Secreted ORF8 is a pathogenic cause of severe COVID-19 and is potentially targetable with select NLRP3 inhibitors

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Abstract: 153 words
Main text: 3972 words
Main Figure: 7
Suppl Figure: 7
Suppl Table: 4
Reference: 35

Scientific Category: inflammation, monocytes, cytokines/chemokines.

33 **Key points:**

34

35 - Secreted glycoprotein ORF8 induces monocytic pro-inflammatory cytokines
36 involving the activation of the NLPR3 inflammasome pathway.

37

38 - ORF8 is prognostically present in the blood of symptomatic patients with covid-
39 19 and is targetable with NLRP3 inhibitor MCC-950.

40

ABSTRACT

COVID-19 is a significant cause of morbidity and mortality in blood cancer patients, especially those on immunosuppressive therapy. Despite extensive research, the specific factor associated with SARS-CoV-2 infection that mediates the life-threatening inflammatory cytokine response in patients with severe COVID-19 remains unidentified. Herein we demonstrate that the virus-encoded Open Reading Frame 8 (ORF8) protein is abundantly secreted as a glycoprotein *in vitro* and in symptomatic patients with COVID-19. ORF8 specifically binds to the NOD-like receptor family pyrin domain-containing 3 (NLRP3) in $CD14^+$ monocytes to induce a non-canonical inflammasomal response, and a canonical response when the second activation signal is present. Levels of ORF8 protein in the blood correlate with severity and disease-specific mortality in patients with acute SARS-CoV-2 infection. Furthermore, the ORF8-induced inflammasome response was readily inhibited by the NLRP3 inhibitor MCC950 *in vitro*. Our study identifies a dominant cause of pathogenesis, its underlying mechanism, and a potential new treatment for severe COVID-19.

57 **INTRODUCTION**

58

59 COVID-19, the global pandemic caused by infection with severe acute
60 respiratory syndrome coronavirus 2 (SARS-CoV-2), has infected more than half a billion
61 people and caused over six million deaths worldwide.[1] Numerous studies have shown
62 that the production of pro-inflammatory cytokines/chemokines including IL1β, IL6, IL8,
63 and CCL2 is responsible for life-threatening symptoms.[2,3] It is also known that the viral
64 load, cytokine levels, and disease severity are tightly associated[4-6], and the virus-
65 neutralizing antibodies and IL1β pathway antagonists could readily mitigate symptoms
66 and improve clinical outcomes.[7,8] However, the intermediate viral factor that directly
67 causes the inflammatory cytokine responses remains unidentified. It has been
68 demonstrated that SARS-CoV-2 infection localizes to nasal and pulmonary epithelial
69 cells,[9,10] while the cytokine response is more systemic. It seems irreconcilable how this
70 cytokine response is initiated given that no live virus has been reported in the blood
71 based on transfusion medicine studies.[11-14] We hypothesized that an inflammatory
72 byproduct of SARS-CoV-2 replication is released into the bloodstream resulting in a
73 systematic cytokine response in severe COVID-19 patients.

74

75 Upon infection of human cells, the SARS-CoV-2 virus replicates its 29.9kb RNA
76 genome and produces up to 29 possible viral proteins, including 16 non-structural
77 proteins (NSP1-16), four structural proteins Spike (SPK), Membrane (MEM), Envelope
78 (ENV) and Nucleocapsid (NUC) and nine accessory proteins (ORF3A, 3B, 6, 7A, 7B, 8,
79 9b, 9c, and 10). While only four structural proteins along with the RNA genome are
80 assembled into new viral particles, the other viral proteins are thought to be left
81 behind[15] which may disrupt host cell functions.[16,17] Herein, we demonstrate that the
82 SARS-CoV-2 encoded ORF8 is abundantly secreted as a glycoprotein into culture
83 supernatant *in vitro* and into the bloodstream in patients with COVID-19. Glycosylated

84 ORF8 stimulates $CD14^+$ monocytes to produce a group of pro-inflammatory cytokines
85 including IL1β through an NLRP3-mediated inflammasome response, and the
86 ORF8/NLRP3 axis is targetable by NLRP3 inhibitor MCC-950.
87

88 **MATERIALS AND METHODS**

89

90 *Study Samples*

91 Human serum samples were collected from Mayo Clinic patients with a
92 documented diagnosis of COVID-19 infection and consented to COVID-19 Research
93 Task Force Specimen Biobank. Samples for these studies were requested and approved
94 by the Task Force Review Committee and the Mayo Clinic Institutional Review Board.
95 Deidentified fresh leukocyte cones of healthy donors were obtained from Mayo Clinic
96 Blood Bank. Use of mouse splenic B cells from C57/B6 mice was approved by the
97 Institutional Animal Care and Use Committee of the Mayo Clinic.

98

99 *SARS-CoV-2 protein constructs and their expression in human cells*

100 Lentiviral constructs expressing SARS-CoV-2 proteins originally described by
101 Gordon, et al [16] were purchased from Addgene (Supplemental Table 1), and used to
102 make stable expression cell lines in HEK293 cells by lentiviral transduction, and
103 transient expression in HEK293F cells with HyCell TransFx media (Cytiva) method for
104 ORF8 protein purification. During the study, we first used the conditioned media (CM)
105 (made by dialyzing the culture supernatants of ORF8-expressing HEK293 cells) for
106 PBMC stimulation, it was later replaced with purified glycosylated ORF8.

107

108 *Stimulation PBMCs or THP-1 and cytokine detection*

109 Fresh PBMCs ($3x10^6$) isolated from healthy donors were stimulated with either
110 20% (v/v) of CM-ORF8 for 72 hours, or 200ng/ml pure ORF8 for 24 hours in a total
111 volume of 1.5ml RPMI/10% FCS based on the titration results (Supplemental Figure 6).
112 The cytokines expression was determined using qPCR (SYBR-green method, primers
113 are listed in Supplemental Table 3), Luminex using a custom procarta Luminex 6-plax-
114 cytokines (IL1β, IL6, IL8, IL18, CCL2, and TNFα) detection kit (ThermoFisher)

115 (Supplemental Fig. 5), intracellular flowcytometry, or Western blotting. For qPCR,
116 housekeeping gene β-Actin or HPRT1 was used for normalization.
117
118 *Detection of ORF8 protein in sera of patients newly infected with SARS-CoV-2*
119 For detection of ORF8 protein in serum samples from patients newly infected
120 with SARS-CoV-2, typically four microliters of serum were directly mixed with 36
121 microliters of 2x Laemmli buffer with 5% of BME, boiled for 3 min before load to 10-
122 20% SDS-PAGE gels, the blots were probed with 1:5000 diluted rabbit anti-ORF8
123 antibody (MyBioSource, Cat# MBS3014575) at 4°C overnight. The images were
124 developed using an ECL reagent kit.
125
126 *Analysis of ORF8 expression and survival in patients with COVID-19*
127 Overall survival was calculated from the time of COVID-19 diagnosis, using the
128 Kaplan-Meir method on JMP 14.0 software (SAS Institute, Cary, NC).
129
130 Additional methods are described in supplemental Materials and methods.
131
132

133 **RESULTS**

134

135 *Secretion of SARS-CoV-2 proteins from human cells*

136 To determine the secretion potential of SARS-CoV-2 encoded proteins from
137 human cells, we transduced HEK293 cells with one of 22 lentiviral constructs available
138 to us each expressing a SARS-CoV-2 protein tagged Strep II (Supplemental Table 1). The
139 secreted viral proteins in the culture supernatants were enriched and analyzed by
140 Western blotting, and their levels were compared to their total cellular expression in
141 whole cell lysates. As shown in Fig. 1a, most viral proteins were robustly expressed
142 with the expected molecular sizes except for NSP13, ORF10, MEM, and NSP4 due to
143 their size or solubility issues. While NSP2, 5, 7, 9, 10, 12, 14, 15, ORF8, and NUC
144 proteins were all detected as secreted proteins, ORF8 was the single most robustly
145 secreted protein (Fig. 1b). This is consistent with *in silico* analysis (Supplemental Fig. 2)
146 showing that all secreted NSPs possess an unconventional protein secretion signal
147 (UPS) while ORF8 carries a classical protein signal sequence (SS) and an N-link
148 glycosylation sites (^{78}NYTV). These results suggest that ORF8 is efficiently secreted
149 through the classical ER/Golgi protein secretion pathway, and its glycosylation explains
150 the up-shifting and smearing of the ORF8 protein band (Fig. 1b).

151

152 *Secreted ORF8 protein induces the production of pro-inflammatory cytokines*

153 We then queried if any of the secreted SARS-CoV-2 proteins could induce the
154 expression of pro-inflammatory cytokines seen in patients with COVID-19. We treated
155 human peripheral blood mononuclear cells (PBMCs) from healthy donors with
156 conditioned media (CM) containing major secreted NSPs or ORF8 proteins followed by
157 an assessment of cytokine expression. We found that none of the secreted NSP proteins
158 induced cytokine expression (Fig. 2a) while ORF8-containing CM (ORF8-CM) induced
159 the expression of IL1β, IL6, IL8, and CCL2 up to 5-fold in PBMCs from select donors

160 (Fig. 2b). Since IL1β, IL6, IL8, and CCL2 are among the key cytokines elevated in
161 patients with severe COVID-19,[18] it is conceivable that secreted ORF8 is the viral factor
162 responsible for inflammatory cytokine response in patients with severe COVID-19.
163
164 To evaluate whether the glycosylation of ORF8 affected cytokine induction, we
165 purified ORF8 from the HEK293 culture supernatant and from ORF8-expressing *E. coli*
166 and designated them as ORF8-glyco^{hi} and ORF8-glyco^{null}, respectively. As shown in Fig.
167 2c that IL1β, IL-6, and IL-8 but not IL-2 were robustly induced by the pure ORF8-glyco^{hi}
168 in a dose-dependent manner confirming that secreted ORF8 is capable of inducing
169 proinflammatory cytokines seen in COVID-19 patients. Interestingly, *E. coli* expressed
170 ORF8-glyco^{null}, had no cytokine induction activity even at higher doses, suggesting that
171 proper ORF8 glycosylation is necessary for cytokine induction.
172
173 We then tested the cytokine induction activities of ORF8 from HEK293 cells
174 treated with the Golgi inhibitor cocktail Brefeldin-A and Monensin (BFA/M) and found
175 that while the glycosylation level was reduced (Supplemental Fig. 3) the cytokine
176 induction activity of BFA/M-treated ORF8 was indeed altered (actually increased),
177 suggesting that the cytokine induction activity of ORF8 is governed by the Golgi
178 complex. Our protein localization data (Supplemental Fig 1, 2) indicate that several
179 SARS-CoV-2 proteins are localized to Golgi membranes and may potentially modify the
180 glycosylation and the cytokine induction activity of ORF8. Indeed, co-expressing MEM,
181 ENV, ORF3A, or ORF7A was able to alter the molecule weight and the cytokine-
182 inducing activities of ORF8 (Supplemental Fig 4).
183
184 *ORF8 stimulates CD14^{+} monocytes to produce pro-inflammatory cytokines/chemokines.*

185 Next, we asked which specific cell subsets in PBMCs are the primary targets of
186 ORF8, and what is the resulting transcriptional signatures in those cells. We performed
187 single-cell RNA sequencing (scRNA-Seq) analysis on three PBMC samples treated with
188 ORF8-CM or control-CM. Single-cell transcriptome-based cell clustering showed that
189 cells in cluster 5 (C5) on the t-SNE plot (Fig. 3a) were induced to express cytokines
190 including IL1β, IL8, and CCL2 and inflammasome pathway components upon ORF8
191 treatment (Fig. 3b) (Supplemental Table 2). The same cytokines are known to be
192 elevated in COVID-19 patients.[19] The expression of CD14, CD16, CD68, and HLA-DR
193 identifies those cytokine secreting cells as activated monocytes.[20,21]
194
195 To identify which monocyte subsets are the targets of ORF8, we analyzed
196 intracellular cytokines in ORF8 stimulated PBMCs from 15 donors by flow cytometry.
197 Significant IL1β expression was detected in classical ($CD14^+/CD16^-$) and intermediate
198 ($CD14^+/CD16^+$) subsets but not non-classical ($CD14^{low}/CD16^+$) monocytes nor cells of
199 other lineages (B or T-lymphocytes, dendritic, NK cells) in all 15 donors (Fig. 3c).
200 Similar results were observed for IL8 and CCL2 expression as well (Fig. 5a). Our data
201 demonstrate that the $CD14^+$ monocyte subsets are the producer of the pro-inflammatory
202 cytokines upon ORF8 stimulation.
203
204 *Secreted ORF8 directly binds to and activates the NLRP3-mediated inflammasome response*
205 To decipher the mechanism by which ORF8 induces pro-inflammatory cytokines
206 in CD14+ monocytes, using the Gene Set Enrichment Analysis (GSEA) and Kyoto
207 Encyclopedia of Gene and Genome (KEGG) tools, we found that the SARS-CoV-2
208 infection pathway, NOD-like receptor signaling pathway, and NFκB pathway were
209 among the top enriched, especially, mRNAs of lysosomal enzymes (LYZ, CTSD, CTSB,
210 CTSS), inflammasomal protein NLPR3, and cytokines IL1β, IL8, CCL2 were among the
211 top expressed (Fig. 3b).

212

213 We then examined the role of the NLRP3-mediated inflammasome pathway in
214 ORF8-mediated cytokine production. Fig. 3d shows that both NLPR3 and IL1β proteins
215 were induced in PBMCs upon ORF8 treatment, and such induction was diminished in
216 NLPR3 knockdown cells (THP1-defNLRP3, InvivoGen) compared to the parental THP-
217 1 cells, demonstrating the NLRP3 dependency of ORF8 mediated cytokine response in
218 human monocytes (Fig. 3e).

219

220 To determine how ORF8 molecules enter $CD14^+$ monocytes, we first incubated
221 PBMCs with pure ORF8 protein on ice for one hour to saturate any potential ORF8
222 binding receptors on the monocyte surface. After extensive washes with ice-cold PBS to
223 remove unbound ORF8 protein, the cells were then incubated at 37°C to initiate ORF8-
224 mediated cell activation in the presence or absence of additional ORF8. Fig. 4a shows
225 robust IL1β mRNA expression was observed only in cells exposed to additional ORF8
226 but not those only pre-incubated with ORF8, suggesting that ORF8 likely enters
227 monocytes through a non-receptor-mediated process, such as phagocytosis.
228 Complementarily, we asked if ORF8 would bind to TLR2, TLR4, CD14, or NLRP3
229 known to be involved in inflammasome activation. We incubated ORF8 protein-coated
230 beads with monocyte lysates from two ORF8 responding and two ORF8 nonresponding
231 healthy donors. Fig. 4b shows that neither TLR2, TLR4, nor CD14 were co-precipitated
232 with ORF8 (CD14 data not shown). However, NLRP3 was readily detected in one of the
233 ORF8-responders, suggesting that indeed ORF8 binds to NLRP3 in primary human
234 monocytes.

235

236 To further dissect how NLRP3 binds to ORF8, we transiently transfected ORF8-
237 expressing HEK293 cells with Flag-tagged NLRP3 constructs Flag-N3-FL (full-length

238 NLRP3), or one of the three deletion mutants Flag-N3-N, Flag-N3-M, or Flag-N3-C (Fig.
239 4c).[22] Cell lysates were then incubated with Strep-Tactin™ beads to pulldown Strep-
240 tagged ORF8 and its binding proteins. In addition to NLRP3-FL (Fig. 4d), both Flag-N3-
241 M, and Flag-N3-C but not Flag-N3-N also strongly co-precipitated with ORF8
242 suggesting both the middle NACHT domain and C-terminal LRR domain of NLRP3
243 each can independently bind to ORF8. These results clearly demonstrate inflammasome
244 protein NLRP3 directly interacts with ORF8.
245
246 *ORF8 activates the NLRP3-mediated inflammasome pathways.*
247 The effect of LPS in inflammasomal activation has been well established;
248 therefore, it was important to demonstrate that our purified ORF8 protein was free of
249 LPS contamination. ORF8 and LPS were compared for their effects on IL1β, IL8, and
250 CCL2 induction in three monocyte subsets (classical, intermediate, and non-classical)
251 from 15 healthy donors. Fig 5a shows that all three subsets responded to LPS while only
252 $CD14^+$ (classical and intermediate) responded to ORF8 with variable induction of IL1β,
253 IL8, and CCL2. These results show that ORF8 and LPS target monocytes with different
254 cell type specificities. We then examined the responsiveness of $CD14^+$ monocytes to LPS
255 or ORF8 treatment using IL1β, IL8, and CCL2 expression as the readout, and we found
256 that intracellular IL1β was detected in the majority (90.1%) of LPS-treated $CD14^+$
257 monocytes but only in a subpopulation (mean=38%) of ORF8 treated $CD14^+$ monocytes.
258 In contrast, CCL2 expression was detected only in a minor population (mean=22.1%) of
259 LPS-treated $CD14^+$ monocytes but in a larger population of ORF8-treated CD14+
260 monocytes (mean=63.7%) (Fig. 5b). These data clearly demonstrate that ORF8 and LPS
261 also have different cytokine induction specificities. In addition, we also examined the
262 cell proliferation and the IgM induction of mouse splenic B cells upon ORF8 and LPS
263 treatment, and we found that the mouse splenic B cells did not proliferate, nor

264 produced any polyclonal IgM 96 hours after ORF8 treatment, while LPS treated cells
265 showed robust proliferation morphology and massive production of IgM (Fig. 5c). In
266 the aggregate, our data clearly demonstrated that the cytokine inducing activity of
267 ORF8 is not due to LPS contamination.
268
269 To examine the activation status of the canonical NFκB pathway necessary for
270 the induction of inflammasomal proteins including NLPR3, Casp-1, and IL1β in ORF8
271 treated monocytes, we examined the GSEA analysis results and found that NFκB
272 pathway components were transcriptionally enriched (NES=1.539662, FDR q-value=0)
273 in ORF8 treated monocytes in cluster 5 (Fig. 6a, left panel). To validate these at the
274 protein level, we treated THP-1 cells with ORF8 for 4 and 24 hours and determined
275 their NFκB pathway protein expression. Fig. 6a (right panels) shows that the increased
276 level of phospho-p65 and the conversion of p100 to p52 were readily detected 4 hours
277 after ORF8 treatment. Similar results were also observed in LPS-treated cells. These data
278 demonstrate that ORF8 stimulation activates the NFκB pathway triggering the
279 activation of the inflammasome pathway in human monocytes.
280
281 Similarly, the GSEA analysis data revealed the mRNAs of inflammasome
282 pathway components were also significantly enriched (NES=1.812154, FDR q-value
283 =0.015483) (Fig. 6b, left panel). We then treated THP-1 cells with ORF8 or LPS alone or
284 in combination with nigericin, a potent agent that provides a second signal for the
285 canonical inflammasomal pathway activation, for 4 or 24 hours. The cell lysates and
286 culture supernatants were analyzed by Western blotting for the expression of NLPR3,
287 Caspase-1, Caspase-5, IL1β, and the pore-forming fragment of gasdermin D (GSDMD)
288 proteins. We found that ORF8 induces the inflammasome pathway by 1) activating the
289 production of IL1β without requiring a separate priming step; 2) signaling through the

290 non-canonical pathway using Casp-1 (early hours) followed by switching to Casp-4/5;
291 3) maintaining cell viability without triggering pyroptosis unless the second signal such
292 as nigericin is present which rapidly initiates pyroptosis (Fig. 6b, right panel). Our data
293 suggest that ORF8 and LPS may represent a family of pyrogens by triggering a non-
294 canonical inflammasomal pathway leading to the production and release of IL1β
295 without activating GSDMD to the level necessary for pyroptosis, a process called
296 hyperactivation for LPS.[23,24] However, ORF8 can also serve as a priming agent for the
297 canonical inflammasome pathway to evoke a prompt pyroptosis in the presence of
298 second signal molecules such as extracellular ATP from damaged cells.
299
300 To validate the dual roles of ORF8 in the activation of canonical and non-
301 canonical inflammasome pathways, we examined ASC speck formation in ORF8- or
302 LPS-treated THP-1 cells by immunofluorescence. As shown in Fig. 6c, ORF8 alone was
303 insufficient to induce ASC speck formation. However, distinct pre-nuclear ASC specks
304 were readily detected when nigericin or extracellular ATP was also present. In addition,
305 the NLPR3 protein was also co-localized with ASC protein to the specks (Fig. 6c), ASC
306 speck formation always precedes cell pyroptosis in our system, and ORF8 is slightly
307 more robust than LPS in mediating ASC formation in the presence of nigericin or
308 extracellular ATP. The results confirm the dual roles of ORF8 in the activation of the
309 non-canonical inflammasome pathway when the second signal is absent, and the
310 canonical inflammasome pathway by priming the cells when the second signal is
311 present. These data further suggest that the mode of action of ORF8 is dependent on the
312 presence or absence of a second signal such as extracellular ATP released from
313 damaged cells/tissues nearby.
314

315 *Blood ORF8 protein levels correlate with the mortality and disease course of severe patients with*
316 *COVID-19*
317 Having demonstrated that ORF8 is secreted as a glycoprotein and capable of
318 inducing inflammatory cytokine responses *in vitro*, we then asked if ORF8 is also
319 secreted and glycosylated in blood from patients infected with SARS-CoV-2. By
320 analyzing serum samples collected from patients within 4-7 days of COVID-19
321 diagnosis using Western blotting, we found that ORF8 protein was readily detected at
322 various levels in 92% (23/25) of newly infected patients. Interestingly, two other major
323 secreted proteins NSP9 and NSP10 were not detected in the same samples suggesting
324 not all the secreted SARS-CoV-2 proteins are present at detectable levels in blood
325 circulation. To determine the glycosylation status of the serum ORF8, we compared the
326 sizes of ORF8 from patients and HEK293 lysate. Our results (Fig. 7A) show that patient
327 serum ORF8 is indeed glycosylated when compared to the ORF8 from HEK293 lysate
328 which has an extra Strep-II tag (28 aa) and possibly an uncleaved signal sequence (16
329 aa). Due to the lack of a quantitative assay, we were unable to determine the absolute
330 quantity of ORF8 in patient samples; however, our semi-quantitative Western blot
331 could readily detect ORF8 in as little as four microliters of patient serum suggesting the
332 protein is present at significant levels in patient blood at the onset of COVID-19.
333
334 Given its activity in inducing inflammatory cytokines *in vitro*, and its significant
335 presence in the blood of newly infected patients, we then asked if the levels of ORF8
336 protein would correlate with disease severity and outcome. To that end, we examined
337 the correlation between blood ORF8 levels and patients' survival outcomes in our
338 cohort of 25 hospitalized patients. Fig. 7b shows that after 120 days of follow-up, all
339 seven fatalities were exclusively associated with the ORF8-high group (scored 2+ or 3+
340 on Western blot) while all patients in the ORF8-low group (scored 0 or 1+) had mild

341 symptoms and quick recovery without any events of death. These results demonstrate a
342 clear correlation between the ORF8 load and disease severity in newly infected patients.
343 We then monitored the ORF8 levels at various time points during the disease course in
344 two patients with prolonged COVID-19, and one patient with long covid lasting more
345 than 17 months. As demonstrated in Fig. 7c, two patients with high blood ORF8 levels
346 had persistent severe symptoms requiring treatment in the intensive care unit (ICU),
347 and their blood ORF8 levels diminished by the time they were recovered and
348 discharged from the hospitals. The long covid patient with lingering symptoms
349 throughout remained ORF8 positive in all three serial blood samples collected over 16-
350 month period (Fig. 7c). These results suggest that ORF8 level is prognostic for COVID-
351 19 outcome, further supporting our hypothesis that the ORF8 protein is a pathogenic
352 cause of severe COVID-19 in patients.
353
354 *ORF8-mediated cytokine induction is targetable by select NLRP3 inhibitors*
355 Given its causative role in COVID-19 pathogenesis, we then tested the
356 targetability of ORF8 mediated cytokine response using three investigational NLRP3
357 inhibitors - MCC950, Tranilast, and OLT117 (Dapansutrile). As shown in Fig. 7d, the
358 NLRP3 inhibitor MCC950 showed effective inhibition on the production of IL1β in both
359 THP-1 cells and PBMCs (blue bars), while Tranilast and OLT117 (hatched bars)
360 exhibited moderate to marginal effect in PBMCs but no effect in THP-1 cells. Our results
361 suggest that ORF8 mediated IL1β production can be effectively inhibited by the NLRP3
362 inhibitor MCC950 at a nanomolar dose range, and possibly by other classes of inhibitors
363 as well, demonstrating that targeting the ORF8/NLRP3 axis is a promising strategy for
364 treating patients with symptomatic COVID-19.
365

366 **DISCUSSION**

367

368 Despite the success in the development and implementation of effective vaccines
369 to prevent SARS-CoV-2 infection, COVID-19 remains a major challenge for many
370 reasons. These include 1) a significant population remains unvaccinated, 2) new
371 variants keep emerging and evading current vaccine protection, 3) patients with
372 compromised immunity including those with blood cancers on immunosuppressive
373 medications are poorly protected, [25,26] and more importantly, 4) many previously
374 infected patients have developed lingering symptoms or long Covid. Therefore, better
375 therapies and clinical management tools for COVID-19 are urgently needed.

376

377 SARS-CoV-2 accessory protein ORF8, a 121 amino acid protein, is the least
378 conserved protein in the beta-coronavirus family.[27,28] Here we report that ORF8 is a
379 major secreted viral glycoprotein *in vitro* and in COVID-19 patients. Our data,
380 summarized in Supplemental Fig. 7, support the concept that the ORF8 protein secreted
381 from locally infected cells in the lung and released to the bloodstream then stimulates
382 circulating $CD14^+$ monocytes to initiate systematic cytokine responses. This is consistent
383 with findings by others that monocytes are the single most affected WBC subset in the
384 blood by SARS-CoV-2 infection.[29] It has been reported that about 6% of monocytes were
385 infected with the virus in COVID-19 patients through CD16-mediated uptake of
386 antibody-opsonized SARS-CoV-2 virus. Whether these cells were truly infected or
387 simply engulfed with infected cells/debris remains to be seen since no live virus was
388 detected.[30] Nevertheless, these monocytes may represent a parallel and complementary
389 process to the ORF8-mediated inflammatory pathway described herein. Our
390 intracellular IL1β data showed a mean of 43% (range, 7.2% - 90.7%) of monocytes that
391 are ORF8 responsive, suggesting that the ORF8-mediated process dominates. We

392 conclude that secreted ORF8 is a key disease-causing viral factor and can be targeted by
393 NLRP3 inhibitors, offering a potential new treatment option.
394
395 NLRP3-mediated inflammasome activation is a critical part of the innate immune
396 response. However, the precise mechanisms for different pathogens have not been fully
397 delineated. Our study indicated that ORF8 may enter monocytes through a non-
398 receptor mediated ORF8 internalization, followed by lysosomal action involving
399 lysozyme (LYZ) and cathepsin proteases (CTSB, CTSD). ORF8 then may bind to the
400 NACHT and/or LRR domain to activate NLRP3 and recruit Casp-1 (early) and Casp-4/5
401 (later). It is not known what the roles of these two different bindings play, and if they
402 are important in pathway choosing (canonical vs. non-canonical). It is also interesting
403 that, like LPS, ORF8 induces the expression/activation of Caspase-1 early (hour 4) but
404 switches to Casp-4/5 later (at hour 24) even when nigericin is present (data not shown).
405 Our data shows that the mode of action of ORF8 is readily changed by the presence of
406 the second signal leading to the switch from non-canonical to canonical inflammasomal
407 response. Therefore, it is conceivable that, in patients with COVID-19, the inflammation
408 may be aggravated by the additional tissue/cell damages that release ATP, leading to
409 the activation of an ORF8-mediated canonical inflammasome pathway and causing
410 pyroptosis.
411
412 Due to the lack of effective COVID-19 animal models, it is challenging to
413 genetically test the pathogenic role and targetability of the ORF8 protein *in vivo*.
414 However, a SARS-CoV-2 variant (Δ382) found in Taiwan and Singapore with a
415 complete loss of the ORF8 gene serves as a tailor-made natural genetic model that
416 validates our findings.[31,32] Young et al. analyzed a cohort of 92 patients infected with the
417 wildtype virus and 29 patients infected with the Δ382 variant and found that patients

418 infected with the wildtype SARS-CoV-2 exhibited much higher levels of pro-
419 inflammatory cytokines than those infected with the Δ382 variant. Furthermore, no
420 patients with the Δ382 variant required supplemental oxygen whereas it was required
421 for 26 (28%) patients infected with wild-type virus. These observations provide yet
422 another line of evidence supporting the link between ORF8 and COVID-19 disease
423 severity, and an *in vivo* genetic proof-of-principle for targeting ORF8 to prevent severe
424 COVID-19 infection. Additionally, various clinical studies and trials have shown that
425 the IL1 receptor antagonist anakinra significantly reduced the mortality risk in
426 hospitalized patients with moderate to severe inflammation symptoms.[33 34,35] The data
427 from our present study suggests that targeting NLRP3 or ORF8 upstream of the IL1
428 receptor would provide greater therapeutic benefit to patients with severe COVID-19
429 symptoms, and could also provide prophylactic benefit to patients at risk of developing
430 severe COVID-19 infection.

431 **ACKNOWLEDGEMENT**

432 We thank The Mayo Clinic Genome Analysis Core, Bioinformatics Core,
433 Microscopy and Cell Analysis Core, and the CMM Electron Microscopy Facility of the
434 University of California San Diego (supported by NIH equipment grant 1S10OD023527-
435 01) for their expert service, Mayo Clinical Blood Bank for providing fresh leukocyte
436 cones of healthy donor blood, and the Mayo Clinic Center for Individualized Medicine
437 for providing access to their biobank data. We also thank the University of Iowa/Mayo
438 Clinic Lymphoma SPORE (NIH/NCI grant P50 CA97274) and the Predolin Foundation
439 Biobank for sharing some of their resources for this project.

440

441 **AUTHOR CONTRIBUTION**

442 XW and TEW designed the experiments, analyzed, and interpreted results, and
443 wrote the manuscript; XW, MKM, GJR, KEN, KAG, XT, TLW, VT, AW, and MJS
444 conducted experiments and analyzed data; XW, JPA, YY, XT, SD, and MJS analyzed
445 data and generated figures; ADB, JPA, GR, JP, SMA, VB, HD, and TEW provided
446 experiment samples; JPA, GJR, JP, TLW, SMA, MJS discussed and interpreted results;
447 all authors read and approved the manuscript.

448

449 **CONFLICT-OF-INTEREST DISCLOSURE**

450 The authors declare no competing financial interests.
451

452 **FIGURE LEGEND**

453

454 **Figure 1. Secretion property of SARS-CoV-2 proteins. a.** Western blot image of cell
455 lysates for validating SARS-CoV-2 protein expression in HEK293 cells. The blots were
456 probed with an anti-Strep II tag antibody and anti-HA (for SPK protein). **b.** Western
457 image of SARS-CoV-2 protein pulldown using StrepTactin™ beads and anti-HA-beads
458 (for SPK) from the culture supernatants for validating SARS-CoV-2 protein secretion
459 from HEK293 cells. The blots were also probed with an anti-Strep II tag antibody and
460 anti-HA (for SPK protein).

461

462 **Figure 2. Secreted ORF8 specifically induces the expression of pro-inflammatory**
463 **cytokines and is glycosylation dependent. a.** Conditioned media containing major
464 secreted NSP proteins did not induce pro-inflammatory cytokines in PBMCs of healthy
465 donors; **b**. Conditioned media containing secreted ORF8 specifically induced pro-
466 inflammatory cytokines (highlighted in yellow) IL1β, IL6, IL8, and CCL2 in PBMCs
467 from unselected healthy donors. **c.** Purified highly glycosylated ORF8 (ORF8-glycol^{hi})
468 from HEK293 but not unglycosylated ORF8 (ORF8-glycol^{null}) from *E. coli* stimulates
469 PBMCs to produce pro-inflammatory cytokines IL1β, IL6, IL8 but not T cell cytokine
470 IL2.

471

472 **Figure 3. Single-cell RNA sequencing of ORF8 treated PBMCs. a.** tSNE maps of cell
473 clusters using concatenated scRNA-Seq data from two ORF8-responsive PBMC
474 samples. Cells in cluster 5 were responsive to ORF8 treatment. **b.** Violin plots show the
475 monocyte markers CD14, CD16, CD68, and HLA-DRB1, key inflammasome pathway
476 components LYZ, CTSB, NLRP3, and IL1β, and pro-inflammatory cytokines IL1β, IL8,
477 and CCL2 mRNA expression in cells of cluster 5. **c.** Flow cytometry analysis of

478 intracellular IL1β protein expression in different PBMC subsets in response to ORF8
479 treatment. The plots show that IL1β was exclusively produced by $CD14^{+}CD16^{-}$
480 (classical) and $CD14^{+}CD16^{+}$ (intermediate) monocyte subsets. **d.** Western blot images
481 validate that the induction of NLRP3 and IL1β in PBMCs upon stimulation with ORF8
482 indeed occurs at the protein level. **e.** Western images showing in response to ORF8
483 treatment, the IL1β induction in parental THP-1 cells became diminished in NLRP3
484 knockdown ($NLRP3^{kd}$) THP-1 cells. The data shown are representative of at least two
485 independent experiments except for the scRNA-Seq.
486
487 **Figure 4. ORF8 induces proinflammatory cytokines through direct binding to NLRP3.**
488 **a.** ORF8 induces IL1β production through a non-surface-receptor-mediated process.
489 PBMCs were pre-incubated on ice with ORF8 (500 ng/ml) for 2 hours to allow ORF8 to
490 bind to its "surface receptors" followed by washing and incubating at 37ºC to activate
491 the cytokine production in the presence or absence of additional ORF8 (200 ng/ml). IL1β
492 expression was measured by qPCR. **b.** ORF8 directly binds to NLRP3 but not TLR2 or
493 TRL4 in primary monocytes shown by affinity pulldown assay. **c.** Schematic drawing
494 of NLRP3 deletion constructs for mapping ORF8 binding domains in NLPR3. **d.**
495 Mapping NLRP3 domains that bind to ORF8 by affinity pulldown assay using ORF8-
496 Strep-Tacin® beads. Western images of total cell lysates (left half) were used as
497 expression controls and Western images of precipitated proteins (right half) showing
498 ORF8 efficiently binds the full-length as well as two NLRP3 deletion mutant proteins.
499
500 **Figure 5. ORF8 is functionally different from LPS. a.** Representative flow data
501 showing LPS could activate multiple cytokine responses in all three monocyte subsets
502 of PMBCs from all 15 healthy donors examined while ORF8 only activates $CD14^{+}$
503 Monocytes but not $CD14^{low}/CD16^{++}$ non-classical monocytes (red boxed) suggesting LPS

504 and ORF8 may target monocytes through different mechanisms. **b.** Intracellular
505 cytokine flow cytometry data summary on 15 healthy donors upon treatment with
506 ORF8 or LPS, suggesting that ORF8 and LPS are different in cytokine induction
507 specificities. **c.** Mouse B cells responded differently to the treatment of ORF8 and LPS as
508 measured by IgM production and the morphological features of cell proliferation status,
509 demonstrating the lack of any detectable contaminating LPS activity in purified ORF8
510 protein.
511
512 **Figure 6. ORF8 induces proinflammatory cytokines through activation of NLRP3-**
513 **mediated inflammasome pathways. a.** Gene enrichment analysis of scRNA-Seq (left
514 panel) and Western confirmation of key NFkB pathway molecules (right panels) upon
515 stimulation of ORF8 for 4 or 24 hours. **b**. Gene enrichment analysis of scRNA-Seq (left
516 panel) and Western confirmation of NLRP3, IL1β, Casp-1, and Casp-5 in THP-1 cell
517 lysates and IL1β and GSDMD in the culture supernatants (right panels) upon
518 stimulation of ORF8 for 4 or 24 hours. **c.** Immunofluorescence images show that, LPS
519 and ORF8 activate ASC speck (arrowhead pointed) formation only when a second
520 signal (nigericin or extracellular ATP) was present. Cells were first treated with 200
521 ng/ml ORF8 or 100 ng/ml LPS for 2.5 hours followed by 90 min treatment with 5 μM
522 Nigericin or 5 μM ATP before cell harvest and staining for immunofluorescence.
523
524 **Figure 7. The levels of ORF8 protein in sera of COVID-19 patients correlate with the**
525 **outcome and disease course, and the ORF8 pathway is targetable with NLRP3**
526 **inhibitors. a.** Representative Western blot images showing ORF8 protein but not NSP9
527 or NSP10 were detectable in as little as 4.0 μl of serum samples from newly infected
528 COVID-19 patients. ORF8 protein in patient sera shown as glycosylated by size
529 estimation; **b.** Kaplan-Meier curve showing fatality in hospital patients is associated

530 with higher serum level of ORF8; **c.** Western images showing ORF8 levels at different
531 time points correlate with disease course in two patients with prolonged clinical
532 courses, and in one patient with long-covid. **d.** Targetability of ORF8 mediated IL1β
533 expression with various NLRP3 inhibitors in THP1 cells and human primary PBMCs.
534 The data shown are representative of two or three independent experiments.
535 Abbreviations: Dx = diagnosis; ED = emergency department; ICU = intensive care unit
536

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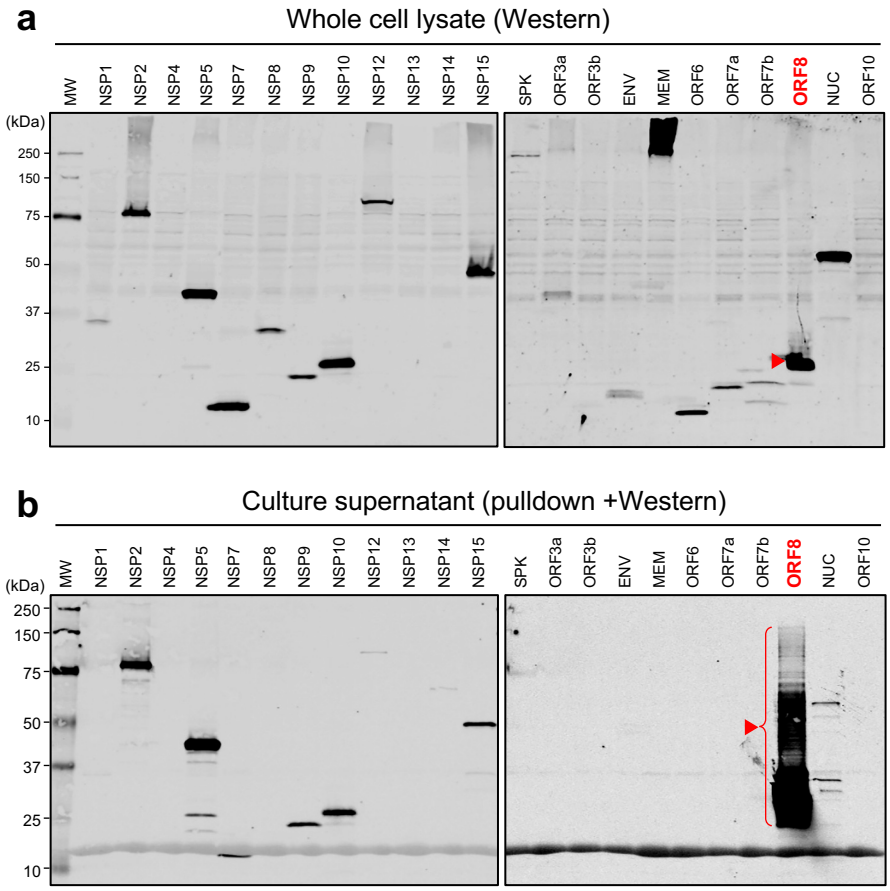

Figure 1. Secretion property of SARS-CoV-2 proteins.

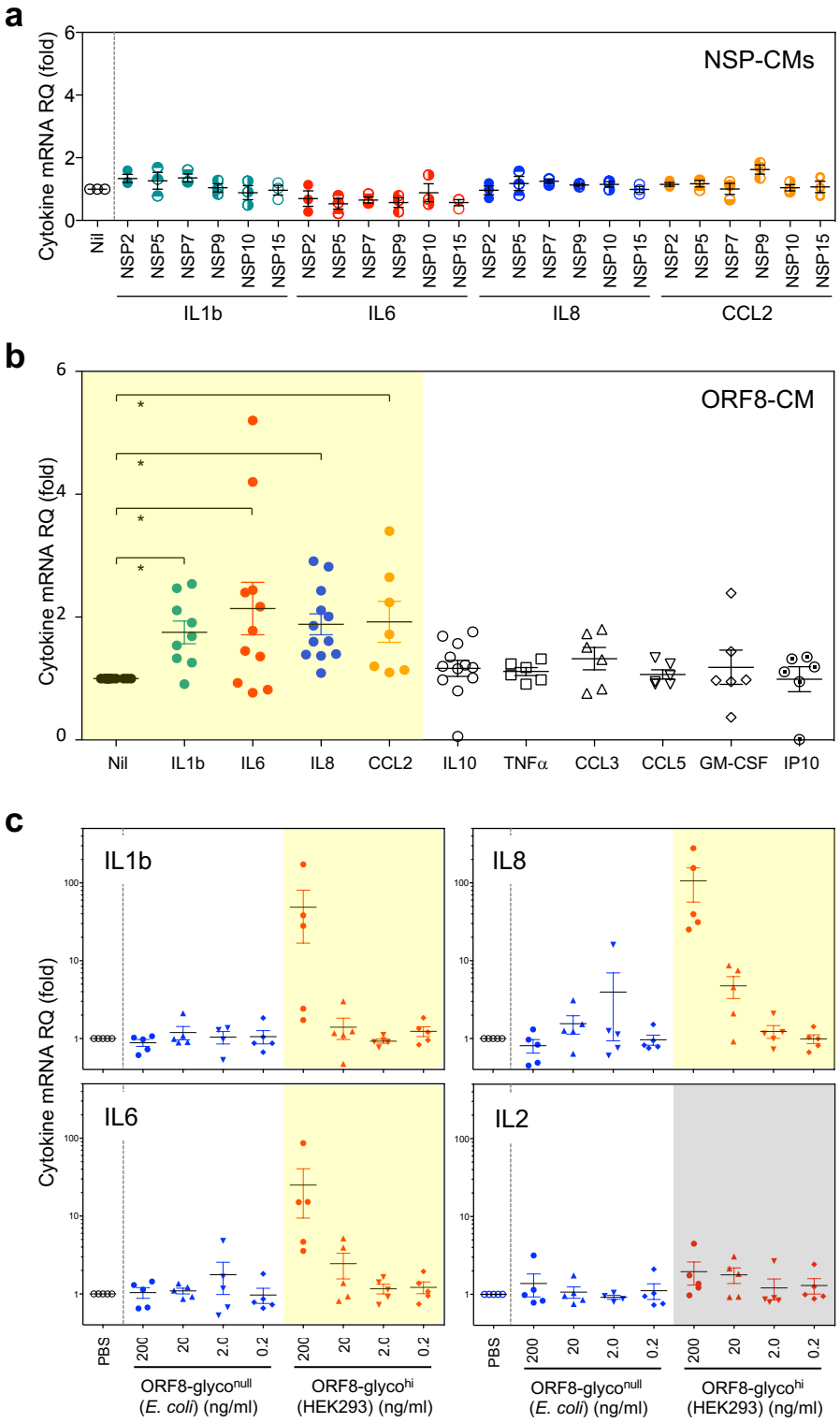

Figure 2. Secreted ORF8 specifically induces the expression of pro-inflammatory cytokines is glycosylation dependent.

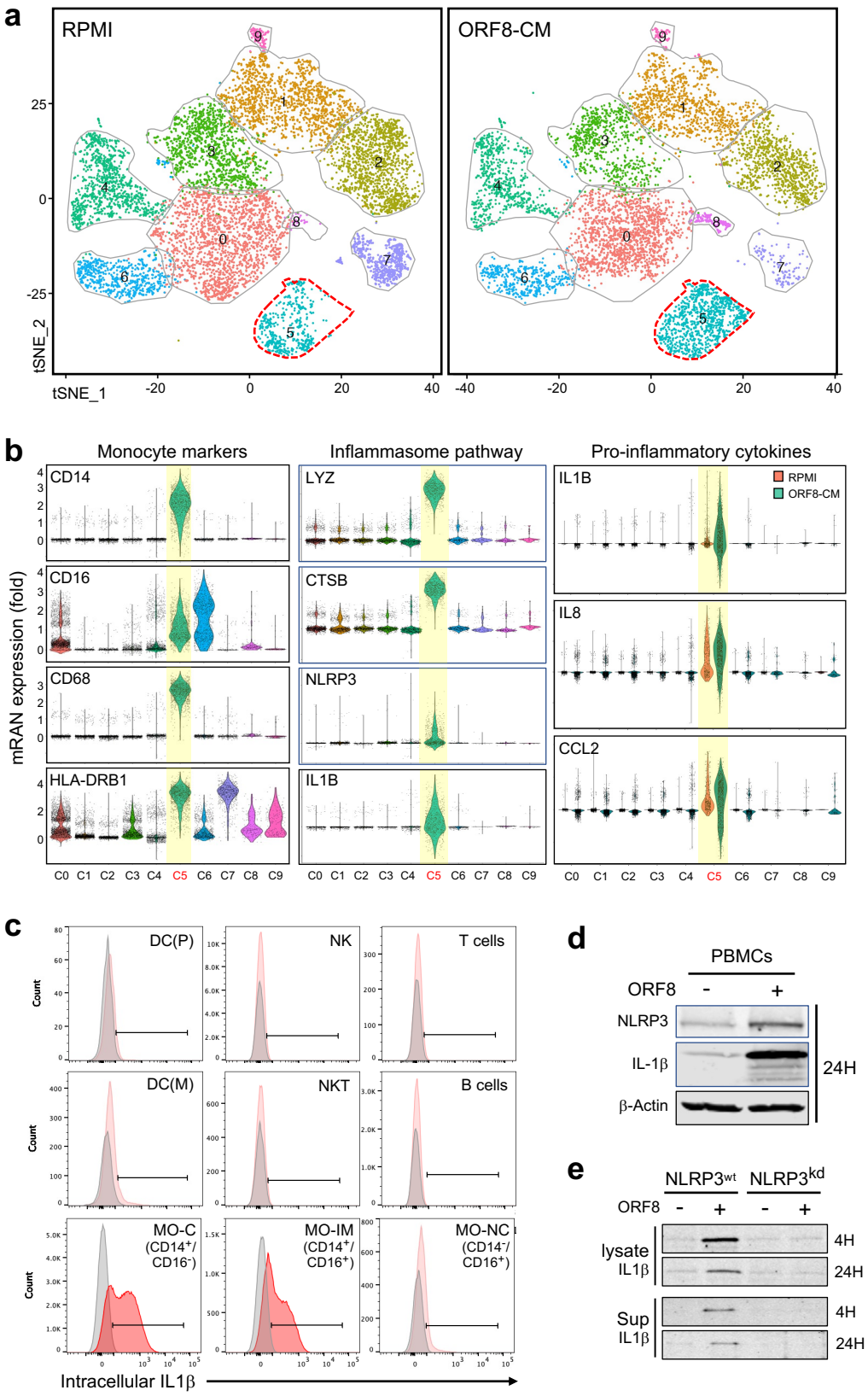

Figure 3. Single cell RNA sequencing of ORF8 treated PBMCs.

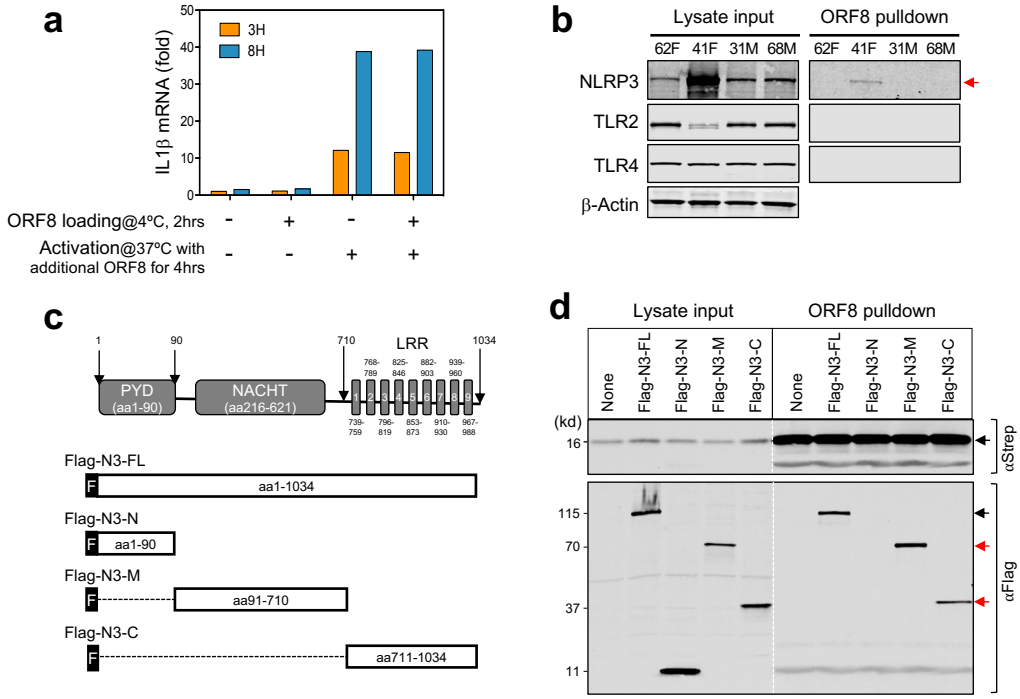

Figure 4. ORF8 induces proinflammatory cytokines through direct binding to NLRP3.

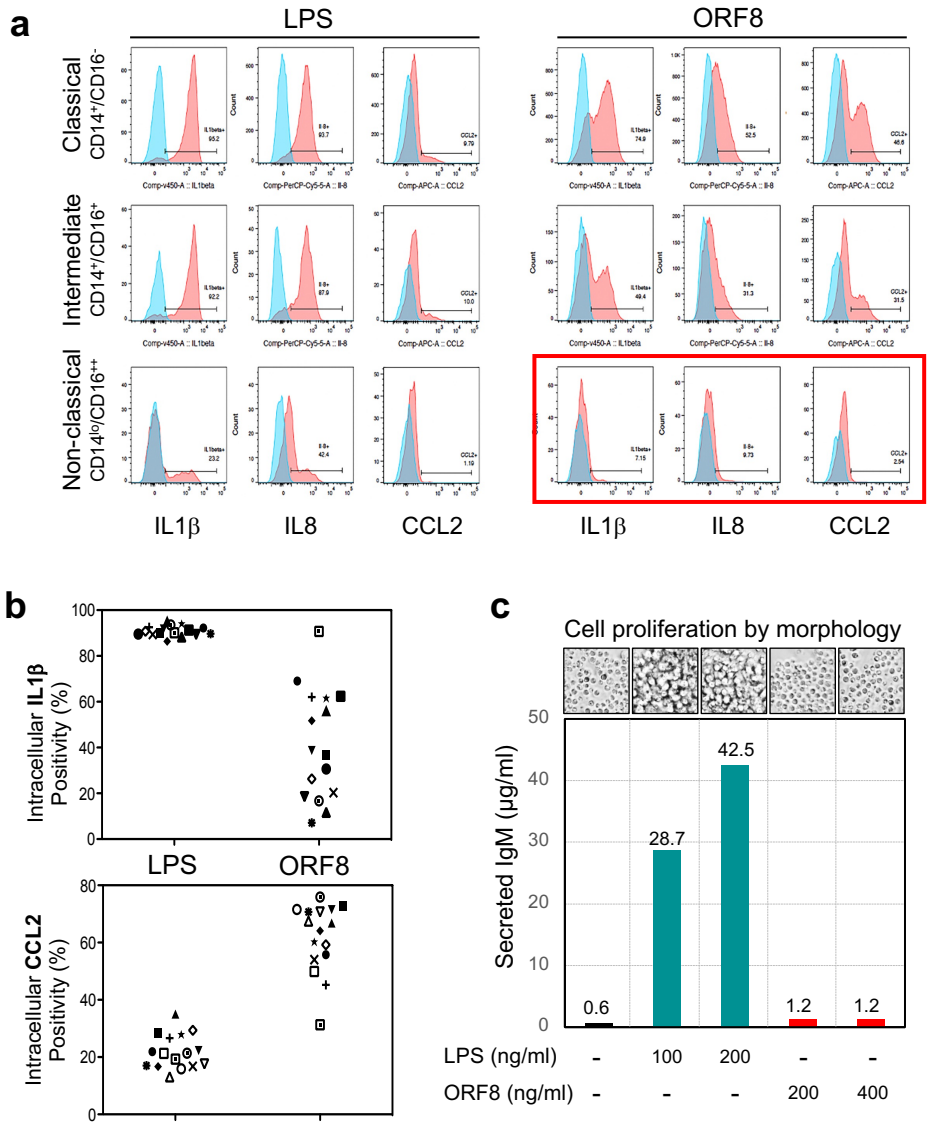

Figure 5. **ORF8 is functionally different from LPS.**

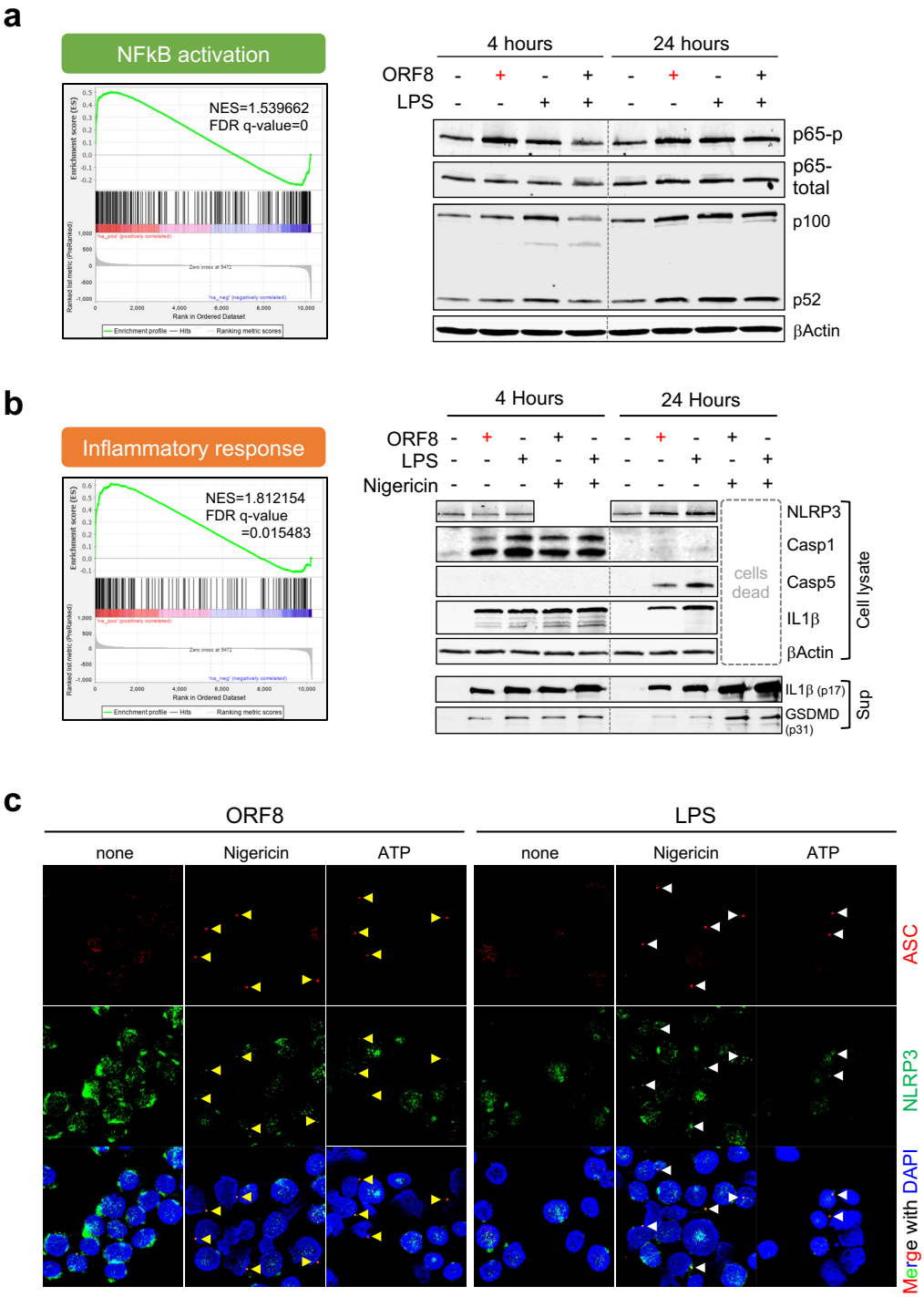

Figure 6. ORF8 induces proinflammatory cytokines through activation of NLRP3 mediated inflammasome pathways.

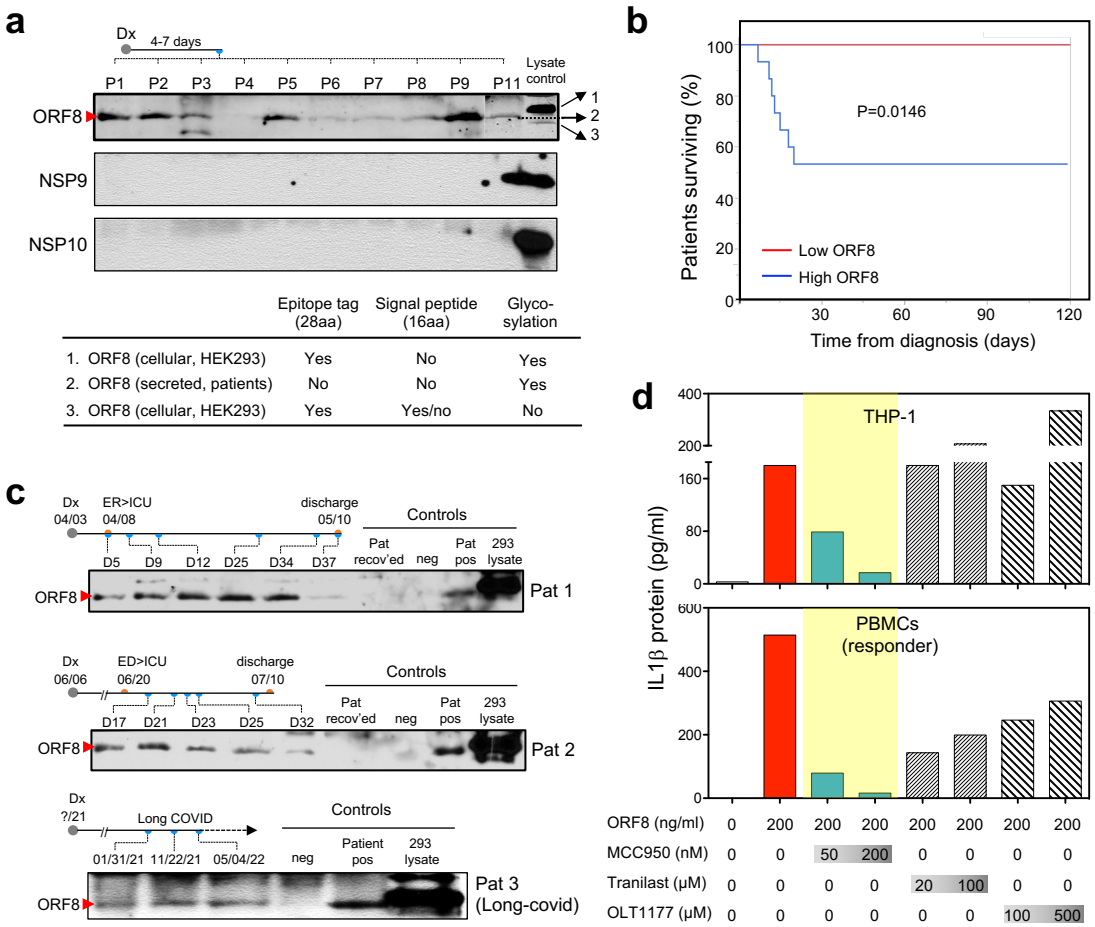

Figure 7. The levels of ORF8 protein in sera of Covid-19 patients correlates with the outcome and disease course, and the ORF8 pathway is targetable with NLRP3 inhibitors.