IgG immune complexes may contribute to neutrophil activation in the course of severe COVID-19

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Less summary

A role for immune complexes in the activation of neutrophils in severe COVID-19 is suggested by the association of circulating neutrophils with immune complexes and the ability of patient sera to activate neutrophils through a mechanism partially depending on FcγRII.

Abstract

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

Key words: COVID-19, SARS-CoV2, neutrophils, CXCL8, inflammation, immune complexes, CD32.

BACKGROUND

SARS-CoV-2 infection induces a wide spectrum of clinical manifestations ranging from asymptomatic to severe disease and death [1]. Disease severity is associated to the induction of a systemic inflammatory response revealed by the high levels of inflammatory cytokines, chemokines and inflammatory markers such as C-reactive protein (CRP), ferritin, D-dimer, and LDH found in patients with severe 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 do 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) being both 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 suggest that neutrophils actually play a major role in the pathogenesis of severe COVID-19 [10,11]. Here, we show that neutrophils from severe COVID-19 patients display an activated phenotype, spontaneously produce CXCL8, and show a strong association with platelets. Moreover, our results suggest that IgG immune complexes (ICs) might contribute to the acquisition of an inflammatory phenotype by neutrophils in the course of severe COVID-19.

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.

Study Participants

Healthy individuals (n=42) were recruited from the blood bank of the "Hospital de Clínicas José San Martín": age range 23-65 years, male 62%. COVID-19 patients (n=67) were recruited from the "Hospital de Clínicas José San Martín" and the "Hospital Fernández". All COVID-19 patients were diagnosed by PCR amplification of SARS-CoV-2 viral RNA obtained from nasopharyngeal or oropharyngeal swabs. Taking into account the World Health Organization´s (WHO) eight-point scale for COVID-19 trial endpoints, patients were classified into three stages, i.e., mild, moderate, and severe [12]. Mild COVID-19 patients (WHO scores 1-2) were ambulatory patients or patients hospitalized with the aim of keeping them isolated; moderate patients (scores 3-4) included those with little or no requirement for supplemental oxygen; and severe patients included those with high-flow oxygen requirement (score 5), intubation and mechanical ventilation (score 6), and multi-organ 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, Argentine) 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 RPMI-1640 medium (Gibco Invitrogen, CA, USA). Neutrophils purity was confirmed by flow cytometry considering the forward (FSC) and side scatter (SSC) 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, CA, USA) (% 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 (CA, USA). Data were acquired using a BD FACSCanto cytometer and BD FACSDiva software (BD Biosciences). Statistical analyses were based on at least 20000 events gated in the neutrophil gate, defined as described above.

Association of Platelets with Neutrophils

It was studied by flow cytometry in whole blood samples by analyzing the platelet markers P-selectin (CD62P) or CD41 in the neutrophil gate. Briefly, whole blood samples were collected by venipuncture into vacutainer heparin tubes and stained less than 30 min after collection. Samples were treated with BD FACS™ lysing solution.

Neutrophil Transmigration Assay

It was carried out using a Corning HTS Transwell 96 permeable supports (5 μM pore size) (Corning, NY, USA). Unstimulated neutrophils (10×10^6/ml) from healthy donors (HD) or severe COVID-19 patients were cultured at 37°C for 18 h, and supernatants were collected. Healthy neutrophils ($1.5x10^5$/100 μl) were added to the upper reservoir and neutrophil supernatants (100 μl), IL-8 (10 ng/ml) or culture medium (control) were added to the lower reservoir. After 1 h at 37°C, neutrophils in the upper and lower chamber were quantified by flow cytometry, and the percentage of neutrophils that have migrated was determined.

Association of IgG Antibodies with Neutrophils

It 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 five times with culture medium. Cells were stained with a FITC-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 h 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

It was performed by polyethyleneglycol (PEG) precipitation [13]. Briefly, 0.2 ml of serum was mixed with 0.4 ml of 0.01 M-borate buffer, pH 8.4. Then, 5.4 of 4.16% PEG 6000 was added (final serum dilution 1/30), and the mixture was incubated at room temperature for 60 min. 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 was expressed as PEG index = OD450 with PEG - OD450 without PEG x 1000.

Quantitation of Neutrophil Apoptosis by Annexin-V Binding and Flow Cytometry

Neutrophils ($2x10^6$/ml) were cultured for 10 h at 37°C in RPMI medium supplemented with 10% 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

It was evaluated by using the indicator dye dihydrorhodamine-123 (Molecular Probes, CA, USA) by flow cytometry. Neutrophils ($2×10^6$/ml) were labeled with dihydrorhodamine-123 and cultured for 30 min at 37°C without stimuli or in the presence of serum (10%) from HD or COVID-19 patients. In some experiments, neutrophils were pre-treated with a blocking antibody directed to FcγRII (clone IV.3, STEMCELL, FL, USA).

Measurement of Cytokines by ELISA

The presence of CXCL8, CCL2, TNF-α and IL6 in cell supernatants was analyzed by ELISA (Biolegend, CA, USA).

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

It was performed by ELISA using the "COVIDAR IgG" kit (Laboratorio Lemos SRL, Buenos Aires, Argentina) that detects IgG antibodies against two viral antigens, trimeric spike and the receptor binding domain (RBD) of the spike protein [14].

Activation of Neutrophils and Monocytes by Immune Complexes Prepared with Serum from Severe or Mild COVID-19 Patients

We screen 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 severe or mild patients, 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 severe and mild patients were incubated for 1 h at room temperature in the plate of COVIDAR IgG kit, that contains two immobilized antigens of SARS-CoV-2, the trimeric spike protein and the RBD domain, prepared as described [14]. Plates were washed five times, and $1.5x10^5$ neutrophils or $1x10^5$ monocytes suspended in 100 µl of culture medium supplemented with 10% FCS were added. Release of hydrogen peroxide, expression of CD11b and production of cytokines were analyzed.

Statistical Analysis

When two groups were present, normally-distributed data were analyzed by two-sided t test and skewed data were analyzed by Mann–Whitney test or Wilcoxon test. For three or more groups, analysis was by one-way ANOVA or Kruskal–Wallis test with correction for multiple comparisons. Normality was assessed by Shapiro–Wilk test. Correlations were tested by Spearman's method. Data analysis was done with GraphPad Prism software version 8. Statistical significance was defined as $p < 0.05$.

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 mild, moderate or severe as described in 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 neutrophils 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 severe COVID-19 patients 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, right panel**). No differences in the rate of apoptosis was observed for neutrophils isolated from patients or HD (**Figure 1D**).

High serum IL6, CXCL8 and TNF-α levels at the time of hospitalization are predictors of COVID-19 patient survival [17]. We evaluated the spontaneous production of IL6, TNF-α and CXCL8 by neutrophils isolated from COVID-19 patients. No production of IL6 and TNF-α was observed (not shown). By contrast, neutrophils from severe COVID-19 patients spontaneously produced CXCL8 (**Figure 2A, left**). This production correlated with two recognized markers of disease severity, the plasmatic concentrations of LDH and D-dimer

(**Figures 2A**) [2, 18]. Consistent with the ability of CXCL8 to induce the internalization of the CXCL8 receptor CXCR1 [19], neutrophils from severe COVID-19 patients showed a lower expression of CXCR1 compared with HD (**Figure 2B**). A higher spontaneous production of the chemokine CCL2 by isolated neutrophils from severe COVID-19 patients was also observed although it did not reach statistical significance when compared with HD (**Figure 2C**). Moreover, we found that neutrophil supernatants from severe COVID-19 patients 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 immune complexes (ICs) in neutrophil activation. Interestingly, flow cytometer analysis performed in whole blood samples revealed high levels of neutrophil-associated IgG antibodies in severe COVID-19 patients (**Figure 3A**). Pre-treatment of whole blood samples with a FcR blocking reagent (Miltenyi Biotec) almost completely displaced IgG antibodies from the neutrophil surface, suggesting that neutrophils bind IgG in a 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 severe COVID-19 patients compared with healthy donors (**Figure 3A, right panel**). We then analyzed whether sera from COVID-19 patients might be able to induce the activation of healthy neutrophils in a FcγR-dependent mode. Incubation of neutrophil isolated from healthy donors with sera from severe COVID-19 patients resulted not only in the binding of IgG ICs (**Figure 3B**), but also in the activation of the neutrophil respiratory burst (**Figure 3C**), being 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 two variables: disease severity and titers of anti-SARS-CoV-2 IgG antibodies. Sera from severe and mild COVID-19 patients 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-titers (<1:100) 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. High titers of specific IgG antibodies were shown to be associated with a high ability to trigger the respiratory burst of neutrophils isolated from healthy donors. Interestingly, sera from severe patients show 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 severe and mild COVID-19 patients 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 h in 96 well plate flat bottom. Then, wells were washed and neutrophils isolated from healthy donors suspended in RPMI medium supplemented with 10% FCS were added, and plates were incubated for 30 min at 37°C. As shown in **Figure 4B**, ICs effectively induced the activation of the 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 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 was observed for neutrophils activated by IgG antibodies from severe patients (**Figure 4 C** and **D left panel**). We then performed a similar analysis using isolated monocytes. ICs failed to induce the production of TNF-α and IL6 (not shown), but efficiently induced the production of CXCL8, being this response significantly higher for ICs prepared with IgG antibodies from severe patients (**Figure 4D right panel**). We conclude that ICs prepared with anti-SARS-CoV2 IgG antibodies from severe COVID-19 patients show a higher inflammatory profile compared with IgG antibodies from mild patients.

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 IL6 concentrations among 10 to 100 fold lower in severe COVID-19 [27]. In contrast, various non-cytokine markers of inflammation and tissue injury such as D-dimer, C-reactive protein, ferritin, and lactate dehydrogenase (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 shown to predict worse outcomes in COVID-19 [29], while increased concentrations of neutrophils products such as neutrophil extracellular traps (NETs) [30], myeloperoxidase (MPO) , 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. NETs 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 died from COVID-19 [32], suggesting that NETs triggering immunothrombosis is 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 severe COVID-19 patients, 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, two recognized markers of disease severity [2, 18]. Consistent with previous reports [32, 38], we found that neutrophil 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: a) severe disease is associated with high levels of circulating anti-SARS-Cov-2 IgG antibodies [23, 24], b) viral antigens have been found in the blood of patients [40, 41], c) systemic complement activation occurs in the course of severe COVID-19 [42]; d) severe infection is associated to 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 (ANAs), and anti-cytoplasmic neutrophil antibodies (ANCAs)[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-IgG antibodies from severe patients express a unique pro-inflammatory 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 hyper-inflammatory response mediated by monocytes, macrophages and NK 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 reveal 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 severe COVID-19 patients. 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 inflammatory mediators such as cytokines and complement components [50]. All these mechanisms might contribute to improve the course of severe COVID-19.

Notes

Acknowledgments.

We thank all 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 the participating in our study and their families.

Financial Support.

This work was supported by grants from the "Fondo Nacional para la Investigación Científica y Tecnológica (FONCyT)" (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 report no potential conflicts.

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LEGENDS TO FIGURES

Figure 1. Peripheral blood neutrophils from severe COVID-19 patients show an activated phenotype. (**A-B**) The expression of CD11b, CD66b, and CD62L by freshly isolated neutrophils from healthy donors (HD) or COVID-19 patients (mild, moderate or severe) was analyzed by flow cytometry. (**C**) Association of platelets with neutrophils was evaluated in freshly 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/ml) were cultured for 10 h at 37°C. Percentages of apoptotic cells were then evaluated by staining with annexin-V FITC/propidium iodide and flow cytometry. Bars represent the mean ± SEM of n (4-16) donors. Histograms and dot-plots show representative experiments. One-way ANOVA was used for comparisons in **A**, **B** and **D**. Kruskal-wallis test was used in **C**. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001.

Figure 2. Spontaneous production of CXCL8 by neutrophils from COVID-19 patients positively correlates with classical markers of disease severity.

(**A, left panel**) Supernatants from unstimulated neutrophils (10×10^6/ml) from HD or COVID-19 patients cultured at 37°C for 18 h were collected, and the presence of CXCL8 was analyzed by ELISA. (**A, center and right panel**) Correlation between the spontaneous production of CXCL8 by neutrophils and plasmatic levels of LDH and D-dimer. (**B**) The expression of CXCR1 by freshly isolated neutrophils from HD or severe COVID-19 patients was analyzed by flow cytometry. (**C**) Supernatants from unstimulated neutrophils (10×10^6/ml) from HD or COVID-19 patients cultured at 37°C for 18 h were collected, and the

presence of CCL2 was analyzed by ELISA. (**D**) Supernatants from unstimulated neutrophils (10×10^6/ml) from HD (n=8) or severe COVID-19 patients (n=13) cultured at 37°C for 18 h were collected. A transwell migration assay was performed by placing healthy neutrophils (1.5×10^5/100 µl medium supplemented with 10% FCS) in the upper chamber and neutrophils supernatants (100 µl), IL-8 (10 ng/ml) or culture medium (control) in the lower chamber. After 1 h of incubation at 37°C, cell migration was analyzed by flow cytometry, and the results expressed as the percentage of neutrophils that have migrated through de membrane. A representative experiment (n=3) is shown. (**A-C**) Bars represent the mean ± SEM of n (7-14) donors. Kruskal-wallis test was used in **A** and **C**. One-way ANOVA was used in **D** and unpaired t test was used in **B**. Histograms show representative experiments. *P<0.05, **P<0.01.

Figure 3. IgG immune complexes may contribute to neutrophil activation in the course of severe COVID-19.

(**A**) IgG binding to neutrophils was evaluated in freshly whole blood samples from HD or severe COVID-19 patients, by staining with a FITC-labeled antibody directed to human IgG. In some experiments, whole blood samples were treated with a FcR blocking reagent before staining. Bars represent the MFI mean ± S.E. (n=4-12) after subtraction of the mean value of the isotype. Right panel shows the concentration of serum ICs from healthy donors (n = 30) and severe COVID-19 patients (n = 37), indicated as PEG index. (**B**) Isolated neutrophils ($2x10^6$/ml) were incubated in culture medium supplemented with 10% serum from HD or severe COVID-19 patients for 1 h at 4°C. Then, cells were stained with a PE-labeled antibody directed to human IgG and analyzed by flow cytometry. A representative experiment (n=3) performed with sera from HD (n=5) or sera from severe COVID patients (n=8) is shown. (**C**) Purified healthy neutrophils ($2x10^6$/ml) were labelled with

dihydrorhodamine-123 and incubated for 30 min 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 sera from mild (n=8) or severe COVID patients (n=26) is shown. (**D**) Neutrophils were pre-treated, or not, with a blocking mAb directed to FcγRII (clone IV.3), and production of hydrogen peroxide induced by serum (10%) from severe COVID-19 patients (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<0.05, **P<0.01, ***P<0.001, and ****<0.0001.

Figure 4. Inflammatory IgG antibodies anti-SARS-CoV-2 in severe COVID-19 patients

(**A**) A panel of sera with high (>1:3200) and low-titers (<1:100) of IgG anti-SARS-CoV-2-antibodies from either severe or mild COVID-19 patients was selected, as described in METHODS. Purified healthy neutrophils ($2x10^6$/ml) were labelled with dihydrorhodamine-123 and incubated for 30 min at 37°C in medium supplemented with 10% of serum from HDs 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 severe and mild COVID-19 patients containing high levels of IgG antibodies anti-SARS-CoV-2 were tittered 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-D**) were incubated with immobilized SARS-CoV-2 antigens (trimer spike protein and RBD domain of spike protein) for 1 h in 96 well plate flat bottom. Then, wells were washed five times with culture medium,

$1.5x10^5$ healthy neutrophils (**C and D left panel**) or $1.x10^5$ healthy monocytes (**D right panel**) suspended in 100 µl of RPMI medium supplemented with 10% FCS 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 min or 6 h of incubation, respectively. The production of CXCL8 by neutrophils (**D left panel**) and monocytes (**D right panel**) were evaluated after 6 and 18 h of incubation, respectively. A representative experiment (n=3) performed with sera (titer 1:3200) from mild patients (n=17-46) or severe patients (25-59) is shown in C-D. Bars represent the mean ± SEM. Kruskal-wallis test was used in **A**, **C** left bar panel and **D**. One-way ANOVA was used in **C** right panel. *P<0.05, **P<0.01, and ***P<0.001.

TABLE 1

	COVID-19 PATIENTS		
	Mild (n=29)	**Moderate (n=19)**	**Severe (n=19)**
Demographic and clinical data			
Age, years, median (range)	42.8 ± 2.7	57.3 ± 6.6	63.4 ± 5.3
Sex, male, n (%)	75.9	78.9	47.4
Fever (%)	17.2	31.6	57.9
ICU admission, n (%)	0	5.3	78.9
Laboratory parameters (mean ± SEM)			
Leukocyte counts/mm3	6724 ± 616	7762 ± 616	8153 ± 2173
Neutrophils counts/mm3	4306 ± 598	6055 ± 1506	6807 ± 2208
Lymphocytes counts/mm3	1476 ± 162	1112 ± 192	514 ± 186
Neutrophil to Lymphocyte ratio	2.84 ± 0.41	5.25 ± 1.07	8.00 ± 3.21
Platelets/mm3	207524±15607	197786±23929	205545±14006
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	1.6 ± 0.4	1.9 ± 0.4	2.8 ± 0.7
Blood sampling (days after diagnosis by PCR)	5.8 ± 1.4	7.0 ± 1.7	11.2 ± 4.2
Days of hospitalization	7.26 ± 0.4	13.5 ± 1.99	21.00 ± 2.9

Abbreviations: ICU, intensive care unit; LDH, lactate dehydrogenase; CRP, C-reactive protein.

Figure 1

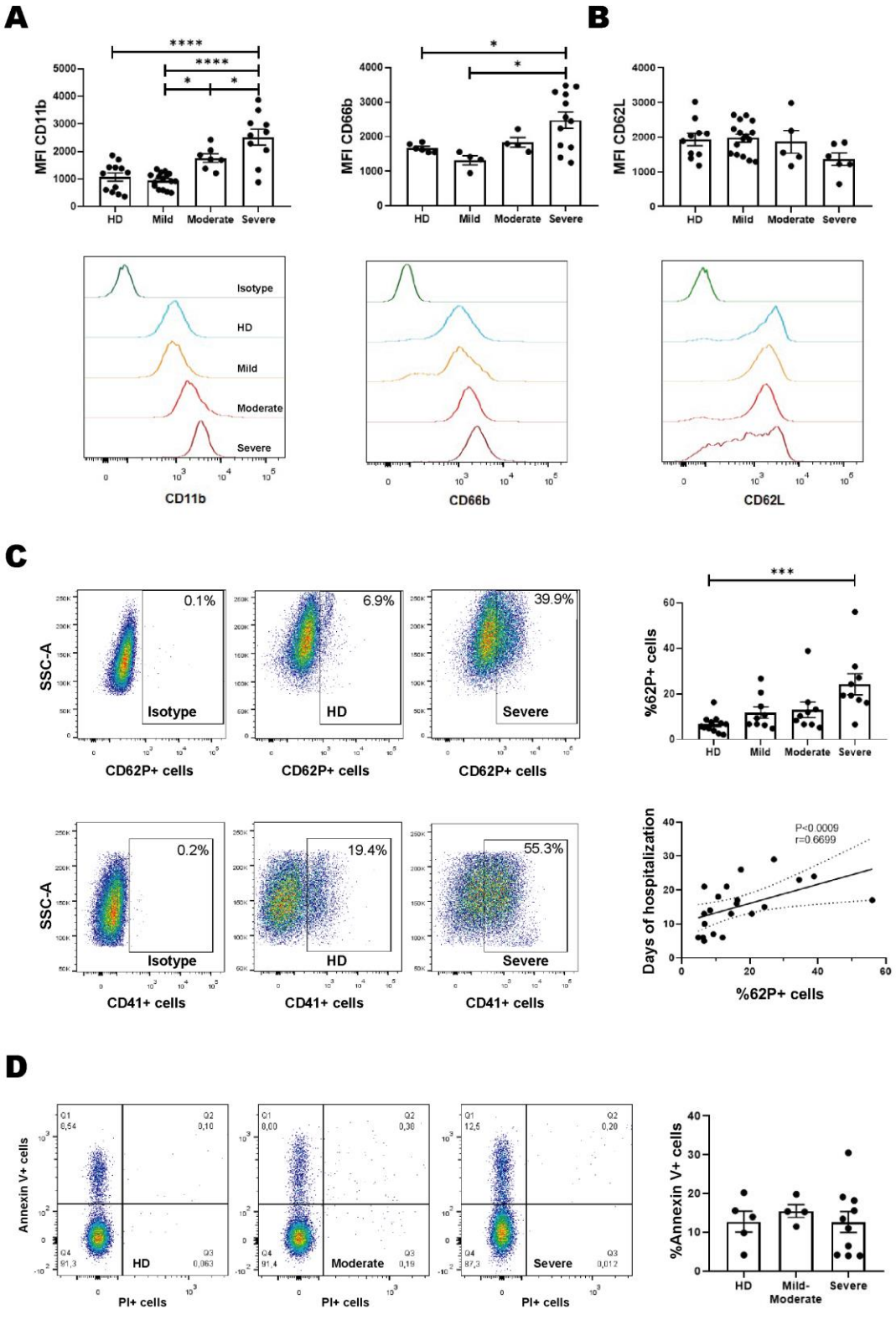

A
B
C
D
MFI CD11b
MFI CD66b
MFI CD62L
HD
Mild
Moderate
Severe
Isotype
CD11b
CD66b
CD62L
SSC-A
0.1%
6.9%
39.9%
CD62P+ cells
%62P+ cells
0.2%
19.4%
55.3%
CD41+ cells
Days of hospitalization
P<0.0009
r=0.6699
Annexin V+ cells
PI+ cells
%Annexin V+ cells
Mild-
Moderate

Figure 2

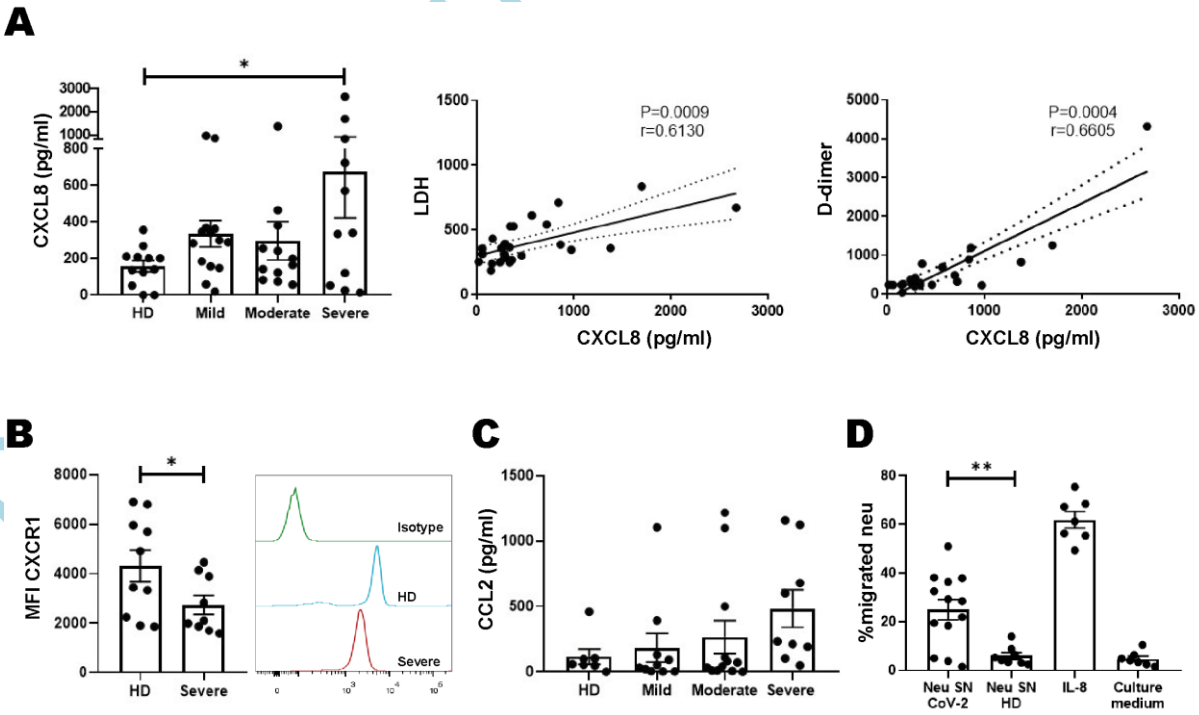

A
CXCL8 (pg/ml)
HD
Mild
Moderate
Severe
LDH
P=0.0009
r=0.6130
CXCL8 (pg/ml)
D-dimer
P=0.0004
r=0.6605
CXCL8 (pg/ml)
B
MFI CXCR1
HD
Severe
Isotype
HD
Severe
C
CCL2 (pg/ml)
HD
Mild
Moderate
Severe
D
%migrated neu
Neu SN CoV-2
Neu SN HD
IL-8
Culture medium

Figure 3

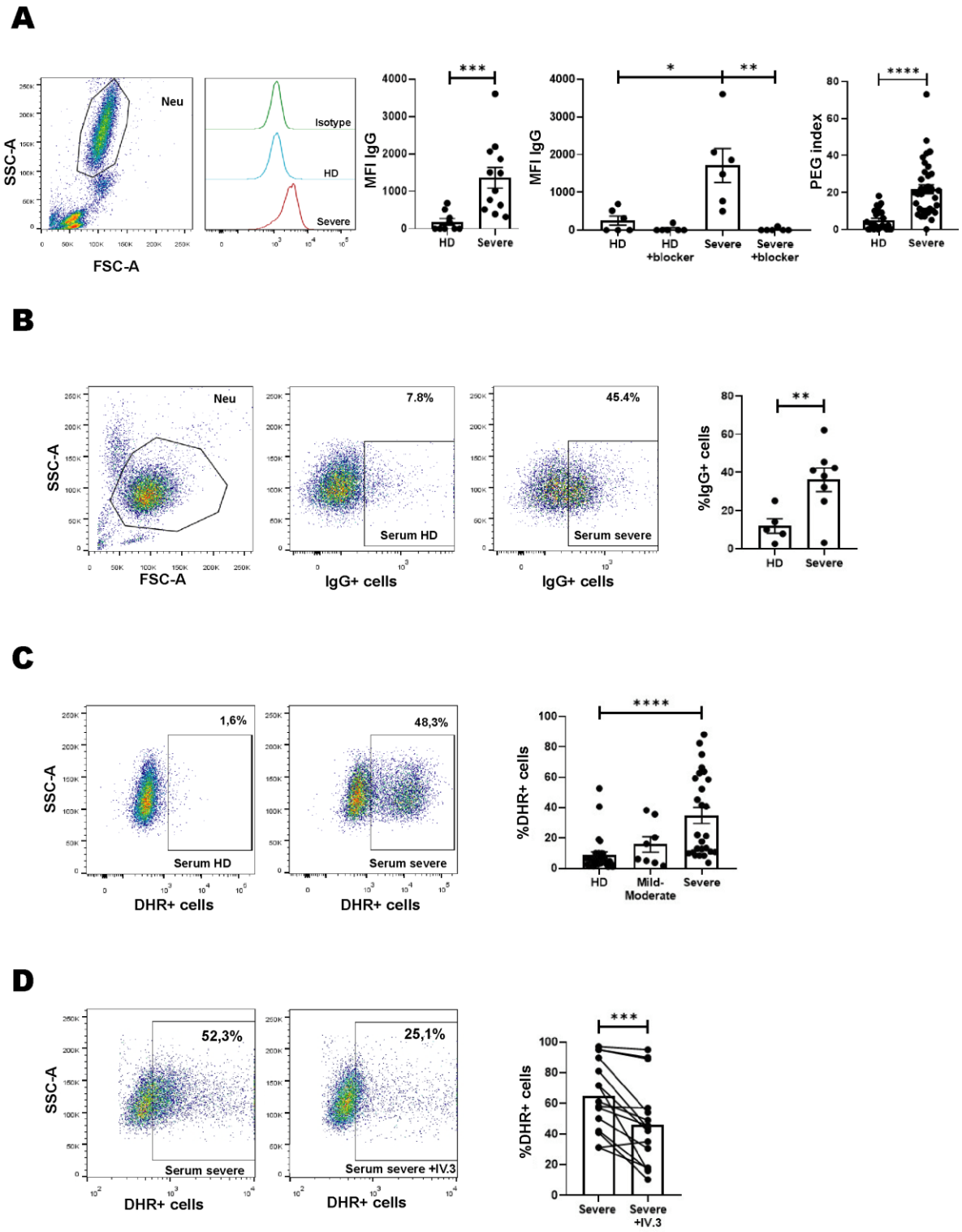
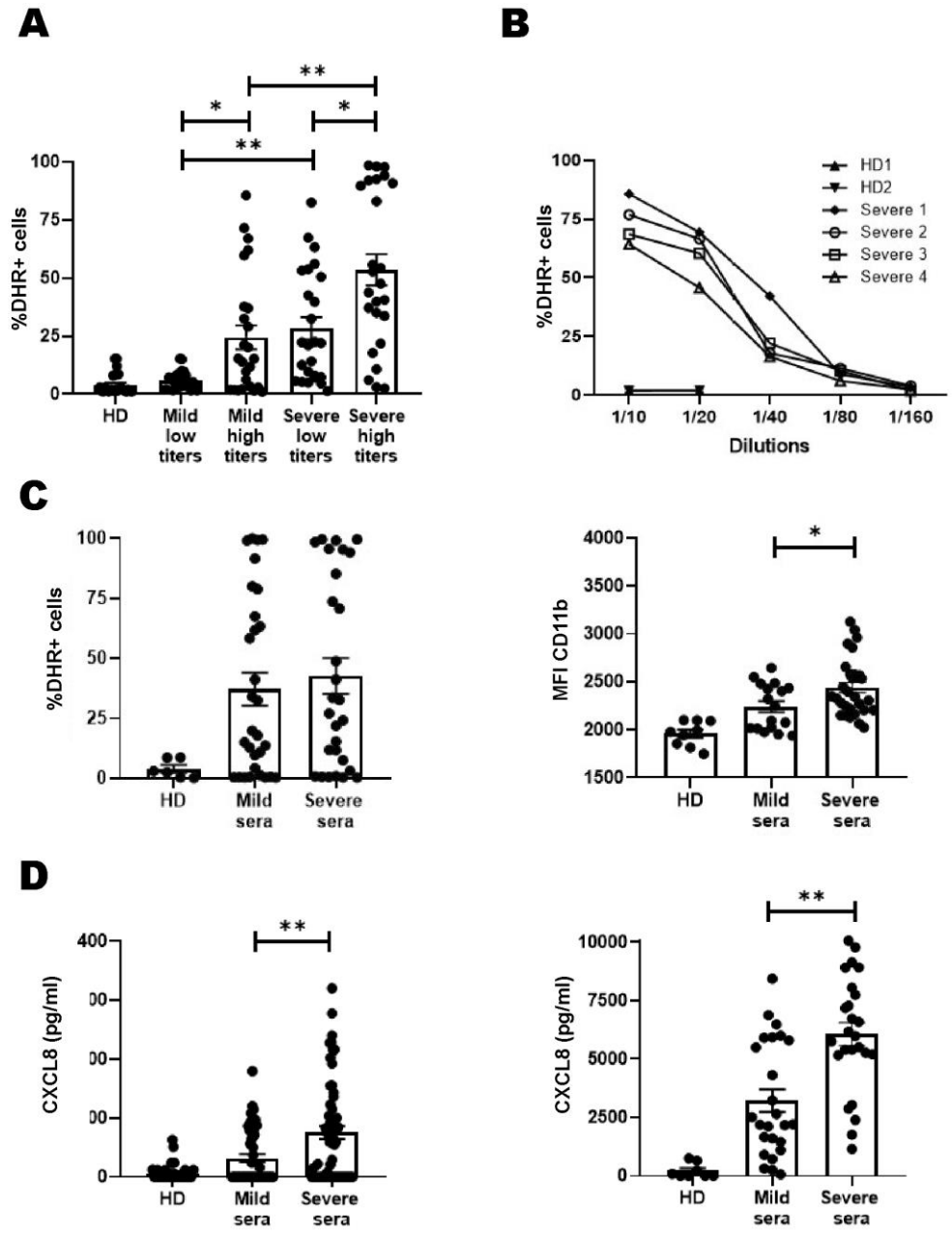

A
Neu
SSC-A
FSC-A
Isotype
HD
Severe
MFI IgG
HD
Severe
HD +blocker
Severe +blocker
PEG index
B
Neu
7.8%
45.4%
Serum HD
Serum severe
IgG+ cells
%IgG+ cells
C
1,6%
48,3%
DHR+ cells
%DHR+ cells
HD
Mild-Moderate
Severe
D
52,3%
25,1%
Serum severe
Serum severe +IV.3
Severe +IV.3

Figure 4


A
B
C
D
%DHR+ cells
HD
Mild low titers
Mild high titers
Severe low titers
Severe high titers
HD1
HD2
Severe 1
Severe 2
Severe 3
Severe 4
1/10
1/20
1/40
1/80
1/160
Dilutions
Mild sera
Severe sera
MFI CD11b
CXCL8 (pg/ml)