The SARS-CoV-2 spike protein primes inflammasome-mediated interleukin-1- beta secretion in COVID-19 patient-derived macrophages

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Abstract

Innate immunity triggers responsible for viral control or hyperinflammation in COVID- 19 are largely unknown. Here we show that the SARS-CoV-2 spike protein primes inflammasome activation and interleukin 1-beta (IL-1β) secretion in macrophages derived from COVID-19 patients but not in macrophages from healthy SARS-CoV-2 naïve controls. Chemical NLRP3 inhibition blocks spike protein-induced IL-1β secretion *ex vivo*. These findings can accelerate research on COVID-19 vaccine design and drug treatment.

Main Text

Since December 2019, coronavirus disease 2019 (COVID-19) has affected more than 3 million people globally 1. The disease is caused by infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a novel coronavirus. In COVID- 19, little is known about protective or detrimental immune responses making rational therapeutic interventions difficult to assess. A subset of patients fails to control initial viral replication some of which present with severe pneumonia, signs of hyperinflammation and excessive release of cytokines in a second phase of the disease 2. This second phase immune response represents a putative target for host directed therapeutic intervention and several approaches such as blockade of the interleukin-6 receptor or inhibition of Janus kinases are being tested in clinical trials 2. However, knowledge on triggers of the SARS-CoV-2 specific inflammatory response and key cytokines that are involved is scarce. Our data and those of others show that the major pro-inflammatory cytokine Interleukin-1-beta (IL-1β) is elevated in plasma from hospitalized COVID-19 patients and its associated signaling pathway seems to drive SARS-CoV-2 pathogenicity 3, 4 (Figure S1A).

IL-1β secretion is primarily initiated by inflammasomes that represent multiprotein signaling platforms responsible for the coordination of the early antimicrobial host defense 5. Inflammasomes are assembled by pattern-recognition-receptors such as the NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) following the detection of pathogenic microorganisms or danger signals in the cytosol of host cells. Upon activation, these receptors initiate the oligomerization of the adaptor protein ASC, serving as an activation platform for caspase 1. Active caspase 1 in turn cleaves pro- IL-1β yielding the mature active IL-1β which can be subsequently secreted. NLRP3 inflammasome activation is a two-step process. In a priming step, cellular receptors recognize conserved pathogen-associated molecular patterns (PAMPs) leading to pro-IL-1β, and pro-IL-18 expression. The activation step required for inflammasome assembly and secretion of mature IL-1β is triggered by a range of intrinsic or pathogen derived stimuli such as ATP, microbial toxins (e.g. nigericin), nucleic acids or vaccine adjuvants 6, 7, 8. For SARS-CoV-2, priming and activation triggers are unknown. We speculated that the ACE-2 receptor-binding spike glycoprotein (S-protein), a major SARS-CoV-2 antigen and focus of therapeutic strategies and vaccine design, may function as a PAMP leading to IL-1β secretion in patient derived macrophages. To test this hypothesis, we affinity purified the SARS-CoV-2 S-protein lacking the transmembrane domain (Fig. 1a) 9. The purified S-protein specifically bound COVID-19 patient-derived IgG but not IgG from SARS-CoV-2 naïve controls confirming selective reactivity with patient derived antibodies (Fig. 1a). Next, we isolated peripheral blood

mononuclear cells (PBMC) of six hospitalized COVID-19 patients and six SARS-CoV-2 naïve healthy controls followed by positive selection of CD14+ monocytes which were differentiated to macrophages by incubation with M-CSF (patient characteristics are provided in the supplementary Tables 1a and 1b and supplementary Fig. S1a). Overall monocyte counts and phenotypes were similar in both patients and controls (Supplementary Fig. 2a and 2b). In a second step, isolated macrophages were stimulated with SARS-CoV-2 S-protein followed by addition of nigericin as the inflammasome activating signal (Fig. 1c). We show that the S-protein potently triggers secretion of IL-1β into the cell supernatants of patient derived macrophages after sequential incubation with nigericin (Fig. 1d). Intriguingly, cells from SARS-CoV-2 naïve healthy controls were non-reactive towards the S-protein (Fig. 1D). In contrast to S-protein, lipopolysaccharide (LPS), a classical PAMP for priming inflammasome activation, induced IL-1β secretion in both groups indicating functional inflammasome signaling pathways (Fig. 1d). Gene expression analysis by quantitative real-time PCR revealed an S-protein induced increase of *IL-1β* mRNA levels in macrophages derived from COVID-19 patients and, to a much lesser extent, from SARS-CoV-2-naïve controls (Fig. 1e). *IL-1β* mRNA levels were significantly higher in COVID-19 patient-derived cells indicating that differential regulation of IL-1β secretion in patients versus SARS-CoV-2 naïve controls occurs on the transcriptional level (Fig. 1e). Macrophage treatment with LPS, nigericin or S-protein alone had no effect on IL- 1β secretion showing that the S-protein solely functions as an NLRP3-inflammasome priming signal requiring a second stimulus for IL-1β secretion (Fig. 1d).

In line with this, we found that MCC950, a selective NLRP3 inhibitor fully blocked IL- 1β secretion in S-protein stimulated patient cells indicating that inflammasome inhibitors may provide valuable therapeutic tools for COVID-19 patients by preventing hyperinflammatory syndromes (Fig. 2a) 10. In addition, hydroxychloroquine, a drug known for its immune-modulatory effects showed similar suppressive effects on S- protein primed patient cells and, to a much lesser extent, on LPS stimulated cells (Fig. 2A, Fig. S2C). Hydroxychloroquine has an ill-defined, most likely host cell-directed inhibitory effect on viral replication *in vitro* 11. Several clinical trials are currently investigating this drug for treatment and prevention of COVID-19.

Furthermore, we show that the S-protein also triggers release of NLRP3-independent pro-inflammatory cytokines such as IL-8, IL-6 and tumor necrosis factor α (TNFα) (Fig. 2b). In contrast to IL-1β, these cytokines were secreted by both COVID-19 patient- derived cells and by cells isolated from SARS-CoV-2 naïve controls in an LPS-like manner (Fig. 2b). This indicates that in macrophages, the S-protein primed NLRP3 inflammasome is differentially regulated depending on previous exposure to SARS- CoV-2 whereas other pro-inflammatory signaling pathways are activated non- selectively.

We revisited our finding of SARS-CoV-2 naïve macrophages being non-reactive towards the S-protein and tested cells derived from COVID-19 convalescent individuals, which had only mild disease manifestations (patient characteristics can be found in the supplementary Table 1c). Interestingly, macrophages from these individuals showed elevated IL-1β secretion upon stimulation similar to hospitalized patients (Fig. 2c). Two convalescent individuals were tested sequentially, 7 days after initial sampling. Here, S-protein dependent IL-1β detected in cell supernatants remained high, but levels had declined in both patients

within the 7-day period while IL-1β levels of LPS treated cells were stable (Fig. 2d). Assuming that the S-protein functions as a classical PAMP, there seems to be a certain degree of trained innate immunity in individuals having survived COVID-19 and only little or no S-protein driven inflammasome activation in cells derived from SARS-CoV-2 naïve individuals. The latter may be a surrogate for failing early viral control in host tissue which is mainly driven by the inflammasome and IL-1β as first lines of defense 8, 12, 13. However, once patients are infected, macrophages become highly reactive, secreting large amounts of IL-1β (Fig. 1C). Here, the NLRP3 inflammasome may contribute to pathophysiology and exuberant inflammation as shown for influenza A virus infection or acute respiratory distress syndrome (ARDS) 14, 15.

In conclusion, we provide first *ex vivo* evidence for a SARS-CoV-2 structural component being a PAMP and driver of pro-inflammatory cytokine secretion. The S- protein as the major antigen of most vaccine constructs currently under investigation seems to have a dual role in both adaptive and innate immunity. Inflammasome formation is crucial for vaccine immunogenicity and for mounting an effective humoral immune response 7. Intriguingly, and possibly relevant for vaccine development, S- protein driven inflammasome activation seems to require prior SARS-CoV-2 *in vivo* priming since naïve individuals failed to secrete IL-1β when their macrophages were exposed to S-protein *ex vivo*. Pathogen or vaccine exposure-dependent inflammasome activation is known in the context of trained immunity and epigenetic reprogramming of monocytes, for example after vaccination with BCG 16, 17. However, it has not been shown yet for a viral infection and a correlating PAMP.

Our data also indicate that patients with severe, SARS-CoV-2-induced hyperinflammatory syndrome may benefit from treatment with IL-1 receptor antagonists or small molecules targeting inflammasomes.

Thus, our findings are highly relevant for further research on SARS-CoV-2 derived triggers of innate immunity pathways required for rational designs of urgently needed therapeutic and preventive measures.

Methods

Patient samples and CD14+ monocyte isolation

Blood samples were obtained from patients with proven SARS-CoV-2 infection at the University Hospital Cologne, Department I of Internal Medicine and from healthy donors. Infections were diagnosed by PCR from respiratory samples. For all samples, written informed consents approved by the ethics committee of Cologne were available in accordance to the Declaration of Helsinki. PBMCs (peripheral blood mononuclear cells) were purified by density gradient centrifugation (Ficoll Plus, GE Healthcare, Chicago, IL, USA). CD14+ cells were isolated from PBMCs by depletion of non- monocytes, using a monocyte isolation kit (Miltenyi Biotech, Bergisch Gladbach, Germany). 5×104 CD14+ cells were seeded into 96-well plates (TPP Techno Plastic Products AG, Trasadingen, Switzerland) and cultured for additional 4 days in Roswell Park Memorial Institute (RPMI) 1640 Medium (Thermo Fisher Scientific) containing 10% fetal

bovine serum (Thermo Fisher Scientific) and 50 ng/ml M-CSF (Miltenyi Biotec) for macrophage differentiation at 37°C and 5 % CO2.

SARS-CoV-2 spike protein expression and purification

A prefusion S ectodomain coding pαH plasmid 9, was used for recombinant protein expression. The coding sequence comprises the aminoacids 1–1208 of 2019-nCoV S (GenBank: MN908947), has two proline substitutions at residues 986 and 987, a “GSAS” substitution at residues 682–685 to remove the furin cleavage site, a C- terminal T4 fibritin trimerization motif, a Twin-Strep-tag, and an 8xHis-tag at the C- terminus. 1 L of HEK293-6E cells in FreeStyle 293 medium (Thermo Fisher Scientific) at a cell density of 0.8×10^6 cells/ml were transfected with polyethylenimine (PEI, Sigma-Aldrich) and 1 µg DNA per 1 mL cell culture medium. Cells were incubated for 7 days at 37°C and 5 % CO2. Supernatants were harvested by centrifugation and filter sterilized through 0.45 µm polyethersulfone (PES) Filter (Thermo Fisher Scientific). Recombinant protein was purified by Strep-Tactin affinity chromatography (IBA lifescience, Göttingen, Germany) according to the Strep-Tactin XT manual at 4°C Buffer of pooled elution fractions was exchanged to PBS pH 7.4 (Thermo Fisher Scientific) by filtrating 4 times with a 100 kDa cutoff regenerated cellulose centrifugal filter (Merck).

SARS-CoV-2 spike binding assay

For analysis of IgG interaction with SARS-CoV-2 protein, high binding 96-well ELISA plates (Corning Inc., Corning, NY, USA) were coated with SARS-CoV-2 spike protein (5 µg/ml) at 4°C overnight, washed 3x with PBS and blocked with PBS, containing 5% BSA (Carl Roth, Karlsruhe, Germany) for 60 min at RT. Thereafter, IgGs were tested at 3-fold dilutions (1:2) starting at concentrations of 166 µg/ml in PBS/5 % BSA for 120 min at RT. IgGs were isolated with Protein G Sepharose® 4 Fast Flow (GE Healthcare). The plates were washed 3x and incubated with horseradish peroxidase- conjugated goat anti-human IgG antibody (Jackson ImmunoResearch West Grove, PA, USA; 1:2500 in PBS/5 % BSA) for 60 min at RT. ELISAs were developed using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) solution (ABTS, Thermo Fisher Scientific) and absorbance (OD 415 nm - 695 nm) was measured with absorbance reader (Tecan, Männedorf, Switzerland).

Inhibitor experiments with *ex vivo* macrophages stimulated with the SARS-CoV- 2 spike protein

Medium of differentiated macrophages was exchanged and macrophages were incubated for 2 h at 37 °C and 5 % CO2 with either DMSO (Sigma-Aldrich, St. Louis, MO, USA), MCC950 (10µM, Sigma-Aldrich), hydroxycholorquinesulfate (HCQ; 10µM and 30µM) (APEXBIO, Houston, TX, USA) for inflammasome inhibition. Afterwards, lipopolysaccharide (0,5 µg/ml, LPS; Sigma-Aldrich) or SARS-CoV-2 spike protein (0,1 µg/ml) were added for additional 4 h in order to prime the inflammasome process. To activate IL-1β secretion nigericin (5 µM, Sigma-Aldrich) was added for 2 h at 37 °C and 5 % CO2. All assays were performed in technical duplicates. Supernatants were frozen down at -80°C for subsequent cytokine analysis. Macrophages seeded in 96-well plates were covered with in total 50 µl RLT buffer (Qiagen, Hilden, Germany) and frozen down at -80 °C for gene expression analysis.

IL-1β ELISA

IL-1β ELISA (BioLegend, San Diego, CA, USA) was performed according to manufactures manual. Briefly, supernatant was diluted 1:50 in IL-1β ELISA Kit diluent and incubated for 2h on previously coated 96-well ELISA plates (Thermo Fisher Scientific). All samples were measured in technical duplicates (inhibition assay was performed in technical duplicates) and concentration was determined with a corresponding standard curve provided by IL-1β ELISA kit. OD was determined with Hidex Sense microplate reader (Hidex, Turku, Finland). Data was analysed with Microsoft Excel (Microsoft, Redmond, WA, USA) and GraphPad Prism 8.0.2 software (GraphPad, San Diego, CA, USA).

Flow cytometry

Single cell suspensions were prepared from PBMCs and surface antigens were stained with fluorescently labelled antibodies: CD14 BV421 (M5E2) (BioLegend), CD16 APC-Cy7 (B73.1) (BioLegend), CD11c FITC (MJ4-27G12) (Miltenyi), HLA-DR PerCP (AC122) (Miltenyi), CD86 PE (IT2.2) (BioLegend), CD206 (19.2) (Becton Dickinson (BD), Franklin Lakes, NJ, USA) (all in a 1:100 dilution). Data was acquired on a MACSQuant 10 flow cytometer (Miltenyi) . FlowJo (v10.6.2, FlowJo, LLC, Ashland, OR, USA) was used for data analysis and presentation.

Cytokine detection assay

Cytokine quantification in EDTA-treated Plasma and supernatants of macrophages stimulation experiments were performed with the Human Inflammatory Cytokine Kit from BD.. Plasma samples and primary cell culture supernatants were diluted (1:2) with assay buffer and incubated with capture beads and PE detection reagent (all BD) for 1.5 h (plasma samples) or 3 h (supernatant samples) according to the manufacturer's instructions. Data was acquired on a MACSQuant 10 flow cytometer (Miltenyi) and analyzed with FlowJo (v10.6.2, FlowJo)) (geometric mean fluorescence intensity (MFI) of each capture bead population). Cytokine concentrations were calculated by Microsoft Excel (Microsoft) with a standard curve of the MFI using provided cytokine standards.

Gene expression analysis

For gene expression analysis, RNA from 1x105 macrophages were isolated using the RNeasy Mini Kit (Qiagen) in accordance to the manufacturer’s instructions. Subsequently, cDNA was generated by reverse transcription with a Quantitect reverse transcription kit (Qiagen).

Quantitative real time PCR was used to measure expression levels of indicated genes. Samples were measured in technical triplicates in a 96 well-plate Multicolor Real-Time PCR Detection System (IQ™5, BIO-Rad) using LightCycler®SYBR-Green I Mix (Roche, Basel, Switzerland). Data analysis was done based on linear regression of the logarithmic fluorescence values/cycle with the pogram LinRegPCR and target gene expression was normalized to the reference gene *Actin*.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 8.0.2 software (GraphPad, San Diego, CA, USA). Statistical parameters (value of n, statistical calculation etc.) are stated in the figure legend. P-values less than or equal to 0.05 were considered statistically significant.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request. The authors declare that the data supporting the findings of this study are available within the paper and its supplementary information files.

Declarations

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Author contributions

S.J.T. and A.S. contributed samples, planned and performed experiments, analyzed data and wrote the manuscript; C.K. and M.Z. performed experiments, analyzed data and wrote the manuscript; J.F., MC.A., J.G., S.W. and U.S. performed experiments, O.C., B.B., P.K., J.M., H.G., I.S., M.H., G.F., N.J., H.K., O.A.C were involved in clinical care of patients, provided biosamples and discussed data, C.L., F.K. planned experiments, analyzed and discussed data; J.R. directed the study and wrote the manuscript.

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Declaration of interests: The authors declare no competing interests

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Figures

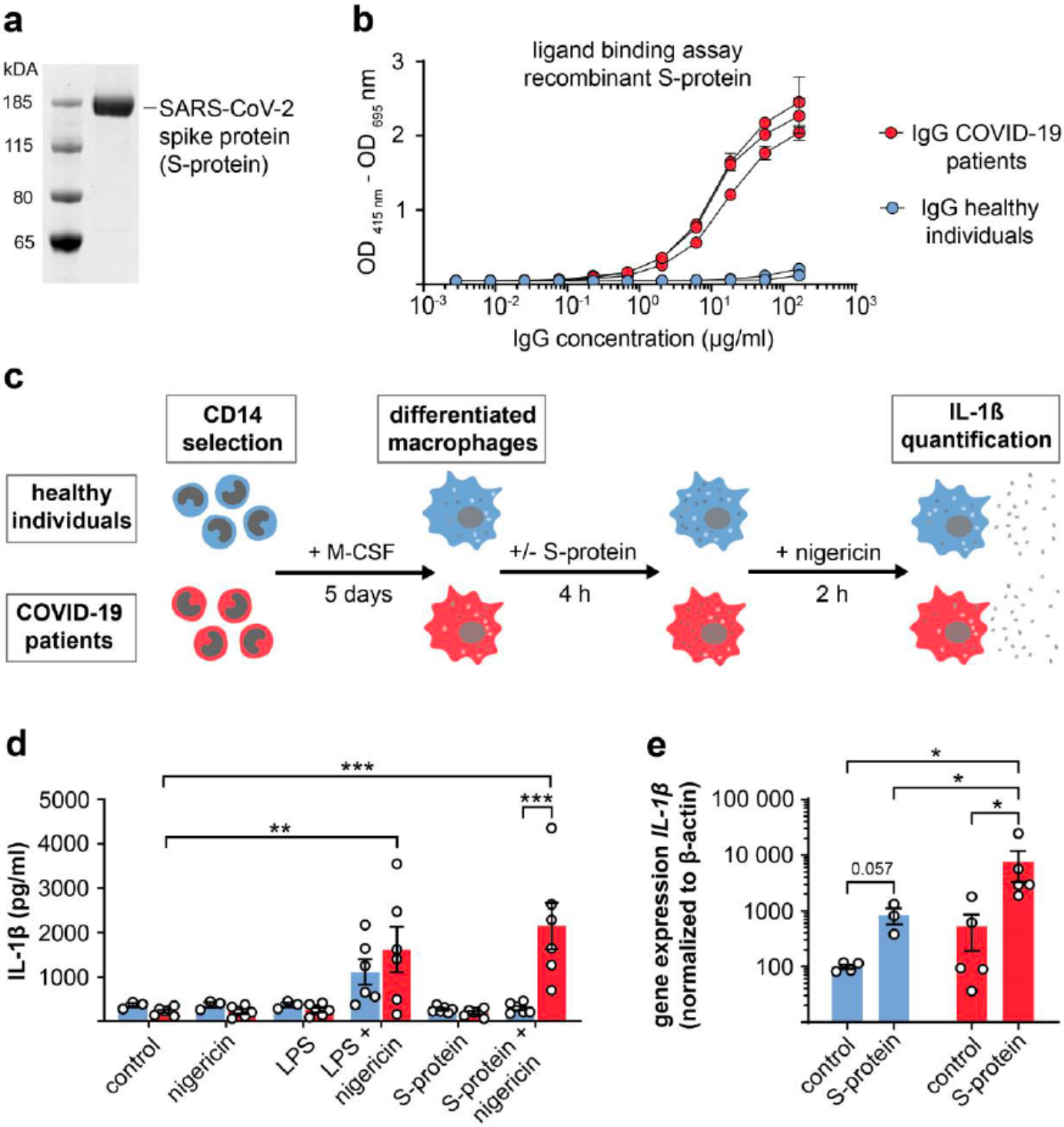

Figure 1

a SDS PAGE of the recombinant 2019-nCoV spike protein (S-protein). b SARS-CoV-2 spike binding assay of IgGs isolated from COVID-19 patients (n = 3; red circles) or healthy individuals (n = 2; blue circles). c Experimental scheme: After PBMCs isolation, CD14+ cells were enriched by negative selection. Subsequently, 5x104 CD14+ cells/well were seeded and incubated in the presence of M-CSF for 5 days. Differentiated macrophages were stimulated with/without recombinant SARS- CoV-2 spike protein or lipopolysaccharide (LPS) for 4h. To activate IL-1β secretion nigericin was added for 2h. Finally, IL-1β secretion was quantified by ELISA. d Quantification of IL-1β concentration in the supernatant of primary macrophage cultures from COVID-19 patients (n = 6; red bars) or healthy individuals (n = 6; blue bars) stimulated with LPS or S-protein. For statistical analysis Two-way ANOVA with tukey post hoc test was used. e IL-1β gene expression of macrophages from COVID- 19 patients (n = 5; red bars) or healthy individuals (n = 4; blue bars) stimulated with/without S-Protein were determined by qRT-PCR. Data are normalized to β-actin. Statistical significance was analyzed using the Kolmogorov-Smirnov test. Graphs show mean ± SEM. *p<0.05; **p<0.01; ***p<0.001.

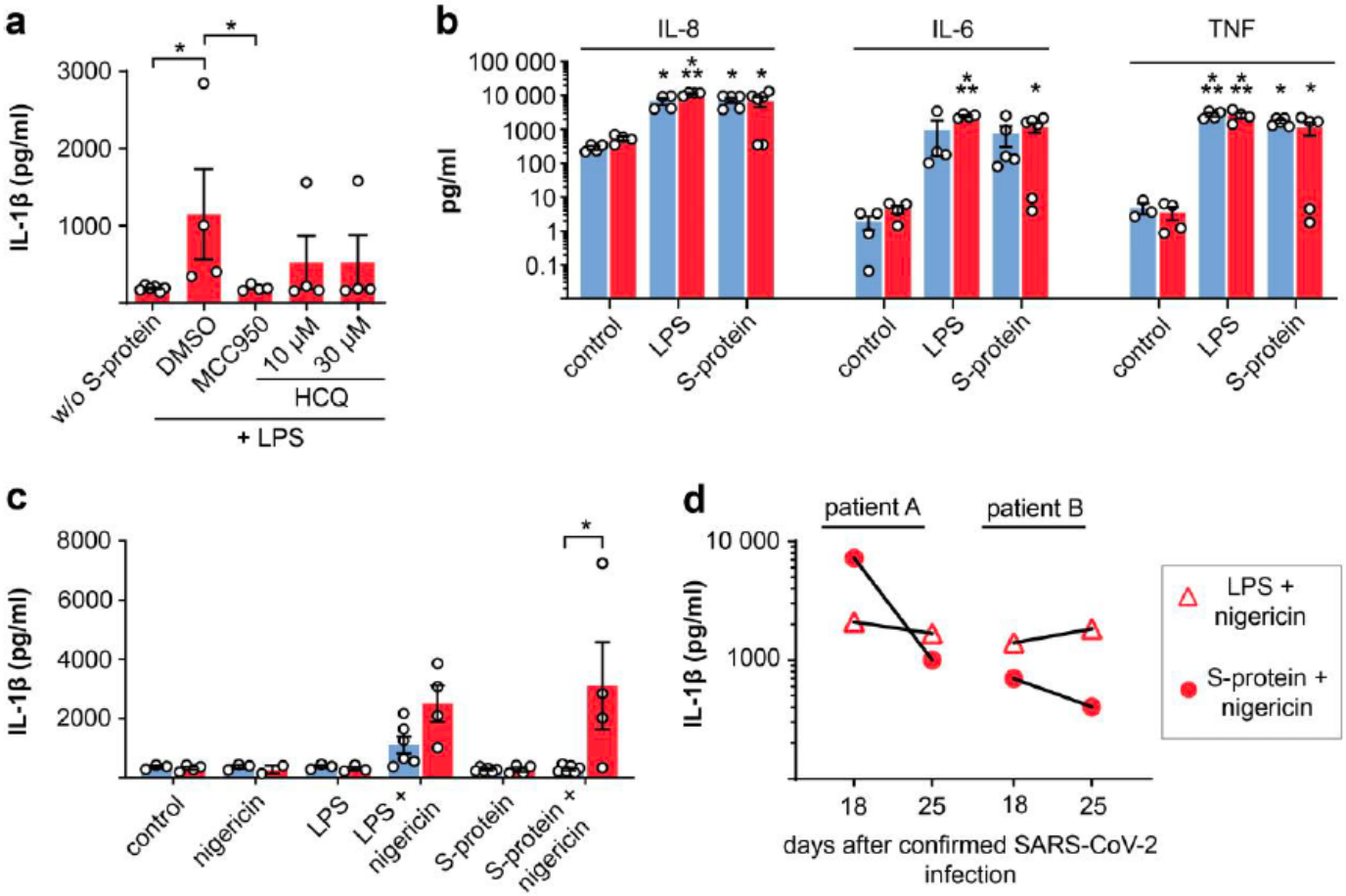

Figure 2

a Macrophages of COVID-19 patients (n = 4) were incubated for 2 h with DMSO (control; solvent of compounds), MCC950 (10µM), or hydroxycholorquinesulfate (HCQ; 10µM and 30µM). Afterwards, macrophages were stimulated with S-protein and IL-1β secretion was induced by nigericin. b Cytometric Bead Array-based quantification of IL-8, IL-6 and TNF in the supernatant of macrophage cultures from

COVID-19 patients (n = 3; red bars) or healthy individuals (n = 2; blue bars) stimulated with LPS or S-protein. For statistical analysis significance between unstimulated control and LPS or S-protein were calculated for each patient group using the Two-way ANOVA with tukey post hoc test based on each technical data point. Each data point represented indicates each replicate. c Quantification of the IL-1β concentration in the supernatant of macrophage cultures from recovered COVID-19 patients with mild disease (n = 4; red bars) or healthy individuals (n = 6; blue bars) stimulated with LPS or S-protein. For statistical analysis Two-way ANOVA with tukey post hoc test were used. d CD14+ cells of two convalescent individuals were isolated sequentially, 7 days after initial sampling (day 18 and day 25 after confirmation of SARS-CoV-2 infection by PCR). After in vitro differentiation with M-CSF, macrophages were stimulated with LPS (red triangles) or S-protein (red circles) and IL-1β was quantified by ELISA. Graphs show mean ± SEM. *p<0.05; **p<0.01; ***p<0.001.

Supplementary Files

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