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MAJOR ARTICLE

Critically Ill Coronavirus Disease 2019 Patients Exhibit Hyperactive Cytokine Responses Associated With Effector Exhausted Senescent T Cells in Acute Infection

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Background. Coronavirus disease 2019 (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.

Methods. In this study, we report that severe acutely infected patients have high levels of both type-1 and type-2 cytokines.

Results. Our results show abnormal cytokine levels upon T-cell stimulation, in a nonpolarized 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. Of note, we demonstrated for the first time an increased frequency of $CD3^{+}CD4^{+}CD28^{-}CD57^{+}$ T cells with expression of programmed death 1, one of the hallmarks of T-cell exhaustion.

Conclusions. 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; exhausted/senescent T cells; immunopathology; SARS-CoV-2.

Coronavirus disease 2019 (COVID-19) is a devastating disease caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) 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, hemophagocytosis, D-dimer, and soluble CD25 (the interleukin [IL]-2 receptor alpha chain) [4, 5]. The presence of activated neutrophils and macrophages in the target tissues has been associated with induction of (1) neutrophil extracellular traps [6] and (2) thrombocytogenesis, promoting vascular collapse, respiratory distress, and multiorgan failure, which are related to the so-called cytokine release syndrome, including excessive productions of granulocyte and macrophage colony-stimulating factor (GM-CSF), IL-2, IL-6, IL-7, IL-10, tumor necrosis factor α (TNF-α), and granulocyte colony-stimulating factor (G-CSF) [7].

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The cytokine storm syndrome is most commonly triggered by viral infections and occurs in 3.7%–4.3% of severe cases of sepsis, which are 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 natural killer 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. More importantly, the presence of high serum levels of IL-2 and CD25s (soluble IL-2 receptor α chain) in severe COVID-19 patients possibly 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 hyperreactivation of T-cell responses in severe patients [10, 11]. In this study, 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, in ages ranging from 18 to 93 years, with an 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.

MATERIALS 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 reverse-transcription polymerase chain reaction (RT-PCR) from nasopharyngeal swab samples based on US Food and Drug Administration-approved ribonucleic acid testing, as well as a serological test for the S antigen. The patients screened were viral P0.1 lineage negative (Gamma), a descendant of B0.1.1.28. To detect the deletion in the ORF1b gene (NSP6: S106del, G107del, F108del) found in the P0.1 variant, nasopharyngeal swab samples were submitted for real-time PCR. We used oligos manufactured by IDT DNA: forward sequence, 5'-GGG TGA TGC GTA TTA TGA CAT GGT TGG-3'; reverse sequence, 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 included the following age- and sex-matched noninfected individuals: those who did not show clinical signs for the disease; those who were prescreened and tested negative for anti-COVID-19 antibodies; and those who had RT-PCR swab tests. The research was approved by the Research Ethics Committee (CEP) from the Brazilian National Health Council, all patients signed a free and informed consent form in accordance with current legislation, and the relevant ethical regulations were approved by the Hospital Naval Marcílio Dias (CAAE no. 31642720.5.0000.5256) and Hospital Universitário Clementino Fraga Filho (CAAE no. 30424020.0.0000.0008).

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 (400 ×*g*). 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 revolutions per minute for 6 minutes, and the cells were resuspended in Roswell Park Memorial Institute (RPMI) medium with 1% Nutridoma (Sigma), counted, and adjusted for each experimental condition. For T-cell stimulation assay, 2×10^5 PBMCs/well were plated in a 96-well plate, in a total volume of 100 µL, and stimulated or not with 5 µg phytohemagglutinin-L ([PHA-L] Sigma-Aldrich), 2 tuberculin units of purified protein derivative (PPD), 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) 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, G-CSF, GM-CSF, interferon gamma

(IFN)-γ, monocyte chemoattractant protein-1 ([MCP-1] monocyte chemotactic and activating factor), macrophage-inflammatory protein (MIP)-1α, MIP-1β, RANTES, TNF-α, and vascular endothelial growth factor (VEGF). Analysis was performed as previously described [12]. In brief, 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-phycoerythrin (PE). Finally, magnetic beads were resuspended and read. The values detected in culture medium without microspheres (background) were subtracted from the samples, allowing us 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 IFN-γ and TNF-α, we used a specific Sandwich-ELISA kit (Elabscience), in which micro ELISA plates were precoated 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 conjugate. After the addition of the colorimetric substrate, the optical density (OD) was measured by spectrophotometry in a wavelength of 450 ± 2 nm, with the OD values being proportional to the concentration of the corresponding cytokine.

Cytofluorometric Analysis of Human Peripheral Blood Mononuclear Cells and Intracellular Perforin Staining

For cytofluorometric analysis, PBMCs were incubated with fluorochrome-conjugated monoclonal antibodies for 45 minutes at 4°C. Freshly isolated PBMCs or in vitro-activated T cells were stained with LIVE-DEAD Aqua (Thermo Fisher Scientific) and surface monoclonal antibodies recognizing CD4 APC-Cy7 (eBioscience), CD8 PE-Cy5 (Beckman Coulter Life Sciences), 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). Then, a minimum of 100 000 cells per sample were acquired on a FACS LSR Fortessa instrument (BD, Franklin Lakes, NJ), and the data were analyzed with FlowJo software (TreeStar, Ashland, OR). To detect perforin, freshly isolated PBMCs from COVID-19 patients were suspended at a density of 1×10^6/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 6 hours at 37°C in a 5% CO_2 incubator, followed by an additional 3 hours 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 antihuman perforin (BD Pharmingen) and antimouse IgG AlexaFluor488 (Sigma). Samples were acquired using BD LSR Fortessa, and the data were analyzed using FlowJo and *FACSDiva* software. The selection of T-cell epitopes spanning the Spike protein sequence was made using the Immune Epitope Database (IEDB) analysis resource Consensus tool, which combines predictions from ANN, also known as NetMHC, SMM, and Comblib. Considering lengths of 9 mers, the prediction score of each length was evaluated against a default panel of 27 most frequent human leukocyte antigen (HLA)-A and HLA-B alleles. Four peptides (FL9 - FVFLVLLPL; FI9 - FTISVTTEI; FF9 - FAMQMAYRF; and 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 ± standard error of the mean and $P \leq .05$ was considered statistically significant. For multiple comparisons, one-way analysis of variance 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-cell 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 patients with severe COVID-19 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 nonspecific 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

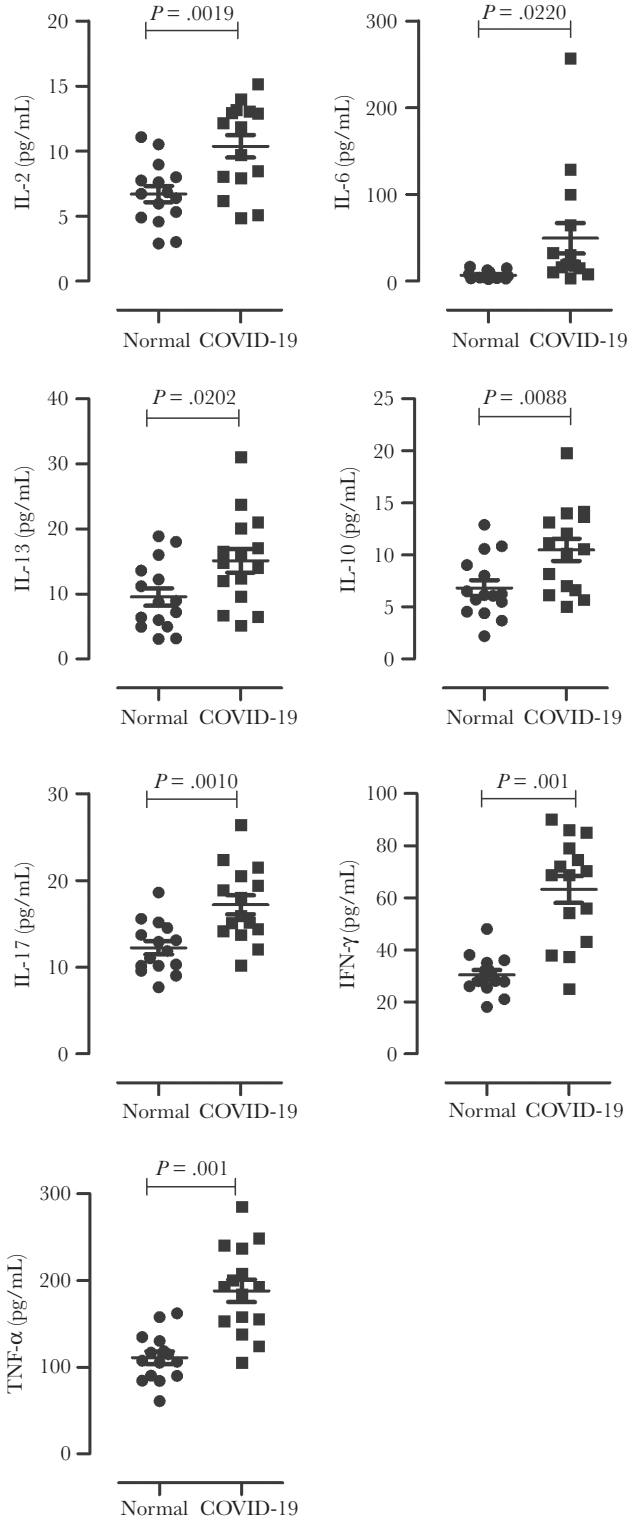

Figure 1. Increased serum interleukin (IL) levels in severe acute respiratory syndrome coronavirus 2 acutely infected severe patients. Scatter plots show individual values for each coronavirus disease 2019 (COVID-19) severe patient (n = 15) and healthy individual (n = 15). Serum interleukins (IL-2, IL-6, 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 was measured spectrophotometrically to a wavelength of 450 nm ± 2 nm. Means of data points for each group ± standard error are shown. Differences between groups are significant. *, $P < .05$; **, $P < .01$; ***, $P < .001$.

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 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, and TNF-α (Figure 2). Corroborating these findings, a broad and nonpolarized profile in the expression of interleukins was also seen through T-cell receptor (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 nonpolarized 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 PPD tuberculin (Figure 5B). Also of note, we found that VEGF was present at significantly increased levels in sera from patients with SARS-CoV-2 infection 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

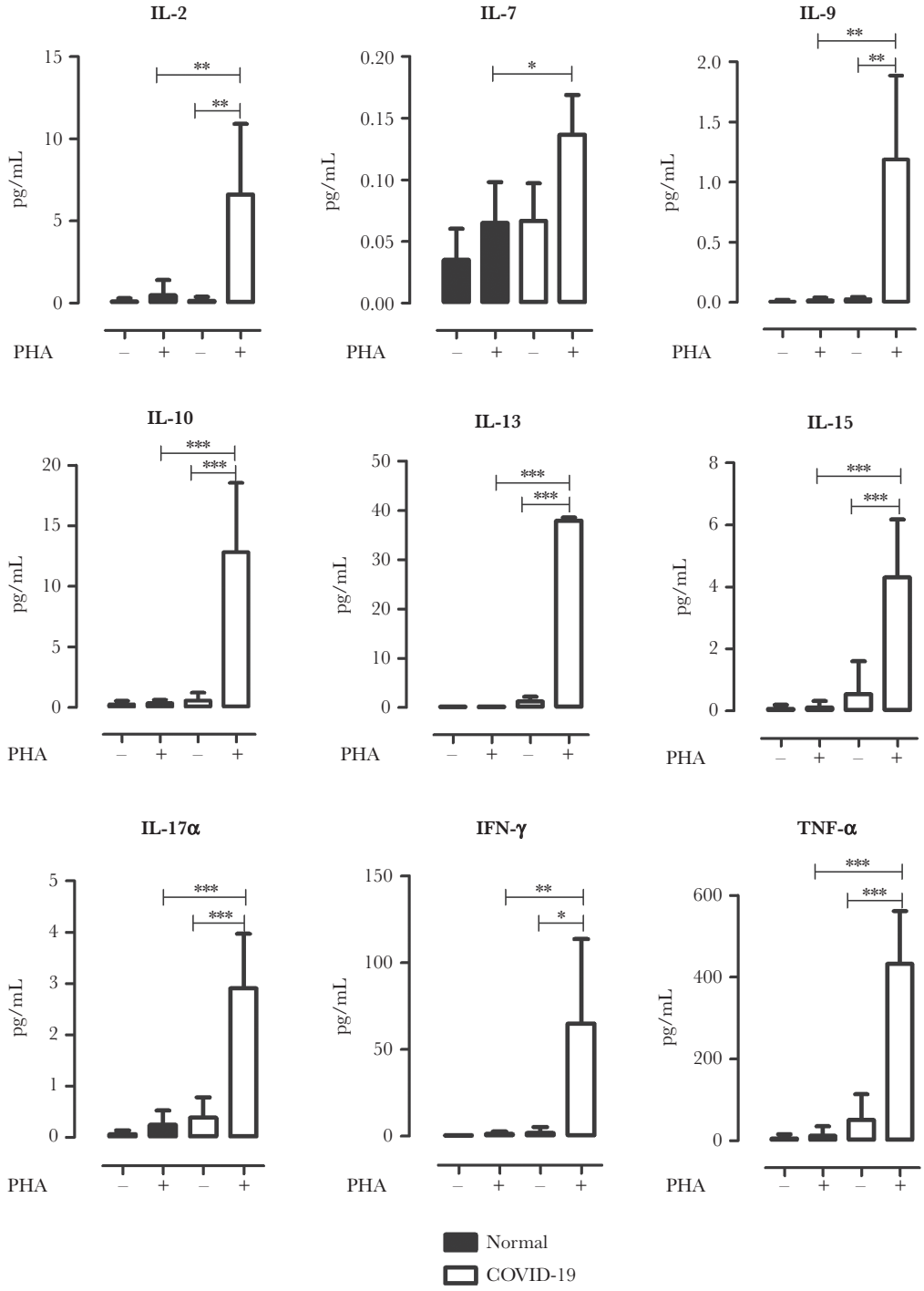

Figure 2. T-cell responses of coronavirus disease 2019 (COVID-19) patients have a broad profile of increased cytokine secretion. Peripheral blood mononuclear cells (PBMCs) (2×10^5 PBMCs/well), obtained from COVID-19 patients (n = 6) and normal donors (n = 6), were stimulated or not with 5 μg of phytohemagglutinin-L (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 ± standard error, and differences between COVID-19 (open squares) and noninfected healthy donors (solid squares) are significant. *, $P < .05$; **, $P < .01$; ***, $P < .001$. IL, interleukin; TNF-α, tumor necrosis factor alpha.

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

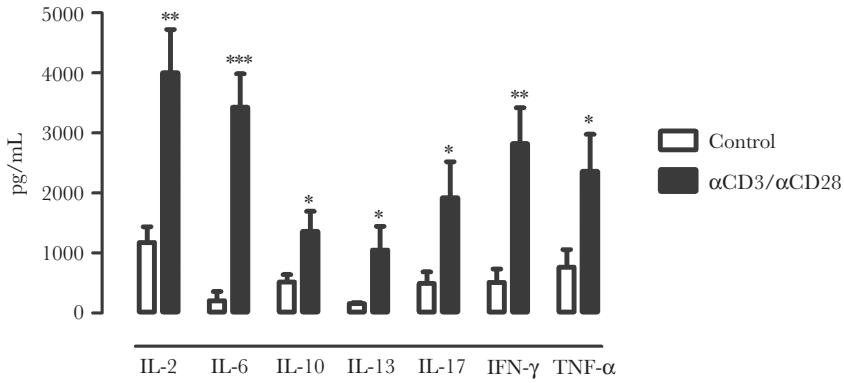

Figure 3. Severe acute respiratory syndrome coronavirus 2 acute infection in aggravated coronavirus disease 2019 (COVID-19) patients is associated with increased levels of interleukin (IL) expression following anti-CD3/CD28 stimulation of T cells. Peripheral blood mononuclear cells (PBMCs) (2×10^5 PBMCs/well) 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, interferon gamma (IFN-γ), and tumor necrosis factor alpha (TNF-α) measure. Data are shown as means ± standard error, and differences between stimulated (solid bars) and nonstimulated controls (open bars) are significant. *, $P < .05$; **, $P < .01$; ***, $P < .001$.

($CD3^+CD28^-CD57^+$) [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 and 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

Severe acute respiratory syndrome coronavirus 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 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, whereas 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 SARS and Middle Eastern respiratory syndrome [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 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

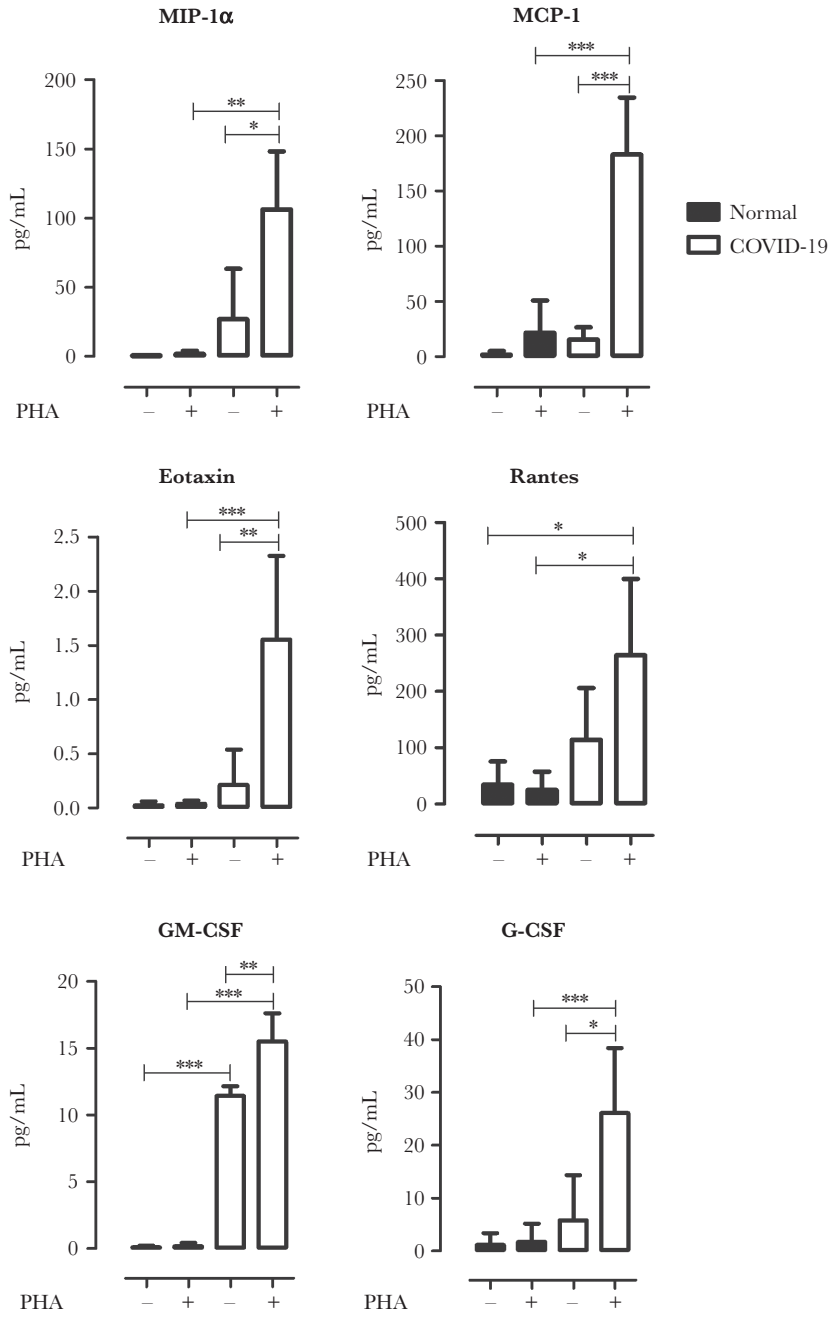

Figure 4. Mitogenic stimulation of T cells induces increased chemokine response and leukocyte growth factors in severe acute respiratory syndrome coronavirus acutely infected severe patients. Peripheral blood mononuclear cells (PBMCs) (2×10^5 PBMC cells/well) obtained from coronavirus disease 2019 (COVID-19) patients (n = 6) and normal donors (n = 6) were stimulated or not with 5 μg of phytohemagglutinin-L (PHA-L) for 3 days, and the supernatants were collected for analysis of secreted chemokines (macrophage-inflammatory protein [MIP]-1α, monocyte chemoattractant protein [MCP]-1, eotaxin, RANTES) and the leukocyte growth factors granulocyte and macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) using Bio-Plex Magpix (Bio-Rad). Data are shown as means ± standard error, and differences between COVID-19 (open bars) and noninfected healthy donors (solid bars) are significant. *, $P < .05$; **, $P < .01$; ***, $P < .001$.

viral infections and mediates 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. Vascular endothelial growth factor promotes vascular neoangiogenesis in physiological as well as in pathophysiological conditions caused by tissue hypoxia. Such an oxygen deprivation promotes the expression of factor inhibiting hypoxia-inducible factor (FIH)-1α, which induces adaptive responses capable of regulating VEGF expression [20]. In fact, in severe cases of the disease, there is a manifestation of disseminated thrombolytic processes, which is possibly involved in the process of respiratory syndrome and multiple organ failure [21]. Of note, our findings demonstrated

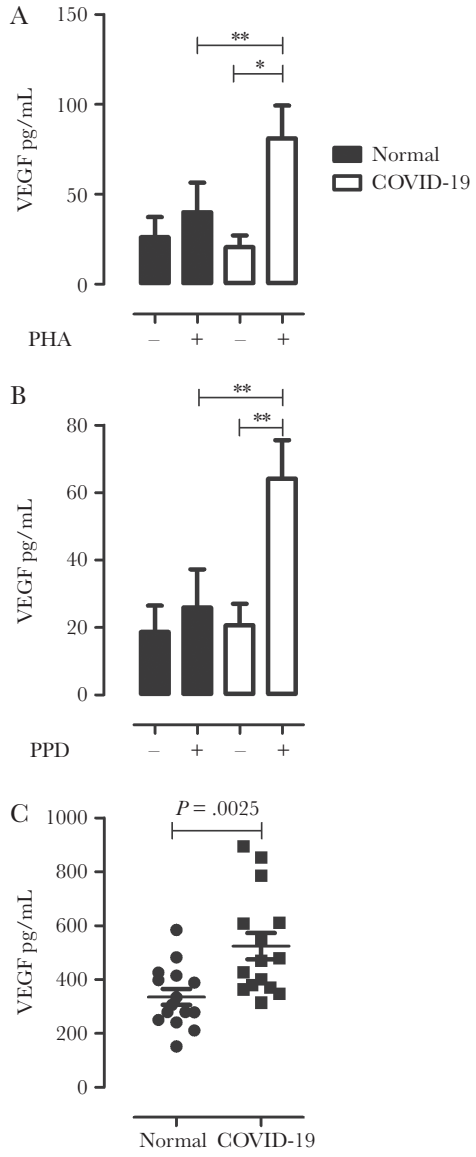

Figure 5. Severe disease in severe acute respiratory syndrome coronavirus 2-infected patients is associated with increased levels of the hypoxia marker VEGF (vascular endothelial growth factor) after mitogenic stimulation of T cells. Peripheral blood mononuclear cells (PBMCs) (2×10^5 PBMCs/well) obtained from coronavirus disease 2019 (COVID-19) patients (n = 6) and normal donors (n = 6) were stimulated or not with (A) 5 µg of phytohemagglutinin-L or (B) tuberculin (purified protein derivative [PPD]) during 3 days, and the supernatants were collected for VEGF measure, using Bio-Plex Magpix (Bio-Rad). Data are shown as means ± standard error (SE), and differences between COVID-19 (open bars) and noninfected healthy donors (solid bars) are significant. *, $P < .05$; **, $P < .01$; ***, $P < .001$. (C) The 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 ($P \leq .05$).

increased VEGF serum levels in critically infected patients, which suggest their relevance in the pathophysiology of the disease.

Vascular endothelial growth factor 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 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]. In this study, we showed a significantly increased frequency of late-differentiated T cells characterized by the particular phenotype $CD28^-CD57^+$ [16]. Although we showed that terminally differentiated SARS-CoV-2-specific $CD3^+CD8^+CD28^-CD57^+$ T cells respond, by producing perforin when stimulated with viral antigens, our findings revealed that severe acute COVID-19 infection is associated with senescence and/or 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, and thus predisposing individuals to secondary infections (Figure 7).

CONCLUSIONS

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.

Supplementary Figure 1. Clinical characteristics of patients with COVID-2019 on admission. We included 22 patients with complete data for all variables (17 nonsurvivors and 5 survivors). On admission to the hospital, all patients had evidence of lung invasion by SARS-CoV-2 as CT showed multiple and peripheral ground-glass opacifications, the most common abnormality consistent with viral pneumonia in patients with COVID-19. All patients had comorbidities for infection and the need for oxygen therapy or mechanical ventilation, with changes in major laboratory markers determined from illness onset, such as levels of D-dimer, neutrophils/lymphocytes ratio (NLR), or C-reactive protein. All of the patients included in this study had acute infection with positive result of the nucleic acid sequence of SARS-CoV-2 by real-time RT-PCR from nasopharyngeal swab samples.

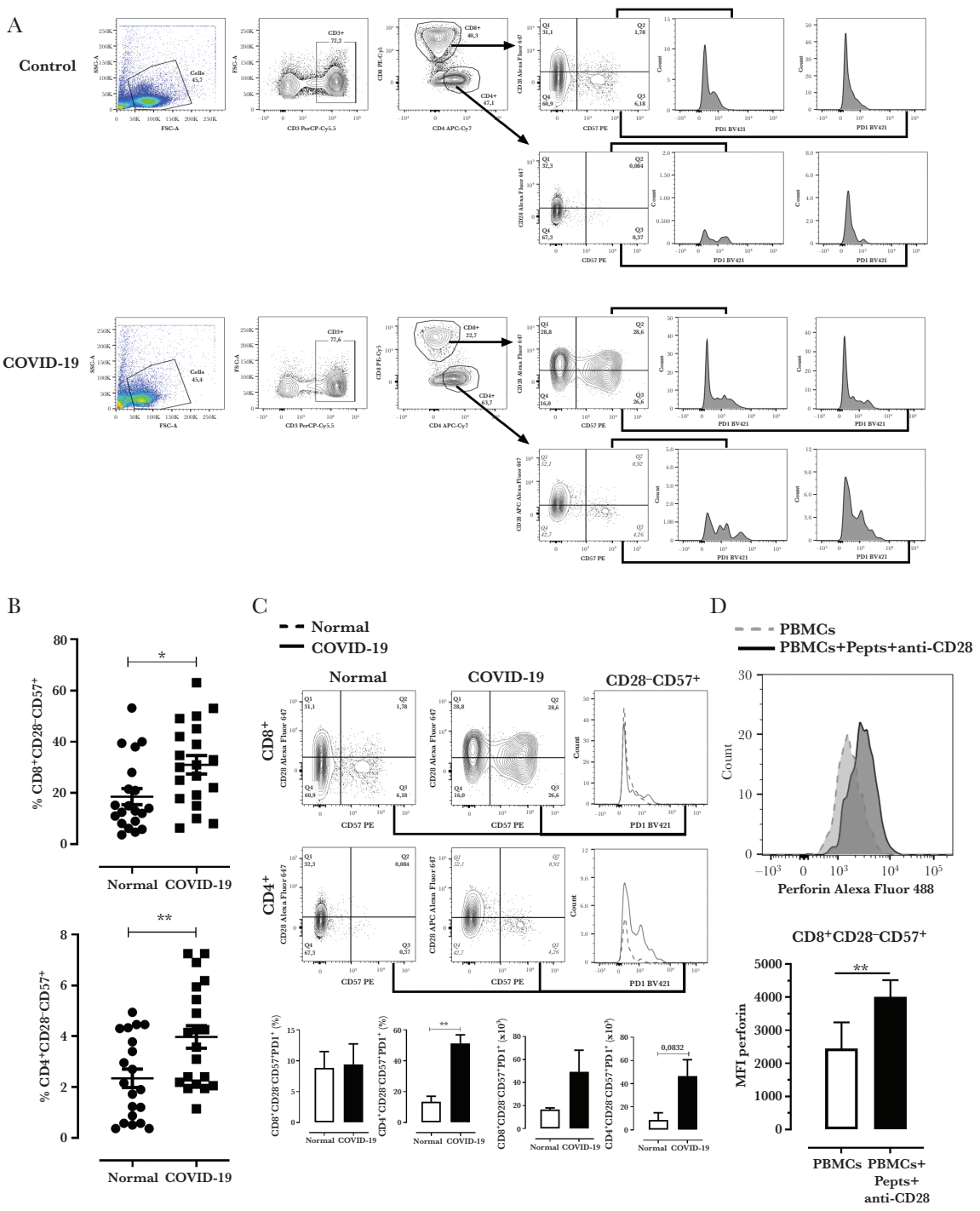

Figure 6. Human late-differentiated effector exhausted/senescent $CD28^{-}CD57^{+}PD1^{+}$ T cells are increased in severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) acutely infected severe patients. Statistical analysis compared the population of $CD28^{-}CD57^{+}$ T cells within the $CD4^{+}$ and $CD8^{+}$ T-cell compartments in severe coronavirus disease 2019 (COVID-19) patients to noninfected healthy control individuals, in peripheral blood mononuclear cells (PBMCs). 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 + 163.6) was significantly higher ($P = .0353$) than in cells obtained from normal donors (MFI = 437.3 + 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) compared with unstimulated control (dashed line). The analyses 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), and the data were analyzed with FlowJo software (TreeStar). The bar chart represents the MFI for the perforin expression values from the gated $CD8^{+}CD28^{-}CD57^{+}$ populations. Data are expressed as mean ± standard error of the mean. Statistical comparisons were performed using 2-tailed unpaired *t* test. *, $P < .05$; **, $P < .01$.

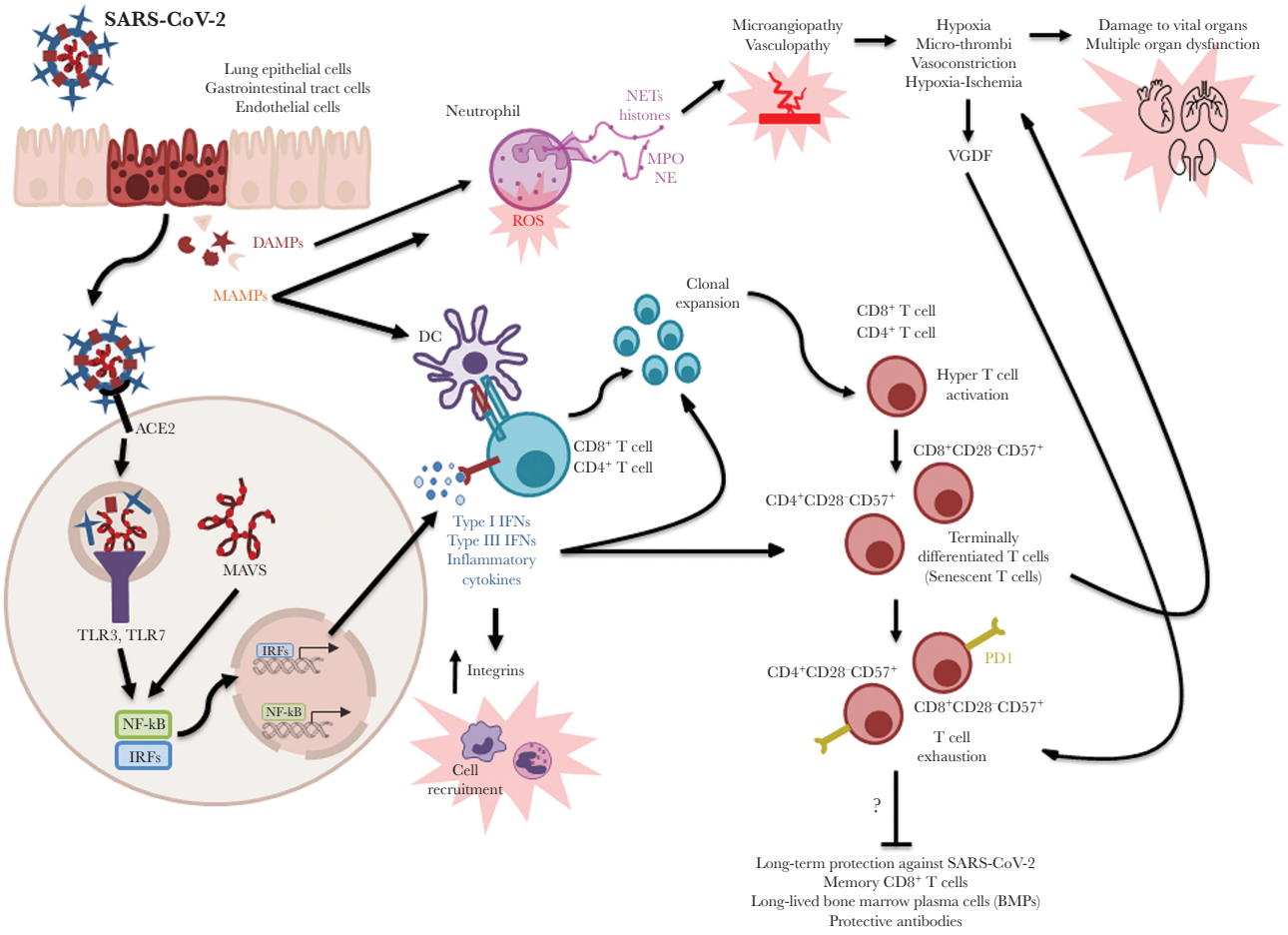

Figure 7. Impact of hyperactive cytokine responses upon the generation of exhausted senescent $CD28^{-}CD57^{+}$ T-cell terminal programs and its implication to severe coronavirus disease 2019 (COVID-19). Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) mainly infects epithelial cells of 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. Severe acute respiratory syndrome coronavirus 2 is a cytopathic virus that induces cell death and local release of various damage-associated molecular pattern proteins (DAMPs) after viral entry into human host cells [23]. Viral ribonucleic acids are released during host cell infection and act as pathogen-associated molecular patterns (PAMPs) and are 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 induce cytosolic translocation of nuclear transcription factors such as NF-κβ and the activating protein (AP-1) to the cell nucleus, initiating the transcription of inflammatory genes and expression of C-reactive protein, proinflammatory cytokines, chemokines, and interferons (IFNs), which is 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 proinflammatory cytokines mainly by the production of interleukin (IL)-1β, IL-2, IL-6, and tumor necrosis factor alpha (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. Coronavirus disease 2019 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 and causes multiorgan 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. Vascular endothelial growth factor 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]. Of note, 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 and thus predisposing individuals to secondary infections.

Notes

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Author contributions. 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 with discussions and revised the manuscript; A. M. designed research studies and wrote the manuscript. All the authors have read and approved the final manuscript.

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Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.

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