Appendix

SARS-CoV-2 nucleocapsid suppresses host pyroptosis through blocking GSDMD cleavage

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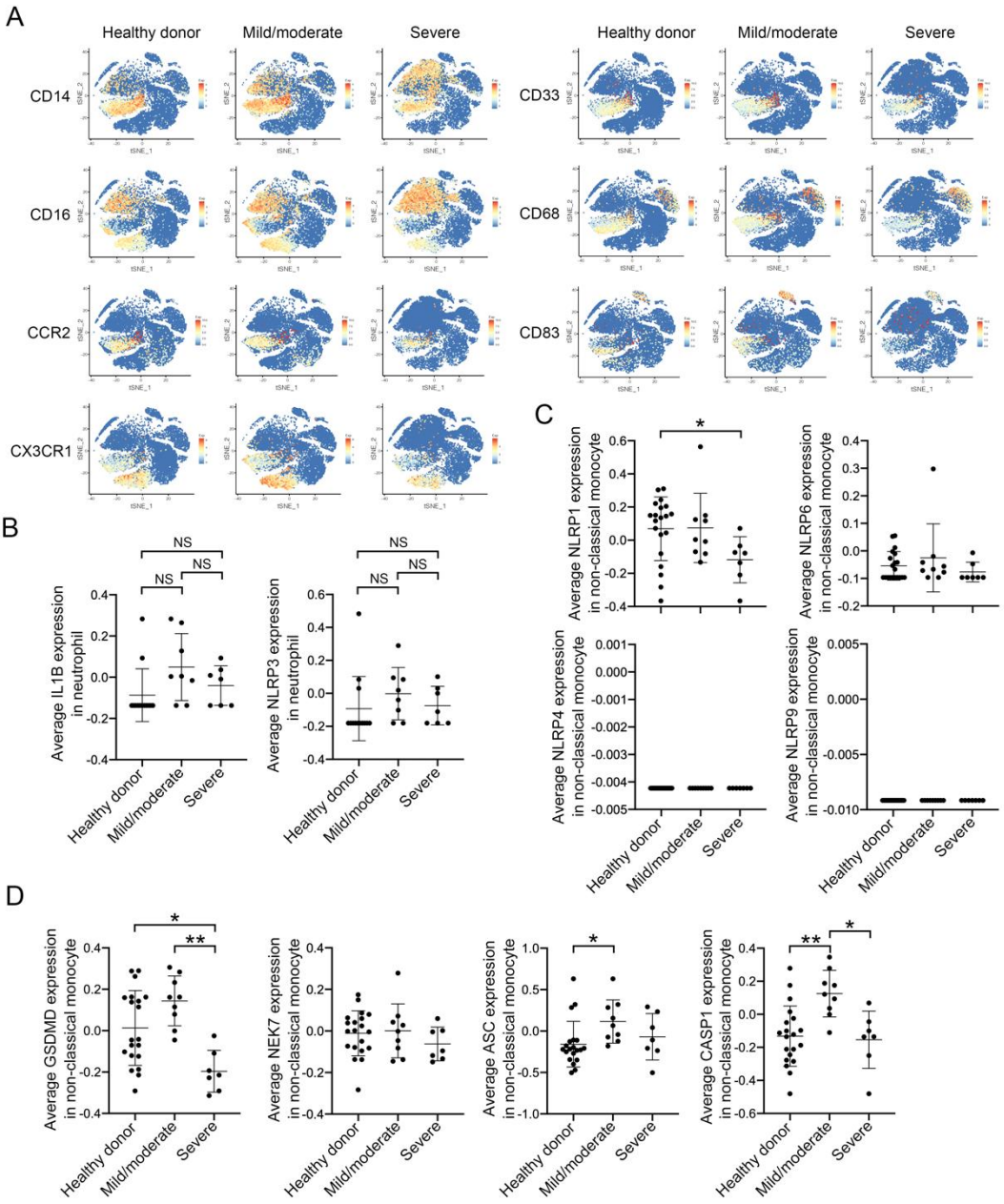
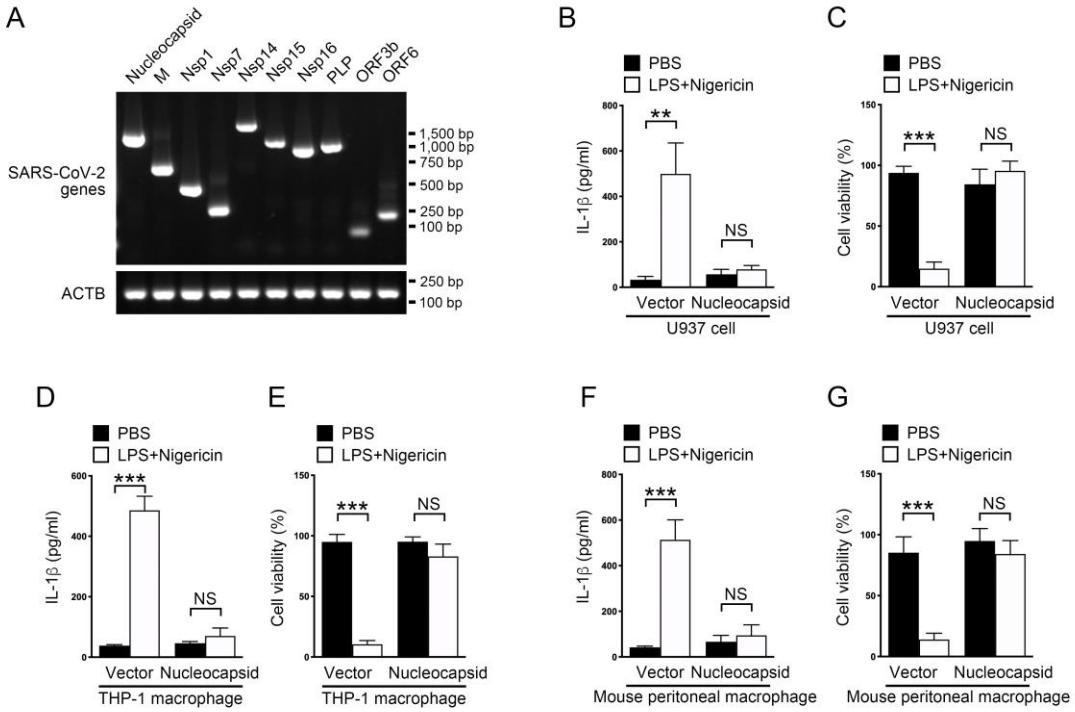


Appendix Figure S1. Genes involved in NLRP3 inflammasome signaling are expressed in COVID-19 patient monocytes.

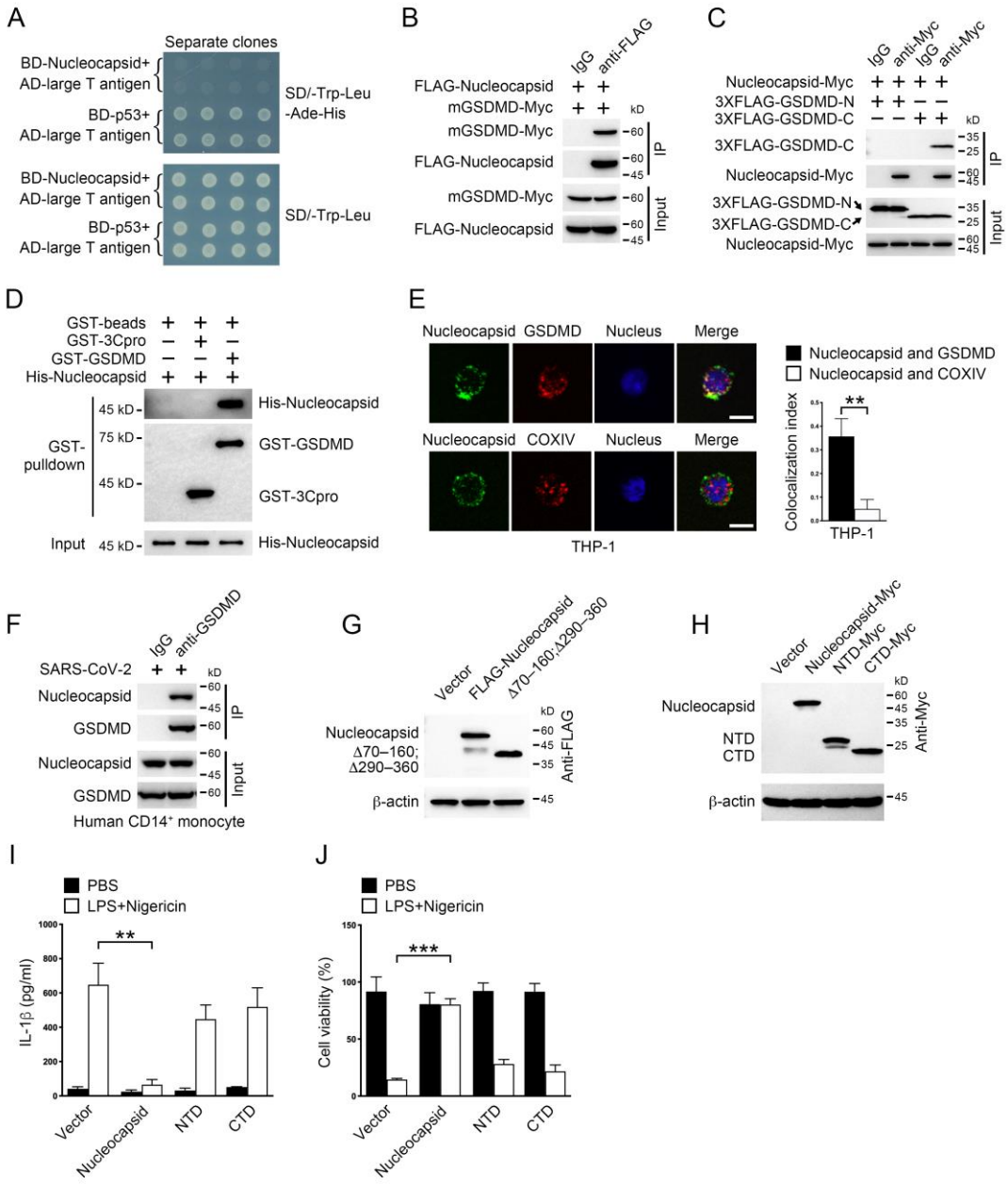
(A–D) Single-cell sequencing data containing PBMCs from health donors, SARS-CoV-2 infected mild/moderate and severe patients (GSE163668) were reanalyzed. n=20 for healthy donor, n=9 for mild/moderate, n=7 for severe. (A) Feature plots of monocyte markers were plotted through projecting their expression levels in each cell to the tSNE distribution map. (B) Average expression levels of IL1B and NLRP3 in neutrophils of healthy donors, mild/moderate and severe patients were calculated. (C) Average expression levels of selected NOD-like receptors in non-classical monocytes of healthy donors, mild/moderate and severe patients were calculated. (D) Average expression levels of the indicated inflammasome participators in non-classical

monocytes of healthy donors, mild/moderate and severe patients were calculated. For B–D, each dot represents an individual. Brown-Forsythe and Welch ANOVA test was used. Data were shown as means±SD. *, $P<0.05$; **, $P<0.01$; NS, non-significant.



Appendix Figure S2. SARS-CoV-2 nucleocapsid protein inhibits pyroptosis in various cell types.

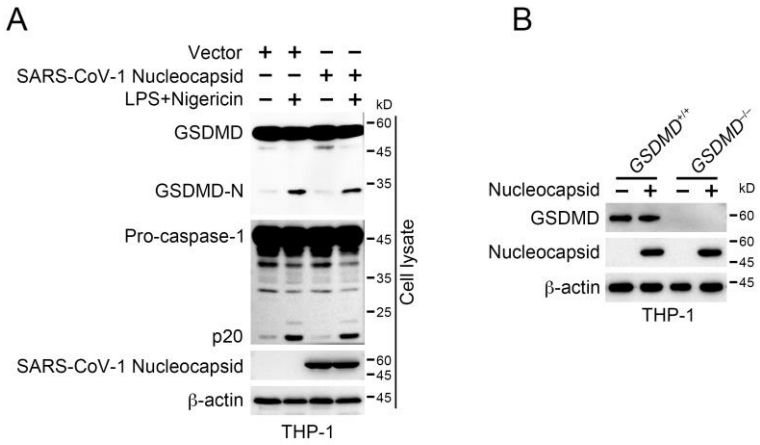
(A) Expressions of the indicated SARS-CoV-2 genes were verified in THP-1 cDNAs through PCR with gene specific primers. (B, C) U937 cells stably expressing the nucleocapsid gene or control vector were stimulated with 10 μM nigericin for 30 min following pretreatment with 1 μg/ml LPS for 3 h. Supernatants were collected and subjected to an ELISA assay for IL-1β levels (B). Cell viability in cell pellets were examined using a cell viability assay through checking ATP levels inside cells (C). (D, E) THP-1 cells stably expressing the nucleocapsid gene or control vector were differentiated into macrophages at the presence of 10 ng/ml PMA for 3 days, followed by stimulation with 1 μg/ml LPS for 3 h and 10 μM nigericin for 30 min. Supernatants were collected and subjected to an ELISA assay for IL-1β levels (D). Cell viability in cell pellets were examined using a cell viability assay through checking ATP levels inside cells (E). (F, G) Mouse peritoneal macrophages were electroporated with plasmids encoding the nucleocapsid gene or control vector for 36 h, followed by stimulation with 1 μg/ml LPS for 3 h and 10 μM nigericin for 30 min. Supernatants were collected and subjected to an ELISA assay for IL-1β levels (F). Cell viability in cell pellets were examined using a cell viability assay through checking ATP levels inside cells (G). For B-G, control vector used here was empty vector corresponded to the nucleocapsid-containing vector. Student's *t*-test was used. Data were shown as means±SD of three technical replicates. **, $P<0.01$; ***, $P<0.001$; NS, non-significant. Similar results were observed for at least three times.



Appendix Figure S3. SARS-CoV-2 nucleocapsid binds GSDMD.

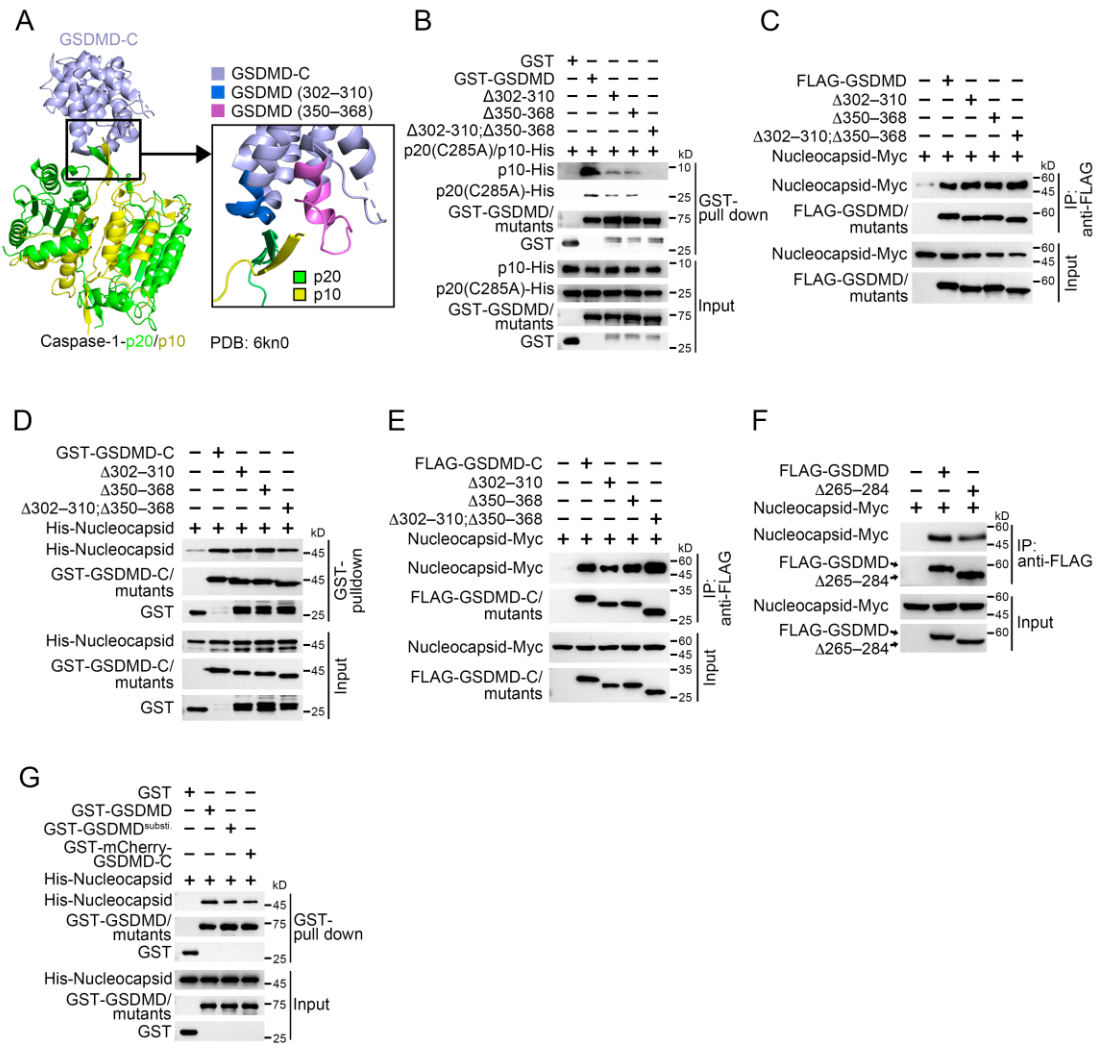
(A) Plasmids encoding DNA binding domain (BD)-tagged nucleocapsid were co-transfected with plasmids encoding activation domain (AD)-tagged large T antigen into yeast strain AH109 and clones grown on medium lacking tryptophan and leucine were further plated into plates lacking another two amino acids adenine and histidine. Plasmids encoding BD-tagged p53 and AD-tagged large T antigen were co-transfected into AH109 as a positive control. Eight separate clones were plated onto the indicated plates. (B) Plasmids encoding FLAG-tagged nucleocapsid and Myc-tagged mouse GSDMD were co-transfected into HEK293T cells for 24 h, followed by immunoprecipitation with antibody against FLAG or isotype IgG. Input and precipitates were immunoblotted as indicated. (C) Plasmids encoding Myc-tagged nucleocapsid and FLAG-tagged GSDMD N-terminus or

C-terminus were co-transfected into HEK293T cells for 15 h, followed by immunoprecipitation with antibody against Myc or isotype IgG. Input and precipitates were immunoblotted as indicated. (D) A GST-pulldown assay was conducted through mixing GST-GSDMD or control GST-3C protease (GST-3Cpro) with His-tagged nucleocapsid, followed by immunoblotting with antibodies against the indicated protein tags. GST-GSDMD, GST-3Cpro and His-nucleocapsid were purified from *E. coli* stain BL21. (E) THP-1 cells were transfected with plasmids encoding nucleocapsid for 24 h, followed by cell fixation, permeabilization and stain with antibodies against nucleocapsid and GSDMD or COXIV. The nucleus was counterstained with DAPI (left panel). Scale bar: 5 μm. Colocalization index between nucleocapsid and GSDMD or COXIV were calculated (right panel). (F) Sorted human $CD14^+$ monocytes were incubated with SARS-CoV-2 virus at an MOI of 1 for 1h before extracellular viruses were washed away. Cells were further cultured for 3 h, followed by immunoprecipitation with antibody against GSDMD or isotype IgG. Input and precipitates were immunoblotted as indicated. (G) THP-1 cells stably expressing full-length nucleocapsid or the truncation lacking amino acids 70–160 and 290–360 were lysed and immunoblotted as indicated. (H) THP-1 cells stably expressing full-length nucleocapsid, the NTD or CTD domain were lysed and immunoblotted as indicated. (I, J) THP-1 cells stably expressing full-length nucleocapsid, the NTD or CTD domain were stimulated with 10 μM nigericin for 30 min following pretreatment with 1 μg/ml LPS for 3 h. An ELISA assay was performed to determine supernatant IL-1β levels (I) and a cell viability assay was conducted to verify the percentage of viable cells through checking cellular ATP levels (J). Control vector used here was empty vector corresponded to the nucleocapsid-containing vector. For E, I, J, Student's *t*-test was used. Data were shown as means±SD of three technical replicates. **, $P<0.01$; ***, $P<0.001$. Results were obtained from at least three independent experiments.



Appendix Figure S4. SARS-CoV-1 nucleocapsid does not inhibit pyroptosis.

(A) THP-1 cells stably expressing SARS-CoV-1 nucleocapsid or control vector were stimulated with 10 μM nigericin for 30 min following pretreatment with 1 μg/ml LPS for 3 h. Cells were immunoblotted with antibodies against the indicated proteins. Control vector used here was empty vector corresponded to the SARS-CoV-1 nucleocapsid-containing vector. (B) *GSDMD*$^{+/+}$ and *GSDMD*$^{-/-}$ THP-1 cells stably expressing SARS-CoV-2 nucleocapsid were lysed and immunoblotted as indicated. Experiments were repeated for three times with similar results.



Appendix Figure S5. SARS-CoV-2 nucleocapsid does not compete with the p20/p10 complex for binding GSDMD.

(A) Crystal structures showed speculated GSDMD domains responsible for p20/p10 binding. (B) The indicated GST-tagged GSDMD variants were incubated with His-tagged p20 (C285A) and p10, followed by a GST-pulldown assay. Input and precipitates were immunoblotted with antibodies against the indicated protein tags. (C) Plasmids encoding FLAG-tagged GSDMD or the indicated truncations were co-transfected with Myc-tagged nucleocapsid into HEK293T cells, followed by immunoprecipitation with antibody against FLAG. Input and precipitates were immunoblotted with antibodies against the indicated protein tags. (D) Recombinant GST-GSDMD C-terminus or GSDMD truncations was incubated with recombinant nucleocapsid, followed by a GST-pulldown assay. Input and precipitates were immunoblotted with antibodies against the indicated protein tags. (E) Plasmids encoding FLAG-tagged GSDMD C-terminus or the indicated truncations were co-transfected with Myc-tagged nucleocapsid into HEK293T cells, followed by immunoprecipitation with antibody against FLAG. Input and precipitates were

immunoblotted with antibodies against the indicated protein tags. (F) Plasmids encoding GSDMD or GSDMD mutant lacking the linker region were co-transfected with nucleocapsid into HEK293T cells, followed by immunoprecipitation with antibody against FLAG. Input and precipitates were immunoblotted with antibodies against the indicated protein tags. (G) GST-tagged Wild-type GSDMD, $GSDMD^{susti.}$ or mCherry-GSDMD-C recombinant proteins were incubated with His-nucleocapsid protein, followed by a GST-pulldown assay. Input and precipitates were immunoblotted with antibodies against the indicated protein tags. Experiments were repeated for three times with similar results.