The Emerging Role of Neutrophil Extracellular Traps in Severe Acute Respiratory Syndrome Coronavirus 2 (COVID-19)

Angélica Arcanjo
Carlos Chagas Filho Biophysics Institute, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil;
Jorgete Logullo
Carlos Chagas Filho Biophysics Institute, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil;
Camilla Cristie Barreto Menezes
Tuberculosis Research Laboratory, Faculty of Medicine, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil;
Thais Chrispim de Souza Cravalho Giangiarulo
Institute of Biomedical Research, Hospital Naval Marcílio Dias, Navy of Brazil, Rio de Janeiro, Brazil
Shana Priscila Coutinho Barroso
Institute of Biomedical Research, Hospital Naval Marcílio Dias, Navy of Brazil, Rio de Janeiro, Brazil
Adriane Todeschini
Carlos Chagas Filho Biophysics Institute, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil;
Leonardo Freire-de-Lima
Carlos Chagas Filho Biophysics Institute, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil;
Debora Ricardo Decoté
Veterinary Institute, Federal Rural University of Rio de Janeiro, Rio de Janeiro, Brazil; Microbiology Institute, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil
Celio Geraldo Freire-de-Lima
Carlos Chagas Filho Biophysics Institute, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil;
Fátima Conceição Silva
Fundação Oswaldo Cruz, FIOCRUZ, Rio de Janeiro, Brazil
Wilson Savino
Fundação Oswaldo Cruz, FIOCRUZ, Rio de Janeiro, Brazil.
Alexandre Morrot (alexandre.morrot@ioc.fiocruz.br)
Tuberculosis Research Laboratory, Faculty of Medicine, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil;

Research Article

Keywords: SARS-CoV2, COVID-19, Extracellular Neutrophils Traps (NETs), Reactive Oxygen Species (ROS), Neutrophils, Immunopathology

Posted Date: July 7th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-40461/v1

Version of Record: A version of this preprint was published at Scientific Reports on November 12th, 2020. See the published version at https://doi.org/10.1038/s41598-020-76781-0.

Abstract

The novel coronavirus SARS-CoV2 causes COVID-19, a highly pathogenic viral infection threatening millions. The majority of those infected are asymptomatic or mildly symptomatic showing typical clinical signs of common cold. However approximately 20% of the patients can progress to acute respiratory distress syndrome (ARDS) and eventually death in about 5% of cases. Recently, angiotensin-converting enzyme 2 (ACE2) has been shown to be a functional receptor for virus entry into host target cells. The upregulation of ACE2 in patients with comorbidities may represent a propensity for increased viral load and spreading of infection to extrapulmonary tissues. This systemic infection is associated with higher neutrophil to lymphocyte ratio in infected tissues and high levels of pro-inflammatory cytokines leading to an extensive microthrombus formation with multiorgan failure. Herein we investigated whether SARS-CoV2 can stimulate extracellular neutrophils traps (NETs) in a process called NETosis. We demonstrated for the first time that SARS-CoV2 in fact is able to activate NETosis in human neutrophils. Our findings indicated that this process is associated with increased levels of intracellular Reactive Oxygen Species (ROS) in neutrophils. The ROS-NET pathway plays a role in thrombosis formation and our study suggest the importance of this target for therapy approaches against disease.

Introduction

There is global health emergency on the threat of a rapidly moving pathogenic SARS-coronavirus 2 (SARS-CoV–2), or COVID–19, highly lethal pandemic of a respiratory pathogen with the potential for killing millions of people (1). Coronaviruses are single-stranded positive-sense RNA viruses belonging to the Coronaviridae family. These viruses mainly infect animals, including mammals. Some recent human coronavirus infections have resulted in lethal epidemics, which include the endemic SARS (Severe Acute Respiratory Syndrome) and MERS (Respiratory Syndrome in the Middle East). In humans, coronavirus infection usually cause mild respiratory infections, like those seen in the common cold including fever, cough, and shortness of breath (2). The majority of those infected in the COVID–19 pandemic are asymptomatic, however about 20% of the patients can progress clinically to acute respiratory distress syndrome (ARDS) in severe patients with an increased risk of vascular hyperpermeability, pneumonia, sepsis and eventually death (1,2).

The overproduction of early response proinflammatory cytokines (TNFa, IL–6, IL–1β and IFN-γ) during innate response against COVID–19 virus results in a cytokine storm, leading to a diffuse alveolar damage evolving with shock and pulmonary dysfunction (3). The disease has been revealed as a systemic disease, the increased expression of ACE2 in patients with comorbidities may represent a propensity for an increased viral load of infection and the spread of the virus to extrapulmonary tissues (4,5). This systemic virus dissemination brings complications to different organs of the body in patients who evolve for the severe clinical form, which may affect central nervous system, eyes, heart and gut, leading to multi-organ dysfunction and extensive microthrombus formation with multiorgan failure (6). Evidences have shown that patients recovering from COVID–19 infection show signs of antiviral T and B cell–mediated adaptive immunity and memory, with clonal expansion and activation (7).

However the deregulated innate immune response and consequent viral dissemination in patients susceptible to COVID–19 infection contributes to events driving a nonresponsive state of T cell responses. Lung-infiltrating CD8+ T cells from severe COVID–19 patients potently exhibit hallmarks of T cell exhaustion, up-regulating PD–1 and Tim–3 markers (8). Analysis of highly expanded T cell populations obtained from peripheral blood of severe COVID–19 patients indicate a pronounced expression of MKI67 and TYMS markers of terminally exhausted T cells (9). The failure of an efficient immunological response early in disease may lead to persistent viral antigen thus contributing to the clinical presentation of patients with severe COVID–19 infections. Histopathologic analysis of COVID–19 patients have revealed the viral persistence in severe clinical forms (10,11). In the absence of a protective immune response, the burst of load promotes sustained levels of neutrophils described as a clinical hallmark of COVID–19. In fact, severe patients show higher neutrophil to lymphocyte ratio (NLR), which is suggested as a marker predictor of death (12).

A mounting body of evidence shows that severe clinical forms are associated to coagulation dysfunction markers, mainly D-dimer, platelet reduction, increases in prothrombin time and fibrin degradation products (6). These factors suggest hyperactivity of the coagulation system in organ failure and death. All these findings have a potential for cross-talk with neutrophil extracellular traps (NETs). NETs are structures made up of intracellular components released by activated neutrophils that discharge DNA, histones and proteins derived from intracellular granules. This process is called NETosis and plays a role in controlling pathogens, but also has a dentrimental effect in cardiovascular and pulmonary diseases (13). Intravascular NETosis in COVID–19 infection could play a role in the vasculature complications, where thrombotic disease can drive organ damage. Therefore in this report we investigate whether COVI–19 leads to NETs formation and the production of reactive oxygen species by neutrophils that could contribute to disease pathogenesis.

Material And Methods

Human samples and SARS-CoV–2 isolation. Serum samples from 20 hospitalized COVID–19 patients were used in this study. Blood were collected into a serum vacutainer tube and allow it to clot at room temperature for 1h. The criteria for confirmed cases with SARS-CoV–2 infection included positive result of the nucleic acid sequence of SARS-CoV–2 by real-time RT-PCR from nasopharyngeal swab samples based on FDA-approved RNA testing. Severe COVID–19 patients were clinically classified as having fever, respiratory infection, respiratory rate of 23 incursions/minute, dyspnea and oxygen saturation <93% at room air. Healthy volunteers and severe COVID–19 patients were recruited from Hospital Naval Marcílio Dias, Rio de Janeiro, Brazil. COVID–19 infected patients were selected with ages ranging from 18 to 78 years. Donors, age and sex matched-non-infected controls were included in the study. Virus was isolated from nasal swab specimens obtained from severe COVID–19 patient. Infection with SARS-CoV–2 was confirmed by performing real-time reverse transcription polymerase chain reaction (RT-PCR) assay, followed by viral M gene sequencing, and virus isolation on Vero E6 cell line. The research was approved by the Research Ethics Committee (CEP) from National Health Concil and all patients signed a free and informed consent form in accordance with current legislation and the relevant ethical regulations

approved by the Hospital Naval Marcílio Dias (CAAE # 31642720.5.0000.5256) and Hospital Universitário Clementino Fraga Filho (CAAE # 30424020.0.0000.0008).

NETosis and phagocytosis assay. Neutrophils were isolated from peripheral blood from the collection of 20 ml of heparinized blood. The blood collected was slowly placed in a 50 mL tube containing 1:2 Ficoll, the gradient was centrifuged for 30 minutes at room temperature without braking and without acceleration (400G). After centrifugation, the upper part containing mononuclear cells was discarded and the neutrophils were collected with a pasteur pipette and, after lysis of the red cells, the cells were resuspended in RPMI medium, counted and adjusted for each experimental condition. For NETosis assays, neutrophils were suspended in RPMI medium containing 1% nutridoma and adjusted to 1 x 10^5 neutrophils/mL. We stimulate or not with (Virus MOI 9.0, PMA 1 nM, serum from normal and severely infected patients, LPS 10 ng/mL). Afterwards, we incubated for 90 minutes at 37 ºC/5% CO_2, the cells were centrifuged at 4 ºC/1600 RPM for 6 minutes, and the supernatant was collected for extracellular DNA measurement. Then 25 µl sample of the supernatant was added to 50 µl of tris-EDTA buffer (1 mM at pH 8.0) and 25 µl of picogreen, a highly sensitive fluorescent DNA dye. The reading was made using a 528 nm emission filter, with 485 nm excitation in a microplate reader (Spectramax M3). For phagocytosis assays, 5 x 10^4 neutrophils were cultured in black 96-well plate treated with 0.001% L-polylysine and incubated in at 37 ^{0}C/5% CO2 for 90 minutes with or without stimuli (COVID–19 Virus at MOI 9.0; serum from normal or severe infected patients at 10%; PMA 1 nM). Phagocytic activity was analyzed from the incubation of Dextran beads conjugated to tetramethyl rhodamine (2000000 MW) added to the cells (200 µg/ml) in the presence of the stimuli. After 90 minutes, the wells were washed and the fluorescence incorporated into the cells was read in fluorometer with 555nm excitation and 580nm emission.

Dosage of reactive oxygen species. For the detection of intracellular Reactive Oxidizing Species (ROS), the dichlorodihydrofluorescin diacetate probe (H2DCFDA) was used. This probe reacts with free radicals in general and emits green fluorescence. For the assays, the 5 x 10^4 neutrophils were cultured in a 96-well plate treated with 0.001% L-polylysine and incubated at 37 ^{0}C/5% CO_2 for 90 minutes in the presence or absence of stimuli: COVID–19 virus (MOI 9.0); 10% serum from normal or severely infected patients; PMA 1 nM. Quantification of ROS was done by adding 20 µM H2DCFDA probe (Invitrogen) 15 minutes before the end of incubation. The plate was read at 530 nm emission and 485 nm excitation using a fluorimeter (Spectramax M3).

Quantification of IL–8. Interleukin 8 (IL–8) was quantified from neutrophil culture supernatants (5 x 10^4/50 µL) in the presence or absence of COVID–19 virus (MOI 9.0) according to the manufacturer's recommendations (Quantikine® Elisa/R&D System). In the assay 100 µL of assay diluent were added to the wells (96-well plate) along with the standards, samples and negative control. After 2 hours of incubation at 37 ^{0}C/5% CO_2, the wells were washed 4x with 200 µL of washing buffer and then 100 µL/well of Human conjugate anti-Interleukin 8 was added. The plate was incubated for one additional hour at room temperature, and after washing with 200 µL/well of washing Buffer, the reaction was revealed by adding 200 µL of substrate solution to each well and reading in a 450 nm plate reader.

Data analysis. Results are expressed as mean ± SEM and P ≤0.05 was considered significant. For multiple comparisons, One-way ANOVA analysis followed by Tukey's least significant difference was performed. Paired t-test analysis was performed for some experiments as indicated in the figure legend. Data analysis was performed by GraphPad Prism 5.03 software.

Results

We sought to investigate whether COVID–19 could activate neutrophils to induce NETosis. Although there is no evidence that viruses can use neutrophils to establish productive infections, many viruses can be detected within neutrophils and even be able to activate neutrophils to produce NETs. However, the exact mechanisms underlying this phenomenon are not fully characterized, it is possible that this process occurs via pattern recognition receptors (PRRs) present in endosomes or even expressed on the surface of neutrophils (17). To assess the induction of NETosis by COVID–19, human neutrophils were stimulated with virus at MOI of 9.0 and the release of DNA was measured 90 minutes later. We strikingly found that COVID–19 was able to induce a significant increase of DNA released to supernatant in the presence of virus as compared to spontaneous release from negative control. Additionally, in a control experiment for rapid NETs release, neutrophils were incubated with PMA for the same time point. Our results show COVID–19 induced the classical NETosis at comparable levels of PMA, after 90 minutes of incubation (Figure 1A).

Besides their ability to control infection of pathogens by inducing NETosis, neutrophils have a plethora of defense mechanisms. In the human blood, neutrophils are the prevalent phagocytic cells, accounting for about 50% of all leukocytes. They are the major phagocyte of the innate immunity and plays a key role in the host defense against infectious pathogens (14,15). We next addressed whether the COVID–19 could activate the phagocytic capacity of neutrophils. To approach this question, we used dextran beads conjugated to fluorescein as a marker of phagocytosis processes. Human neutrophils were plated in monolayers in 96-well plates and stimulated with COVID–19 virus at MOI of 9.0 in the presence of dextran beads conjugated to fluorescein. Phagocytosis was assessed 90 minutes later by measuring the intracellular neutrophil uptake. Our results indicate a reduction in the incorporation of beads in cells treated with COVID–19, as compared to the phagocytic activity of control neutrophils. This magnitude of reduction was observed in neutrophils treated with PMA for the same time point, indicating that NETosis processes in these groups partially impaired the ability of the cells to phagocyte (Figure 1B).

Next we tested whether SARS-CoV–2 could induce neutrophilic Reactive Oxygen Species (ROS). ROS generated by NADPH oxidase play an important role in clearance of RNA virus by neutrophils. Neutrophils release large amounts of ROS at the site of infection following the activation of G-protein-coupled receptors (GPCRs), toll-like receptors or IL–8-induced priming of the oxidative burst. ROS released by the NADPH oxidase complex can also activate granular proteases to induce NETosis (16,17). Our findings demonstrate that neutrophils are capable of inducing the production and release of ROS when incubated with COVID–19 for 90 minutes (MOI of 9.0). This same response was observed in the positive controls treated with PMA (Figure 1C). Furthermore, neutrophils have a preformed capacity to produce the

cytokine IL–8, considering that IL–8 mRNA is produced constitutively by these cells. The production of this proinflammatory cytokine could have a possible autocrine effect in stimulating ROS by neutrophils (18), however our results indicate that treatment with COVID–19 had no modulation in the levels of secreted IL–8, when compared to other control groups (Figure 1D).

Detection of active viral particles in the peripheral blood is linked to disease severity (19–21). Serum derived from these patients presents several thrombolytic factors and inflammatory cytokines that could activate NETosis in neutrophils. According to our hypothesis, our results demonstrate that addition of sera from severe COVID–19 patients to neutrophil monolayer induced the release of DNA compatible with NETs, as compared with heterologous control serum. Our results indicate that this increase was progressive over the 120 minutes of kinetics analyzed. Both serum groups had a higher DNA release index than spontaneous release of negative controls. The addition of LPS to sera did not confer any synergistic effect on DNA release by activated neutrophils (Figure 2A). In a larger cohort, using 20 individuals per group, our data corroborate the previous analysis showing a significant increase in the release of DNA by neutrophils treated with severe serum COVID–19 patients when compared with normal serum, as a control (Figure 2B). In an independent experimental set using this cohort, we showed that the neutrophil response do COVID–19 serum is not synergistically modulated by the addition of LPS (Figure 2C).

Discussion

The ongoing pandemic COVID–19 is a respiratory viral infection associated in about 20% of individuals infected with severe acute respiratory syndrome, predisposing to thrombosis both in veins and arteries due to excessive inflammation, platelet activation, endothelial dysfunction and stasis. The most frequent hemostatic change due to infection is thrombocytopenia and elevation of D-dimer (6). In Case-control studies using a cohort of 183 patients it was found that the degree of activation of D-dimers was much higher in patients who did not survive than in those who survived. In addition, in 71% of those who died, clots were found. The increase of D-dimer was related to an increased need for invasive ventilation, intensive care and death (6,18). It is still unclear whether these changes are direct consequences of viral virulence or the effect of the systemic inflammatory response. Recent studies have shown that many critically ill patients admitted with Covid–19 have generalized microthrombi processes formed in veins or arteries, which can be directly related to the severity and lethality of the disease. These studies revealed that 31% of 184 patients hospitalized for the new pneumonia virus had abnormally clotted blood, a percentage consider extraordinarily high compared to hospitalizations for others causes (22).

Despite all the data converging to a strong relationship between the new coronavirus and the formation of microthrombus, the mechanisms leading this process remain incompletely understood. It has been revealed that severe COVID–19 patients share characteristics common to the development of Acute Respiratory Distress Syndrome with the presence of thick mucus secretions in the airways and the development of blood clots (23). These symptoms are similar to those of diseases already known as being caused by NETs, including chronic obstructive pulmonary disease and pneumonia induced by

pathogen infections. The pathological effect of NETs is not restricted only to airway obstruction in lung injuries but also play a role in the occlusion of arteries and vessels in degenerative cardiovascular disease (24). Recent studies have demonstrated elevated levels of markers of NETs such as cell-free DNA, myeloperoxidase (MPO) -DNA, and citrullinated histone H3 (Cit-H3) in serum samples from patients with severe COVID–19 but not in healthy controls (25). Their investigation showing that the infected patients serum could trigger NET formation by healthy neutrophils were corroborate in our study using a cohort of 20 severe COVID–19 patients with acute respiratory distress syndrome. It is possible that the activation of NET in these sera is due to the presence of active virus in the blood of patients in the severe form of the disease (19,20).

We further show for the first time in the literature in this study that COVID–19 virus in fact is able to activate NETosis in human neutrophils. Our findings demonstrate that this process is associated with increased levels of intracellular Reactive Oxygen Species (ROS) of neutrophils incubated in the presence of COVID–19. Reactive oxygen species can kill pathogens directly by causing oxidative damage or indirectly in neutrophils by stimulating pathogen elimination via extracellular neutrophil trap formation (15,16). ROS also have a detrimental role promoting venous thrombus formation through modulation of the enzymatic cascade of fibrinolysis systems coagulation and the complement system (26). These findings undoubtedly point to a critical role for neutrophils in the pathology of infection. It will be important to determine whether the presence of NETs in immunohistochemistry analysis of lung tissue from autopsy samples are associated with disease severity and/or particular clinical characteristics of COVID–19. In other severe or persistent viral infections, neutrophil-mediated alveolar damage leads to interstitial edema, ventilation/perfusion mismatch and respiratory failure. Recent studies have identified neutrophil infiltration in the pulmonary capillaries in autopsy reports of COVID–19 patients (27). This further supports the hypothesis that neutrophils may be responsible for the severity of the disease.

Taking into account that the severity of the COVID–19 infection has linked to NET formation, it is possible that compounds that degrade NETs or block their formation could relieve ARDS associated with disease. This is seen in cases of cystic fibrosis in which the therapeutic use of dornase alfa that dissolves NETs by cleaving DNA provides loosen sputum and relieve symptoms (28). The protocol for administering these medications cannot be organ-specific since the disease has been revealed as a systemic disease. The upregulation of ACE2 in patients with comorbidities may represent a propensity for an increased viral load and spreading of infection to extrapulmonary tissues (4,5). The systemic infection would lead to a storm of pro-inflammatory cytokines leading to an extensive microthrombus formation with multiorgan failure in severe patients. As an alternative to this therapeutic target, studies have revealed that the use of heparin, a low-cost anticoagulant, was associated with an improvement in the prognosis of severe cases in Covid–19 resulting in increased oxygen levels in the patients' blood (29). Our studies also suggest the importance of using antioxidant treatments that aim to abrogate the possible participation of ROS generated by thrombosis in neutrophils activated by the COVID–19 infection. Further clinical studies are needed to bring clarity to this issue. Despite the limitations in our capacity in responding promptly to the clinical demand for this disease, a comprehensive understanding of the viral pathogenesis is needed in order to eliminate this devastating pandemic virus.

Declarations

Substantial Significance Statement

Coronaviruses are a large family of viruses that cause diseases ranging from the common cold to more serious infections, such as the Middle East Respiratory Syndrome (MERS-CoV), the Severe Acute Respiratory Syndrome (SARS-CoV), e recentemente o Coronavirus disease 2019 (COVID–19). There is an urgent need to better understand the pathophysiology of COVID–19, the global pandemic caused by SARS-CoV–2. We demonstrated for the first time that SARS-CoV2 stimulate extracellular neutrophils traps (NETs) in a process called NETosis. Our findings indicated that this process is associated with increased levels of intracellular Reactive Oxygen Species (ROS) in neutrophils. The ROS-NET patway plays a role in thrombosis formation and our study suggest the importance of this target for therapy approaches against disease.

Acknowledgments

This work was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico do Brasil (CNPq), Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

1. A. Rothan *et al.*, The epidemiology and pathogenesisof coronavirus disease (COVID-19) outbreak. *J Autoimmun.* 109:102433 (2020).
2. Jin *et al.*, Virology, Epidemiology, Pathogenesis, and Control of COVID-19. *Viruses* 27;12(4):372 (2020).
3. Ye *et al.*, The pathogenesisand treatment of the `Cytokine Storm' in COVID-19. *J Infect.* 80(6):607-613 (2020).
4. R. Bourgonje *et al.*, Angiotensin-converting enzyme-2(ACE2), SARS-CoV-2 and pathophysiology of coronavirus disease 2019 (COVID-19). *J Pathol.* May 17 (2020).
5. Raj *et al.*, SARS-CoV-2 Shedding From Asymptomatic Patients: Contribution of Potential Extrapulmonary Tissue Reservoirs. *Am J Trop Med Hyg.* 2020 Mat 13 (2020).
6. M. Connors *et al.*, COVID-19 and its implications for thrombosisand anticoagulation. *Blood* 135(23):2033-2040 (2020).

7. G. Alba *et al*., Targets of T Cell Responses to SARS-CoV-2 Coronavirus in Humans With COVID-19 Disease and Unexposed Individuals. *Cell* 20;S0092-8674(20)30610-3 (2020).
8. Diao *et al*., Reduction and Functional Exhaustion of T Cells in Patients With Coronavirus Disease 2019 (COVID-19). *Front Immunol.* 11:827 (2020).
9. Wen *et al*., Immune Cell Profiling of COVID-19 Patients in the Recovery Stage by Single-Cell Sequencing. *Cell Discov.* 6:31 (2020).
10. A. Vardhana *et al*., The Many Faces of the anti-COVID Immune Response. *J Exp Med.*217(6):e20200678 (2020).
11. H. Yao *et al*., A Pathological Report of Three COVID-19 Cases by Minimal Invasive Autopsies. *Zhonghua Bing Li Xue Za Zhi*49(5):411-417 (2020).
12. Liu Y *et al*., Neutrophil-to-lymphocyte ratio as an independent risk factor for mortality in hospitalized patientswith COVID-19. *J Infect.* S0163-4453(20)30208-5 (2020).
13. Papayannopoulos, Neutrophilextracellular traps in immunity and disease. *Nat Rev Immunol.* 18(2):134-147 (2020).
14. Kubes, The enigmatic neutrophil: what we do not know. *Cell Tissue Res.* 371(3):399-406 (2018).
15. L. Reshi *et al*., RNAViruses: ROS-Mediated Cell Death. *Int J Cell Biol.* 2014:467452 (2014).
16. T. Nguyen *et al*., Neutrophils to the ROScue: Mechanisms of NADPH Oxidase Activation and Bacterial Resistance. *Front Cell Infect Microbiol.*7:373 (2017).
17. Bréchard *et al*., Interleukin-8primes oxidative burst in neutrophil-like HL-60 through changes in cytosolic calcium. *Cell Calcium* 37(6):531-40 (2005).
18. Li *et al*., Dynamic relationship between D-dimerand COVID-19 severity. *J Haematol.* May 18 (2020).
19. Chen *et al*., Detectable serum SARS-CoV-2 viralload (RNAaemia) is closely correlated with drastically elevated interleukin 6 (IL-6) level in critically ill COVID-19 patients. *Clin Infect Dis.* ciaa449 (2020).
20. F. Shi *et al*., Association of viral load with serum biomakers among COVID-19 cases. *Virology* 546:122-126 (2020).
21. G. Schönrich *et al*., Neutrophil Extracellular Traps Go Viral. *Front Immunol.* 7:366 (2016).
22. F.A. Klok *et al.,* Incidence of thrombotic complications in critically ill ICU patients with COVID-19. *Thromb Res.* 191:145-147 (2020).
23. J. Betsy *et al*., Targeting potential drivers of COVID-19: Neutrophil extracellular traps. *J Exp Med.* 217(6): e20200652 (2020).
24. V. Papayannopoulos, Neutrophil extracellular traps in immunity and disease. *Nat Rev Immunol.* 18(2):134-147 (2018).
25. Y. Zuo *et al*., Neutrophil extracellular traps in COVID-19. *JCI Insight.* 5(11):138999 (2020).
26. C.G.R. Siow *et al*., Reactive Oxygen Species in Venous Thrombosis. *Int J Mol Sci.* 21(6):1918 (2020).
27. K.E. Konopka *et al*., Postmortem Lung Findings in an Asthmatic Patient With Coronavirus Disease 2019. *Chest* S0012-3692(20)30775-3 (2020).

28. C. Yang *et al.*, Dornase alfa for cystic fibrosis. *Cochrane Database Syst Rev.* 9(9):CD001127 (2018).
29. N. Tang *et al.*, Anticoagulant treatment is associated with decreased mortality in severe coronavirus disease 2019 patients with coagulopathy. *J Thromb Haemost.* 18(5):1094-1099 (2020).

Figures

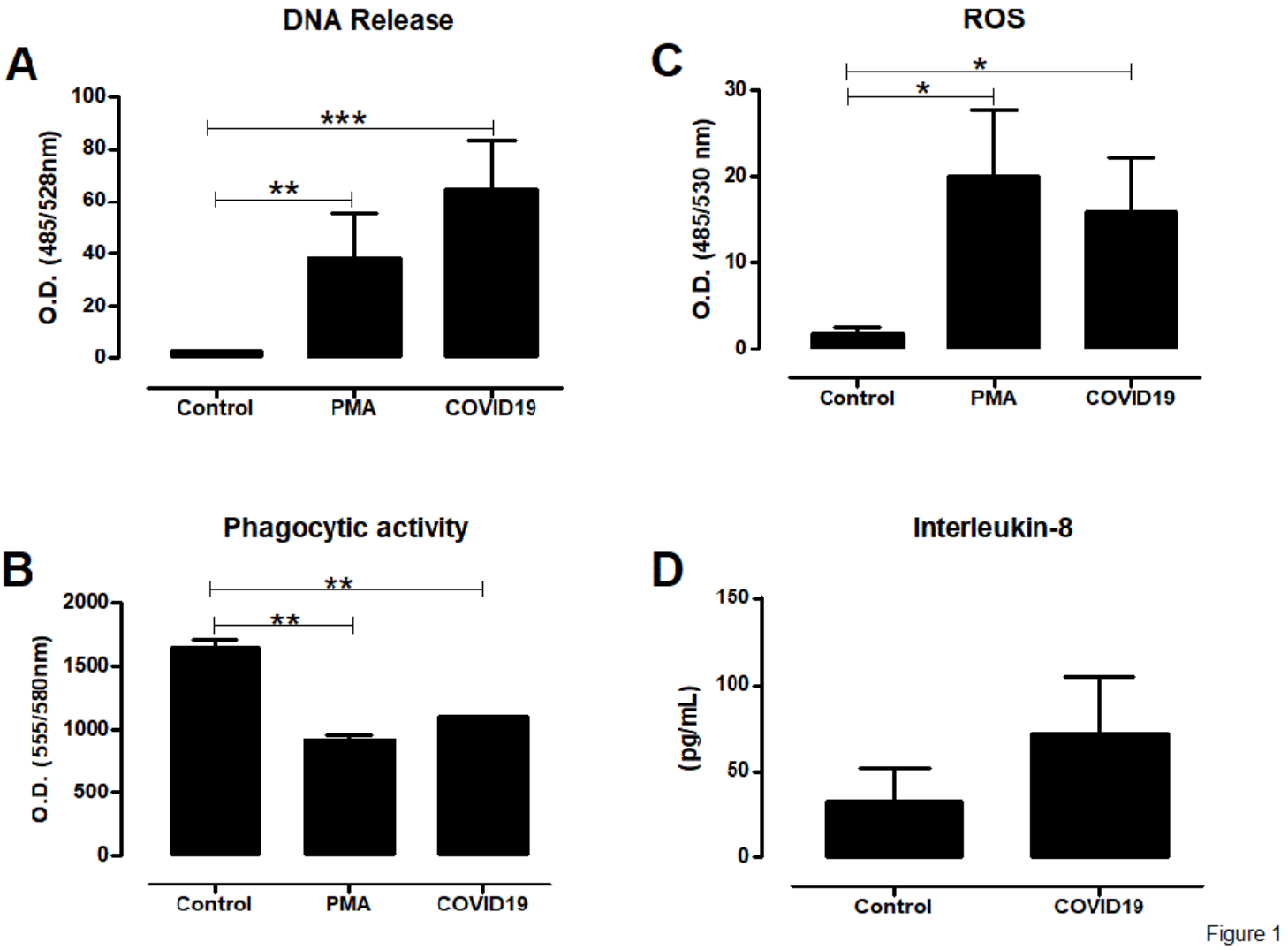

Figure 1

Figure 1

Neutrophils release extracellular DNA in response to serum from severe COVID-19 patients. Neutrophils (5 x 104/well) were stimulated or not with LPS (10 mg/mL) in the presence of 10% serum from normal or infected patients. After the different stimulus times indicated in the kinetics (A), or at a time point of 90 minutes (B, C), the supernatants were collected and the NETs were quantified by the Quant-iT ™ PicoGreen dsDNA method, using specific reagent for DNA detection double tape by optical density (528 nm). The data are representative of three independent experiments. (A) Sera from two donors (healthy control and severe COVID-19 patient) were used; (B, C) Sera from a cohort of 40 donors (20 healthy controls and severe 20 COVID-19 patients) were used. (D) Secretion of IL-8 from neutrophils stimulated by COVID-19

virus. Neutrophils (5 x 104/well) were stimulated or not with COVID-19 virus (MOI 9.0) and, after 90 minutes, the supernatants were collected for interleukin assay by ELISA. The quantification of interleukin production 8 (IL-8) was performed from the culture supernatant according to the recommendations of the manufacturer Quantikine Elisa/ R & D System (see materials and methods). The results were analyzed using One-way ANOVA followed by Tukey's. Differences between groups are significant * $p \leq 0.05$, ** $p \leq 0.0025$, *** $p \leq 0.0001$.

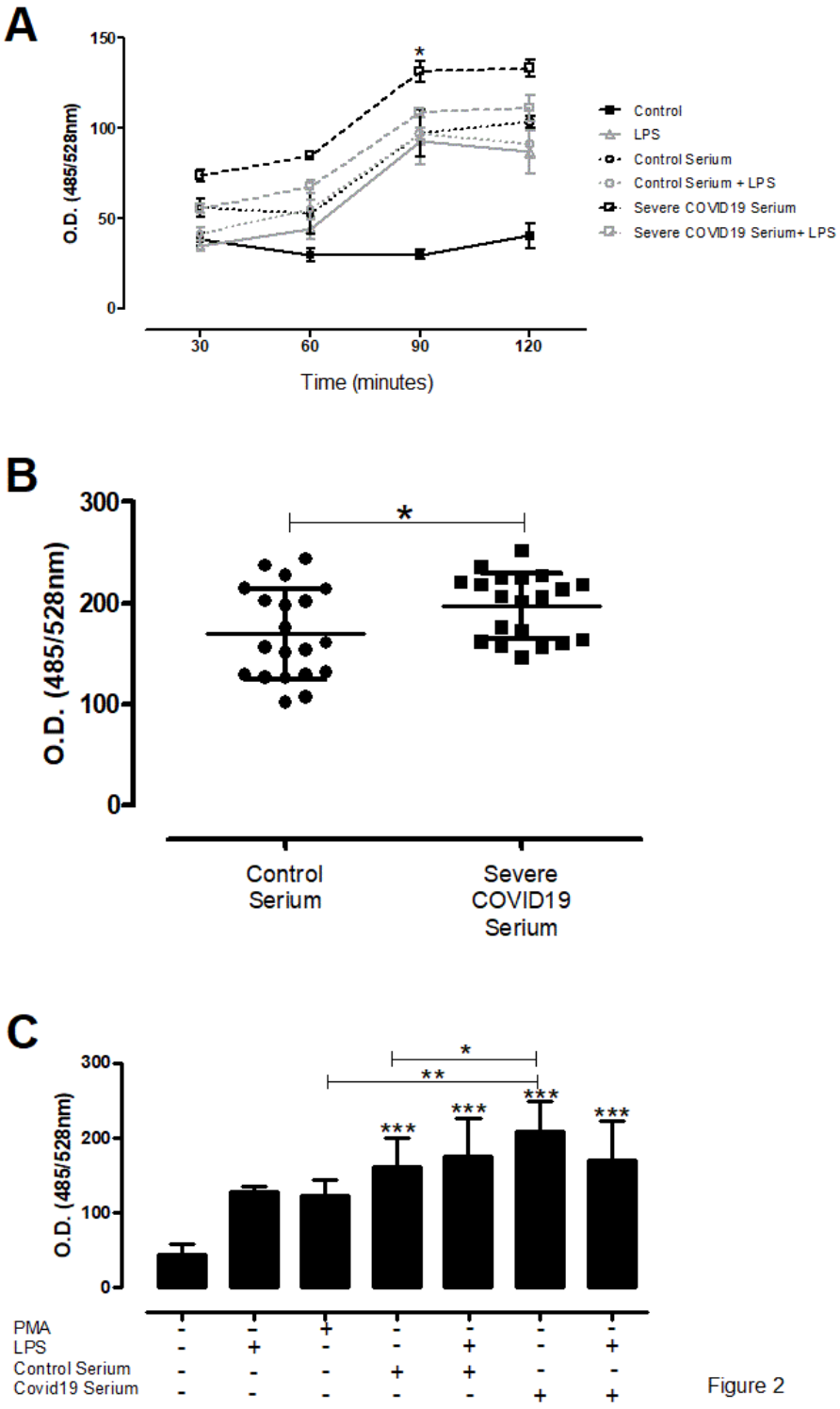

Figure 2

Figure 2

Neutrophils releases extracellular DNA and activates reactive oxygen species (ROS) in response to COVID-19 virus. Neutrophils (5 x 104/well) were stimulated or not with COVID-19 virus or PMA (positive control) and after 90 minutes (A) the supernatants were collected and the extracellular DNA release quantified by the Quant-iT ™ PicoGreen dsDNA method, specific reagent for double-stranded DNA labeling in optical density assay (528 nm). (B) Neutrophils (5 x 104/well) were stimulated or not with COVID-19 virus or PMA (positive control), and the phagocytic activity was analyzed by uptake of Dextran beads conjugated to tetramethyl rhodamine (2000000 MW) added to the cells (200 µg/ml) in the presence of the stimuli. The values expressed in optical density (580 nm) refer to the incorporated florescence after 90 minutes of incubation. (C) Quantification of reactive oxygen species (ROS). Neutrophils (5 x 104/well) were stimulated or not in the presence of 10% serum from normal donors or severe COVID-19 patients. After 90 minutes of stimulation, intracellular ROS was quantified using H2DCFDA probe (see materials and methods), and the values expressed refer to the quantification of florescence incorporated by the probe (530 nm). The data are representative of three independent experiments. The results were analyzed using One-way ANOVA followed by Tukey's. Differences between groups are significant * $p \leq 0.05$, ** $p \leq 0.0025$, *** $p \leq 0.0001$.