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Supplemental information

Secreted ORF8 induces monocytic pro-inflammatory cytokines through NLRP3 pathways in patients with severe COVID-19

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Table S1. Top enriched genes expressed in ORF8 treated monocytes (cluster 5). Related to Figure S3

Top 20 genes	Function	ORF8 treated Avg_log2FC	Induction (fold)
CXCL8 (IL8)	Chemokine	5.14	2.01
CCL2	Chemokine	4.92	2.61
IL1β	Pro-inflammatory cytokine	3.53	4.29
CD14	Monocyte marker	3.66	1.16
CD68	Monocyte marker	3.38	0.86
	Inflammasome related		
LYZ	Lysozyme	5.07	0.83
MMP9	Modulator	5.01	1.69
S100A9	Cytokine secretion	4.55	1.38
S100A8	Cytokine secretion	3.82	2.05
FTH1	Ferritin (Acute reactant)	4.39	1.08
FTL	Ferritin (Acute reactant)	3.77	0.82
CTSD	Lysosomal A1 family peptidase	3.82	0.85
CTSB	Lysosomal C1 family peptidase	3.69	0.88
CTSS	Lysosomal C1 family peptidase	3.59	0.80
ACP5	Acid phosphatase 5	3.68	0.96
AIF1	Allograft inflammatory factor 1	3.53	0.96
FUCA1	Lysosomal α-L-fucosidase 1	3.58	0.70
IFI30	Lysosomal thiol reductase	4.50	0.90
CYBB	Microbicidal oxidase subunit	3.79	1.14
FCER1G	Fc receptor subunit	3.77	1.15

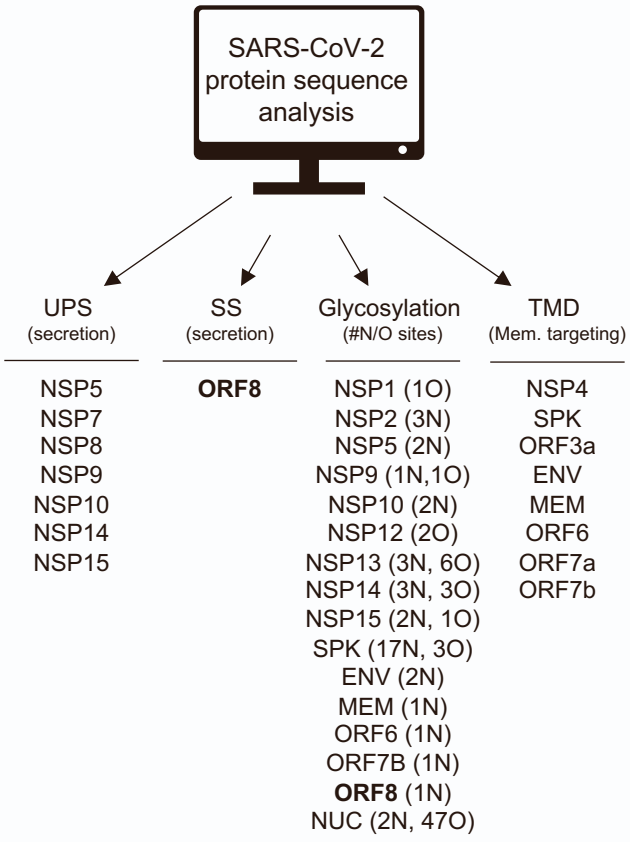

Figure S1. *In silico* analysis of SARS-CoV-2 proteins. Related to Figure 1. Various online tools were used for these analyses, including Outcyte 1.0 [for unconventional protein secretion (UPS) analysis], SignalP 5.0 [for signal sequence (SS) analysis], NetNGlyc 1.0 [for N-linkage glycosylation site prediction], NetOGlyc 4.0 [for O-linkage glycosylation site prediction], and TMHMM 2.0 [for transmembrane domains (TMD) analysis]. Each of their URLs can be found in the key resource table. The number of predicted N or O glycosylation sites are listed in parentheses as (3N) and (2O), respectively.

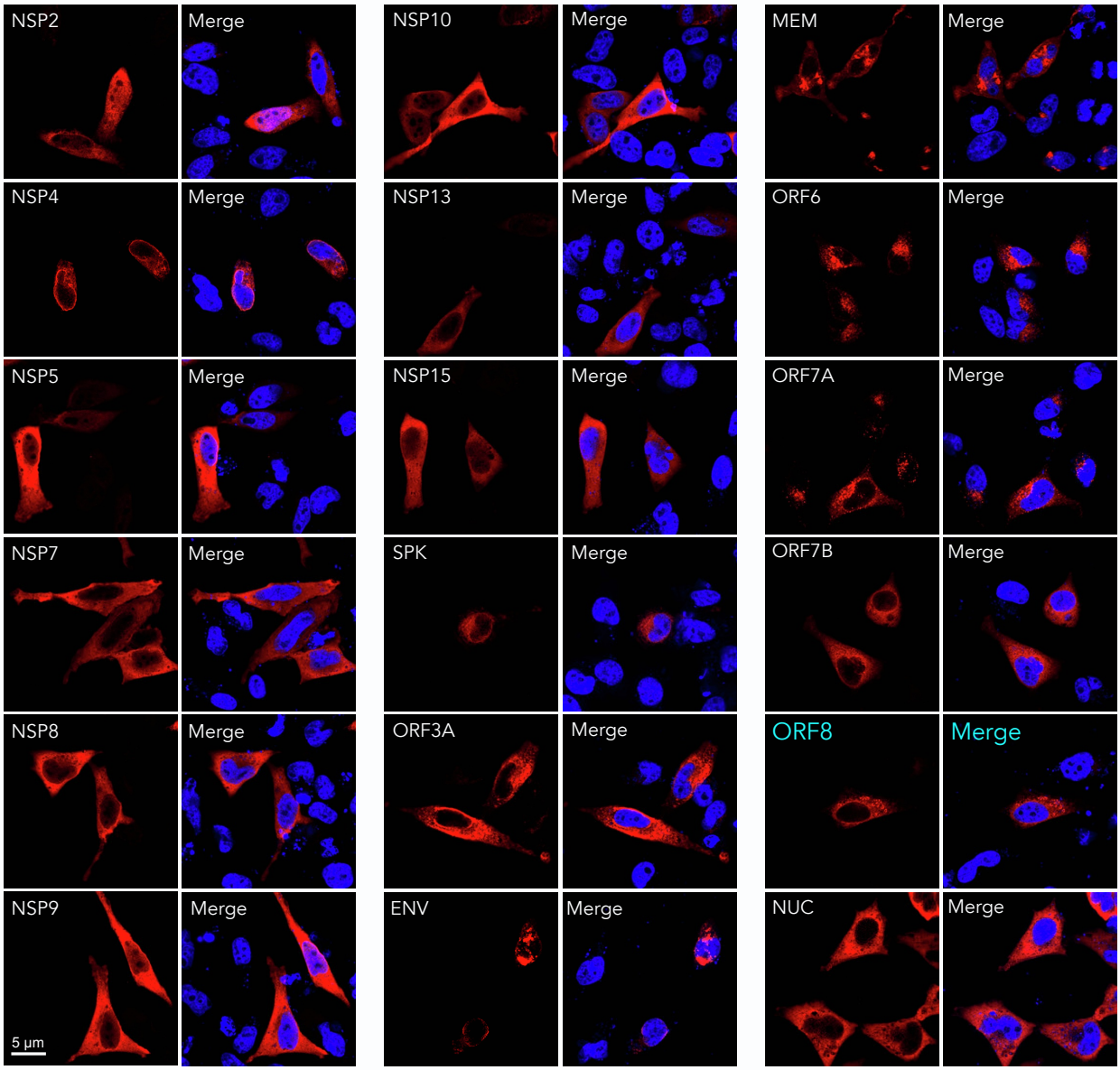

Figure S2. Intracellular localization of major SARS-CoV-2 proteins in HeLa cells. Related to Figure 1. Some of these proteins were not detected by Western blotting likely due to their small sizes or insolubility issues since they were readily detected by immunofluorescence here. ORF8 localized to cytoplasmic vesicular structures is consistent with its secretory nature. Scale bar: 5 µm

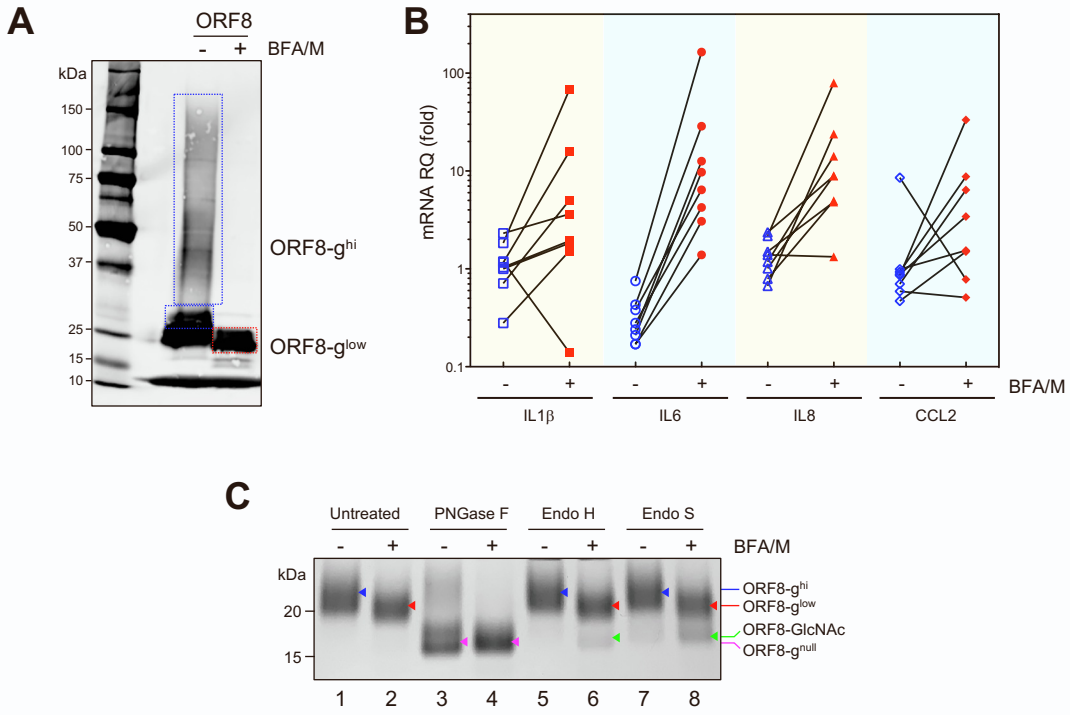

Figure S3. Golgi inhibitors alter the glycosylation and the cytokine induction activity of ORF8. Related to Figure 2C. A. ORF8 protein mobility was affected by the treatment of Golgi inhibitors BFA/M; **B.** Cytokine induction activity of ORF8 was affected by the treatment of BFA/M; **C.** ORF8 glycosylation was validated by treating the pure ORF8 protein with various glycosylases.

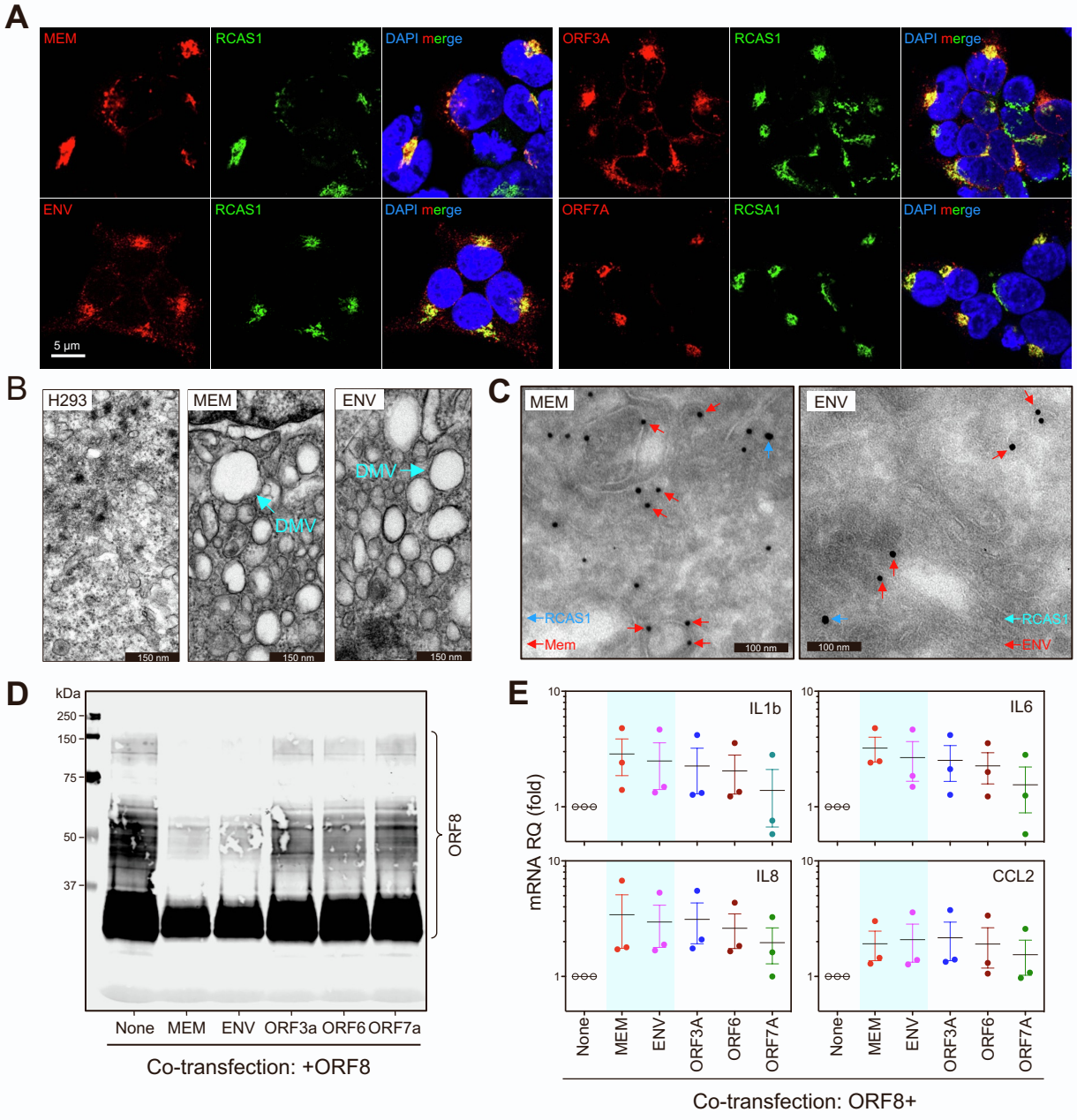

Figure S4. Golgi-localized SARS-CoV-2 proteins affect the glycosylation and cytokine induction activity of ORF8. Related to Figure 2C. (**A**) SARS-CoV-2 proteins MEM, ENV, ORF3A, and ORF7A colocalizing with Golgi protein RCAS1 by immunofluorescence; (**B**) MEM or ENV induces the formation of double-membrane vesicles (DMV) by transmission electron microscopy; (**C**) MEM, ENV co-localized with Golgi protein RCAS1 by immunogold labeling. (**D**) Co-expression of the Golgi-targeting proteins of SARS-CoV-2 affects the glycosylation of ORF8. (**E**) Co-expression of the Golgi-targeting proteins alters the cytokine induction activity of ORF8. Scale bars: 5 μm (**A**), 150 nm (**B**), and 100 nm **(C).**

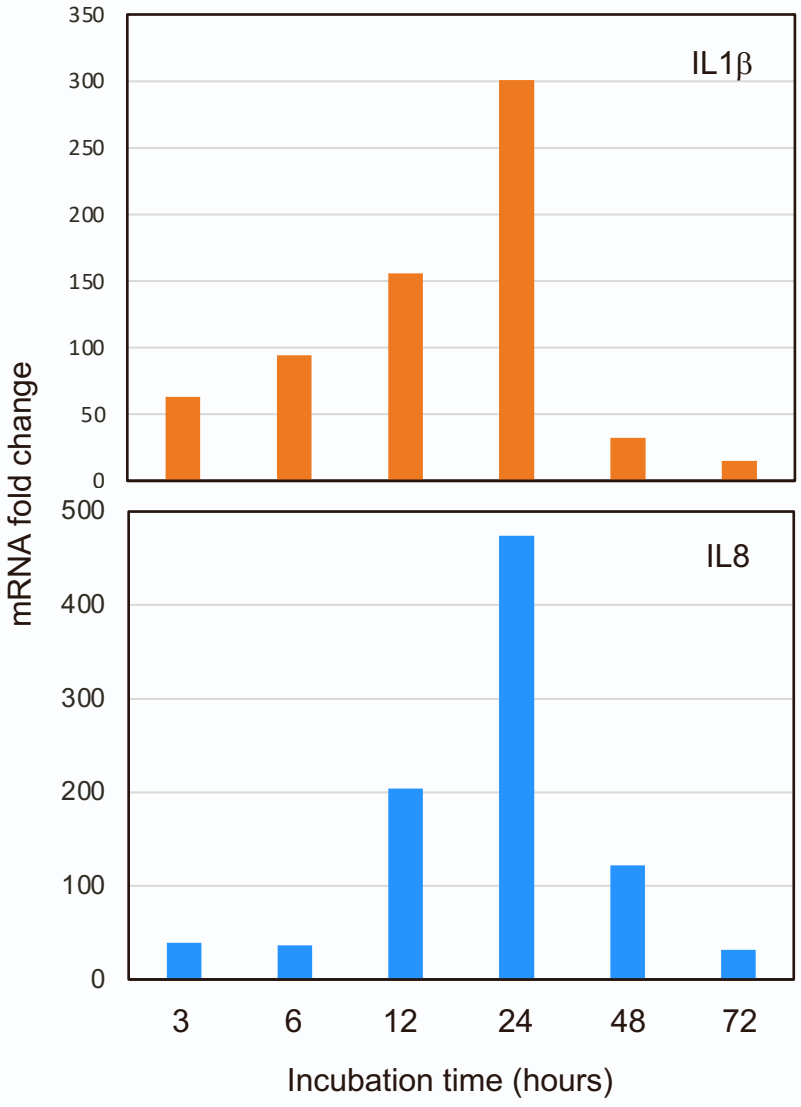

Figure S5. IL1β and IL8 expression kinetics in human monocytes upon stimulation of ORF8. **Related to the STAR methods section.** The mRNA levels of IL1β and IL6 in PBMCs treated with ORF8 (200 ng/ml) at various time points were determined by qPCRs and normalized with *HPRT1* gene expression. Data is representative of six samples.

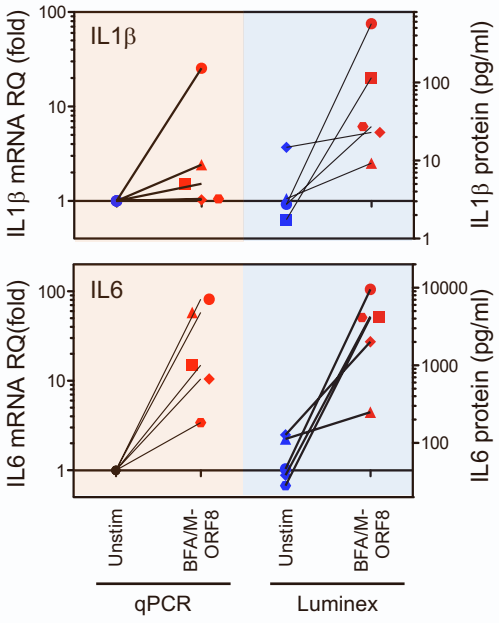

Figure S6. Sensitivity comparison between cytokine protein and mRNA detections. Related to the STAR methods section. Cytokine IL1β and IL6 proteins in the culture supernatant of PBMCs unstimulated or stimulated with ORF8 were quantitated by Luminex assay, while IL1β and IL6 mRNAs isolated from the cells were quantitated by qRT-PCR.