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COVID-19

Spontaneous NLRP3 inflammasome-driven IL-1-β secretion is induced in severe COVID-19 patients and responds to anakinra treatment

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GRAPHICAL ABSTRACT

Background: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection may result in a severe pneumonia associated with elevation of blood inflammatory parameters, reminiscent of cytokine storm syndrome. Steroidal anti-inflammatory therapies have shown efficacy in reducing mortality in critically ill patients; however, the mechanisms by which SARS-CoV-2 triggers such an extensive inflammation remain unexplained.

Objectives: To dissect the mechanisms underlying SARS-CoV-2–associated inflammation in patients with severe coronavirus disease 2019 (COVID-19), we studied the role of IL-1β, a pivotal cytokine driving inflammatory phenotypes, whose maturation and secretion are regulated by inflammasomes.

Methods: We analyzed nod-like receptor protein 3 pathway activation by means of confocal microscopy, plasma cytokine measurement, cytokine secretion following in vitro stimulation of blood circulating monocytes, and whole-blood RNA sequencing. The role of open reading frame 3a SARS-CoV-2 protein was assessed by confocal microscopy analysis following nucleofection of a monocytic cell line.

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Results: We found that circulating monocytes from patients with COVID-19 display ASC (adapter molecule apoptotic speck like protein–containing a CARD) specks that colocalize with nod-like receptor protein 3 inflammasome and spontaneously secrete IL-1β in vitro. This spontaneous activation reverts following patient’s treatment with the IL-1 receptor antagonist anakinra. Transfection of a monocyte cell line with cDNA coding for the ORF3a SARS-CoV-2 protein resulted in ASC speck formation.

Conclusions: These results provide further evidence that IL-1β targeting could represent an effective strategy in this disease and suggest a mechanistic explanation for the strong inflammatory manifestations associated with COVID-19. (J Allergy Clin Immunol 2022;150:796-805.)

Key words: NLRP3 inflammasome, IL-1β, inflammation, SARS-CoV-2

Coronavirus disease 2019 (COVID-19) is an acute respiratory illness caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). COVID-19 severe pneumonia has been associated with systemic inflammation and elevation of parameters such as ferritin, lactate dehydrogenase, and soluble IL-2 receptor evocative of clinical inflammatory phenotypes known as macrophage activation syndrome (MAS) or hemophagocytic lymphohistiocytosis and cytokine storm syndromes. MAS is a life-threatening condition seen in pediatric patients affected by systemic-onset juvenile idiopathic arthritis, which is also observed in previously healthy children and known as secondary macrophage lymphohistiocytosis, often triggered by viral infection. Anticytokine therapies targeting IL-1β (canakinumab) or IL-1 receptor antagonist (anakinra) have demonstrated the central role of this molecule in MAS and systemic hemophagocytic lymphohistiocytosis, markedly improving the outcome. Recently, several reports have highlighted the role of innate immune cells, particularly monocytes, and of monocyte-derived cytokines such as IL-1β, in COVID-19, and showed a correlation between the degree of involvement of innate immunity and the disease severity and outcome. Stimulation of PBMCs from patients with severe COVID-19 has shown an increased secretion of IL-1β compared with PBMCs from patients with severe H1N1 influenza, confirming gene expression data extracted by whole blood or single-cell transcriptomic. In support of a key role of IL-1β in patients with severe COVID-19, we and others have recently reported the potential efficacy of blocking IL-1β by using anti–IL-1 drugs in patients with severe COVID-19.

In mononuclear cells from healthy subjects, IL-1β is translated as an inactive precursor, pro–IL-1β, which is processed and secreted as bioactive IL-1β upon activation of the nod-like receptor protein 3 (NLRP3) inflammasome. NLRP3, activated by inflammatory signals, oligomerizes and recruits pro–caspase-1 and the adaptor molecule apoptotic speck-like protein–containing a CARD (ASC), resulting in activation of caspase-1, processing of pro–IL-1β, and secretion of the mature cytokine. NLRP3 inflammasome also mediates processing and secretion of IL-18, a leading cytokine in MAS. IL-1β production, processing, and secretion require a first signal, named signal 1, which induces transcription of pro–IL-1β and inflammasome genes, and a second signal, named signal 2, which triggers the assembly of the NLRP3 inflammasome. In human monocytes, signal 1 is sufficient to trigger the cascade of events leading to inflammasome activation and IL-1β secretion, low but sustained in time. Signal 2, although unnecessary, strongly speeds up and enhances these processes. Recently, NLRP3 and absent in melanoma 2 protein inflammation–some activation and ASC speck formation in peripheral blood cells and CD14+ cells from autopic lung tissue of patients with COVID-19 have been reported. The involvement of viral proteins N18 and open reading frame 3a (ORF3a) in NLRP3 activation has also been demonstrated in vitro.

METHODS

Study design and participants

The study was approved by our regional institutional review board (Immunocovid-19 project). Samples were collected on informed consent from SARS-CoV-2–infected patients with a positive nasopharyngeal swab and a clinical presentation including dyspnea associated with fever, systemic inflammation, rapidly worsening respiratory distress, and pathognomonic lung abnormalities on chest computed tomography. In patients treated with anakinra, samples were collected before treatment (day 0) and at 3 and 7 days of treatment.

Patient and public involvement

Patient and public were not involved in the design, or conduct, or reporting, or dissemination plans of the research.

Isolation and stimulation of cells

PBMCs from peripheral blood were isolated by gradient centrifugation (FicollHystopaque). Then, monocytes were selected by adhesion, incubating them for 1 hour in RPMI without FCS. Monocytes were cultured in RPMI 1640 (Thermo Fisher Scientific, Waltham, Mass), supplemented with 5% FCS and in the presence or absence of LPS (100 ng/mL; Sigma Aldrich, St Louis, Mo), and MCC950 (10 μM; Sigma Aldrich). Human monocytic cell line THP1 was cultured in RPMI-1640 medium. Monocytes were differentiated in macrophages using phorbol 12-myristate 13-acetate 100 nM for 24 hours.

Secretion of cytokines

In vitro secretion of cytokines by monocyte was quantified by ELISA. Data were normalized by seeded monocytes per well. The number of plated monocytes was calculated on the basis of hospital cell blood count and plated PBMCs and finally related to 1 × 10⁶ cells. All cytokine dosages were normalized to this value. The BD CBA assays for Human Soluble Protein (BD Biosciences 558264; Franklin Lakes, NJ) was used to assess cytokine profile in blood plasma. All data were analyzed using the FCAP Array software (Soft Flow Ltd, Pécs, Hungary).

RNA and real-time PCR

Blood samples were collected in Paxgene Blood RNA. cDNA was retrotranscribed using Superscript VILO cDNA Synthesis Kit (Invitrogen,
Carlsbad, Calif). Selected IFN-stimulated gene expression was quantified briefly, IFN-stimulated gene (IFI27, IFI44L, IFIT1, ISG15, RSAD2, SIGLEC1) expression was quantified by real-time PCR using gene-specific primers and probes (custom designed by TibMolBiol, Genova, Italy) using HPRT and G6PD as reference genes, as previously described with modifications.20 ORF3a was detected in monocyte isolated from patient’s PBMCs and THP-1 by quantitative PCR (CGGATGGCTTATTGTTGGCG, Forward, TGAACACCCTTGGAGAGTGC, Reverse).

### TABLE I. Patients’ clinical characteristics

| Patient ID | Age (y) | Sex | Comorbidities | Clinical | T (°C) | Sat. O₂ | PaO₂/FiO₂ | SARS-CoV-2 nasal swab | SARS-CoV-2 serology | Day after disease onset | Days of Steroid at first sampling | Days of anakinra at first sampling | Other therapies administered |
|------------|---------|-----|----------------|----------|--------|---------|------------|-----------------------|----------------------|----------------------|-----------------------------|----------------------------|---------------------------------|
| COVID-01   | 55      | F   | CD, HT         | Fever, cough, dyspnea | 37     | 97%     | 177       | POS                   | NA                   | 5                    | 2                           | 0                            | MPred (0.5-1 mg/kg/d for 4 d, 2 d before anakinra), EXP, AZT |
| COVID-02   | 25      | M   |                | Fever, cough, dyspnea | 39     | 96%     | 210       | POS                   | NA                   | 19                   | 0                           | 0                            | MPred (2.3 mg/kg/d for 8 d with anakinra), HCQ, EXP, AZT |
| COVID-03   | 67      | M   | HT             | Fever, cough, dyspnea, arthralgia | 36.8   | 94%     | 226       | POS                   | NA                   | 6                    | 2                           | 0                            | HCQ, EXP, remdesivir, AZT |
| COVID-04   | 59      | M   | CD             | Fever, anosmia, headache | 36     | 94%     | 116       | POS                   | NA                   | 11                   | 0                           | 0                            | MPred (0.5-1 mg/kg/d for 3 d), EXP, AZT |
| COVID-05   | 72      | F   | CD, HT         | Cough | 39.2   | 96%     | 223       | POS                   | NA                   | 11                   | 0                           | 0                            | MPred (1-2 mg/kg/d for 7 d, with anakinra), HCQ, EXP, AZT |
| COVID-06   | 38      | M   | —              | Fever, headache | 39     | 97%     | 381       | POS                   | NA                   | 7                    | 0                           | 0                            | EXP, AZT |
| COVID-07   | 71      | M   | HT, emphysema  | Fever, dyspnea | 36.5   | 93%     | 218       | POS                   | NA                   | 6                    | 2                           | 0                            | MPred (1-2 mg/kg/d for 7 d), EXP, AZT |
| COVID-08   | 59      | F   | No             | Fever, dyspnea, cough, dysgeusia | 38     | 93%     | 118       | POS                   | NA                   | 9                    | 1                           | 0                            | MPred (1-2 mg/kg/d for 7 d), EXP, AZT |
| COVID-09   | 63      | M   | No             | Fever, dyspnea | 36     | 92%     | 82        | POS                   | NA                   | 10                   | 3                           | 0                            | MPred (1-2 mg/kg/d for 10 d), EXP, remdesivir |
| COVID-10   | 76      | M   | HT, CD, HPT    | Fever, dyspnea | 36.5   | 88%     | 170       | POS                   | NA                   | 14                   | 7                           | 0                            | MPred (1-2 mg/kg/d for 7 d), EXP, AZT |
| COVID-11   | 59      | M   | HT             | Fever, dyspnea | 36.7   | 96%     | 65        | POS                   | NA                   | 15                   | 5                           | 0                            | MPred (1-2 mg/kg/d for 7 d), EXP, AZT |
| COVID-12   | 90      | M   | HT, CD         | Fever, dyspnea | 36.5   | 95%     | 220       | POS                   | NA                   | 6                    | 2                           | 0                            | MPred (1-2 mg/kg/d for 7 d), EXP, AZT, remdesivir |
| COVID-13   | 74      | M   | HT             | Fever, dyspnea | 36     | 94%     | 121       | POS                   | NA                   | 18                   | 7                           | 0                            | MPred (1-2 mg/kg/d for 7 d), EXP, AZT |
| COVID-14   | 66      | F   | No             | Nausea, hyporexia, diarrhea, fever | 36.4   | 98%     | 342       | POS                   | NA                   | 7                    | 4                           | 0                            | MPred (2 mg/kg/d for 4 d), EXP |
| COVID-15   | 76      | M   | CD, T2D        | Dyspnea | 36.6   | 98%     | 417       | POS                   | NA                   | 12                   | 0                           | 0                            | AZT, apixaban |
| COVID-16   | 57      | F   | Psychosis      | Fever, dyspnea | 36.9   | 95%     | 327       | POS                   | NA                   | 9                    | 1                           | 0                            | MPred (1-2 mg/kg/d for 7 d), EXP, AZT |
| COVID-17   | 71      | F   | HT, HPT, cholecystectomy | Fever | 35     | 93%     | 259       | POS                   | NA                   | 2                    | 2                           | 0                            | MPred (1-1.5 mg/d/kg for 2 d), remdesivir, EXP |
| COVID-18   | 72      | M   | CD, CKD, T2D, hepatopathy | Delirium, bilateral edema | 36     | 98%     | 303       | POS                   | NA                   | 2                    | 2                           | 0                            | Dexamethasone (6 mg/d for 2 d), remdesivir, AZT |
| BB4569209  | 42      | M   | No             | Dyspnea, fever | 36     | 98%     | 276       | POS                   | NA                   | 3                    | 1                           | 0                            | MPred (0.5-1 mg/kg/d for 1 d), levofloxacin, EXP, 4000 d, remdesivir started in the following days |
| BB8873982  | 61      | M   | No             | Severe pneumonia | 38     | 93%     | 142       | POS                   | NA                   | 11                   | 5                           | 0                            | Remdesivir, steroids, EXP, Anakinra, steroids, insulin, EXP |
| COVID-C5   | 57      | M   | No             | Severe pneumonia | 38     | 89%     | 78        | POS                   | NA                   | 10                   | 6                           | 0                            | Steroids, EXP, anakinra, EXP |
| COVID-C2   | 43      | M   | No             | Severe pneumonia | 38     | 92%     | 301       | POS                   | NA                   | 9                    | 6                           | 0                            | Remdesivir, steroids, EXP |
| BB4988699  | 69      | F   | No             | Severe pneumonia | 39     | 80%     | 73        | POS                   | IgG and IgM POS        | 4                   | 1                           | 0                            | Fentanyl, anakinra, steroids, insulin, EXP |
| BB6716896  | 64      | M   | HT, obesity    | Severe pneumonia | 36     | 60%     | 280       | POS                   | IgG and IgM POS        | 5                   | 1                           | 0                            | Anakinra, steroids, insulin, EXP |
| BB5535728  | 51      | M   | No             | Severe pneumonia | 38     | 90%     | 211       | POS                   | NEG                   | 6                    | 1                           | 0                            | Anakinra, steroids, EXP, AZT |
| BB3726697  | 88      | M   | HT             | Severe pneumonia | 37     | 90%     | 68        | POS                   | NEG                   | 4                    | 1                           | 0                            | Anakinra, steroids, EXP, AZT |

AZT, Azithromycin; CD, cardiovascular disease; CKD, chronic kidney disease; EXP, enoxaparin; F, female; HCQ, hydroxychloroquine; HPT, hypothyroidism; HT, hypertension; M, male; MPred, methylprednisolone; NA, not available; NEG, negative; POS, positive; T2D, type 2 diabetes.
DNA transfection

PCMV6-AC-rfp-ORF3a and PCMV6-AC-rfp were purchased from Ori- gene (Rockville, Md). 10^6 THP-1 cells were transfected with nucleofector de-vice II (Amaxa, Nordrhein-Westfalen, Germany) and nucleofector kit V (Lonza, Basel, Switzerland) using U-001 program with 5 μg of plasmid DNA (% of transfected cells: 24.20 ± 4.384 for PCMV6-AC-rfp-ORF3a and 39.40 ± 0.848 for PCMV6-AC-rfp) and 20 μg of plasmid DNA, respec- tively (% of transfected cells: 33.66 ± 3.951 for PCMV6-AC-rfp-ORF3a and 50.05 ± 1.484 for PCMV6-AC-rfp). Transfection efficiency and cell viability were monitored by fluorescence-activated cell sorting.
Imaging

THP-1 macrophages were cultured on glass coverslip and transfected as described. After 16 hours, cells were stained with anti-ASC (TMS1) (Hu mAb-Alexa Fluor 647 conjugate (Voden, Monza e Brianza, Lombardia, Italy) and anti-NLRP3 (Rabbit, Life Technologies, Carlsbad, Calif) overnight and acquired using a Leica TCS-SP8 confocal microscope; Z-sectioning images were acquired with a z-slice thickness of about 0.15 μm.

Detection of ASC speck by fluorescence-activated cell sorting

Monocytes were isolated from PBMCs by magnetic selection using CD14 MicroBeads (MiltenyiBiotec, Bergisch Gladbach, North Rhine-Westphalia, Germany) according to manufacturer’s instructions. Cells were resuspended at 1 × 10⁷/mL in RPMI1640 supplemented with 5% FCS, left untreated or primed with 100 ng/mL LPS (Sigma) for 3 hours, and stimulated with 5 mM ATP (Sigma Aldrich) for another 30 minutes. Cells were fixed and permeabilized using BD Cytofix/Cytoperm reagents (BD Biosciences) and incubated overnight with anti-ASC (TMS1) phycoerythrin (BioLegend, San Diego, Calif) at 4°C. Samples were analyzed using the FACSCanto A (BD Biosciences) flow cytometer and Kaluza Software 2.0 (Beckton Coulter, Brea, Calif). ASC speck formation was detected as reduction in the ASC fluorescent pulse width with a concomitant increase in the ASC pulse area, as described.²⁻³

FLICA-Caspase-1 staining

Caspase-1 activation was evaluated on frozen PBMCs by flow cytometry. On thawing, cells were allowed to recover for 1 hour at 37°C in RPMI 5% FBS. Cells were labeled for 1 hour with FLICA 660-YV AD-FMK caspasi-1 inhibitor reagent (ImmunologyChemistry Technologies, LLC, Davis, Calif) according to manufacturer’s instructions. Cells were then stained with CD14 PE-Cy7 (eBioscience, San Diego, Calif), fixed, and permeabilized using BD Cytofix/Cytoperm reagents, and incubated overnight with anti-ASC (TMS1) PE at 4°C. Active caspase-1 was analyzed on gated CD14⁺ASC⁺ monocytes or on gated CD14⁺ASC speck-forming cells.

RNA-sequencing data processing

Poly-A⁺-enriched libraries were generated with the Illumina TruSeq mRNA Stranded kit. Library quality control was performed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, Calif). Indexed libraries were sequenced with HiSeq 3000 instrument to generate approximately 30 M 150-bp paired-end reads per sample. Reads were trimmed for low-quality ends with TrimGalore, and transcript abundance was estimated with Kallisto.³⁻⁴ Kallisto counts were normalized by the regularized log transformation using the DeSeq²³ R package (Bioconductor software). Data are accessible at gene expression omnibus database, accession code GSE163317.

Bioinformatic analysis for RNA sequencing

Analysis was performed by gene enrichment analysis (GSEA) implemented in the cluster Profiler R package²⁴ and GSEA prerank method based on log-fold change score²⁵ (Hallmark (H) gene set collection).²⁶ Day 7 and day 0 were used to estimate log-fold change. Curated collection was retrieved from the Molecular Signature Database (SigDB) v7.2 database.¹⁹.²⁶–³⁰ Custom collection (see Online Repository file 1 in this article’s Online Repository at www.jacionline.org) was created, retrieving the genes from the literature.²⁶–³⁰ Normalized enrichment score (NES) and nominal P-value (Nom P value) were calculated by GSEA.²⁶,²⁵ Gene sets between 5 and 5000 genes were retained for analysis. P value lower than .05 and false discovery rate (FDR) q value lower than 0.15 were considered significant. Functional enrichment results were visualized using dot plots implemented in the clusterProfiler R package (Bioconductor software).²⁵ NES and −log10(q-value) were encoded in color palette, ranging from red (positive) to blue (negative), and dot size, respectively.

Statistical analysis

Statistical analysis was performed with GraphPad Prism (5.0, La Jolla, Calif). Mann-Whitney test was used to compare samples, and results were reported as median with interquartile range.

All data relevant to the study are included in the article or uploaded as Online Repository information or accessible in open access repository. Data source are provided in Table E1 in this article’s Online Repository at www.jacionline.org. RNA-seq data are accessible at gene expression omnibus database, accession GSE163317.

There was no patient and public involvement.

RESULTS

To characterize IL-1β pathway activation in COVID-19, we analyzed blood samples from patients affected by severe COVID-19 and displaying strong elevation of inflammatory markers (clinical data reported in Tables I and II), before and after treatment with anakinra. Blood samples were collected at baseline and at day 3 and day 7 of treatment. Before treatment, RNA-sequencing analysis revealed as expected the activation of innate immune pathways linked to viral infection such as type I IFN response together with activation of inflammatory pathways involving IL-6 (see Fig E1 in this article’s Online Repository at www.jacionline.org). FACS and confocal microscopy analysis of PBMCs from patients revealed the presence of CD14⁺ monocytes displaying ASC specks colocalizing with NLRP3 (Fig 1, A and B). Caspase-1 activation in ASC speck⁺ monocytes confirmed the involvement of NLRP3 (Fig 1, A; see Fig E2 in this article’s Online Repository at www.jacionline.org). Specks are absent in CD14⁺ monocytes from healthy donors, supporting the activation of NLRP3 inflammasome in patients with COVID-19 but not in healthy subjects. We therefore isolated peripheral blood monocyte from patients with COVID-19 and healthy donors and cultured them in vitro for 18 hours with or without LPS, which in healthy monocytes induces pro–IL-1β expression, inflammasome activation, and secretion of both IL-1β and IL-18.⁵⁻⁻⁷ Spontaneous secretion of IL-1β and IL-18 by unstimulated patients’ monocytes, which was partially inhibited by the NLRP3 inhibitor MCC950, was detected (Fig 1, C). These results, although consistent with the presence of ASC specks in patients’ monocytes, were quite unexpected because in our experience IL-1β and IL-18 are not secreted in vitro by unstimulated monocytes either from healthy subjects or from patients with well-established IL-1–driven genetic conditions such as cryopyrin-associated periodic syndromes (CAPSs), caused by gain-of-function mutations of NLRP3.⁶⁻⁻⁷ LPS stimulation of monocytes from patients with COVID-19 further increased the secretion of IL-1β over the levels secreted by unstimulated cells (in 6 of 8 patients, 75%). Also, IL-18 secretion was induced, although at a lesser extent (Fig 1, C). The NLRP3 blocker MCC950 inhibited secretion, indicating that secretion of both IL-1β and IL-18 is dependent on the NLRP3 inflammasome. Like in healthy monocytes, the inflammasome-independent cytokines TNF-α, IL-6, and IL-1RA were not spontaneously secreted by monocytes but induced by exposure to LPS (Fig 1, C). We previously showed that few days of treatment of patients with CAPS with anakinra result in inhibition of LPS-induced IL-1β secretion by patient’s monocytes in vitro.¹³ Accordingly, we observed a decrease in the spontaneous and LPS-induced IL-1β and IL-18 secretion by monocytes from patients with COVID-19 under therapy with anakinra (Fig 2, A), more evident at 7 days from the beginning of the treatment. Secretion of TNF-α and IL-6 was
### TABLE II. Anakinra-treated patients’ inflammatory parameters

| Patient ID | Treatment days | CRP (0-0.5 mg/dL) | Ferritin (30-400 ng/mL) | LDH(135-200 U/L) |
|------------|----------------|-------------------|------------------------|------------------|
| COVID-01   | Day 0          | 5.9               | 2760                   | 443              |
|            | Day 3          | 0.35              | 308                    | 254              |
|            | Day 7          | NA                | 294                    | 226              |
| COVID-02   | Day 0          | 5.62              | 2948                   | 392              |
|            | Day 3          | 1.45              | 1053                   | 265              |
|            | Day 7          | 0.14              | NA                     | 248              |
| COVID-03   | Day 0          | 16.5              | 1346                   | 322              |
|            | Day 3          | 1.22              | 1417                   | 413              |
|            | Day 7          | 0.92              | NA                     | 339              |
| COVID-04   | Day 0          | 3.8               | 460                    | 493              |
|            | Day 3          | 0.68              | 504                    | 205              |
|            | Day 7          | 0.19              | 405                    | 186              |
| COVID-05   | Day 0          | 12.18             | 1637                   | 296              |
|            | Day 3          | 7.29              | 552                    | 382              |
|            | Day 7          | 2.42              | 1352                   | 379              |
| COVID-49   | Day 0          | 10.78             | 662                    | 382              |
|            | Day 3          | 1.07              | NA                     | 425              |
|            | Day 7          | NA                | NA                     | 320              |
| COVID-53   | Day 0          | 5.88              | 621                    | 376              |
|            | Day 3          | 0.67              | NA                     | 335              |
|            | Day 7          | 0.34              | NA                     | 316              |
| COVID-54   | Day 0          | 8.01              | 900                    | 303              |
|            | Day 3          | 2.31              | NA                     | 269              |
|            | Day 7          | 0.73              | 1079                   | NA               |

CRP, C-Reactive protein; LDH, lactic dehydrogenase; NA, not applicable/available.

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**Fig 1.** NLRP3 and caspase-1 activation, and IL-1β and IL-18 spontaneous secretion, by monocytes of patients with COVID-19. **A.** Above: ASC speck detection in monocytes from patients with severe COVID-19 by FACS. Representative dot plots of CD14+ monocytes from HDs (left), patients with COVID-19 (center), and HDs treated with LPS + ATP, used as a positive control. ASC specks are detected gating as ASC pulse width vs ASC pulse area. Percentages of ASC speck + cells are shown. Below: left, quantification of ASC speck CD14+ monocytes as identified by FACS; right, percentage of cells positive for active caspase-1 quantified by FACS, using FLICA-Caspase-1. **B.** Representative confocal images of NLRP3 and ASC speck formation in monocytes from HDs and patients with COVID-19. Images are a result of a 3-dimensional reconstruction of Z stack. Cells were stained with anti-ASC antibody (yellow), NLRP3 (green), and DAPI (blue). Scale bar is 5 μm. **C.** In vitro inflammatory cytokine secretion by monocytes as measured in culture supernatant after 18 hours with or without stimulation with the indicated stimuli. BF, Brightfield; DAPI, 4’-6-diamidino-2-phenylindole, dihydrochloride; FACS, fluorescence-activated cell sorting; FLICA, fluorochrome-labeled inhibitors of caspas; HD, healthy donor; IQR, interquartile range; MCC, MCC950 NLRP3 inhibitor. Data are expressed as median ± IQR. *P < .05, **P < .01 as assessed by Mann-Whitney t test.
not decreased in agreement with their direct induction after Toll-like receptor triggering via activation of the nuclear factor-kappa B signaling pathway independently of IL-1β receptor signaling. Other inflammatory markers did not show a significant reduction in supernatant (Fig 2, A) and in the serum (Fig 3, A and B). Type I IFN, as measured by IFN RNA signature in peripheral blood, showed a decrease (Fig 3, A). Interestingly, when we compared through RNA sequencing the whole-blood transcriptome of patients before and after treatment with anakinra, we observed a significant downregulation of IL-1β–induced genes and upregulation of genes shown to be downregulated in patients with severe COVID-19 (Fig 2, B, and Fig E1). More importantly, following anakinra therapy, we observed the same modulation of gene sets observed in patients with CAPS treated by the same drug (Fig 2, B). These results were evident in patients with COVID-19 who recovered from the disease after treatment but not in the patient who died (P5). Together, these data suggest that activation of NLRP3 inflammasome and IL-1β secretion have a major role in the systemic inflammation in patients with severe COVID-19. Moreover, the modulation of IL-1β–induced or –repressed genes by IL-1 receptor blockade supports the exploitation of anakinra as an efficient therapeutic approach.33-35 The increased secretion of IL-1β by patients with COVID-19 is likely due to extrinsic and intrinsic factors. SARS-CoV-2 lung infection may induce the release in the microenvironment by viral-infected injured cells of damage-associated molecular patterns such as ATP and IL-1α and pathogen-associated molecular patterns including ssRNA and other viral proteins. These pathogen-associated molecular patterns and damage-associated molecular patterns are able to induce IL-1β production and/or secretion36 and thus, contribute extrinsically to the inflammatory response in patients. In addition, considering the high frequency of the hyperinflammatory state observed in patients with COVID-19 compared with other viral respiratory lung infections,8 and the reported ability of SARS-CoV-2 to infect myeloid cells,16,17 we speculated that SARS-CoV-2 could intrinsically activate the NLRP3 pathway in monocytes. Studies in SARS-CoV have demonstrated that the viroprotein 3, encoded by the ORF3a viral gene, is able to induce both signal 1 and 2 in monocytes, activating IL-1β transcription through nuclear factor-kB binding and NLRP3 through TNF receptor–associated factor 3 binding.37 SARS-CoV-2 encodes the homologue protein ORF3a. We then transfected the monocytic cell line THP1 with a plasmid containing ORF3a gene tagged with red fluorescent
protein. After 24 hours, we obtained confocal images of transfected cells and observed the formation of ASC specks that colocalized with ORF3a protein and NLRP3 in cells transfected with the ORF3a plasmid but not in cells transfected with the empty plasmid (Fig 4, A-C). These data indicate that ORF3a protein of SARS-CoV-2 activates NLRP3, inducing ASC speck formation. We assessed the presence of viral ORF3a mRNA in monocytes from 3 patients with COVID-19 analyzed for IL-1β secretion in Fig 1, C: 2 of 3 tested samples expressed ORF3a. Interestingly, the 2 positive samples were from P2 and P4, who showed spontaneous secretion of IL-1β in vitro (Fig 1, C), whereas P1 monocytes, negative for ORF3a mRNA, did not secrete IL-1β spontaneously. We then assessed viral ORF3a mRNA in monocytes from other 8 patients with COVID-19 and did not detect it, suggesting that SARS-CoV-2 mRNA is only marginally present in peripheral blood patient’s monocytes.

DISCUSSION

In summary, our study presents 2 main findings: (1) circulating monocytes from patients with SARS-CoV-2 severe infection present activation of the NLRP3-IL-1β/IL-18 pathway as shown by detection of ASC specks and spontaneous in vitro secretion of IL-1β and IL-18 and (2) NLRP3 inflammasome, with consequent ASC speck formation, is directly activated by the ORF3a SARS-CoV-2 viral protein, which might be present in circulating monocytes from patients with COVID-19.

IL-1β cytokine is one of the major players in the systemic inflammatory response seen in sepsis caused by bacterial infections, where LPS induces pro–IL-1β production, NLRP3 activation, and IL-1β processing and secretion. Similarly, it has a predominant role also in the hyperinflammatory state observed in MAS, where treatment with anti–IL-1 drugs has dramatically improved patients’ outcome. An important role in MAS is also played by IL-18, whose secretion is also increased by monocytes from patients with COVID-19. Although COVID-19 is not an inflammatory syndrome per se, it is frequently associated with an inflammatory state more similar to bacterial-induced systemic inflammatory response and MAS than the one observed in response to other viral respiratory infections.

It remains to be understood when and how is IL-1β secreted by monocytes from patients with COVID-19. We previously showed that 2 different routes of secretion are activated depending on the strength of monocyte activation, a vesicular pathway for weak stimuli and a gasdermin D–dependent pathway for strong stimuli, leading to a dramatic inflammatory response. Notably, in patients with CAPS also, a weak stimulus is sufficient to trigger gasdermin D–mediated massive IL-1β secretion. It is tempting to speculate that in COVID-19–infected monocytes ORF3a and other viral proteins trigger NLRP3 inflammasome activation, leading to an abnormal IL-1β secretion with consequent severe inflammation. The concurrent presence in the infected lungs of other pathogen-associated molecular patterns and damage-associated molecular patterns may amplify the inflammatory response.
response and promote the so-called cytokine storm. Our findings further support the importance of targeting IL-1β activity with anti–IL-1 drugs in severe COVID-19.

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Key messages

- Monocytes from SARS-CoV-2–infected individuals present NLRP3 inflammasome activation and spontaneous secretion of IL-1β. This activation is blocked by administration of the IL-1R blocker anakinra.
- SARS-CoV-2 protein ORF3a activates NLRP3 inflammasome inducing ASC-spike formation in patients’ monocytes.
- Drugs targeting the NLRP3 IL1-β pathway might have a therapeutic role in the treatment of patients with severe COVID-19 with evidence of inflammation.

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