YIF1B-deficient mice resist to SARS-CoV-2-induced cytokine storm**

1179 (A) AECs obtained from *Yif1b*<sup>-/-</sup> mice and their littermates (*Yif1b*<sup>+/+</sup>) were infected  
1180 with SARS-CoV-2 for 12 hours at a dosage of 10<sup>5</sup> TCID<sub>50</sub>/mL. The interaction of  
1181 ORF8 and YIF1B was detected by co-immunoprecipitation (A). HA-tagged YIF1B  
1182 was transfected into cells to restore YIF1B expression. The secretion of ORF8 was  
1183 detected by ELISA (B). Representative images from n = 3 biological replicates are  
1184 shown (A). Data are shown as the mean  $\pm$  s.e.m. of n = 3 biological replicates (B).

1185 (C) AECs obtained from *Yiflb*<sup>-/-</sup> mice and their littermates (*Yiflb*<sup>+/+</sup>) were infected  
1186 with SARS-CoV-2, or transfected with ORF8-Flag plasmids. After 12 hours, the  
1187 supernatant was collected to purify ORF8 protein. After PNGase F digestion, ORF8  
1188 protein was detected by western blotting. Representative images from n = 3 biological  
1189 replicates are shown.

1190 (D, E) AECs obtained from *Yiflb*<sup>-/-</sup> mice and their littermates (*Yiflb*<sup>+/+</sup>) were infected  
1191 with SARS-CoV-2 at a dosage of 10<sup>5</sup> TCID<sub>50</sub>/mL. After 12 hours, the supernatant was  
1192 collected to purify ORF8 protein, which was used to stimulate THP-1 DM cells for  
1193 another 12 hours. The interaction of ORF8 and IL17RA was detected by  
1194 co-immunoprecipitation (D). The activation of IL-17 pathway was evaluated by  
1195 testing NF-κB activity (E). Representative images from n = 3 biological replicates are  
1196 shown (D). Data are shown as the mean ± s.e.m. of n = 3 biological replicates (E).

1197 (F) Survival of *Yiflb*<sup>-/-</sup> mice (n = 16) and their littermates (*Yiflb*<sup>+/+</sup>, n = 14) after  
1198 intranasal infection with SARS-CoV-2 with 4×10<sup>5</sup> PFU. Data are shown as  
1199 Kaplan-Meier curves.

1200 (G) Lung lesions of *Yiflb*<sup>-/-</sup> mice and their littermates (*Yiflb*<sup>+/+</sup>) were detected by H&E  
1201 staining at 7 dpi. Representative images from n = 6 biological replicates are shown.  
1202 Scale bar = 500 μm.

1203 (H) Viral loads in lungs obtained from *Yiflb*<sup>-/-</sup> mice and their littermates (*Yiflb*<sup>+/+</sup>) at 7  
1204 dpi. Data are shown as the mean ± s.e.m. of n = 6 biological replicates.

1205 (I, J) The secretion of cytokines and chemokines in lungs (I) and livers (J) obtained  
1206 from *Yiflb*<sup>-/-</sup> mice and their littermates (*Yiflb*<sup>+/+</sup>) at 7 dpi, was detected by ELISA.

1207 Data are shown as the mean  $\pm$  s.e.m. of  $n = 6$  biological replicates.

1208 Two-way ANOVA followed by Bonferroni post *hoc* test (B, E, I, J), Log-rank

1209 (Mantel-Cox) test (F) and unpaired two-tailed Student *t* test (H) were used for data

1210 analysis. \*,  $p < 0.05$ , \*\*,  $p < 0.01$ . Abbreviations: n.s., not significant.

1211

1212 **Figure 10 Schematic representation of conventional and unconventional secretion**  
1213 **of ORF8 during SARS-CoV-2 infection**

1214

1215 **Figure S1 SARS-CoV-2 ORF8 can be secreted without the presence of complete**  
1216 **virus**

1217 (A) Validation of Calu-3 *Ace2*<sup>+/+</sup>, Calu-3 *Ace2*<sup>-/-</sup>, THP-1 DM *Il17ra*<sup>+/+</sup> and THP-1 DM  
1218 *Il17ra*<sup>-/-</sup> cells. The expressions of ACE2 and IL17RA were detected by western  
1219 blotting. Representative images from  $n = 3$  biological replicates are shown.

1220 (B) Schematic diagram of THP-1 DM cells stimulation model. HEK-293FT cells were  
1221 transfected with Flag-tagged ORF8, 3CL pro, or Nef. After 12 hours, the supernatant  
1222 was collected and divided into two parts. One part was used to purify secretory  
1223 proteins, followed by western blotting; the other part was used to stimulate THP-1  
1224 DM cells for 12 hours. The release of cytokines and chemokines was detected by  
1225 ELISA.

1226 (C) Secretory proteins obtained from HEK-293FT cell supernatant in (B) were  
1227 detected by western blotting. Representative images from  $n = 3$  biological replicates  
1228 are shown.

1229 (D) The release of cytokines and chemokines from THP-1 DM cells in (B) was  
1230 detected by ELISA. Data are shown as the mean  $\pm$  s.e.m. of n = 3 biological  
1231 replicates.  
1232 One-way ANOVA followed by Bonferroni post *hoc* test (D) was used for data analysis.  
1233 \*, p < 0.05, \*\*, p < 0.01.

1234

# 1235 Figure S2 **SARS ORF8a cannot be glycosylated**

1236 (A, B) Brefeldin A (3  $\mu$ g/mL) or Monensin (2  $\mu$ M) was used to pretreat Calu-3 cells  
1237 for 2 hours, followed by SARS-CoV-2 infection for 12 hours at a dosage of  $10^5$   
1238 TCID<sub>50</sub>/mL (A), or Flag-tagged ORF8 plasmid transfection for 12 hours (B). The  
1239 supernatant was collected to stimulate THP-1 DM cells within the specified time. The  
1240 release of cytokines and chemokines was detected by ELISA. Data are shown as the  
1241 mean  $\pm$  s.e.m. of n = 3 biological replicates.

1242 (C) PNGase F (1,000 units/ $\mu$ g protein), O-Glycosidase (4,000 units/ $\mu$ g protein) or  
1243  $\alpha$ 2-3, 6, 8, 9 Neuraminidase A (4 units/ $\mu$ g glycoprotein) was added into purified  
1244 SARS ORF8a protein to release glycans. Western blotting was used to detect the  
1245 glycosylation of ORF8a protein. Representative images from n = 3 biological  
1246 replicates are shown.

1247 (D) Calu-3 cells were transfected with SARS ORF8a-Flag plasmids. Tunicamycin (2  
1248  $\mu$ g/mL) was added into Calu-3 cells for 2 hours to prevent N-linked glycosylation, or  
1249 PNGase F (1,000 units/ $\mu$ g protein) was used to remove the N-linked glycosylation in  
1250 purified ORF8 protein. After deglycosylation assays, the cell culture supernatant or

1251 purified ORF8a protein was used to stimulate THP-1 DM cells. After 12 hours, the  
1252 release of cytokines and chemokines was detected by ELISA. Data are shown as the  
1253 mean  $\pm$  s.e.m. of n = 3 biological replicates.

1254 Two-way (A, B) or one-way (D) ANOVA followed by Bonferroni post *hoc* test was  
1255 used for data analysis. Abbreviations: n.s., not significant.

1256

# 1257 Figure S3 **ORF8 N78 glycosylation blocks the activation of IL-17 pathway**

1258 (A) Calu-3 cells were infected with SARS-CoV-2 ORF8-N78Q variant, or transfected  
1259 with ORF8-N78Q plasmids. Twelve hours later, the supernatant was used to stimulate  
1260 THP-1 DM cells for another 12 hours. The activation of IL-17 pathway was evaluated  
1261 by testing NF- $\kappa$ B activity. Data are shown as the mean  $\pm$  s.e.m. of n = 3 biological  
1262 replicates.

1263 (B, C) Calu-3 cells were infected with SARS-CoV-2 ORF8-N78Q variant (B), or  
1264 transfected with ORF8-N78Q plasmids (C). The supernatant was collected to purify  
1265 ORF8 protein. After PNGase F digestion, the ORF8 protein was used to stimulate  
1266 THP-1 DM cells. After 12 hours, the activation of IL-17 pathway was evaluated by  
1267 testing NF- $\kappa$ B activity. Data are shown as the mean  $\pm$  s.e.m. of n = 3 biological  
1268 replicates.

1269 (D, E) Brefeldin A (3  $\mu$ g/mL) or Monensin (2  $\mu$ M) was used to pretreat Calu-3 cells  
1270 for 2 hours, followed by infection with SARS-CoV-2 ORF8-N78Q variant (D) or  
1271 transfection with ORF8-N78Q plasmids (E) for 12 hours. The supernatant was used to  
1272 stimulate THP-1 DM cells for 12 hours. The secretion of ORF8 was detected by

1273 ELISA. Data are shown as the mean  $\pm$  s.e.m. of  $n = 3$  biological replicates.  
 1274 (F, G) The activation of IL-17 pathway in (D, E) was evaluated by testing NF- $\kappa$ B  
 1275 activity. Data are shown as the mean  $\pm$  s.e.m. of  $n = 3$  biological replicates.  
 1276 Two-way ANOVA followed by Bonferroni post *hoc* test was used for data analysis. \*,  
 1277  $p < 0.05$ , \*\*,  $p < 0.01$ . Abbreviations: n.s., not significant.

1278

1279 **Figure S4 SARS-CoV-2 ORF8 can be secreted through an unconventional**  
 1280 **pathway**

1281 (A) Calu-3 cells were infected with SARS-CoV-2 for 12 hours at a dosage of  $10^5$   
 1282 TCID<sub>50</sub>/mL. Starvation assay (12 hours) or Rapamycin (50 nM) was used to induce  
 1283 autophagy. The secretion of ORF8 was detected by western blotting. Brefeldin A (3  
 1284  $\mu$ g/mL) was used to block conventional secretion of ORF8. Representative images  
 1285 from  $n = 3$  biological replicates are shown.

1286 (B) Calu-3 cells were infected with SARS-CoV-2 for 12 hours at a dosage of  $10^5$   
 1287 TCID<sub>50</sub>/mL. 3-MA (10 mM) or Wtm (20 nM) was used to inhibit the autophagy  
 1288 induced by starvation. The secretion of ORF8 was detected by western blotting.  
 1289 Brefeldin A (3  $\mu$ g/mL) was used to block conventional secretion of ORF8.  
 1290 Representative images from  $n = 3$  biological replicates are shown.

1291 (C) Calu-3 cells were infected with SARS-CoV-2 for 12 hours at a dosage of  $10^5$   
 1292 TCID<sub>50</sub>/mL. siRNA of *Atg5*, *Atg2a*, or *Atg2b* was transfected into cells to inhibit the  
 1293 autophagy induced by starvation. The secretion of ORF8 was detected by western  
 1294 blotting. Brefeldin A (3  $\mu$ g/mL) was used to block conventional secretion of ORF8.



1295 Representative images from n = 3 biological replicates are shown.

1296 (D) Calu-3 cells were infected with SARS-CoV-2, or transfected with ORF8-Flag.

1297 After 12 hours, the co-localization of ORF8 and ERGIC-53 was observed by

1298 immunofluorescence. Brefeldin A (3 µg/mL) was used to block conventional secretion

1299 of ORF8. Representative images from n = 3 biological replicates are shown. Scale bar

1300 = 10 µm.

1301 (E) Calu-3 cells were infected with SARS-CoV-2 for 12 hours at a dosage of 10<sup>5</sup>

1302 TCID<sub>50</sub>/mL. Whole cell lysates were collected for Proteinase K protection assay. PDI

1303 and SEC22B were used as positive control and negative control, respectively.

1304 Brefeldin A (3 µg/mL) was used to block conventional secretion of ORF8.

1305 Representative images from n = 3 biological replicates are shown.

1306 (F) Schematic diagram of screening channel proteins secreted by ORF8 through an

1307 unconventional pattern. Brefeldin A (3 µg/mL) or Monensin (2 µM) was used to

1308 pretreat HEK-293FT cells for 2 hours, followed by transfection with

1309 DHFR/Flag-tagged ORF8 for 12 hours. Aminopterin was used to inhibit DHFR

1310 unfolding. Whole cell lysates were collected for HPLC-MS/MS analysis.

1311 Transmembrane proteins interacting with ORF8 both in Monensin and Brefeldin A

1312 treatment groups were considered as candidates.

1313 (G) Calu-3 cells were infected with SARS-CoV-2 for 12 hours at a dosage of 10<sup>5</sup>

1314 TCID<sub>50</sub>/mL. siRNAs of potential channel proteins were transfected into cells, and the

1315 secretion of ORF8 was detected by western blotting. Representative images from n =

1316 3 biological replicates are shown.

1317

1318 **Figure S5 Validation of *Yif1b*<sup>-/-</sup> mice**

1319 (A) Schematic diagram of knockout strategy in generation of *Yif1b*<sup>-/-</sup> hACE2 mice

1320 using CRISPR-Cas9. Two sgRNAs were designed to delete 3-5 exons of *Yif1b*.

1321 (B) PCR validation of *Yif1b*<sup>-/-</sup> mice genotype. Representative images from n = 3

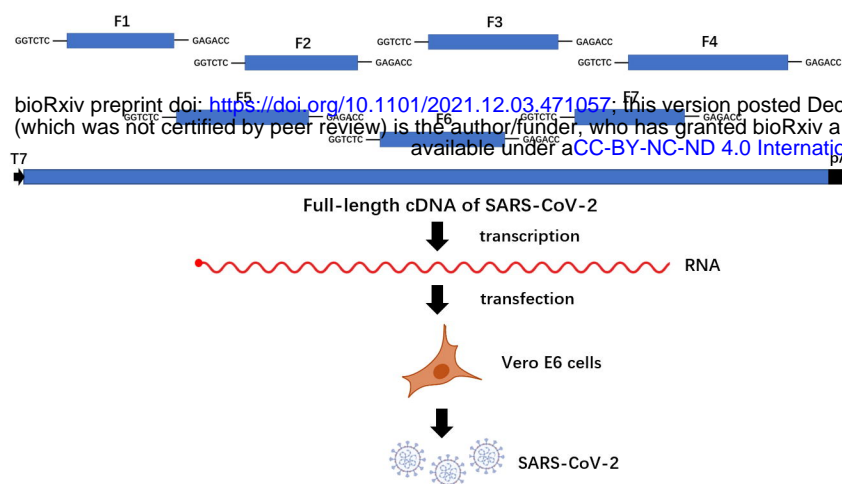
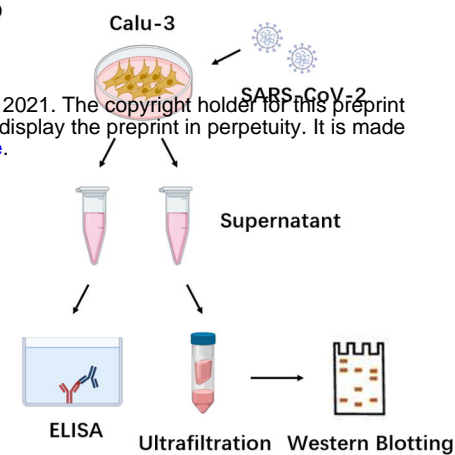
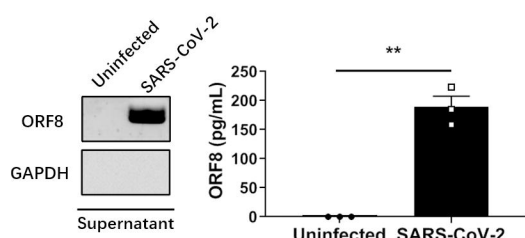
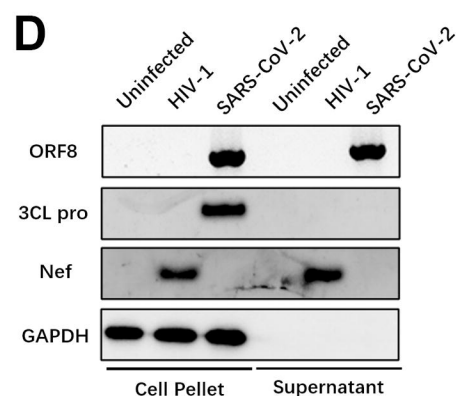
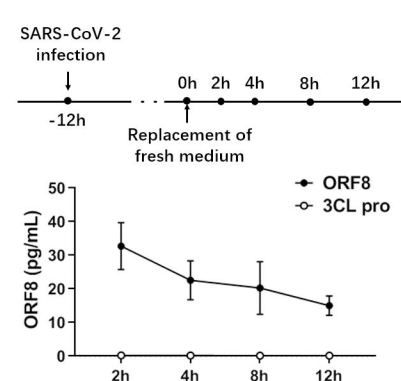
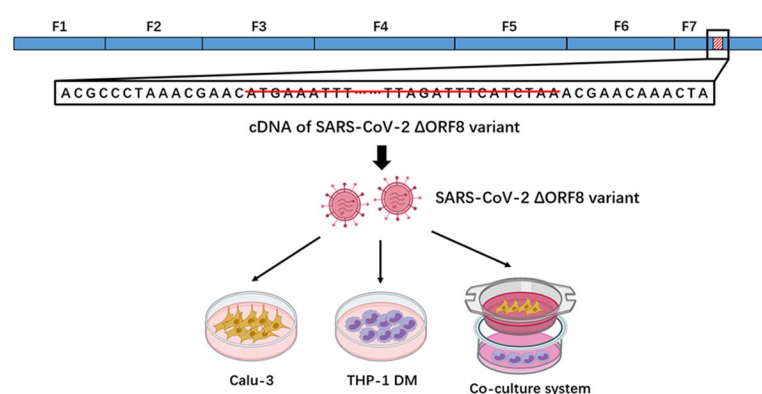
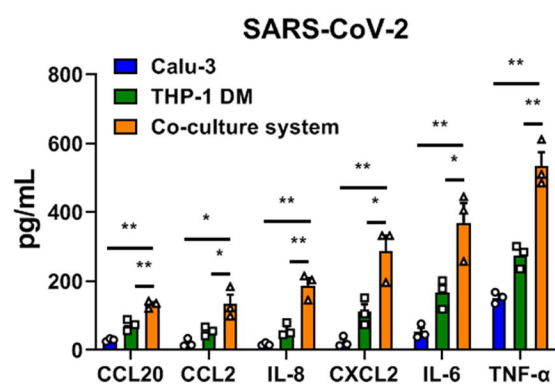
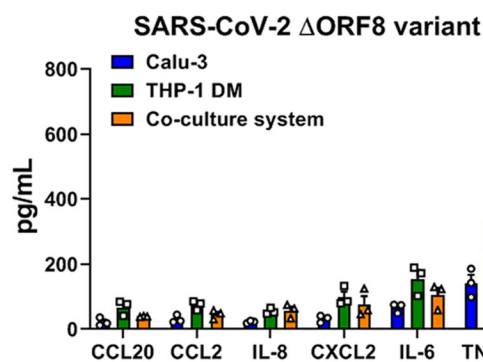
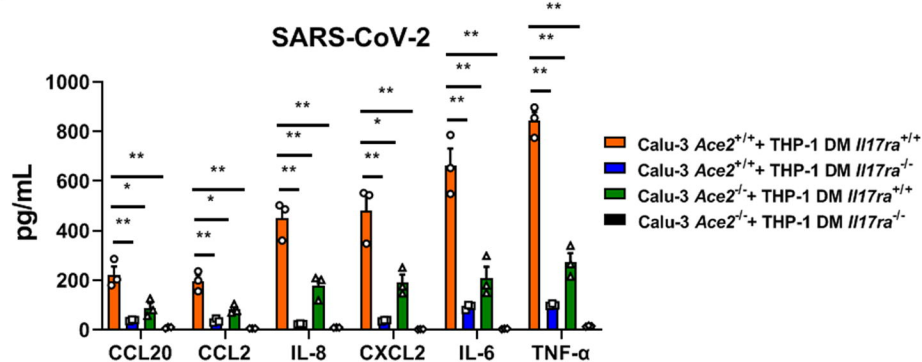
1322 biological replicates are shown.

1323 (C) The expressions of YIF1B in lungs obtained from *Yif1b*<sup>+/+</sup> and *Yif1b*<sup>-/-</sup> mice were

1324 detected by western blotting. Representative images from n = 3 biological replicates

1325 are shown.

1326

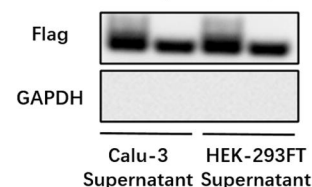
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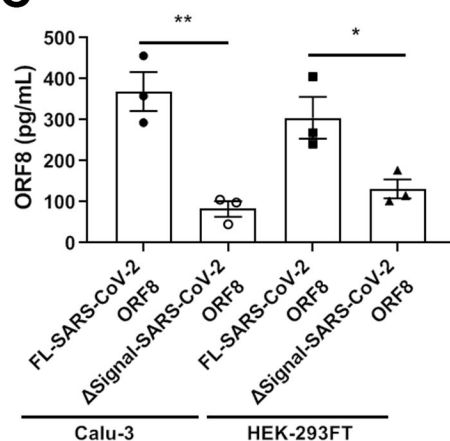
bioRxiv preprint doi: <https://doi.org/10.1101/2021.12.03.471057>; this version posted December 28, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

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 ΔSignal-SARS-CoV-2 ORF8 : ..... Flag

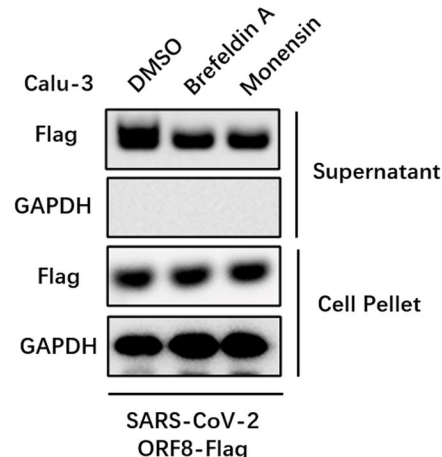
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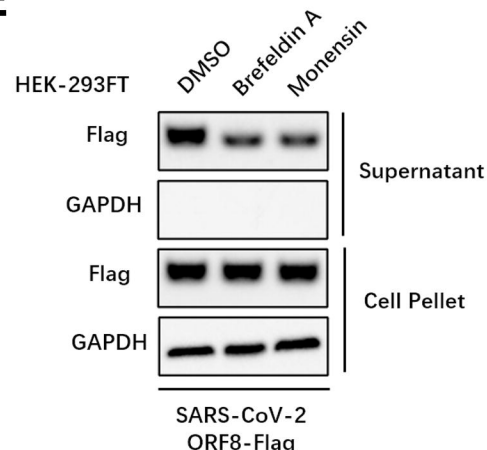
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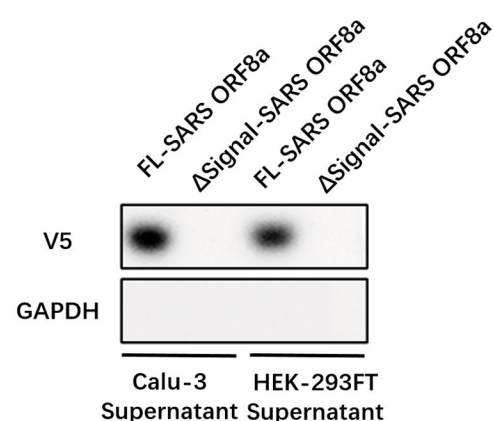
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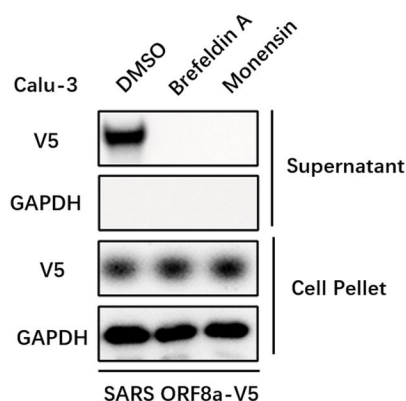
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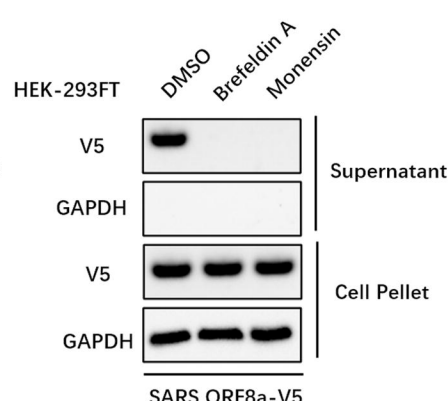
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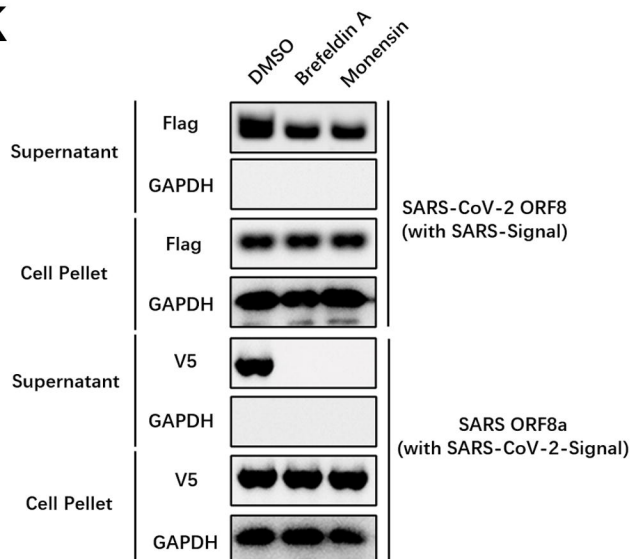
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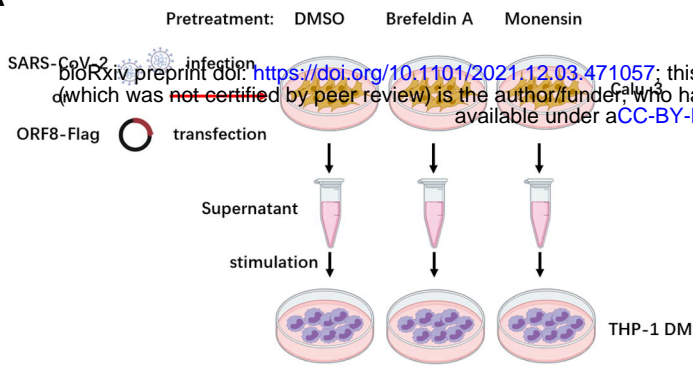
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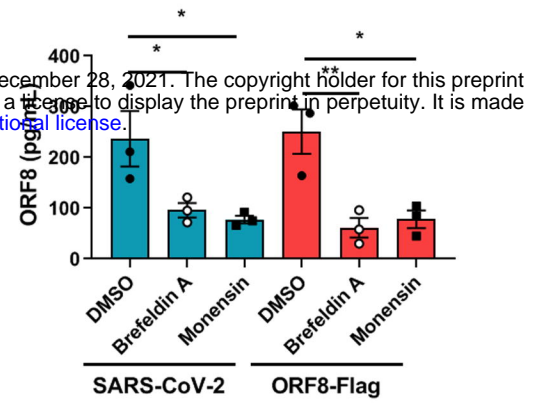
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SARS-CoV-2 ORF8 (with SARS-Signal) : M K L L I V L T C I S L C S C I C ..... Flag  
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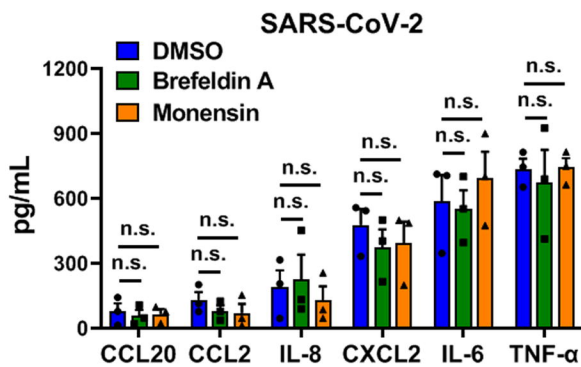
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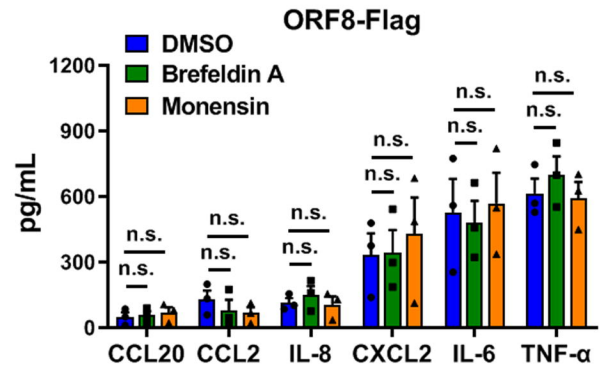
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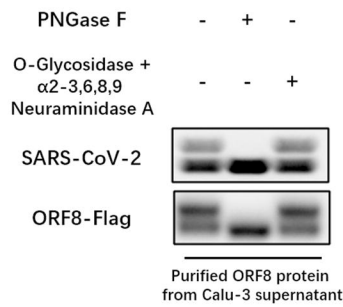
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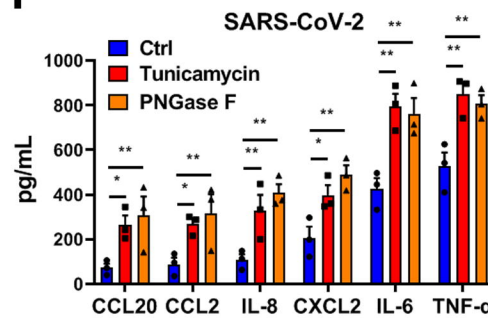
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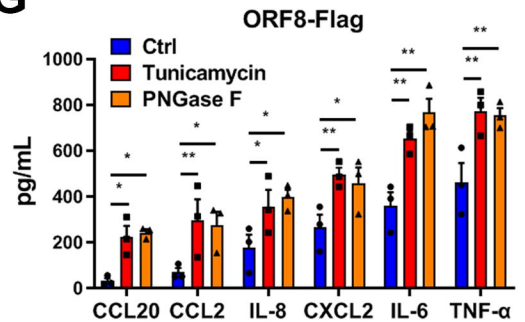
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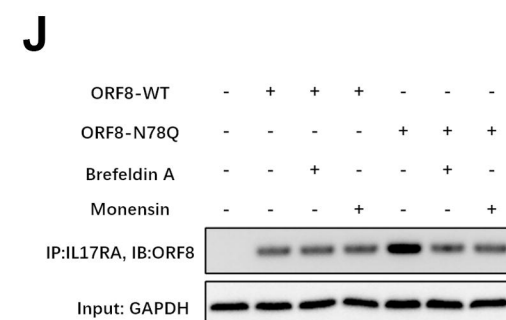
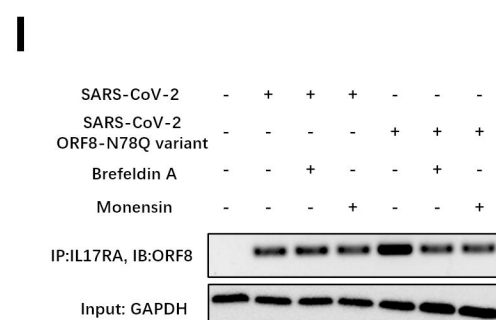
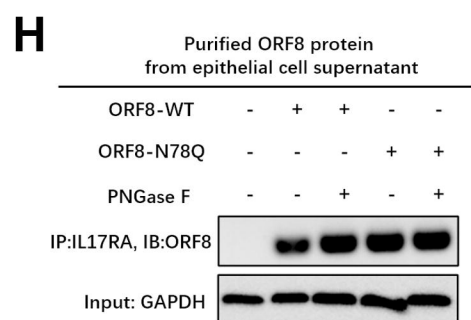
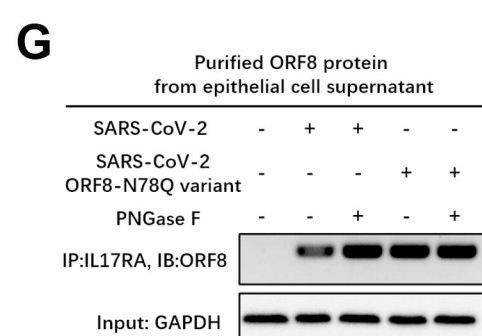
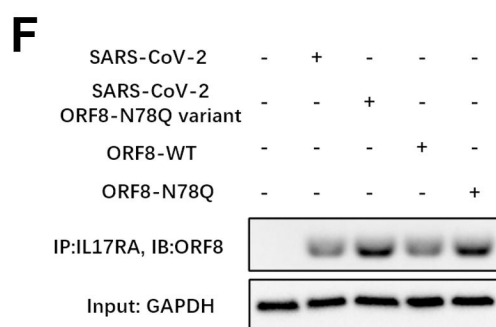
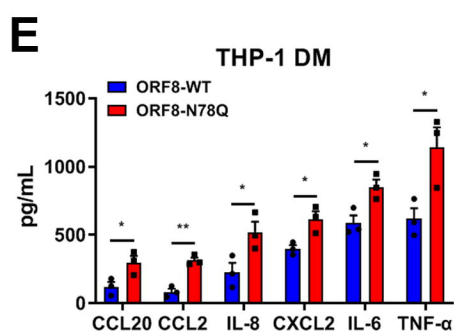
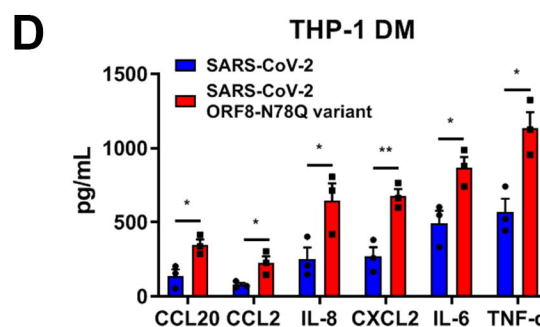
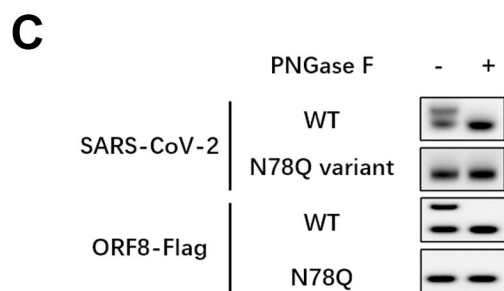
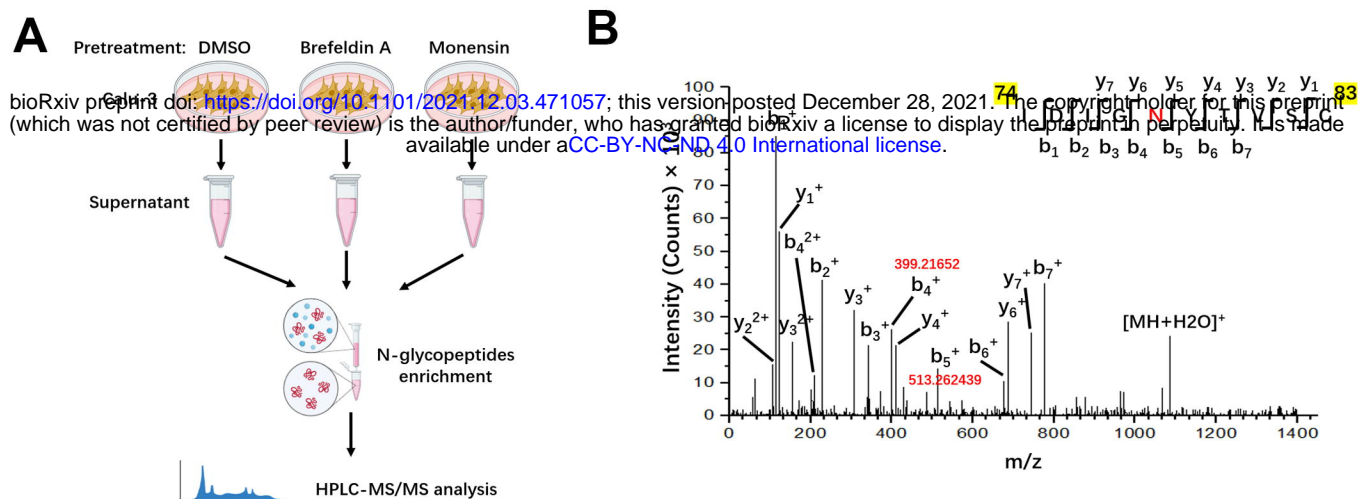


**F**



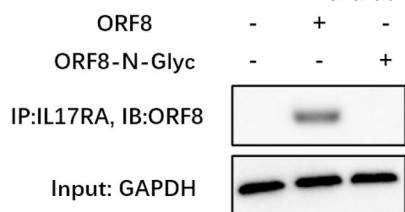
**G**



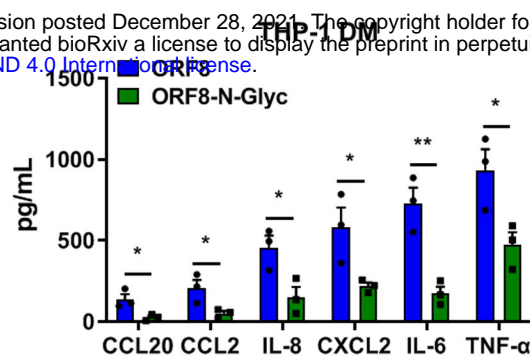




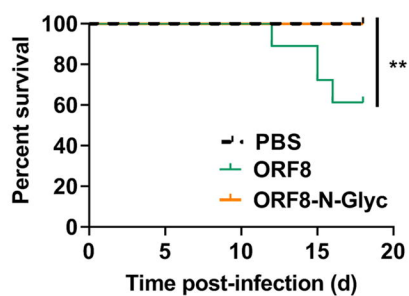
**A**



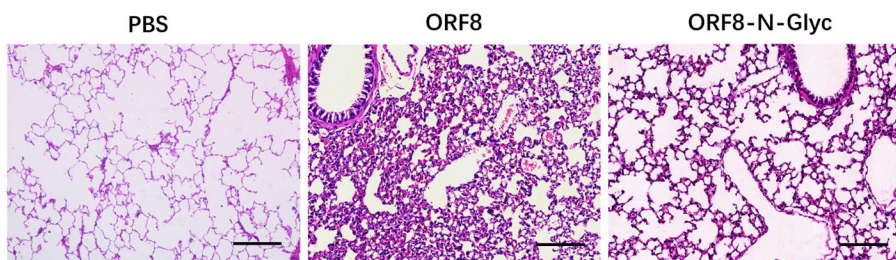
**B**



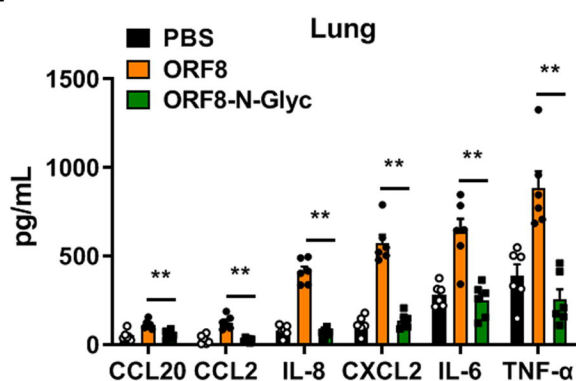
**C**



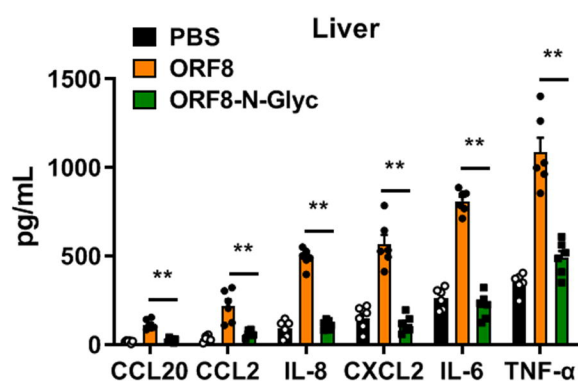
**D**

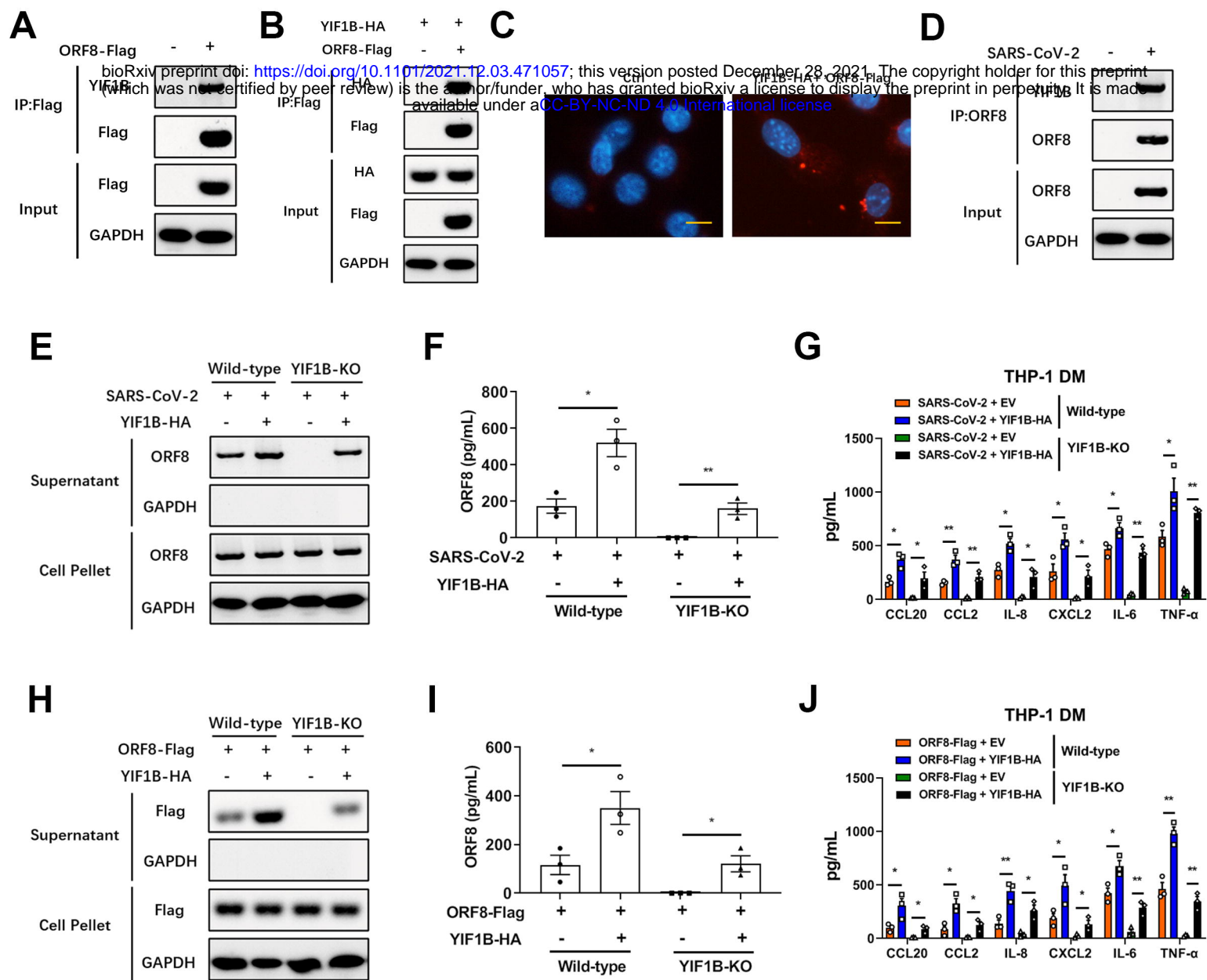


**E**

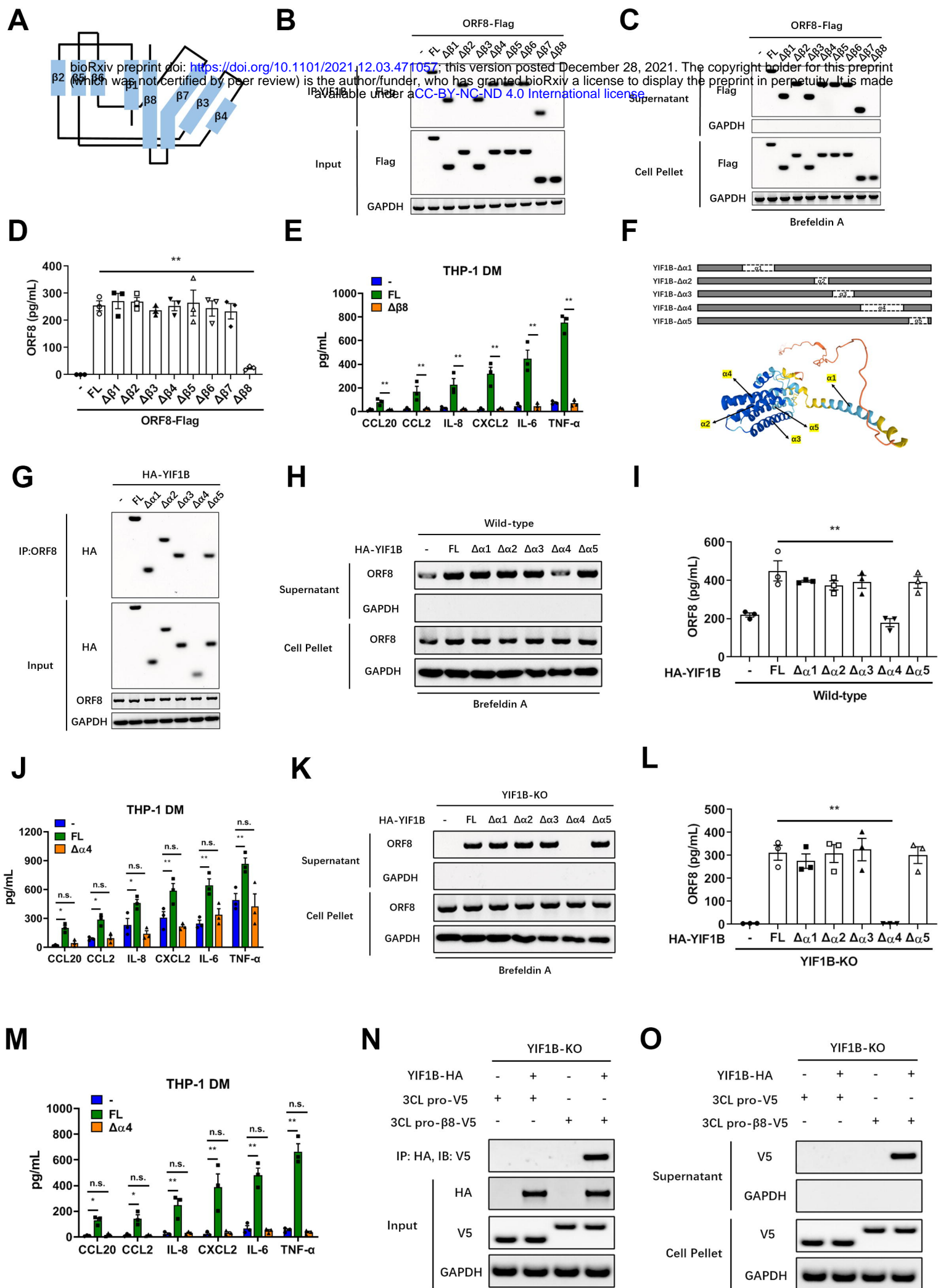


**F**

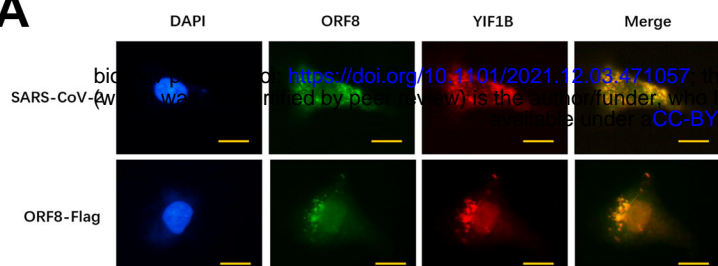




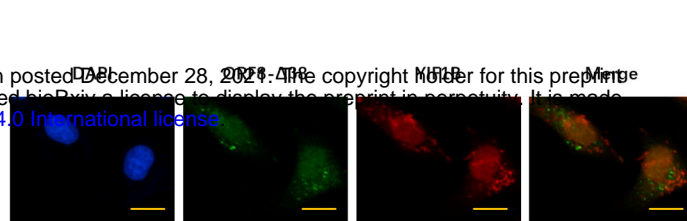




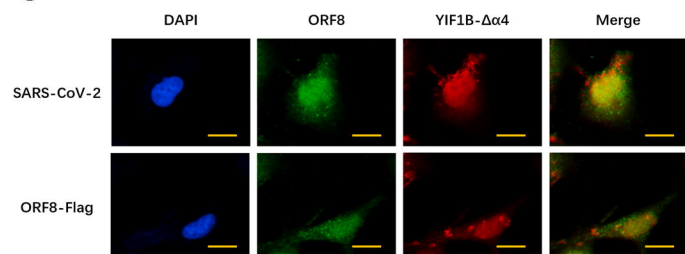
**A**



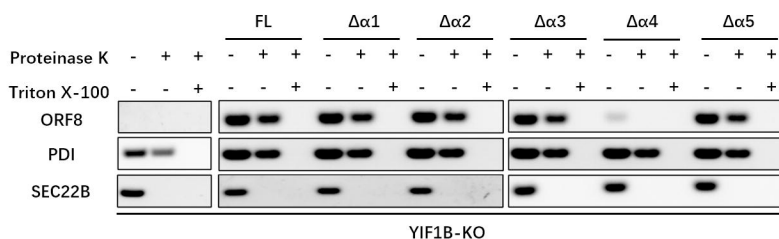
**B**



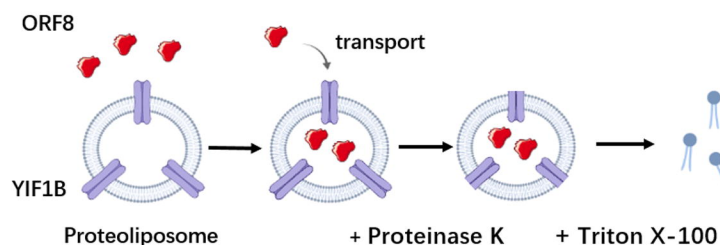
**C**



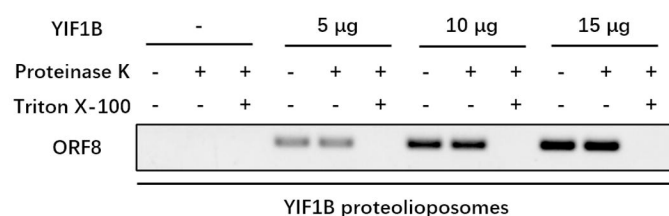
**D**



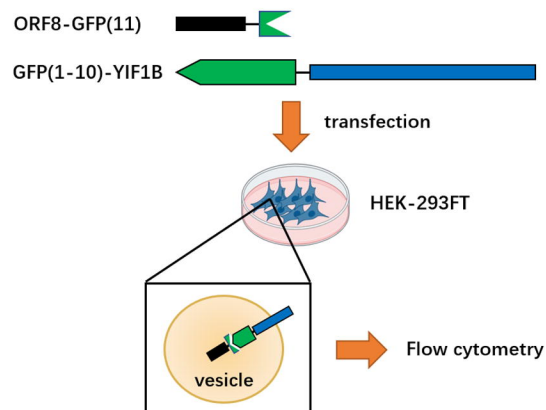
**E**



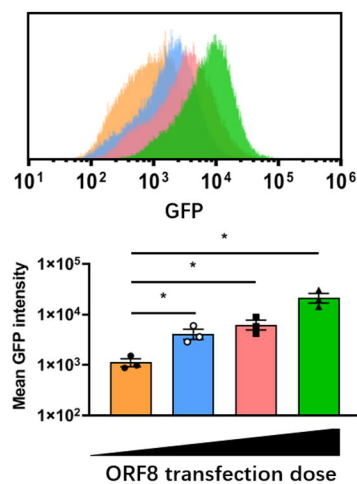
**F**



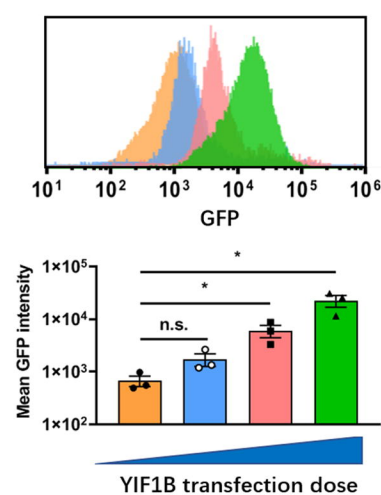
**G**

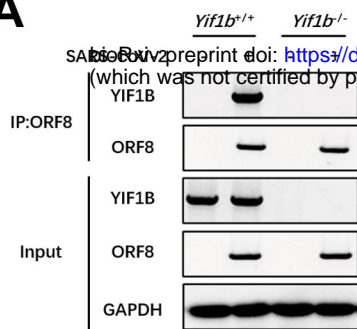
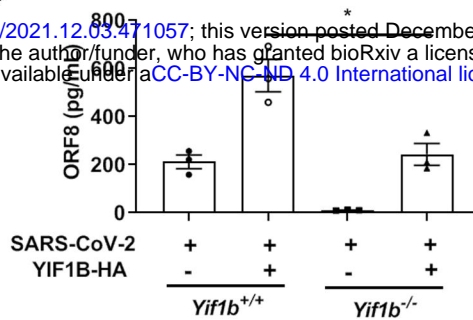
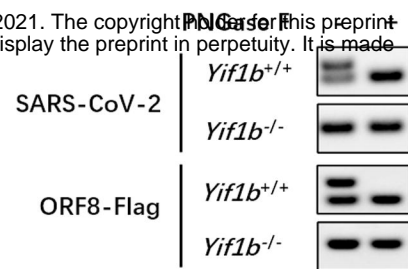
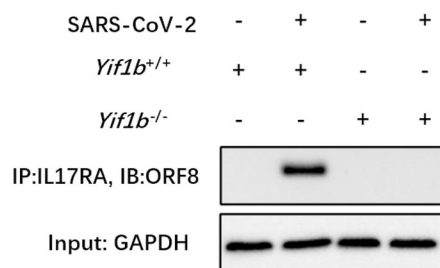
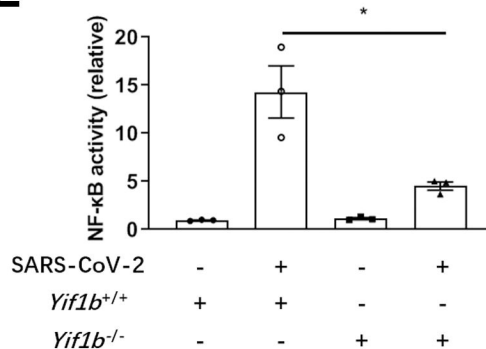
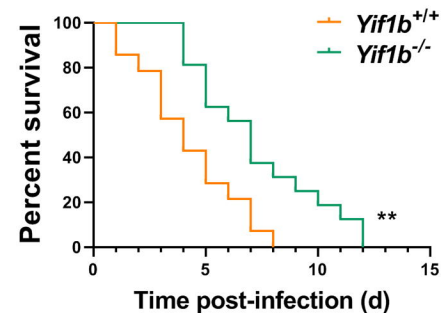
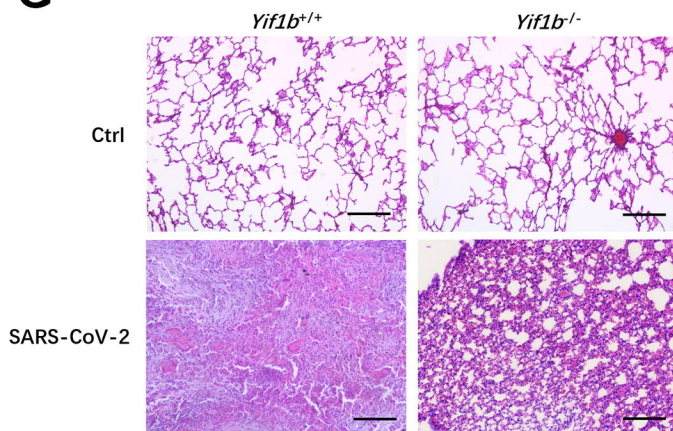
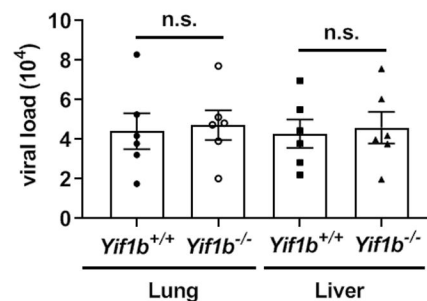
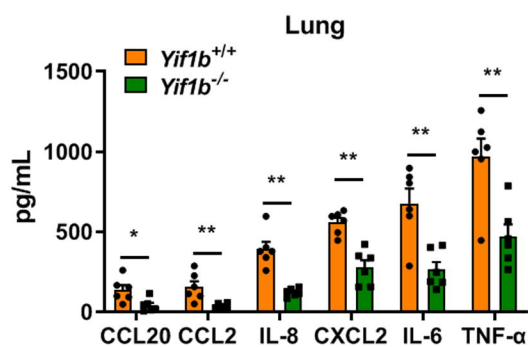


**H**



**I**



**A****B****C****D****E****F****G****H****I****J**