Critically ill COVID-19 patients exhibit hyperactive cytokine responses associated with effector exhausted senescent T cells in acute infection

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Summary: Our findings reveal that severe COVID-19 infection is marked by hyperactive T cell responses resulting in an aging process of the immune system and acute immunodeficiency due to clonal exhaustion of CD4 T helper responses.

Abstract

COVID-19 can progress to severe pneumonia with respiratory failure and is aggravated by the deregulation of the immune system causing an excessive inflammation including the cytokine storm. We herein report that severe acutely infected patients have high levels of both type-1 and type-2 cytokines. Our results show abnormal cytokine levels upon T cell stimulation, in a non-polarized profile. Furthermore, our findings indicate that this hyperactive cytokine response is associated with a significantly increased frequency of late-differentiated T cells with particular phenotype of effector exhausted/senescent $CD28^-$ $CD57^+$ cells. Interestingly, we demonstrated for the first time an increased frequency of $CD3^+CD4^+CD28^-CD57^+$ T cells with expression of programmed death 1 (PD-1), *one* of the hallmarks of T cell exhaustion. These findings reveal that COVID-19 is associated with acute immunodeficiency, especially within the $CD4^+$ T cell compartment and points to possible mechanisms of loss of clonal repertoire and susceptibility to viral relapse and reinfection events.

Keywords: COVID-19; SARS-CoV-2 coronavirus; Immunopathology; Exhausted/senescent T cells.

Introduction

COVID-19 is a devastating disease caused by the SARS-CoV-2 coronavirus infection, originally classified as a severe acute respiratory syndrome coronavirus (SARS-CoV). Most SARS-CoV-2 infected individuals are asymptomatic or exhibit an influenza-like inflammatory reaction. However, 5-20% of infected subjects develop a mild to severe condition whose major symptoms range from shortness of breath, vascular thrombosis and pulmonary obstruction (1). The severity of the infection is related to the presence of reduced immunological repertoire in elderly patients and the presence of comorbidities such as diabetes, obesity and cardiovascular dysfunction associated with increased expression of the angiotensin-converting enzyme 2 (ACE2) receptor, used by the virus to infect epithelial cells in the upper and lower airways (2). The binding of SARS-CoV-2 to ACE2 occurs through its spike (S) protein and viral entry is enhanced by the type II transmembrane serine protease TMPRSS2, which cleaves a portion of the S protein, exposing its fusion domain (3).

The high mortality rate seen in COVID-19 is related to the unregulated activation of the immune system. Patients who evolve to the severe form of the infection have a high neutrophil/ lymphocyte rate, acute pulmonary neutrophilic infiltration showing elevated serum cytokines, ferritin, haemophagocytosis, D-dimer, and soluble CD25 (the IL-2 receptor alpha chain) (4,5). The presence of activated neutrophils and macrophages in the target tissues has been associated with induction of neutrophil extracellular traps (6) and of thrombocytogenesis, promoting vascular collapse, respiratory distress and multiorgan failure, which are related to the so-called cytokine release syndrome (CRS), including excessive productions of granulocyte and macrophage colony stimulating factor (GM-CSF), interleukin (IL) -2, IL-6, IL-7, IL-10, tumor necrosis factor α (TNF-α) and granulocyte colony stimulating factor (G-CSF) (7).

The cytokine storm syndrome is most commonly triggered by viral infections and occurs in 3.7-4.3% of severe cases of sepsis; being associated with a hyperinflammatory response. The clinical characteristics of the syndrome consist of sustained elevated fever, abnormally high levels of serum ferritin and triglycerides, pancytopenia, disseminated intravascular coagulation, liver dysfunction and splenomegaly (8). Other changes are also present, such as decreased or absent NK cell activity, elevated serum levels of interleukin receptor chains, as well as hemophagocytosis, defined as phagocytosis of blood cells such as erythrocytes, leukocytes or platelets (9). In general, the predisposing factors for the development of the cytokine storm consist of a different combination, varying from viral escape mechanisms, preventing the antiviral immune response, associated with genetic defects or acquired in host defense and other immunological abnormalities, such as low levels of interferon. All of this culminates in impaired viral clearance, leading to unregulated activation of the immune system and Severe Acute Respiratory Syndrome (SARS) (8).

The underlying molecular mechanisms implicated in inducing the cytokine storm in critically ill patients with COVID-19 remain poorly understood. Importantly, the presence of high serum levels of IL-2 and CD25s (soluble IL-2 receptor a chain) in severe COVID-19 patients possible implies the participation of T cells in this immunopathology. Both IL-2 and CD25s are produced by activated T cells, suggesting a possible event of hyper reactivation of T cell responses in severe patients (10,11). Herein, we investigated the activation status of T cells in severe COVID-19 patients and demonstrated that these cells present a hyperactivation profile of cytokine responses induced by mitogens, as well as by heterologous antigens not associated with infection. Our cohort included all patients with clinical presentation of severe acute respiratory syndrome in SARS-CoV-2 infection, with comorbidities, ages ranging from 18-93 years, with average survival-to-discharge rate of 22.7% (Supplementary Figure 1). Our results indicate an increase in the frequency of T cells presenting a phenotype compatible

with clonal exhaustion and senescence in severe infection, corroborating the notion of an exacerbation and hyperactivation of T cell responses.

Material and Methods

Human samples. Blood samples from 22 hospitalized severe acutely infected COVID-19 patients (Supplementary Figure 1) and 22 healthy donors were collected into a heparinized vacutainer tube. The criteria for the infection diagnosis 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, as well as serological test for the S antigen. The patients screened were viral P.1 lineage negative (Gamma), descendant of B.1.1.28. To detect the deletion in the ORF1b gene (NSP6: S106del, G107del, F108del) found in P.1 variant, nasopharyngeal swab samples was submitted to Real Time PCR. We used oligos manufactured by IDT DNA, forward sequence 5'- GGG TGA TGC GTA TTA TGA CAT GGT TGG - 3', reverse 5'- CTA GCA CCA TCA TCA TAC ACA GTT CTT GC - 3' and probe sequence 5'(ZEN) - TGGTTGATACTAGTTTGAAGCTAAAA) - 3'. Real Time PCR was performed using Taqman Probe (Promega/IDT). Severe COVID-19 patients were clinically classified as having respiratory rate of 23 incursions/minute, dyspnea and oxygen saturation <93% at room air. Patients were recruited from Hospital Naval Marcílio Dias, Rio de Janeiro, Brazil. Healthy donors include age and sex matched-non-infected individuals who were eligible for not showing clinical signs for the disease, being prescreened to be negative for anti-COVID-19 antibodies and RT-PCR Swab Tests. The research was approved by the Research Ethics Committee (CEP) from Brazilian National Health Council 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 #

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Peripheral blood mononuclear cell purification and T-cell stimulation assay. Peripheral blood mononuclear cells (PBMCs) were purified from heparinized blood from COVID patients and normal donors using a FICOLL gradient (Histopaque-1077 Sigma) in a 1:2 ratio. The gradient was centrifuged for 30 minutes at room temperature without braking or acceleration (400G). After centrifugation, the upper part containing PBMCs was collected with a Pasteur pipette, and the red cells were lysed in lysis buffer. The cell suspension was then centrifuged at 1500 RPM for 6 minutes and the cells resuspended in RPMI medium with 1% nutridoma (Sigma), counted and adjusted for each experimental condition. For T cell stimulation assay, $2x10^5$ PBMCs/well were plated in a 96-well plate, in a total volume of 100 µL and stimulated or not with 5µg PHA-L (Sigma Aldrich), 2U PPD (Tuberculin) or anti-CD3/CD28 beads (1 µg/mL). After 3 days, the supernatants were collected for analysis of secreted cytokines.

Analysis of multiple secreted mediators and serum interleukin levels. Determination of cytokines, chemokines and growth factors secreted by stimulated PBMC cultures was carried through Luminex (Austin TX, USA) xMAP magnetic technology for the following analytes: IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-13, IL-15, IL-17, eotaxin, GCSF, GM-CSF, IFN-γ, MCP-1 (MCAF), MIP-1α, MIP-1β, RANTES, TNFα and VEGF. Analysis was performed as previously described (12). Briefly, after calibration and validation of Bio-Plex Magpix (Bio-Rad), reagent reconstitution and standard curve preparation, magnetic beads were added to each well. Each step was preceded by washing, using an automated Bio-Plex Pro wash station (Bio-Rad). Then, samples, standard and controls were added, followed by detection

antibodies and streptavidin-PE. Finally, magnetic beads were re-suspended and read. The values detected in culture medium without microspheres (background) were subtracted from the samples, allowing to access the protein levels secreted by cultures. For the analysis of serum interleukins (IL-2, IL-6, IL-10, IL-13 and IL-17), we used the multiplex biometric immunoassay containing fluorescent microspheres conjugated with target-specific monoclonal antibodies (Bio-Plex Pro Human Cytokine Screening, Bio-Rad). The tests were done according to the manufacturer's instructions and the fluorescence levels were detected on the Luminex 200 system. To measure Interferon gamma (IFN-γ) and Tumor necrosis factor alpha (TNF-α), we used a specific Sandwich-ELISA kit (Elabscience), in which micro ELISA plates were pre-coated with antibodies specific to the respective human cytokines. Standards or samples were added to the wells and combined with the specific antibodies. The presence of immunocomplex is revealed by the addition of biotinylated antibodies specific for Human TNF-α or IFN-γ, plus Avidin-Horseradish Peroxidase (HRP) conjugate. After the addition of the colorimetric substrate, the optical density (OD) was measured by spectrophotometry in a wavelength of 450 nm ± 2 nm, with the optical density (OD) values being proportional to the concentration of the corresponding cytokine.

Cytofluorometric analysis of human PBMCs and intracellular perforin staining. For cytofluorometric analysis, PBMCs were incubated with fluorochrome-conjugated monoclonal antibodies for 45 min at 4 °C. Freshly isolated PBMCs or *in vitro* activated T cells were stained with LIVE-DEAD Aqua (ThermoFisher Scientific) and surface mAbs recognizing CD4 APC-Cy7 (eBioscience, USA), CD8 PE-Cy5 (Beckman Coulter Life Sciences, USA), CD3 PerCP-Cy5.5, CD28 Alexa Fluor 647, CD57 PE and PD1 BV421 (EXBIO Antibodies). Cells were washed with stain buffer, and fixed with the cytofix buffer (Becton Dickinson, USA). Then, a minimum of 100,000 cells per sample were acquired on a FACS LSR Fortessa

instrument (BD, Franklin Lakes, USA), and the data were analyzed with FlowJo software (TreeStar; Ashland, USA). To detect perforin, freshly-isolated PBMCs from COVID-19 patients were suspended at a density of $1x10^6$/mL in complete RPMI medium. Cells were incubated in the presence of anti-CD28 (2 µg/ml) plus specific COVID-19 peptide mix (2 µg/ml of each MHCI peptide FL9, FL19, FF9 and FWF9) and anti-CD28 for 6h at 37°C in a 5% CO_2 incubator, followed by an additional 3 h in the presence of Brefeldin A (10 µg/mL). Lymphocytes were stained for cell membrane markers (anti-CD28, anti-CD57, anti-CD8, anti-CD4, anti-CD3), and permeabilized for intracellular perforin staining using purified mouse anti-human perforin (BD Pharmingen) and anti-mouse IgG AlexaFluor488 (Sigma). Samples were acquired using BD LSR Fortessa and the data were analyzed using Flow Jo and DIVA softwares. The selection of T-cell epitopes spanning the Spike protein sequence was made using the IEDB analysis resource Consensus tool, which combines predictions from ANN aka NetMHC, SMM and Comblib. Considering lengths of 9 mers, the prediction score of each length was evaluated against a default pannel of 27 most frequent HLA-A and HLA-B alleles. Four peptides (FL9 - FVFLVLLPL, FI9 - FTISVTTEI, FF9 - FAMQMAYRF, FWF9 - FVSNGTHWF) with median consensus percentile rank lower than 1 and predicted binders in at least 60% of HLA binding frequency were synthesized (95%) and used as T-cell epitopes.

Data analysis. Results were expressed as mean ± SEM and $p \leq 0.05$ was considered statistically significant. For multiple comparisons, One-way ANOVA analysis followed by Tukey's least significant difference was applied. Paired *t*-test analysis was performed for some experiments as indicated in the figure legend. Data analysis was performed by using the GraphPad Prism 5.03 software.

Results

High levels of cytokines related to T cell-dependent responses have been reported in sera of patients infected with the severe form of COVID-19, such as IL-2 and soluble CD25 (IL-2 receptor α chain) (10,11). These cytokines point to a hyperactive state of T responses in these individuals. Although the pathogenetic mechanisms remain largely unknown, these findings point to a possible role of T-cells in the pathogenesis of severe SARS-CoV-2. Our data corroborate this line of evidence showing increased levels of T cell-associated cytokines, including IFN-γ, IL-2, IL-6, IL-7, IL-10, IL-13 and IL-17 in sera from severe COVID-19 patients as compared to healthy controls (Figure 1).

Viral infections can be associated to hyperactivation events preceding the development of T cell exhaustion. A significant number of non-specific T lymphocytes can be activated by cytokine-dependent manner mechanisms, a phenomenon referred to as bystander activation. These cells can nevertheless impact the course of the immune response to the infection, not only participating in protective immunity, by secreting cytokines, but also due to their potential roles in responses related to the immunopathology of the disease. To identify whether there is a role for T cells in the quantitative and qualitative contribution of the cytokine storm profile characteristic of severe forms of infection in COVID-19 patients, we stimulated PBMCs from acutely-infected symptomatic patients with phytohemagglutinin-L (PHA-L), the lectin extract from the red kidney bean, consisting of only L-type subunits (isolectin L4). The subunits L (leukocyte reactive) have a high affinity for lymphocyte surface receptors and are appropriate for high-efficiency induction and functional analysis of human T-lymphocyte responses (13). Pananalysis of the cytokine and chemokine profile of PBMCs after mitogen stimulation indicates that severely infected patients significantly produce more cytokines than healthy controls, as ascertained by the levels of IL-2, IL-7, IL-9, IL-10, IL-13, IL-15, IL-17α, IFN-γ, TNF-α (Figure 2). Corroborating these findings, a

broad and non-polarized profile in the expression of interleukins was also seen through TCR-dependent activation with anti-CD3/CD28 in samples derived from COVID-19 patients (Figure 3). Moreover, the mitogenic activation of PBMCs indicated a more pronounced profile in the expression of chemokines and leukocyte colony-stimulating factor in severe infected patients, including MIP-1α, MCP-1, Eotaxin, RANTES, GM-CSF and G-CSF (Figure 4).

This mitogenic driven T-cell activation reveals a non-polarized profile of differentiation, suggesting a possible bystander TCR-independent activation event, possibly due to the action of cytokines produced during the infection. As compared to healthy individuals, we further showed that mitogen-activated T cells from patients with severe COVID-19 infection secrete more VEGF, an angiogenesis stimulator present in hypoxia conditions, being responsible for the suppression of immunity by inhibiting the maturation of dendritic cells, induction of regulatory T lymphocytes and myeloid-derived suppressor cells (Figure 5A). Our results indicate that such an enhanced VEGF production is present in heterologous antigenic responses of antigens not associated with infection, given that T cell responses in peripheral blood mononuclear cells obtained from symptomatic patients in the acute phase of COVID-19 secrete high levels of this cytokine upon stimulation with soluble antigen purified protein derivative (PPD) tuberculin (Figure 5B). Interestingly, we also found that VEGF was present at significantly increased levels in sera from patients with severe respiratory syndrome coronavirus 2 (SARS-CoV-2) infection as compared to healthy controls (Figure 5C).

Recent studies have reported an association between VEGF and programmed death-ligand 1 (PDL-1) in T cell exhaustion pathways in several malignancies (14). This issue is particularly relevant considering that the low count of $CD4^+$ and $CD8^+$ lymphocytes is a hallmark finding in COVID-19 disease, and both T cell subtypes are shown to express significantly higher PD-1 levels in COVID-19 patients (15). These findings suggest a higher susceptibility of these

cells to apoptosis and exhaustion during SARS-CoV-2 infection, which may account for the heterogeneity in immune responses to SARS-CoV-2, including in $CD8^+$ T cells. The importance of respiratory $CD8^+$ T cell responses is critical in both the protection of asymptomatic and convalescent individuals, as well as in immunopathological responses in severe cases, and may be related to disease features. Persistent antigenic stimulation leads to gradual accumulation of late-differentiated T-cells with particular phenotype ($CD3^+CD28^-CD57^+$) (16). In fact, our results demonstrated increased frequencies of both terminally differentiated $CD3^+CD4^+CD28^-CD57^+$ and $CD3^+CD8^+CD28^-CD57^+$ senescent T cells in severely infected COVID-19 patients in the acute phase of the disease (Figure 6A,B). Cytofluorometric analysis identified increased frequencies and size of $CD3^+CD4^+CD28^-CD57^+$ T cell subset expressing PDL-1 in patients with severe disease compared to healthy controls (Figure 6C), showing senescence/exhaustion events in the infection of patients in COVID-19. Moreover, our findings further indicated that the compartment of $CD3^+CD8^+CD28^-CD57^+$ T cells had SARS-CoV-2-specific $CD8^+$ T-cell responses producing perforin when stimulated with viral antigens (Figure 6D).

Discussion

SARS-CoV-2 is highly pathogenic in humans, causing severe acute respiratory syndrome, a pandemic pneumonia with immeasurable public health challenges to the world (1). It has been reported that the viral ORF6, ORF8 and nucleocapsid proteins play an important role in modulating the host innate immunity. They are potential inhibitors of type I interferon (IFN-β) and NF-κβ-responsive promoter, an innate immune signaling pathway critical for the host defense against viral infections (17). Low levels of type I interferons probably lead the immune system to compensate with unregulated activation of responses in the acute phase of infection, as exemplified by cytokine storm (7). In general, the predisposing factors for development of the cytokine storm consist of a diverse combination of mechanisms,

involving viral escape associated with genetic defects of host defense, as well as other immunological abnormalities, such as high rate of neutrophil infiltration into target tissues. The infection and consequent activation of neutrophilic network and thrombocytogenesis lead to multiple organ failure (6,8). This mechanism of immunopathogenesis has been proposed as being determinant in the worsening of infection, contributing to the high morbidity and lethality seen in COVID-19 (1).

Studies have shown that high serum levels of the cytokines sIL-2R (a soluble form of the IL-2 receptor) and IL-6 are prognostic markers for disease severity (18). Seriously infected patients had the highest serum levels of both cytokines, while those with mild condition had lowest indexes, showing that the disease severity is positively correlated with the expression levels of sIL-2R and IL-6. These two cytokines are part of the inflammatory mediators present in the COVID-19-associated cytokine storm, a possible leading cause of death in the Spanish flu pandemic of 1918 and other respiratory diseases caused by coronavirus, such as severe acute respiratory syndrome (SARS) and middle eastern respiratory syndrome (MERS) (7). The presence of sIL-2R points to the participation of T lymphocytes in the contribution of the inflammatory cytokine storm. In fact, our studies showed a significant increase of the T cell-associated cytokine levels, namely IFN-γ, IL-2, IL-6, IL-7, IL-10, IL-15 and IL-17, in sera obtained from severe symptomatic patients as compared to healthy controls.

Furthermore, human T-lymphocyte responses from severely infected symptomatic patients indicated an increased production of cytokines IL-2, IL-6, IL-7, IL-9, IL-10, IL-13, IL-15, IL-17α, IFN-γ, TNF-α, MIP-1α, MCP-1, Eotaxin, RANTES, GM-CSF and G-CSF by stimulation with high affinity mitogen for lymphocyte surface receptors. It should be pointed out, however, that the revealed profile obtained via the *TCR*/CD3 complex stimulation does not indicate polarizing cytokine T cell responses, rather corresponding to a broad spectrum of inflammatory mediators. This suggests a possible effect of the systemic inflammatory

environment characteristic of the acute phase in driving a T-cell receptor-independent and cytokine-dependent manner. This mechanism is common in viral infections, being responsible for pronounced unspecified T cell-dependent responses, the phenomenon referred to as bystander activation (19). In addition, our results revealed increased levels of VEGF in response to the mitogenic stimulation of PBMCs from severe COVID-19 patients. VEGF promotes vascular neoangiogenesis in physiological as well as in pathophysiological conditions caused by tissue hypoxia. Such an oxygen deprivation promotes the expression of FIH-1α, responsible for inducing adaptive responses capable of regulating VEGF expression (20). In fact, in severe cases of the disease there is a manifestation of disseminated thrombolytic processes, possibly being involved in the process of respiratory syndrome and multiple organ failure (21). Interestingly, our findings demonstrated increased VEGF serum levels in critically infected patients, suggesting their relevance in the pathophysiology of the disease.

VEGF induces the expression of the transcription factor TOX in T cells to drive a clonal exhaustion program in these lymphocytes (14). This can be decisive given that our findings corroborate the demonstration that both $CD4^+$ T and $CD8^+$ T cells in severe acute COVID-19 patients present significantly higher PD-1 expression suggesting a propensity of these cells to apoptosis and exhaustion during SARS-CoV-2 infection (15). The mechanisms of clonal deletion as a result of the processes of antigenic or apoptotic ligand-mediated hyperactivation are characteristic of infections with systemic inflammation. In these conditions, an induction of terminal differentiation programs in which clonal senescence processes of activated lymphocytes is observed (22). We showed herein a significantly increased frequency of late-differentiated T-cells characterized by the particular phenotype $CD28^-CD57^+$ (16). Although we showed herein that terminally differentiated SARS-CoV-2-specific $CD3^+CD8^+CD28^-CD57^+$ T cells respond, producing perforin when stimulated with viral antigens, our findings

revealed that severe acute COVID-19 infection is associated with senescence/exhaustion of T cells, especially within the $CD4^+$ T cell compartment since we observed a high frequency of $CD3^+CD4^+CD28^-CD57^+$ T cells expressing PD-1. The clonal loss of $CD4^+$ lymphocytes could limit the repertoire of the memory T cell compartment, affecting B cell responses by limiting their duration and affinity thus predisposing individuals to secondary infections (Figure 7).

In conclusion, our study highlights the need for anti-inflammatory therapeutic approaches to prevent T-cell hyperactivation and paralysis, as an attempt to avoid the observed extensive T cell loss in severely affected individuals. The better understanding of mechanism of T cell dysfunction will help the development of targeted therapy against severe COVID-19 by providing a better approach to vaccine designs involving T cell response for the long-term control of viral infection.

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Authorhip Contributions

The *authors* confirm *contribution* to the paper as follows: **A.A, K.G.P., J.L., P.E.C.L., C.C.B.M., I.D.L.** and **J.M.G.** conducted the experiments, acquired and analyzed data; **L.F.d.L., D.D.R., R.N.R.d.S., C.G.F.d.L., A.A.F., J.d.C.L.J., A.L.B., P.M.D.L., S.P.C.B. and F.C.S.** provided reagents; **W.S.** helped discussion and revised the manuscript; **A.M.** designed research studies and wrote the manuscript. All the authors have read and approved the final manuscript.

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.

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Figure Legends

Figure 1. Increased serum interleukin levels in SARS-CoV-2–acutely infected severe patients. Scatter plots show individual values for each COVID-19 severe patient (n=15) and healthy individual (n=15). Serum interleukins (IL-2, IL-6, IL-10, IL-13 and IL-17) were analyzed using the multiplex biometric immunoassay containing fluorescent microspheres conjugated with target-specific monoclonal antibodies, according to the manufacturer's instructions and the fluorescence levels were detected on the Luminex 200 system. For the detection of Interferon gamma (IFN-γ) and Tumor necrosis factor alpha (TNF-α), we used Sandwich-ELISA kit and an optical density (OD) was measured spectrophotometrically to a wavelength of 450 nm ± 2 nm Means of data points for each group ± SE are shown. Differences between groups are significant * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$).

Figure 2. T cell responses of COVID-19 patients have a broad profile of increased cytokine secretion. Peripheral blood mononuclear cells ($2x10^5$ PBMCs/well), obtained from COVID-19 patients (n = 6) and normal donors (n=6) ,were stimulated or not with 5µg PHA-L for 3 days, and the supernatants were collected for analysis of secreted cytokines using Bio-Plex Magpix (Bio-Rad). Data are shown as means ± SE and differences between COVID-19 (open squares) and non-infected healthy donors (solid squares) are significant * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$).

Figure 3. Sars-Cov-2 acute infection in aggravated COVID-19 patients is associated with increased levels of interleukin expression following anti-CD3/CD28 stimulation of T cells. Peripheral blood mononuclear cells ($2x10^5$ PBMCs/well) obtained from COVID patients (n=6) and normal donors (n=6) were stimulated or not with anti-CD3/CD28 beads (1 µg/mL) during 3 days, and the supernatants were collected for IL-2, IL-6, IL-10, IL13, IL17,

IFN-γ and TNF-α measure. Data are shown as means ± SE and differences between stimulated (solid bars) and non-stimulated controls (open bars) are significant * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$).

Figure 4. Mitogenic stimulation of T cells induces increased chemokine response and leukocyte growth factors in SARS-CoV-2–acutely infected severe patients. Peripheral blood mononuclear cells ($2x10^5$ PBMC cells/well) obtained from COVID patients (n=6) and normal donors (n=6) were stimulated or not with 5µg PHA-L for 3 days, and the supernatants were collected for analysis of secreted chemokines (MIP-1α, MCP-1, Eotaxin, Rantes), and the leukocyte growth factors GM- and G-CSF using Bio-Plex Magpix (Bio-Rad). Data are shown as means ± SE and differences between COVID-19 (open bars) and non-infected healthy donors (solid bars) are significant * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$).

Figure 5. Severe disease in SARS-CoV-2 – infected patients is associated with increased levels of the hypoxia marker VEGF (vascular endothelial growth factor) following mitogenic stimulation of T cells. Peripheral blood mononuclear cells ($2x10^5$ PBMCs/well) obtained from COVID patients (n=6) and normal donors (n=6) were stimulated or not with (A) 5µg PHA-L or (B) tuberculin (PPD) during 3 days, and the supernatants were collected for VEGF measure, using Bio-Plex Magpix (Bio-Rad). Data are shown as means ± SE and differences between COVID-19 (open bars) and non-infected healthy donors (solid bars) are significant * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$). (C) VEGF serum levels were evaluated using the multiplex biometric immunoassay containing fluorescent microspheres conjugated with target-specific monoclonal antibodies, according to the manufacturer's instructions and the fluorescence levels were detected on the Luminex 200 system. Scatter plots show individual values for each COVID-19 severe patient (n=15) and healthy individual (n=15). Means ± SE are shown for each group. Differences between groups are significant ($p \leq 0.05$).

Figure 6. Human late-differentiated effector exhausted/senescent $CD28^{-}CD57^{+}PD1^{+}$ T cells are increased in SARS-CoV-2 – acutely infected severe patients. Statistical analysis compared the population of $CD28^{-}CD57^{+}$ T cells within the $CD4^{+}$ and $CD8^{+}$ T cell compartments in severe COVID-19 patients *versus* non-infected healthy control individuals, in peripheral blood mononuclear cells. The panels represent flow cytometry analysis of freshly-isolated PBMCs from a normal volunteer and from a COVID-19 patient. **(A)** The gating strategy used to identify the activation profile of T $CD8^{+}$ and T $CD4^{+}$ activated or senescent based on PD1 expression is shown above. T cells were characterized based on their CD3, CD8/CD4 expression, and further categorized by CD28 and CD57 expression. Effector senescent ($CD28^{-}CD57^{+}$) $CD8^{+}$ T and $CD4^{+}$ T were assessed for their PD1 expression. **(B)** Frequencies of $CD8^{+}CD28^{-}CD57^{+}$ and $CD4^{+}CD28^{-}$ $CD57^{+}$ T cells and **(C)** histogram plots showing their expression levels of PD1 on both populations from assessed normal volunteers (dotted line) and COVID-19 patients (solid line). The mean fluorescence intensity (MFI) of PD-1 expression on $CD4^{+}CD28^{-}CD57^{+}$ T cells of COVID-19 patients (MFI = 1038 $\pm$ 163.6) was significantly higher (*p* value 0.0353) than in cells obtained from normal donors (MFI = 437.3 $\pm$ 64.83). Bar charts represent the percentages and total number of cells in the gated population expressing PD-1 on the surface of $CD4^{+}CD28^{-}CD57^{+}$ and of $CD8^{+}CD28^{-}CD57^{+}$ cells in normal and COVID-19 patients. **(D)** $CD8^{+}CD28^{-}CD57^{+}$ T cells of COVID-19 patients exhibit a differential increase in perforin expression determined by intracellular staining when stimulated with SARS-CoV-2 antigen Spike-derived peptides (FVFLVLLPL, FTISVTTEI, FAMQMAYRF and FVSNGTHWF) in the presence of anti-CD28 (solid line) as compared with unstimulated control (dashed line). The analysis were obtained using direct staining with *the fluorochrome-conjugated* monoclonal antibodies CD3 PerCP-Cy5.5, CD4 APC-Cy7, CD8 PE-Cy5, CD28 Alexa Fluor 647, CD57 PE, PD1 BV421, and indirect

intracellular staining for perforin using *Alexa Fluor 488-conjugated* monoclonal antibodies on a FACS LSRFortessa instrument (BD, Franklin Lakes, USA), and the data were analyzed with FlowJo software (TreeStar; Ashland, USA). The bar chart represents the mean fluorescence intensity (MFI) for the perforin expression values from the gated $CD8^{+}CD28^{-}CD57^{+}$ populations. Data are expressed as mean ± SEM. Statistical comparisons performed using two-tailed unpaired *t*-test. *P<0.05; **P<0.01.

Figure 7. Impact of hyperactive cytokine responses upon the generation of exhausted senescent $CD28^{-}CD57^{+}$ T cell terminal programs and its implication to severe COVID-19. Sars-Cov-2 mainly infects epithelial cells of the respiratory airways, in which the virus is able to replicate. However, some studies have shown that alveolar macrophages and dendritic cells can also be infected, acting as viral reservoirs. SARS-CoV-2 is a cytopathic virus which induces cell death and local release of various damage-associated molecular pattern proteins (DAMPs) following viral entry into human host cells (23). Viral RNAs are released during host cell infection and act as pathogen-associated molecular patterns (PAMPs), being then recognized by pattern receptors (PRRs) such as toll-like receptors and retinoic acid-inducible (RIG-I) type I receptors. Signaling these classes the innate cell receptors induces cytosolic translocation of nuclear transcription factors such as NF-kB and the activating protein (AP-1) to the cell nucleus, initiating the transcription of inflammatory genes and expression of C-Reactive Protein, pro-inflammatory cytokines, chemokines and interferons (IFNs), necessary for the induction of antiviral responses for infection control (23,24). However, due to the critical viral mass in the transmission of the infection and comorbid conditions, patients susceptible to worsening the infection develop an exacerbated acute inflammatory response responsible for an imbalance of the innate immune system with massive expression of characterized pro-inflammatory cytokines mainly by the production of IL-1β, IL-2, IL-6 and TNF-α and chemokines such as CCL2/MPC-1, CXCL8/IL-8 and CXCL10/IP-10 at a

systemic pathological level that can contribute to the severity of the disease (7). Studies have demonstrated that SARS-CoV-2 is unable to activate NETosis in human neutrophils. This process is associated with increased levels of intracellular Reactive Oxygen Species (ROS) in neutrophils. The ROS-NET pathway plays a role in thrombus formation, a host defense mechanism that can become deregulated in COVID-19 (6). The distinct COVID-19-associated coagulopathy is the result of this characteristic proinflammatory milieu along activation of platelets and complement, and impairment of the microvasculature. COVID-19 patient autopsies have revealed thrombi in the microvasculature, a pathophysiology mechanism that contributes to vessel occlusion and hypoxia induction, which is more frequent with worsening disease severity causing multi-organ failure during the clinical course (21,25). Our findings demonstrate increased serum VEGF (vascular endothelial growth factor) levels in critically infected patients, showing their relevance in the pathophysiology of the disease. VEGF, promotes neoangiogenesis in physiological and pathophysiological conditions (promoted by tissue hypoxia) and is involved in a clonal exhaustion program in T cells by inducing the expression of the transcription factor TOX, driving a clonal exhaustion program in these lymphocytes (14,20). Interestingly, our findings revealed a significantly increased frequency of both late-differentiated $CD4^+$ and $CD8^+$ T cells with a particular phenotype of exhausted/senescent $CD28^-CD57^+$ effector cells, possibly resulting from the T-cell hyperactivation process observed in the acute phase of infection in critically infected patients. We demonstrated an increased frequency of $CD4^+CD28^-CD57^+$ with a high expression of programmed death 1 (PD-1), one of the hallmarks of T cell exhaustion, in severe COVID-19 infection. The clonal loss of $CD4^+$ lymphocytes could limit the repertoire of the memory $CD8^+$ T cell compartment, also affecting B cell responses by limiting their duration and affinity thus predisposing individuals to secondary infections.

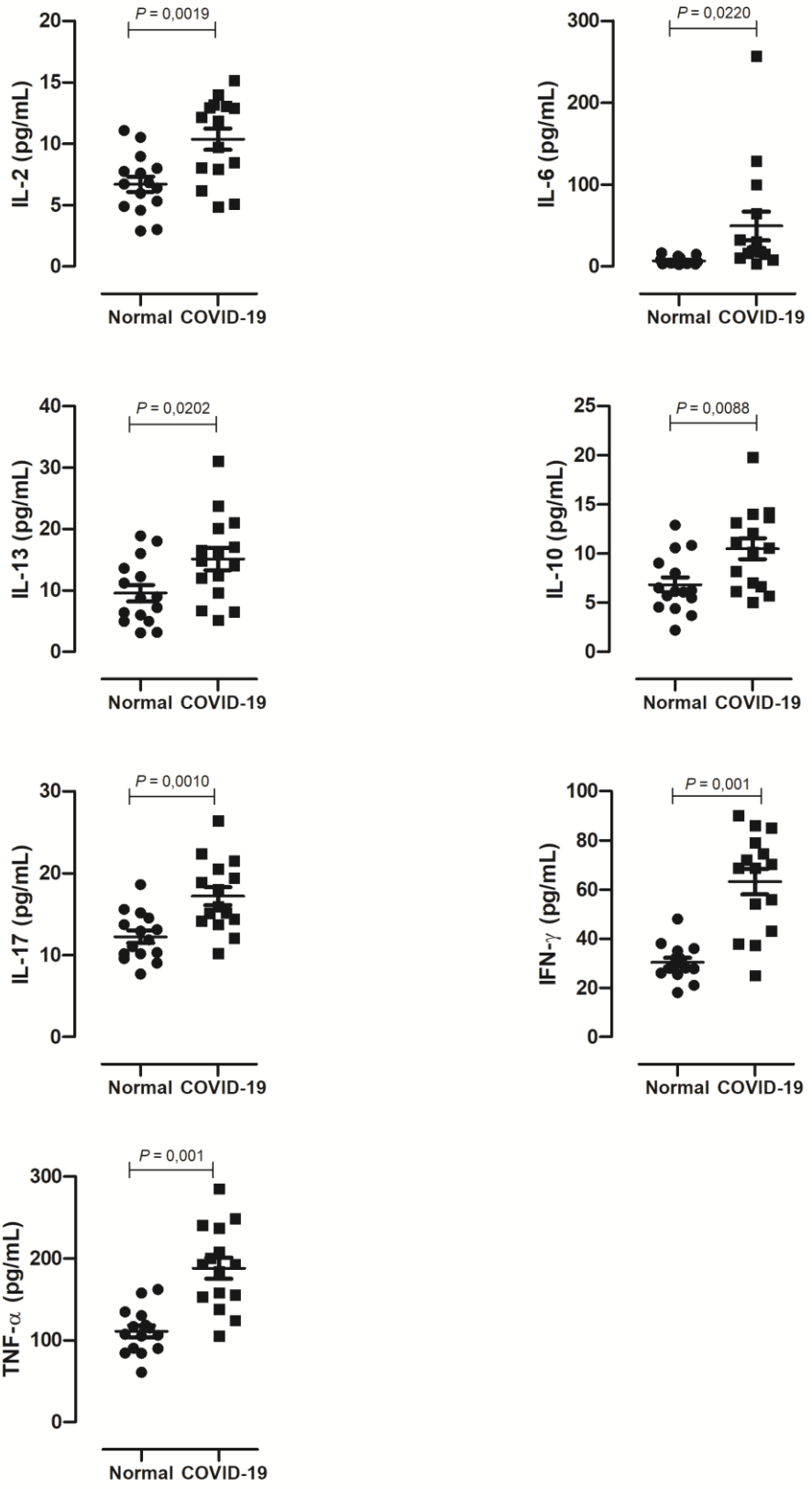

Figure 1

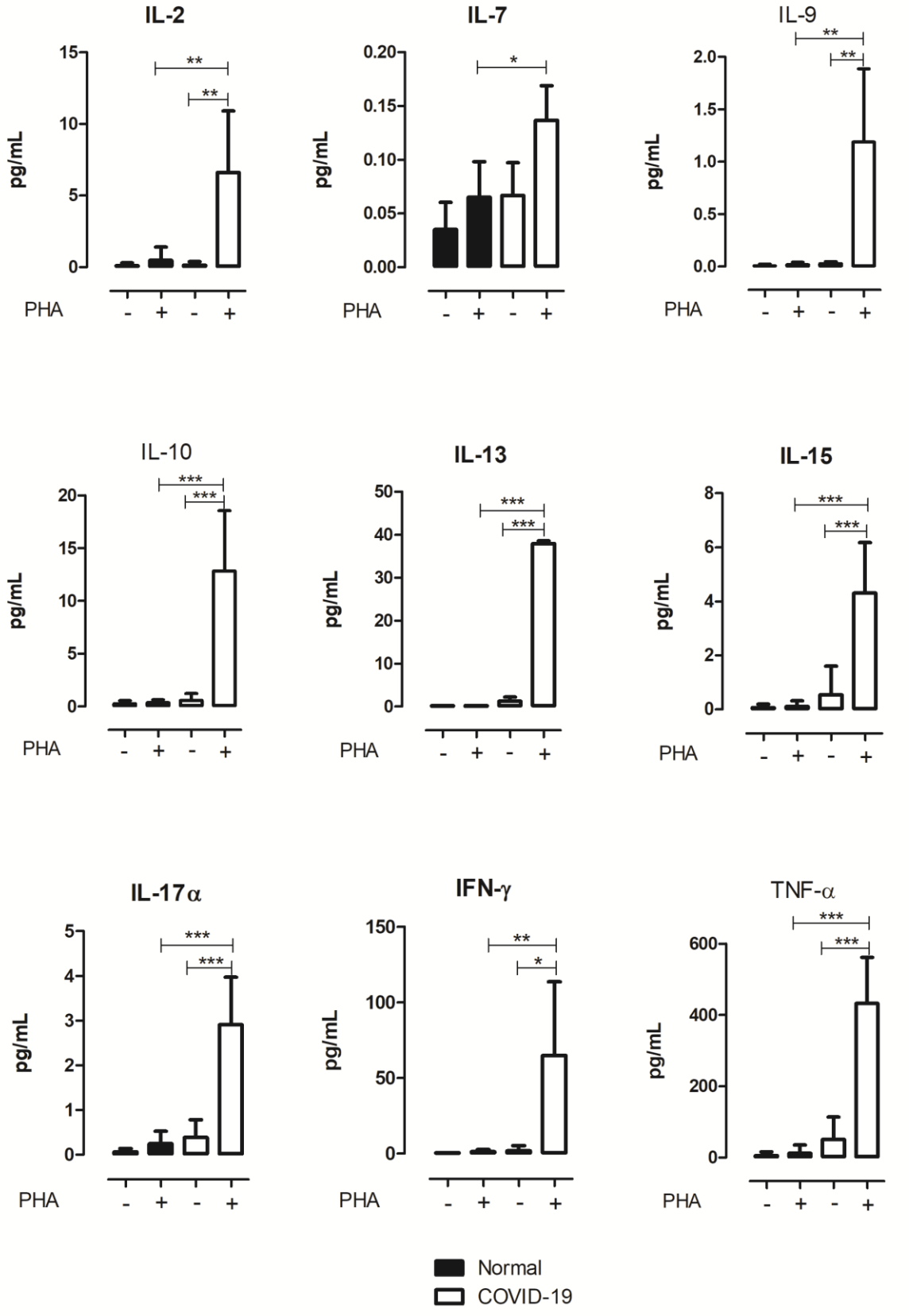

Figure 2

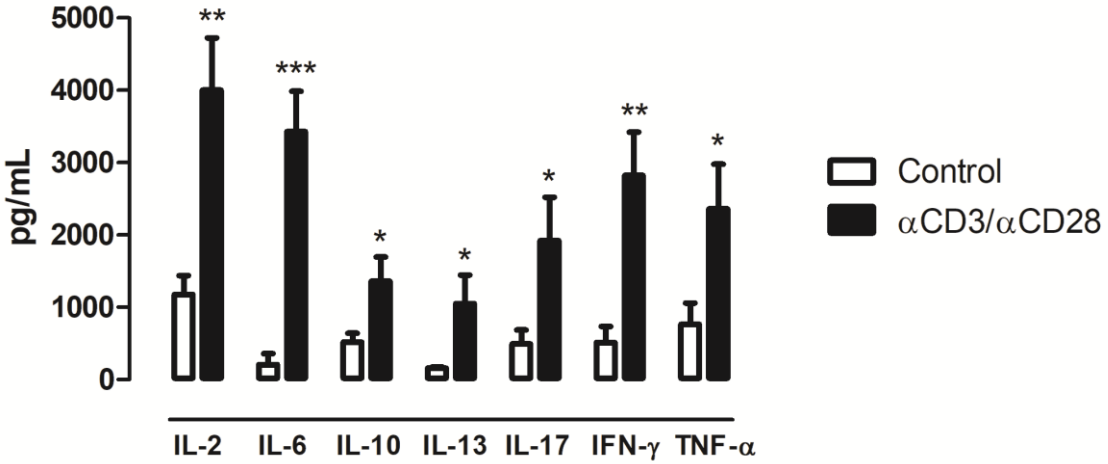

Figure 3

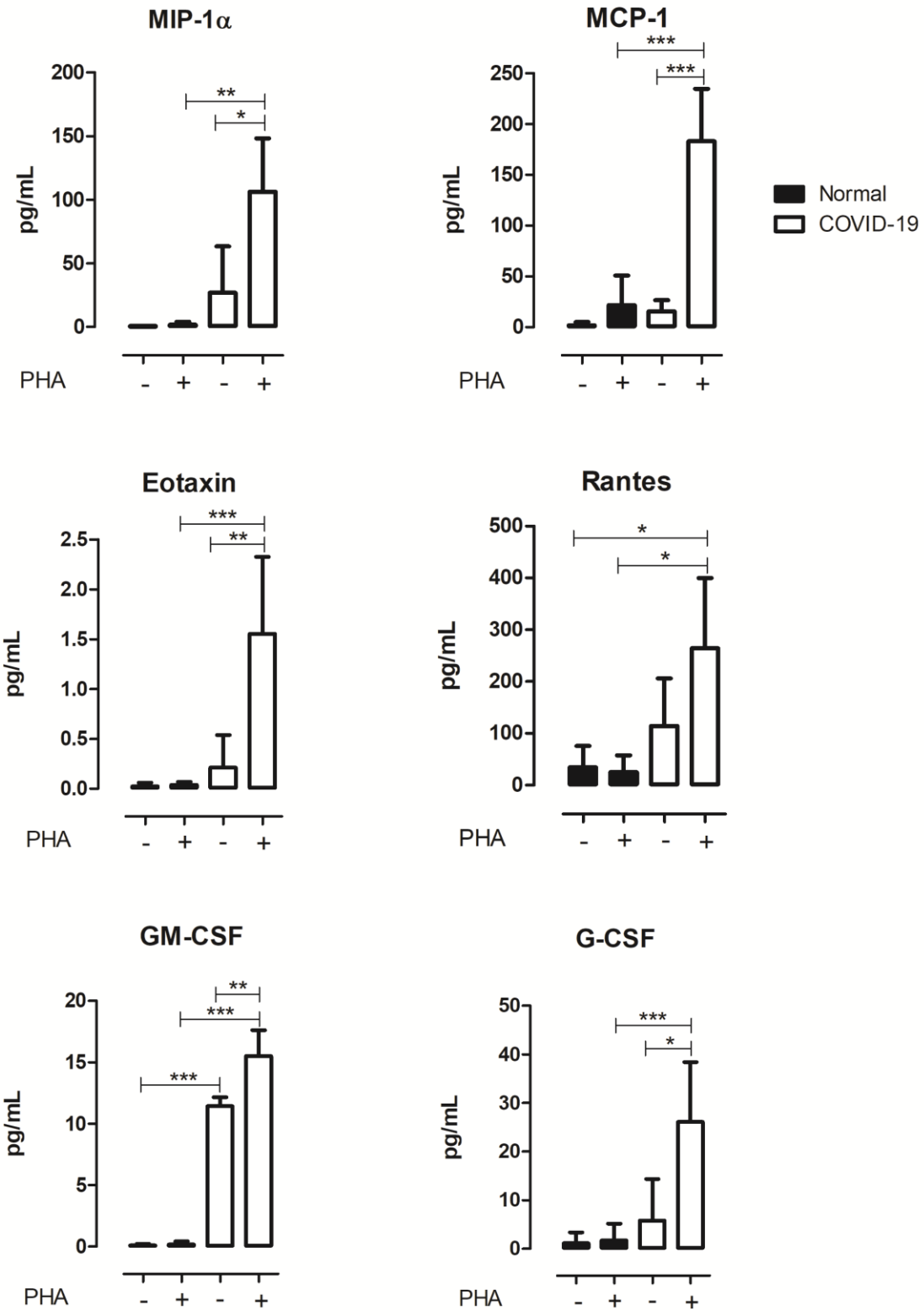

Figure 4

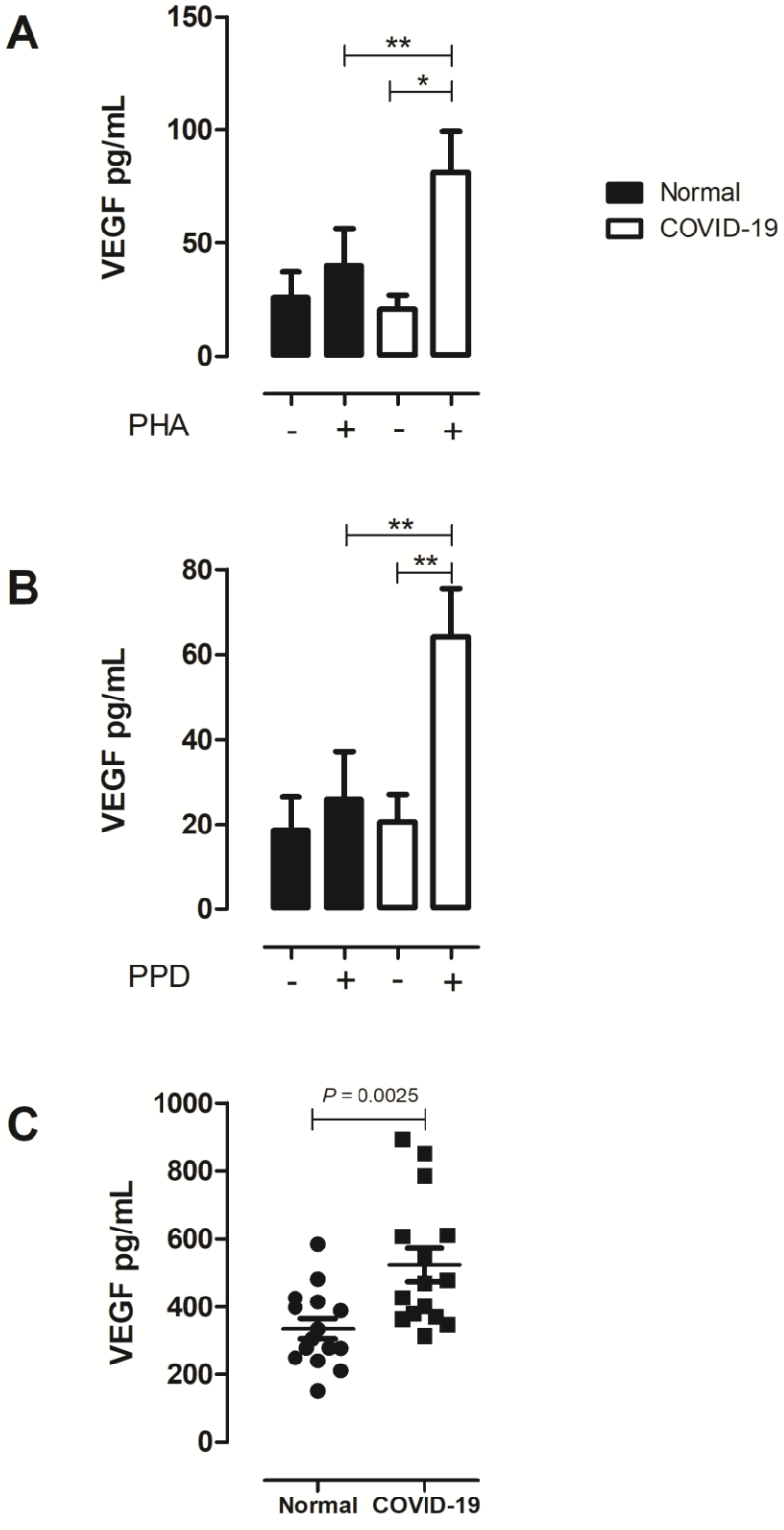

Figure 5

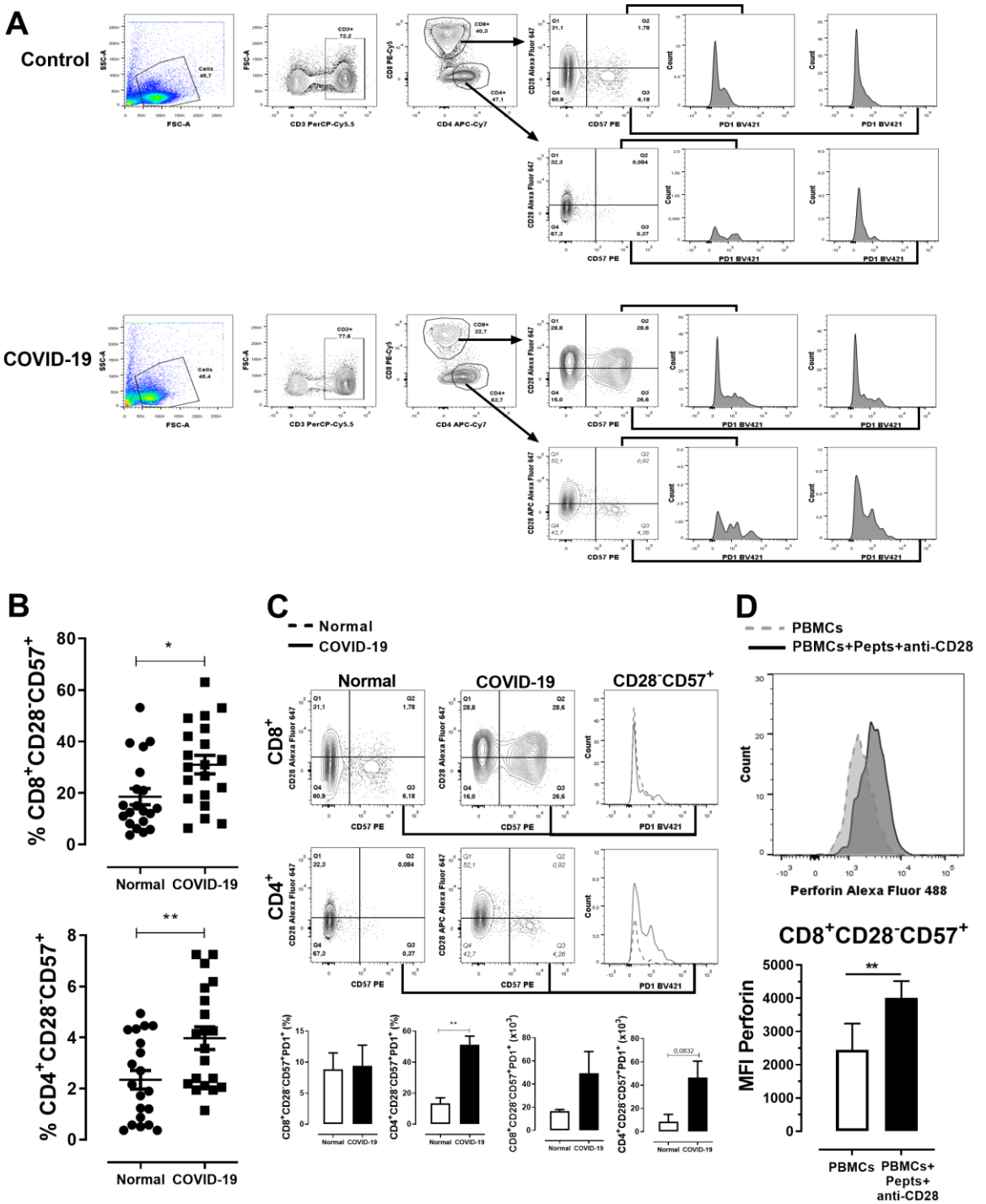

Figure 6

Figure 7