The Journal of Infectious Diseases
MAJOR ARTICLE

Immunoglobulin G Immune Complexes May Contribute to Neutrophil Activation in the Course of Severe Coronavirus Disease 2019

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Severe coronavirus disease 2019 (COVID-19) is associated with an overactive inflammatory response mediated by macrophages. Here, we analyzed the phenotype and function of neutrophils in patients with COVID-19. We found that neutrophils from patients with severe COVID-19 express high levels of CD11b and CD66b, spontaneously produce CXCL8 and CCL2, and show a strong association with platelets. Production of CXCL8 correlated with plasma concentrations of lactate dehydrogenase and D-dimer. Whole blood assays revealed that neutrophils from patients with severe COVID-19 show a clear association with immunoglobulin G (IgG) immune complexes. Moreover, we found that sera from patients with severe disease contain high levels of immune complexes and activate neutrophils through a mechanism partially dependent on FcγRII (CD32). Interestingly, when integrated in immune complexes, anti–severe acute respiratory syndrome coronavirus 2 IgG antibodies from patients with severe COVID-19 displayed a higher proinflammatory profile compared with antibodies from patients with mild disease. Our study suggests that IgG immune complexes might promote the acquisition of an inflammatory signature by neutrophils, worsening the course of COVID-19.

Keywords. COVID-19; CD32; CXCL8; neutrophils; inflammation; immune complexes; SARS-CoV-2.

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection induces a wide spectrum of clinical manifestations ranging from asymptomatic to severe disease and death [1]. Disease severity is associated with the induction of a systemic inflammatory response revealed by high levels of inflammatory cytokines, chemokines, and inflammatory markers such as C-reactive protein (CRP), ferritin, D-dimer, and lactate dehydrogenase (LDH) found in patients with severe coronavirus disease 2019 (COVID-19) [2]. Aberrant immune response in the lung is associated not only with severe COVID-19 but also with a variety of respiratory viral infections including those mediated by respiratory syncytial virus (RSV) and influenza A virus (IAV) [3, 4]. Neutrophils are the predominant cell type infiltrating the lung during severe RSV infection, and it has been demonstrated that they not only promote epithelial cell damage and mucus overproduction, but also enhance susceptibility to RSV infection [4, 5]. Regarding IAV infection, it has been shown that patients with severe disease show a massive infiltration of the airway by neutrophils and an excessive production of neutrophil extracellular traps (NETs), both being responses associated with fatal outcome in experimental models and humans [6].

Macrophages are the major source of inflammatory cytokines in the course of severe COVID-19 [7]. By contrast, the contribution of neutrophils is not well defined. While contradictory reports have been published regarding the degree of airway infiltration by neutrophils in severe COVID-19 [8, 9], a large body of evidence suggests that neutrophils actually play a major role in the pathogenesis of severe COVID-19 [10, 11]. Here, we show that neutrophils from patients with severe COVID-19 display an activated phenotype, spontaneously produce CXCL8, and show a strong association with platelets. Moreover, our results suggest that immunoglobulin G (IgG) immune complexes (ICs) might contribute to the acquisition of an inflammatory phenotype by neutrophils in the course of severe COVID-19.

MATERIALS AND METHODS

Ethics Statement

Our study was approved by the Ethics Committees of the Hospital de Clínicas José San Martín and Hospital Fernández (Argentina), in accordance with the Declaration of Helsinki (Fortaleza 2013). Written informed consent was obtained from all donors or legal guardians.

Received 27 November 2020; editorial decision 26 March 2021; accepted 30 March 2021; published online April 2, 2021.

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The Journal of Infectious Diseases® 2021;224:575–85

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DOI: 10.1093/infdis/jiab174

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Study Participants

Healthy individuals (n = 42) were recruited from the blood bank of the Hospital de Clínicas José San Martín; the age range of participants was 23–65 years, and 62% were male. Patients with COVID-19 (n = 67) were recruited from the Hospital de Clínicas José San Martín and the Hospital Fernández. All COVID-19 patients were diagnosed by polymerase chain reaction (PCR) amplification of SARS-CoV-2 viral RNA obtained from nasopharyngeal or oropharyngeal swabs. Taking into account the World Health Organization's (WHO) 8-point scale for COVID-19 trial endpoints, patients were classified into 3 stages: mild, moderate, and severe [12]. Patients with mild COVID-19 (WHO scores 1–2) were ambulatory patients or patients hospitalized with the aim of keeping them isolated; patients with moderate COVID-19 (scores 3–4) included those with little or no requirement for supplemental oxygen; and patients with severe COVID-19 included those with high-flow oxygen requirement (score 5), intubation and mechanical ventilation (score 6), or multiorgan support (score 7). Disease severity was recorded at the time of sampling. Patient characteristics are shown in Table 1.

Isolation of Neutrophils and Monocytes

Neutrophils were isolated from heparinized blood samples by centrifugation on Ficoll–Paque (GE Healthcare) and Dextran (Sigma-Aldrich) sedimentation. Contaminating erythrocytes were removed by hypotonic lysis. After washing, the cell pellets (>98% of neutrophils on May-Grunwald-Giemsa–stained cytopreparations) were suspended in RPMI1640 medium (Gibco Invitrogen). Neutrophil purity was confirmed by flow cytometry considering the forward and side scatter neutrophil properties, the high expression of CD66b, and the very low expression of CD14 that allows to distinguish neutrophils from monocytes. Peripheral blood mononuclear cells were isolated from the Ficoll-Paque gradient and monocytes were purified by positive selection using anti-CD14–coated magnetic beads (Miltenyi Biotec; purity 89%–97%).

Analysis of Neutrophil Phenotype

Labeled monoclonal antibodies (mAbs) directed to CD11b, CD14, CD41, CD66b, CD62L (L-selectin), CXCR1, CD62P (P-selectin), IgG, and isotype controls were from BD Biosciences. Data were acquired using a BD FACSCanto cytometer and BD FACSDiva software (BD Biosciences). Statistical analyses were based on at least 20 000 events gated in the neutrophil gate, defined as described above.

Association of Platelets With Neutrophils

The association of platelets with neutrophil was studied by flow cytometry in whole blood samples by analyzing the platelet markers P-selectin (CD62P) or CD41 in the

Table 1. Patients With Coronavirus Disease

Characteristic	Mild (n = 29)	Moderate (n = 19)	Severe (n = 19)
Demographic and clinical data			
Age, y, mean ± SEM	42.8 ± 2.7	57.3 ± 6.6	63.4 ± 5.3
Sex, male, %	75.9	78.9	47.4
Fever, %	17.2	31.6	57.9
ICU admission, %	0	5.3	78.9
Laboratory parameters, mean ± SEM			
Leukocyte count, cells/µL	6724 ± 616	7762 ± 616	8153 ± 2173
Neutrophil count, cells/µL	4306 ± 598	6055 ± 1506	6807 ± 2208
Lymphocyte count, cells/µL	1476 ± 162	1112 ± 192	514 ± 186
Neutrophil to lymphocyte ratio	2.84 ± 0.41	5.25 ± 1.07	8.00 ± 3.21
Platelets/µL	207 524 ± 15 607	197 786 ± 23 929	205 545 ± 14 006
LDH, U/L	302.0 ± 13.1	384.9 ± 42.0	640.0 ± 142.2
D-dimer, ng/mL	403.1 ± 66.5	577.6 ± 179.5	1391.0 ± 269.1
CRP, mg/L	2.96 ± 0.67	4.84 ± 1.22	12.72 ± 2.57
Ferritin, ng/mL	405.6 ± 90.0	572.0 ± 136.6	1622.0 ± 485.2
Comorbidities, %			
Hypertension	6.9	21.0	26.3
Obesity	13.8	26.3	36.8
Diabetes	17.2	21.1	26.3
Stroke	0	0	0
Cardiovascular disease	10.3	21.1	15.8
Chronic obstructive pulmonary disease	3.4	5.3	10.5
Chronic kidney disease	3.4	5.3	0
Days of symptoms at admission, mean ± SEM	1.6 ± 0.4	1.9 ± 0.4	2.8 ± 0.7
Blood sampling (days after diagnosis by PCR), mean ± SEM	5.8 ± 1.4	7.0 ± 1.7	11.2 ± 4.2
Days of hospitalization, mean ± SEM	7.26 ± 0.4	13.5 ± 1.99	21.00 ± 2.9

Abbreviations: CRP, C-reactive protein; ICU, intensive care unit; LDH, lactate dehydrogenase; PCR, polymerase chain reaction; SEM, standard error of the mean.

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neutrophil gate. In brief, whole blood samples were collected by venipuncture into Vacutainer heparin tubes and stained <30 minutes after collection. Samples were treated with BD FACS lysing solution.

Neutrophil Transmigration Assay

A neutrophil transmigration assay was carried out using a Corning HTS Transwell 96 permeable support (5 μM pore size). Unstimulated neutrophils (10×10^6/mL) from healthy donors (HD) or patients with severe COVID-19 were cultured at 37°C for 18 hours, and supernatants were collected. Healthy neutrophils (1.5×10^5/100 μL) were added to the upper reservoir and neutrophil supernatant (100 μL), interleukin 8 (10 ng/mL), or culture medium (control) was added to the lower reservoir. After 1 hour at 37°C, neutrophils in the upper and lower chamber were quantified by flow cytometry, and the percentage of neutrophils that migrated was determined.

Association of IgG Antibodies With Neutrophils

The association of IgG antibodies with neutrophils was analyzed by flow cytometry in whole blood samples or isolated neutrophils. Whole blood assays were performed by analyzing the presence of IgG antibodies associated to the neutrophil surface in freshly heparinized blood samples previously washed 5 times with culture medium. Cells were stained with a fluorescein isothiocyanate–labeled mAb directed to IgG, and samples were treated with BD FACS lysing solution prior to acquisition. Assays with isolated healthy neutrophils were performed by incubating neutrophils with serum (10%) from HD or COVID-19 patients for 1 hour at 4°C. Experiments were performed at 4°C to avoid the internalization of IgG antibodies attached to the neutrophils' surface. Then, cells were washed and IgG binding was revealed by staining with a labeled mAb-directed to IgG.

Estimation of Circulating ICs

Estimation of circulating ICs was performed by polyethyleneglycol (PEG) precipitation [13]. In brief, 0.2 mL of serum was mixed with 0.4 mL of 0.01 M-borate buffer, pH 8.4. Then, 5.4 ml of 4.16% PEG 6000 was added (final serum dilution 1:30), and the mixture was incubated at room temperature for 60 minutes. Turbidity was measured spectrophotometrically at 450 nm against a control for each serum that contains 1:30 diluted serum in borate buffer without PEG. Levels of ICs were expressed as PEG index = (OD_{450} with PEG – OD_{450} without PEG) × 1000.

Quantitation of Neutrophil Apoptosis by Annexin V Binding and Flow Cytometry

Neutrophils (2×10^6 cells/mL) were cultured for 10 hours at 37°C in RPMI medium supplemented with 10% fetal calf serum (FCS), and annexin V binding to apoptotic neutrophils was carried out using an apoptosis detection kit (BD Biosciences).

Flow Cytometric Determination of Hydrogen Peroxide Production by Neutrophils

The flow cytometric determination of hydrogen peroxide production by neutrophils was evaluated by using the indicator dye dihydrorhodamine-123 (Molecular Probes) by flow cytometry. Neutrophils (2×10^6 cells/mL) were labeled with dihydrorhodamine-123 and cultured for 30 minutes at 37°C without stimuli or in the presence of serum (10%) from HD or COVID-19 patients. In some experiments, neutrophils were pretreated with a blocking antibody directed to FcγRII (clone IV.3, STEMCELL Technologies).

Measurement of Cytokines by Enzyme-Linked Immunosorbent Assay

The presence of CXCL8, CCL2, tumor necrosis factor alpha (TNF-α), and interleukin 6 (IL-6) in cell supernatants was analyzed by enzyme-linked immunosorbent assay (ELISA) (Biolegend).

Determination of Serum Titers of IgG Antibodies Against SARS-CoV-2

The determination of serum titers of IgG antibody was performed by ELISA using the COVIDAR IgG kit (Laboratorio Lemos SRL), which detects IgG antibodies against 2 viral antigens, trimeric spike and the receptor binding domain (RBD) of the spike protein [14].

Activation of Neutrophils and Monocytes by ICs Prepared With Serum From Patients With Severe or Mild COVID-19

We screened a large number of sera from COVID-19 patients by ELISA (COVIDAR IgG) and selected a panel of sera with high titers (>1:3200) of anti–SARS-CoV-2 IgG antibodies from patients with severe or mild disease, that were used to prepare ICs and stimulate healthy neutrophils and monocytes. The titers of all sera were normalized to 1:3200. Then, sera from patients with severe and mild disease were incubated for 1 hour at room temperature in the plate of the COVIDAR IgG kit, which contains 2 immobilized antigens of SARS-CoV-2, the trimeric spike protein and the RBD domain, prepared as described elsewhere [14]. Plates were washed 5 times, and 1.5×10^5 neutrophils or 1×10^5 monocytes suspended in 100 μL of culture medium supplemented with 10% FCS was added. Release of hydrogen peroxide, expression of CD11b, and production of cytokines were analyzed.

Statistical Analysis

When 2 groups were present, normally distributed data were analyzed by 2-sided *t* test and skewed data were analyzed by Mann–Whitney test or Wilcoxon test. For 3 or more groups, analysis was by 1-way analysis of variance or Kruskal–Wallis test with correction for multiple comparisons. Normality was assessed by Shapiro–Wilk test. Correlations were tested by

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Spearman method. Data analysis was done with GraphPad Prism software version 8. Statistical significance was defined as $P < .05$.

RESULTS

All patients were diagnosed with COVID-19 by PCR amplification of SARS-CoV-2 viral RNA obtained from nasopharyngeal or oropharyngeal swabs. Patients were classified as having mild, moderate, or severe disease as described in the Methods. Neutrophil activation is associated with phenotype changes such as the up-regulation of CD11b and CD66b, and L-selectin loss. We found that neutrophils isolated from severe COVID-19 patients, but not from patients with mild or moderate disease, showed a higher expression of CD11b and CD66b compared with HD (Figure 1A). A lower expression of L-selectin was also observed in severe patients, although it did not reach statistical significance when compared with HD (Figure 1B). Considering previous studies showing that the interaction with platelets promotes neutrophil activation [15, 16], we analyzed whether neutrophils from COVID-19 patients showed a greater tendency to interact with platelets. It was analyzed by flow cytometry in whole blood samples by studying the presence of the platelet markers P-selectin (CD62P) or CD41 in the neutrophil gate. Neutrophils from patients with severe COVID-19 showed a higher association with platelets compared with healthy donors (Figure 1C). Moreover, a positive correlation was found between the degree of neutrophil–platelet association and the days of hospitalization (Figure 1C). No differences in the rate of apoptosis were observed for neutrophils isolated from patients or HD (Figure 1D).

High serum IL-6, CXCL8, and TNF-α levels at the time of hospitalization are predictors of COVID-19 patient survival [17]. We evaluated the spontaneous production of IL-6, TNF-α, and CXCL8 by neutrophils isolated from COVID-19 patients. No production of IL-6 and TNF-α was observed (not shown). By contrast, neutrophils from severe COVID-19 patients spontaneously produced CXCL8 (Figure 2A). This production correlated with 2 recognized markers of disease severity: the plasmatic concentrations of LDH and D-dimer (Figure 2A) [2, 18]. Consistent with the ability of CXCL8 to induce the internalization of the CXCL8 receptor CXCR1 [19], neutrophils from patients with severe COVID-19 showed a lower expression of CXCR1 compared with HD (Figure 2B). A higher spontaneous production of the chemokine CCL2 by isolated neutrophils from patients with severe COVID-19 was also observed, although it did not reach statistical significance when compared with HD (Figure 2C). Moreover, we found that neutrophil supernatants from patients with severe COVID-19 induced a strong chemotactic response on healthy neutrophils (Figure 2D).

Considering that severe disease is associated not only with the production of high levels of anti-SARS CoV-2 IgG antibodies but also with the production of IgG autoantibodies [20–22], we analyzed the role of IgG antibodies and ICs in neutrophil activation. Interestingly, flow cytometry analysis performed in whole blood samples revealed high levels of neutrophil-associated IgG antibodies in patients with severe COVID-19 (Figure 3A). Pretreatment of whole blood samples with an FcR blocking reagent (Miltenyi Biotec) almost completely displaced IgG antibodies from the neutrophil surface, suggesting that neutrophils bind IgG in an FcγR-dependent mode and not through the recognition of neutrophil surface antigens by the Fab portion of IgG antibodies. Consistent with these observations, we found that serum concentrations of ICs were significantly higher in patients with severe COVID-19 compared with healthy donors (Figure 3A). We then analyzed whether sera from COVID-19 patients might be able to induce the activation of healthy neutrophils in an FcγR-dependent mode. Incubation of neutrophils isolated from healthy donors with sera from patients with severe COVID-19 resulted not only in the binding of IgG ICs (Figure 3B), but also in the activation of the neutrophil respiratory burst (Figure 3C), being that this response significantly prevented by a blocking antibody directed to FcγRII (Figure 3D).

To gain insight into the mechanisms underlying the activation of healthy neutrophils by sera from COVID-19 patients, we analyzed the impact of 2 variables: disease severity and titers of anti-SARS-CoV-2 IgG antibodies. Sera from patients with severe and mild COVID-19 were titrated by an ELISA to detect anti–SARS-CoV-2 IgG antibodies (see Methods). In agreement with previous reports [23, 24], we observed that sera from severe patients showed higher antibody titers compared with mild patients (not shown). By screening a large number of sera, we could select a panel of sera with high (>1:3200) and low (<1:100) titers of IgG anti–SARS-CoV-2 antibodies from either severe or mild patients, and analyzed their ability to activate the respiratory burst of healthy neutrophils. Sera with high titers of specific IgG antibodies showed a higher ability to trigger the respiratory burst of neutrophils isolated from HD. Interestingly, sera from patients with severe disease showed a higher ability to induce the activation of the neutrophil respiratory burst when compared with sera from mild patients with similar titers of anti–SARS-CoV-2 IgG antibodies (Figure 4A).

It has been shown that a decreased fucosylation of the Fc portion of IgG antibodies increases its affinity for FcγRIII [25]. Recent reports indicated that anti–SARS-CoV-2 IgG antibodies in severely ill COVID-19 patients show a unique ability to trigger FcγRIIIa-dependent inflammatory responses by macrophages due to a reduced fucosylation at the Fc portion of IgG [26]. We analyzed whether IgG antibodies directed to the SARS-CoV-2 spike protein from patients with severe and mild COVID-19 differed in their ability to induce neutrophil activation. To this aim, all sera were adjusted to a final titer of 1:3200, and different sera dilutions were incubated with immobilized S protein for 1 hour in 96-well flat-bottom plates. Then, wells

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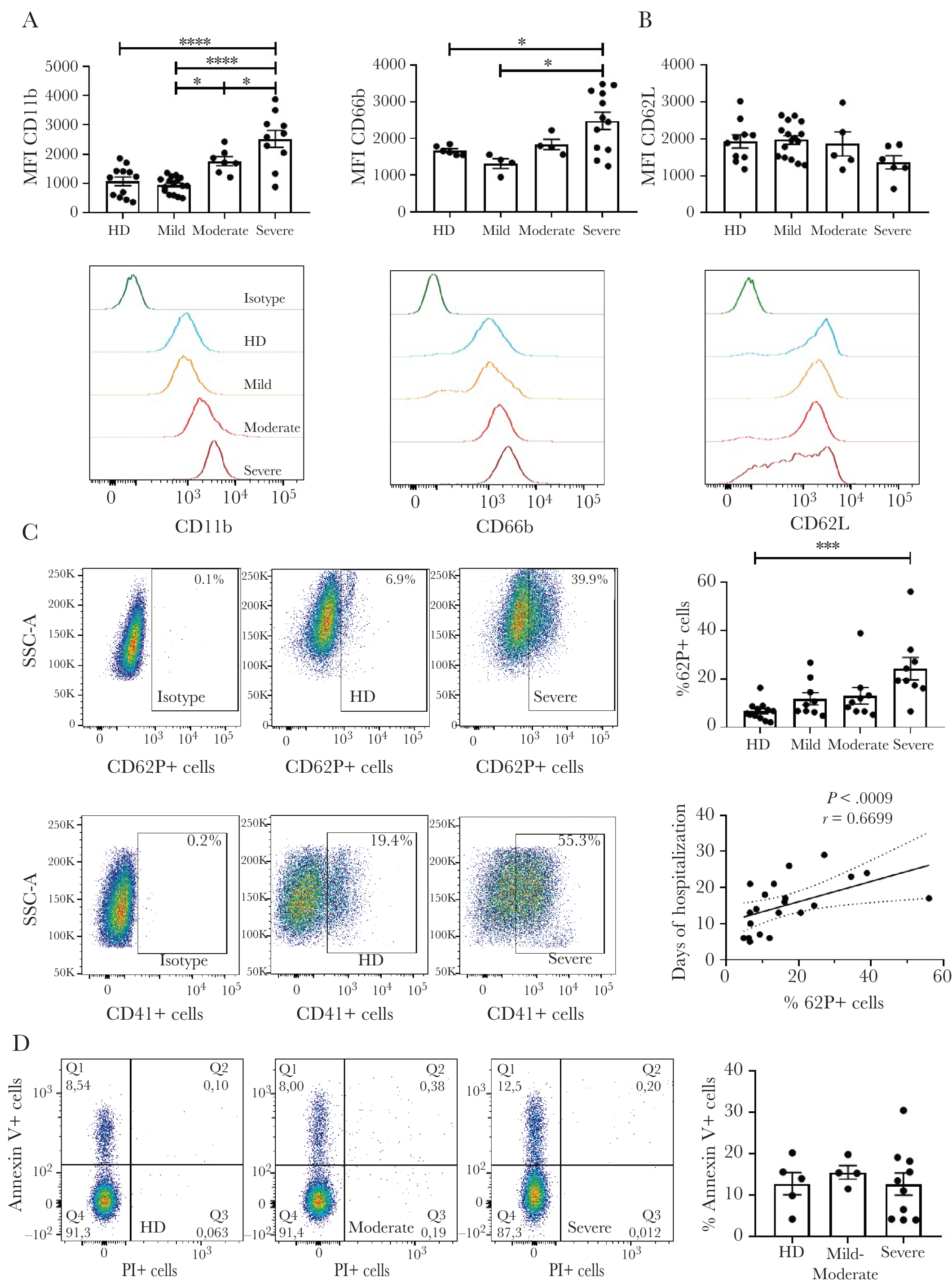

Figure 1. Peripheral blood neutrophils from patients with severe coronavirus disease 2019 (COVID-19) show an activated phenotype. *A* and *B*, The expression of CD11b, CD66b, and CD62L by freshly isolated neutrophils from healthy donors or COVID-19 patients (mild, moderate, or severe) was analyzed by flow cytometry. *C*, Association of platelets with neutrophils was evaluated in fresh whole blood samples by flow cytometry by analyzing the presence of the platelet marker CD62-P (upper panels) or CD41 (lower panels) in the neutrophil gate. Lower right panel shows the correlation between the days of hospitalization and the degree of neutrophil-platelet association. *D*, Neutrophils (2×10^6 cells/mL) were cultured for 10 hours at 37°C. Percentages of apoptotic cells were then evaluated by staining with annexin V fluorescein isothiocyanate/propidium iodide and flow cytometry. Bars represent the mean ± standard error of the mean of 4–16 donors. Histograms and dot-plots show representative experiments. One-way analysis of variance was used for comparisons in (*A*), (*B*), and (*D*); Kruskal–Wallis test was used in (*C*). **P* < .05, ****P* < .001, *****P* < .0001. Abbreviations: HD, healthy donors; MFI, mean fluorescence intensity; PI+, propidium iodide positive; SSC-A, side scatter area.

were washed and neutrophils isolated from healthy donors suspended in RPMI medium supplemented with 10% FCS were added, and plates were incubated for 30 minutes at 37°C. As shown in Figure 4B, ICs effectively induced the activation of the

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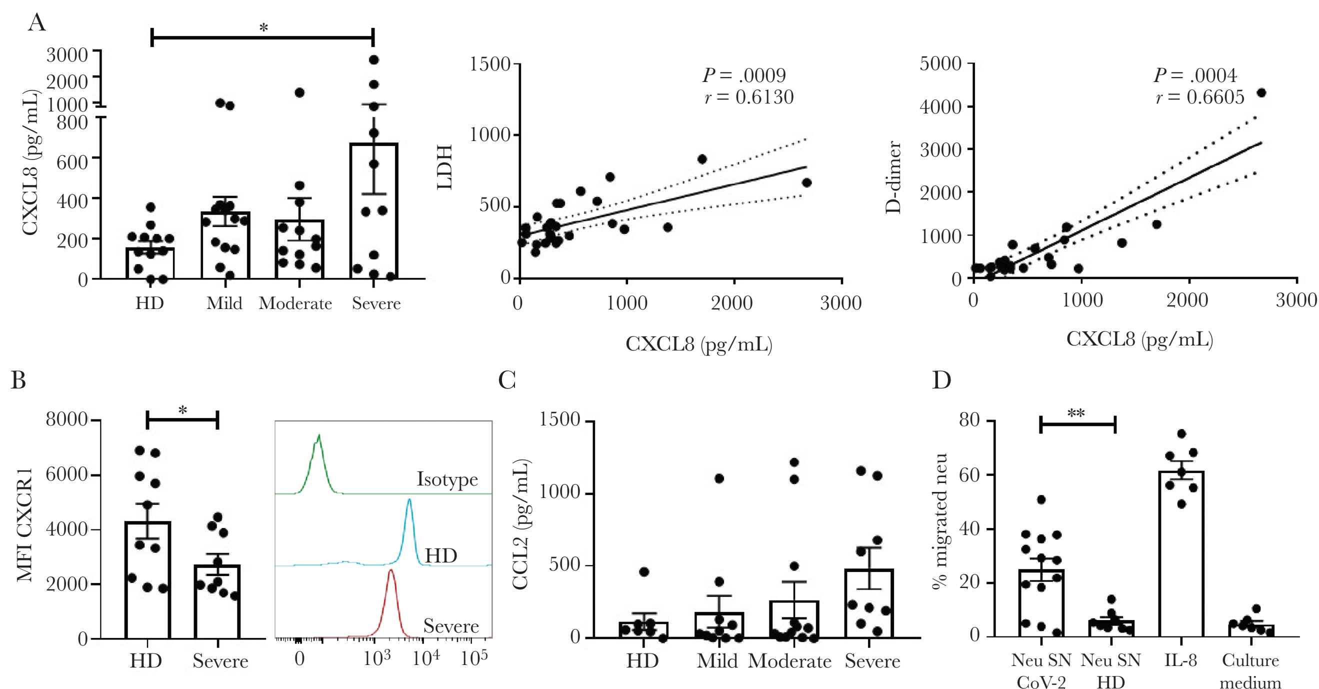

Figure 2. Spontaneous production of CXCL8 by neutrophils from patients with coronavirus disease 2019 (COVID-19) positively correlates with classical markers of disease severity. *A*, Supernatants from unstimulated neutrophils (10×10^6 cells/mL) from healthy donors (HD) or COVID-19 patients cultured at 37°C for 18 hours were collected, and the presence of CXCL8 was analyzed by enzyme-linked immunosorbent assay (ELISA) (left panel). Correlation between the spontaneous production of CXCL8 by neutrophils and plasma levels of lactate dehydrogenase and D-dimer (center and right panels). *B*, Expression of CXCR1 by freshly isolated neutrophils from HD or patients with severe COVID-19 was analyzed by flow cytometry. *C*, Supernatants from unstimulated neutrophils (10×10^6 cells/mL) from HD or COVID-19 patients cultured at 37°C for 18 hours were collected, and the presence of CCL2 was analyzed by ELISA. *D*, Supernatants from unstimulated neutrophils (10×10^6 cells/mL) from HD (n = 8) or patients with severe COVID-19 (n = 13) cultured at 37°C for 18 hours were collected. A transwell migration assay was performed by placing healthy neutrophils (1.5×10^5 cells/100 µL medium supplemented with 10% fetal calf serum) in the upper chamber and neutrophil supernatant (100 µL), interleukin 8 (10 ng/mL), or culture medium (control) in the lower chamber. After 1 hour of incubation at 37°C, cell migration was analyzed by flow cytometry, and the results are expressed as the percentage of neutrophils that have migrated through the membrane. A representative experiment (n = 3) is shown. *A–C*, Bars represent the mean ± standard error of the mean of n = 7–14 donors. Kruskal–Wallis test was used in (*A*) and (*C*). One-way analysis of variance was used in (*D*) and unpaired *t* test was used in (*B*). Histograms show representative experiments. $^*P < .05$, $^{**}P < .01$. Abbreviations: HD, healthy donors; IL-8, interleukin 8; LDH, lactate dehydrogenase; MFI, mean fluorescence intensity; Neu, neutrophil; SN, supernatant.

neutrophil respiratory burst when serum dilutions of 1:10 and 1: 20 were used. Using sera at a final dilution of 1:20, neutrophil activation was then analyzed by measuring respiratory burst activation, up-regulation of CD11b expression, and CXCL8 production. No differences were observed between the responses induced by IgG antibodies from severe and mild COVID-19 patients regarding neutrophil respiratory burst activation; by contrast, a higher CD11b expression and an increased stimulation of CXCL8 production were observed for neutrophils activated by IgG antibodies from patients with severe COVID-19 (Figure 4C and 4D). We then performed a similar analysis using isolated monocytes. ICs failed to induce the production of TNF-α and IL-6 (not shown), but efficiently induced the production of CXCL8, with this response being significantly higher for ICs prepared with IgG antibodies from patients with severe disease (Figure 4D). We conclude that ICs prepared with anti–SARS-CoV2 IgG antibodies from patients with severe COVID-19 show a higher inflammatory profile compared with IgG antibodies from patients with mild disease.

DISCUSSION

Inflammation is a hallmark of severe COVID-19, but the players and the mechanisms involved in the onset and exacerbation of the inflammatory response remain poorly defined. It was assumed that SARS-CoV-2–induced lung dysfunction is associated with a strong storm of inflammatory cytokines, but a rigorous comparison with other inflammatory syndromes such as sepsis, cytokine release syndrome, and acute respiratory distress revealed serum IL-6 concentrations among 10- to 100-fold lower in severe COVID-19 [27]. In contrast, various noncytokine markers of inflammation and tissue injury such as D-dimer, CRP, ferritin, and LDH are increased in a similar fashion in severe COVID-19 and other critical inflammatory diseases [27], suggesting that systemic inflammation in severe COVID-19 could not be only attributed to the acquisition of an inflammatory signature by macrophages.

High numbers of neutrophils have been found in the bronchoalveolar lavage fluid of patients with severe COVID-19 [8, 9, 28]. Elevated blood neutrophil counts have been shown to

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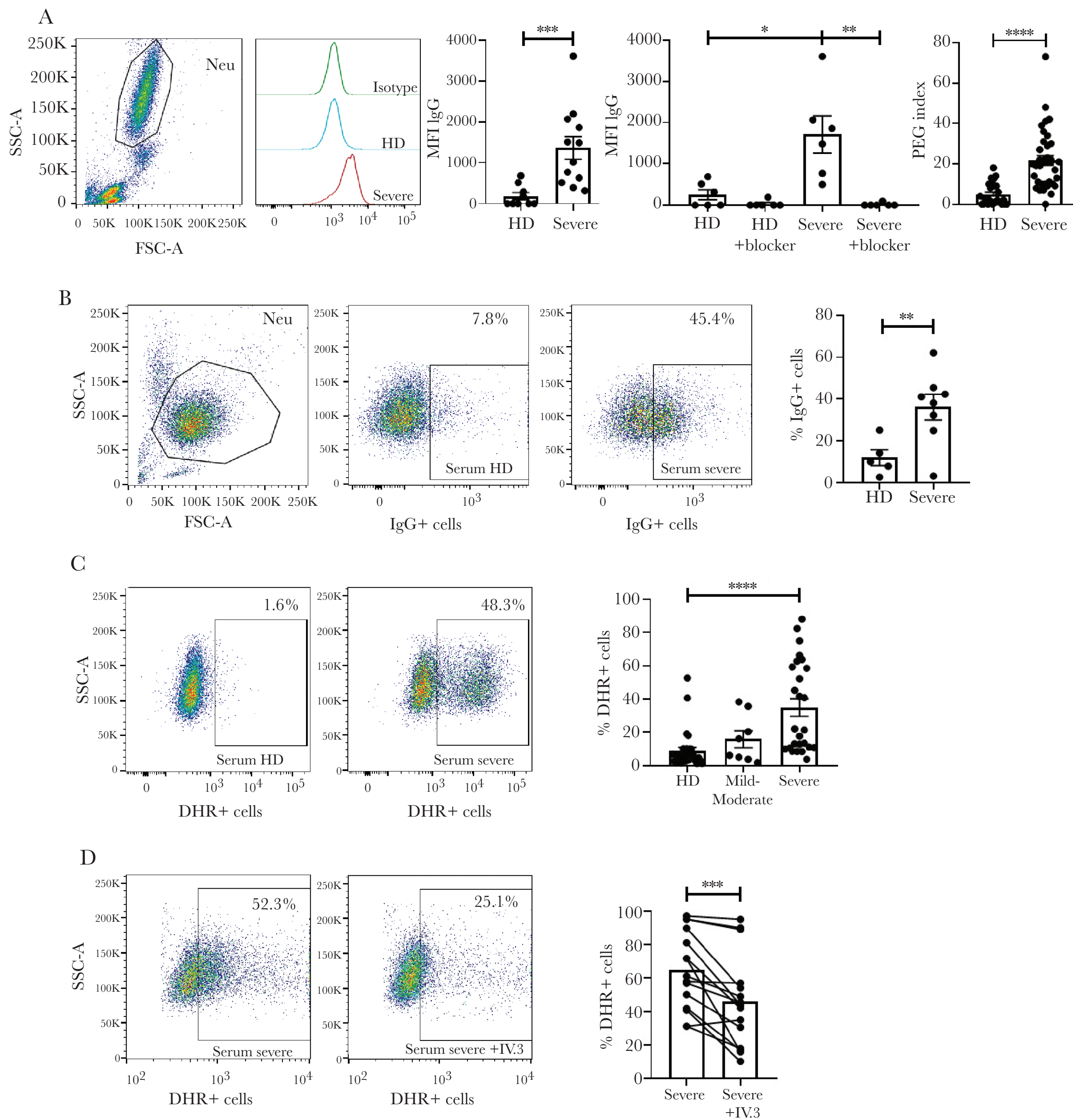

Figure 3. Immunoglobulin G (IgG) immune complexes may contribute to neutrophil activation in the course of severe coronavirus disease 2019 (COVID-19). *A*, IgG binding to neutrophils was evaluated in fresh whole blood samples from healthy donors (HD) or patients with severe COVID-19, by staining with a fluorescein isothiocyanate–labeled antibody directed to human IgG. In some experiments, whole blood samples were treated with an FcR blocking reagent before staining. Bars represent the mean fluorescence intensity ± standard error (n = 4–12) after subtraction of the mean value of the isotype. Right panel shows the concentration of serum immune complexes from healthy donors (n = 30) and patients with severe COVID-19 (n = 37), indicated as polyethyleneglycol index. *B*, Isolated neutrophils (2×10^6 cells/mL) were incubated in culture medium supplemented with 10% serum from HD or patients with severe COVID-19 for 1 hour at 4°C. Then, cells were stained with a Phicoerythrin-labeled antibody directed to human IgG and analyzed by flow cytometry. A representative experiment (n = 3) performed with sera from HD (n = 5) or from patients with severe COVID-19 (n = 8) is shown. *C*, Purified healthy neutrophils (2×10^6 cells/mL) were labeled with dihydrorhodamine-123 and incubated for 30 minutes at 37°C in medium supplemented with 10% of serum from HD or COVID-19 patients. Production of hydrogen peroxide was analyzed by flow cytometry. A representative experiment (n = 3) performed with sera from HD (n = 29) or from patients with mild (n = 8) or severe (n = 26) COVID-19 is shown. *D*, Neutrophils were pretreated, or not, with a blocking monoclonal antibody directed to FcγRII (clone IV.3), and production of hydrogen peroxide induced by serum (10%) from patients with severe COVID-19 (n = 15) was analyzed as described in (*C*). A representative experiment (n = 3) is shown. Kruskal–Wallis test was used for comparisons in (*A*) center right bar panel and (*C*); unpaired *t* test was used in (*B*). Mann–Whitney test was used in (*A*) center left panel and right panel; and paired *t* test was used in (*D*). Histograms and dot-plots show representative experiments. **P* < .05, ***P* < .01, ****P* < .001, *****P* < .0001. Abbreviations: DHR, Dihydrorhodamine; FSC-A, forward scatter area; HD, healthy donors; IgG, immunoglobulin G; MFI, mean fluorescence intensity; Neu, neutrophils; PEG, polyethyleneglycol; SSC-A, side scatter area.

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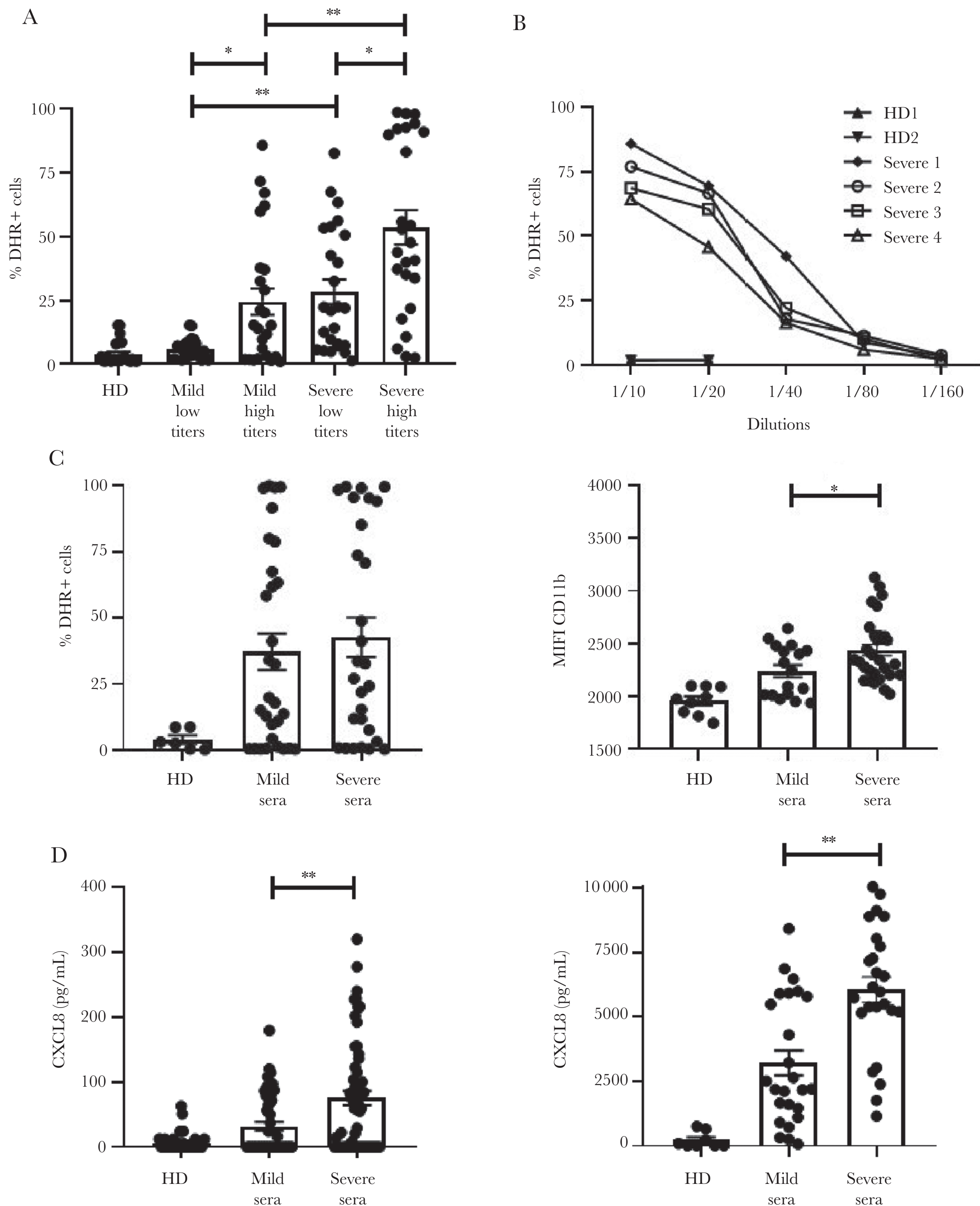

Figure 4. Inflammatory immunoglobulin G (IgG) anti–severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) antibodies in patients with severe coronavirus disease 2019 (COVID-19). *A*, Panel of sera with high (>1:3200) and low (<1:100) titers of IgG anti–SARS-CoV-2 antibodies from patients with either severe or mild COVID-19 was selected, as described in the Methods. Purified healthy neutrophils (2×10^6 cells/mL) were labeled with dihydrorhodamine-123 and incubated for 30 minutes at 37°C in medium supplemented with 10% of serum from healthy donors (HD) or COVID-19 patients. Production of hydrogen peroxide was analyzed by flow cytometry as described above. A representative experiment (n = 3) performed with sera from HD (n = 24) or mild patients/low titers (n = 23), mild patients/high titers (n = 23), severe patients/low titers (n = 24), and severe patients/high titers (n = 24) is shown. *B–D*, Sera from patients with severe and mild COVID-19 containing high levels of IgG anti–SARS-CoV-2 antibodies were titered and adjusted to a final titer of 1:3200. Sera from HDs were used as controls. Different sera dilutions (*B*) or 5% of normalized sera (*C* and *D*) were incubated with immobilized SARS-CoV-2 antigens (trimer spike protein and receptor-binding domain domain of spike protein) for 1 hour in 96-well flat-bottom plates. Then, wells were washed 5 times with culture medium, 1.5×10^5 healthy neutrophils (*C* and *D*, left panel) or 1×10^5 healthy monocytes (*D*, right panel) suspended in 100 µL of RPMI medium

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predict worse outcomes in COVID-19 [29], whereas increased concentrations of neutrophil products such as NETs [30], myeloperoxidase, and calprotectin [31] have been described in patients with severe COVID-19. A large body of evidence suggests that NETs play a major role in the pathogenesis of severe COVID-19. NET components have been detected in the plasma of patients with severe COVID-19 and correlated with disease severity [11, 32, 33]. Moreover, NET-containing microthrombi with neutrophil-platelet infiltration has been described in pulmonary autopsies from patients who died from COVID-19 [32], suggesting that NETs triggering immunothrombosis are involved in the pathogenesis of severe COVID-19. Neutrophils could also contribute to thrombotic events in the course of COVID-19 by releasing neutrophil-derived microparticles [33]. They have been associated to thrombosis in different diseases [34, 35] and have been found at high concentrations in the plasma of COVID-19 patients [33]. Interestingly, the activation of neutrophils and platelets in the course of COVID-19 could be induced not only by inflammatory mediators, but also directly by interacting with SARS-CoV-2 [30, 36, 37].

We here show that neutrophils from patients with severe COVID-19, but not from patients with mild or moderate disease, display an activated phenotype and spontaneously produce CXCL8 and CCL2. Production of CXCL8 correlated with the plasmatic concentrations of LDH and D-dimer, 2 recognized markers of disease severity [2, 18]. Consistent with previous reports [32, 38], we found that neutrophils from severe COVID-19 patients interact with platelets, and a correlation between the degree of neutrophil-platelet interaction and the days of hospitalization was found.

Neutralizing IgG antibody titers have shown to be higher in severe COVID-19 compared with mild or moderate disease [23, 24], calling into question whether IgG antibodies might also be involved in the pathogenesis of COVID-19. No evidence has been published supporting a role for antibody-dependent enhancement of infection in COVID-19. However, IgG antibodies might contribute to disease severity not only by promoting viral infection, but also by inducing inflammatory responses. In fact, studies performed in SARS-CoV/macaque models showed that IgG antibodies against the SARS-CoV-2 spike protein increases the severity of lung injury by promoting an inflammatory response mediated by macrophages [39].

The drivers of inflammation in COVID-19 remain to be clarified. Little attention has been paid to the possible role of IgG ICs. However, different observations suggest that IgG ICs might in fact be involved in the pathogenesis of severe COVID-19: (1) severe disease is associated with high levels of circulating anti–SARS-CoV-2 IgG antibodies [23, 24]; (2) viral antigens have been found in the blood of patients [40, 41]; (3) systemic complement activation occurs in the course of severe COVID-19 [42]; and (4) severe infection is associated with an exaggerated extrafollicular B-cell response [43] and the production of a variety of autoantibodies directed to red blood cells [22], platelets [21], type I interferons [20], self-carbohydrates [44], self-phospholipids [45], antinuclear antibodies, and anti-cytoplasmic neutrophil antibodies [46]. Our present results suggest not only that IgG ICs promote neutrophil activation in the course of severe COVID-19 but also that ICs might play a role in the induction of the systemic inflammatory and thrombotic responses associated with severe COVID-19. Interestingly, it has been recently reported that sera from severe COVID-19 patients contain ICs that activate platelets through FcγRIIA [47]. Our results also suggest that anti–SARS-CoV-2 IgG antibodies from patients with severe COVID-19 express a unique proinflammatory profile when incorporated into ICs. This observation is consistent with previous studies showing that ICs containing anti-spike IgG antibodies from severely ill COVID-19 patients induce a hyperinflammatory response mediated by monocytes, macrophages, and natural killer cells, due to a reduced fucosylation of the Fc fragment of IgG antibodies directed to SARS-CoV-2, resulting in an enhanced affinity to FcγRIII [26, 48].

A careful look of our experimental data reveals substantial heterogeneity among severely ill COVID-19 patients. Some of them show a dramatic increase in the neutrophil expression of CD11b and CD66b as well as in the spontaneous production of CXCL8 and CCL2, while other patients show values comparable to HD. This observation could reflect that severe COVID-19 could progress through different underlying mechanisms involving, or not, the participation of either neutrophils or ICs. Beyond this heterogeneity, our results suggest that ICs might contribute to the induction and maintenance of the inflammatory response in patients with severe COVID-19. Our observations suggest that the administration of intravenous immunoglobulin (IVIG) might represent a useful therapeutic tool to prevent disease progression. In fact, different reports have shown that IVIG treatment improves the course of severe COVID-19 [49]. The anti-inflammatory effects mediated by IVIG involve different mechanisms including the blockade of activating FcγRs, the induction of inhibitory signals through FcγRIIb, a decreased half-life of IgG autoantibodies due to the blockade of the neonatal receptor for IgG (FcRn), and the neutralization of

supplemented with 10% fetal calf serum were added, and the production of hydrogen peroxide by neutrophils (*B* and *C*, left panel) and the expression of CD11b by neutrophils (*C*, right panel) was evaluated after 30 minutes or 6 hours of incubation, respectively. The production of CXCL8 by neutrophils (*D*, left panel) and monocytes (*D*, right panel) were evaluated after 6 and 18 hours of incubation, respectively. A representative experiment (n = 3) performed with sera (titer 1:3200) from patients with mild (n = 17–46) or severe (n = 25–59) COVID-19 is shown in (*C*) and (*D*). Bars represent the mean ± standard error of the mean. Kruskal–Wallis test was used in (*A*), (*C*, left bar panel) and (*D*). One-way analysis of variance was used in (*C*, right panel). $^{*}P < .05$, $^{***}P < .01$. Abbreviations: DHR, Dihydrorhodamine; HD, healthy donors; MFI, mean fluorescence intensity.

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inflammatory mediators such as cytokines and complement components [50]. All of these mechanisms might contribute to improve the course of severe COVID-19.

Notes

Acknowledgments. We thank all of the team members of the Departamento de Medicina Interna, Hospital de Clínicas José San Martín and the Servicio de Clínica Médica, Hospital Fernández, Argentina. Most of all, we are indebted to all of the participants in our study and their families.

Financial support. This work was supported by grants from the Fondo Nacional para la Investigación Científica y Tecnológica (PICT 2017-1616 and PICT 2018–02844 to J. G.) and the Universidad de Buenos Aires (20020170100573BA to J. G.).

Potential conflicts of interest. All authors: No reported conflicts of interest.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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