Hyperactive cytokine T-cell response is associated with immunosenescence in severe acute COVID-19 infection

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Abstract

COVID-19 is a disease caused by the novel SARS-CoV-2 coronavirus, originally classified as a severe acute respiratory syndrome coronavirus (SARS-CoV). The most severe cases of COVID-19 can progress to severe pneumonia with respiratory failure, septicemia, multiple organ failure and death. The severity of the disease is aggravated by the deregulation of the immune system causing an excessive initial inflammation including the cytokine storm, compring interleukins characteristic of the T-dependent adaptive response. In the present study we show that severe patients have high levels of T helper type-1 and type-2 cytokines, as well as VGEF. Furthermore, our show abnormal cytokine levels upon T-cell mitogen stimulation, in a non-polarized response profile. This response is not specific, given that the stimulus with the heterologous tuberculin antigen was able to induce high levels of cytokines compared to healthy controls, including the vascular endothelial growth factor VEGF, which promotes neoangiogenesis in physiological and pathophysiological conditions, caused by tissue hypoxia, and involved in a clonal exhaustion program in T cells. This can be decisive given our findings demonstrating for the first time a significantly increased frequency of late-differentiated $CD8^+$ T cells characterized by critically shortened telomeres with particular phenotype ($CD57^+CD28^-$) in severe acute COVID-19 infection. These findings reveal that severe COVID-19 is associated with senescence of T cells, especially within the $CD8^+$ T cell compartment and points to possible mechanisms of loss of clonal repertoire and susceptibility to recurrences of COVID-19 symptoms, due to viral relapse and reinfection events.

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 individuals 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

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 to 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 to prevent 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 and heterologous antigens not associated with infection. Our results indicate an increase in the frequency of T cells presenting a phenotype compatible with clonal exhaustion and senescence in severe infection, corroborating with a possible exacerbation and hyperactivation of T cell responses.

Materials And Methods

Human samples. Blood samples from 15 hospitalized severe acutely infected COVID-19 patients and 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 and serological test for the S antigen. 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; and healthy donors include age and sex matched-non-infected controls. 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 # 31642720.5.0000.5256) and Hospital Universitário Clementino Fraga Filho (CAAE # 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 (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) or 2U PPD (Tuberculin). 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-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.

FACS analysis of human PBMCs. For cytofluorometry analysis, PBMCs were incubated with fluorochrome-conjugated monoclonal antibodies for 45 min at 4 °C. The antibodies used in this study were anti-CD3-PerCP-Cy5.5, anti-CD8-PE, anti-CD28-APC, anti-CD57-FITC (eBioscience, San Diego, CA, USA). Multicolor flow cytometry was performed using a BD FACSCanto II Flow Cytometer (BD Biosciences, San Jose, CA, USA), and the data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

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-7, IL-10, IL-15 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 is appropriate for high-efficiency induction and the 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 ascertaien by the levels of IL-2, IL-7, IL-9, IL-10, IL-13, IL-15, IL-17α, IFN-g, TNF-α (Figure 2). 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 3).

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 the infection. As compared to than 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 cells and myeloid-derived suppressor cells (Figure 4A). 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 4B). Interestingly, we also found that VEGF is 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 4C).

Recent studies have reported the associations 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 may be related to disease features. Persistent antigenic stimulation leads to gradual accumulation of late-differentiated $CD8^+$ T-cells characterized by critically shortened telomeres typical of senescent cells with particular phenotype ($CD57^+CD28^-$) (16). Cytofluorometric analysis identified increased frequencies of this $CD8^+$ T cell subset in patients with severe disease compared to healthy controls (Figure 5), suggesting senescence/exhaustion events in the infection of severe patients in COVID-19.

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 a determinant in the worsening of infection, contributing to the high morbidity and lethality seen in COVID-19 (1).

Studies show 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 (7), 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). 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 of IFN-γ, IL-2, IL-7, IL-10, IL-15 and IL-17 from 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-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 by the mitogenic T cell stimulation does not demonstrate 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 show increased levels of VEGF in response to the mitogenic stimulation of peripheral blood mononuclear cells 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 demonstrate increased serum VEGF levels in critically infected patients, suggesting their relevance in the pathophysiology of the disease.

VEGF induces the expression of 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 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 $CD8^+$ T-cells characterized by critically shortened telomeres with particular phenotype ($CD57^+CD28^-$) (16). Our study reveals that severe acute COVID-19 infection is associated with senescence of T cells, especially within the $CD8^+$ T cell compartment. The clonal loss of CD8 lymphocytes could limit the repertoire of the memory T cell compartment, thus predisposing individuals to secondary infections. In conclusion, our study highlights the usefulness of anti-inflammatory therapeutic approaches to prevent T-cell hyperactivation and paralysis, in an attempt to avoid the observed extensive T cell loss in severely affected individuals.

Declarations

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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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Figures

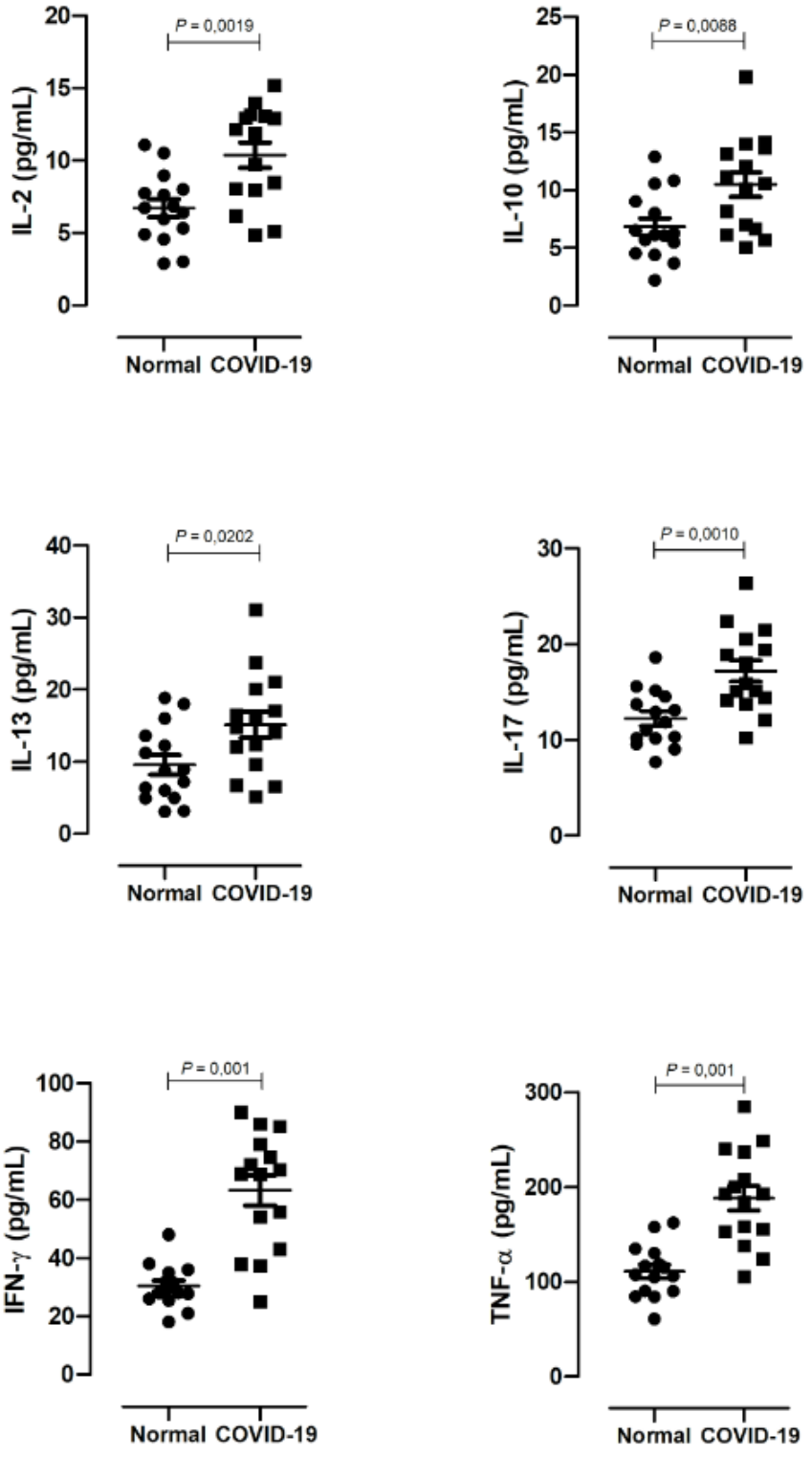

Figure 1

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-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 ($p \leq 0.05$).

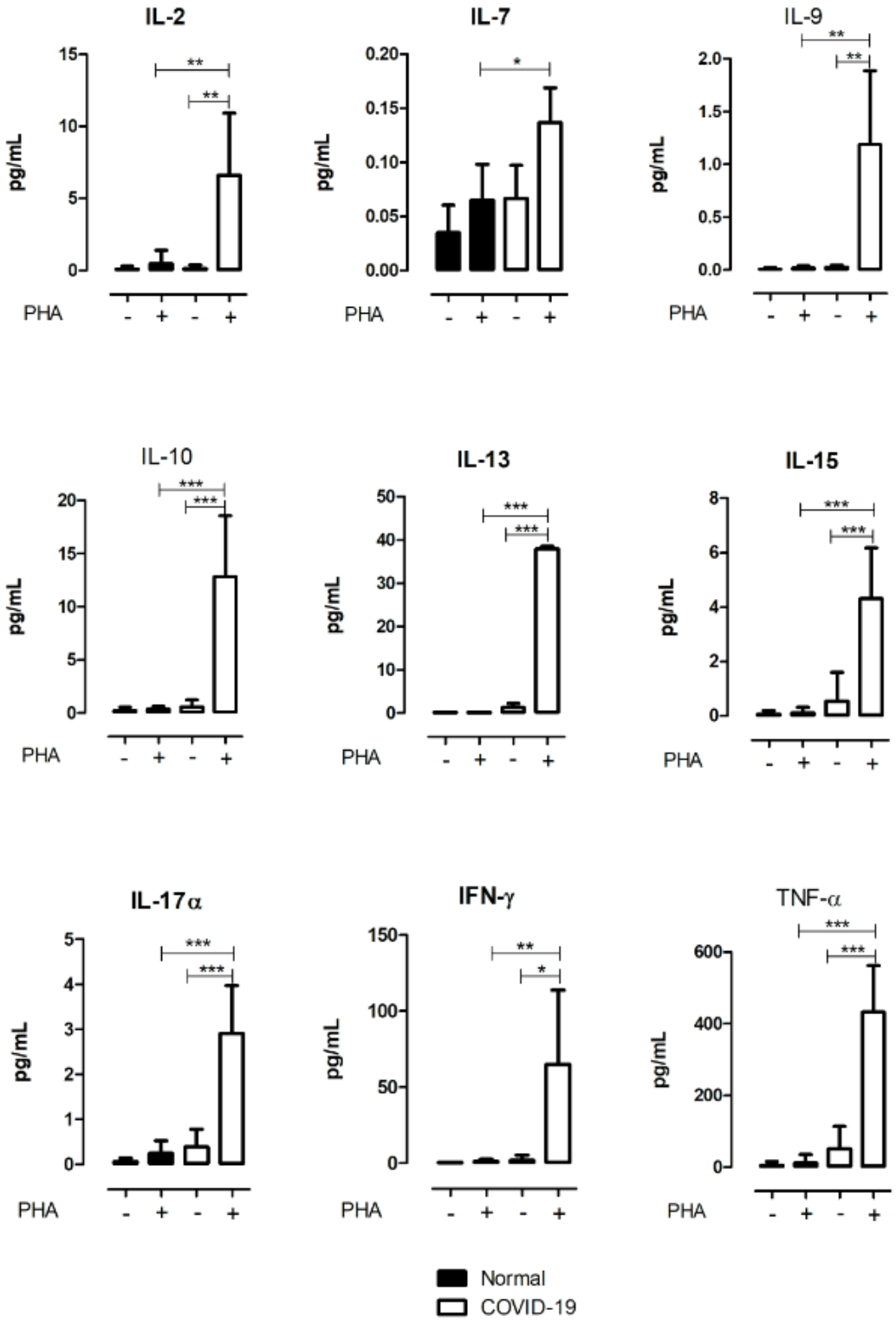

Figure 2

T cell responses of COVID-19 patients have a broad profile of increased cytokine secretion. Peripheral blood mononuclear cells (2x105 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 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).

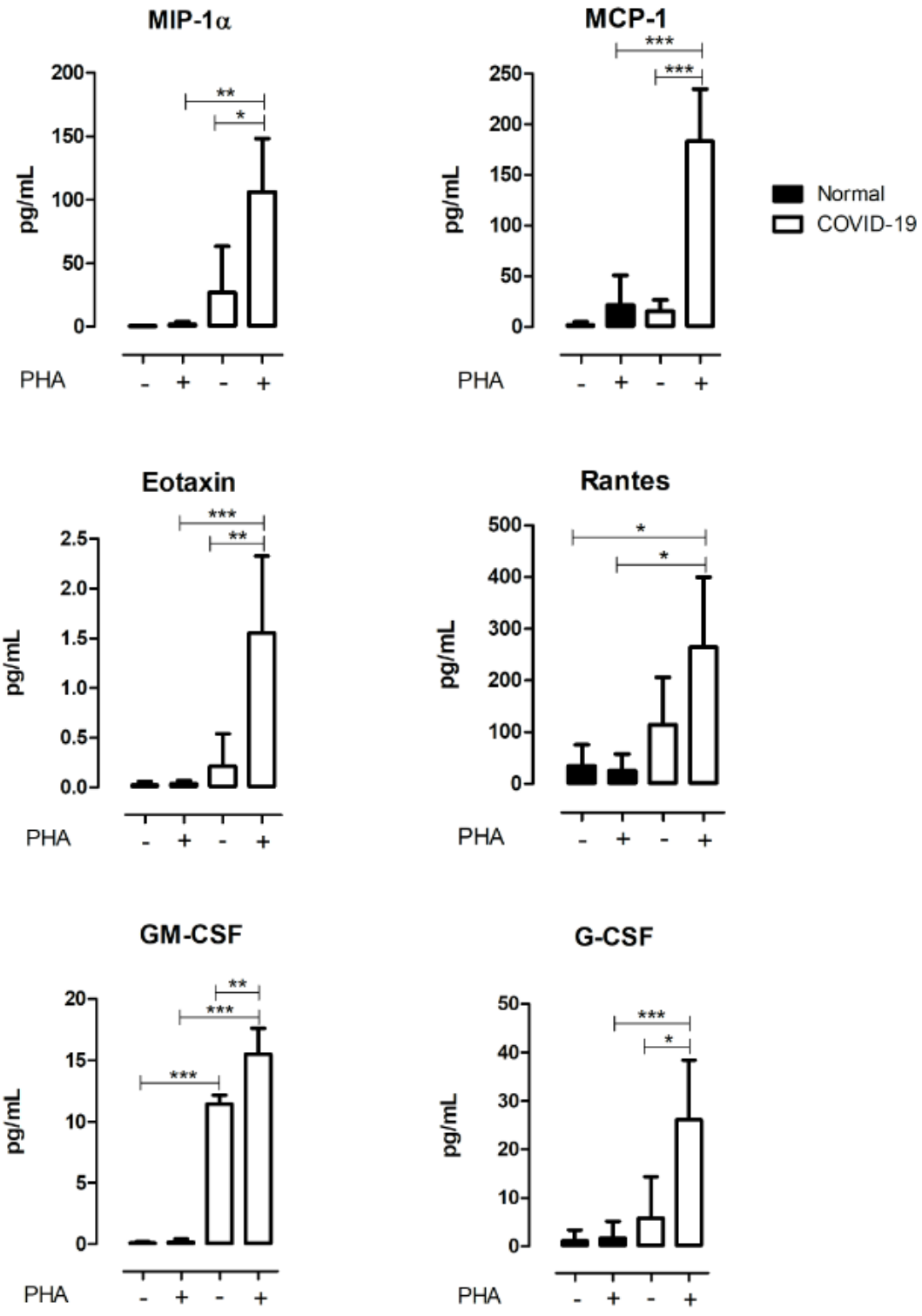

Figure 3

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 (2x105 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 squares) and non-infected healthy donors (solid squares) are significant * (p <0.05), ** (p <0.01), *** (p < 0.001).

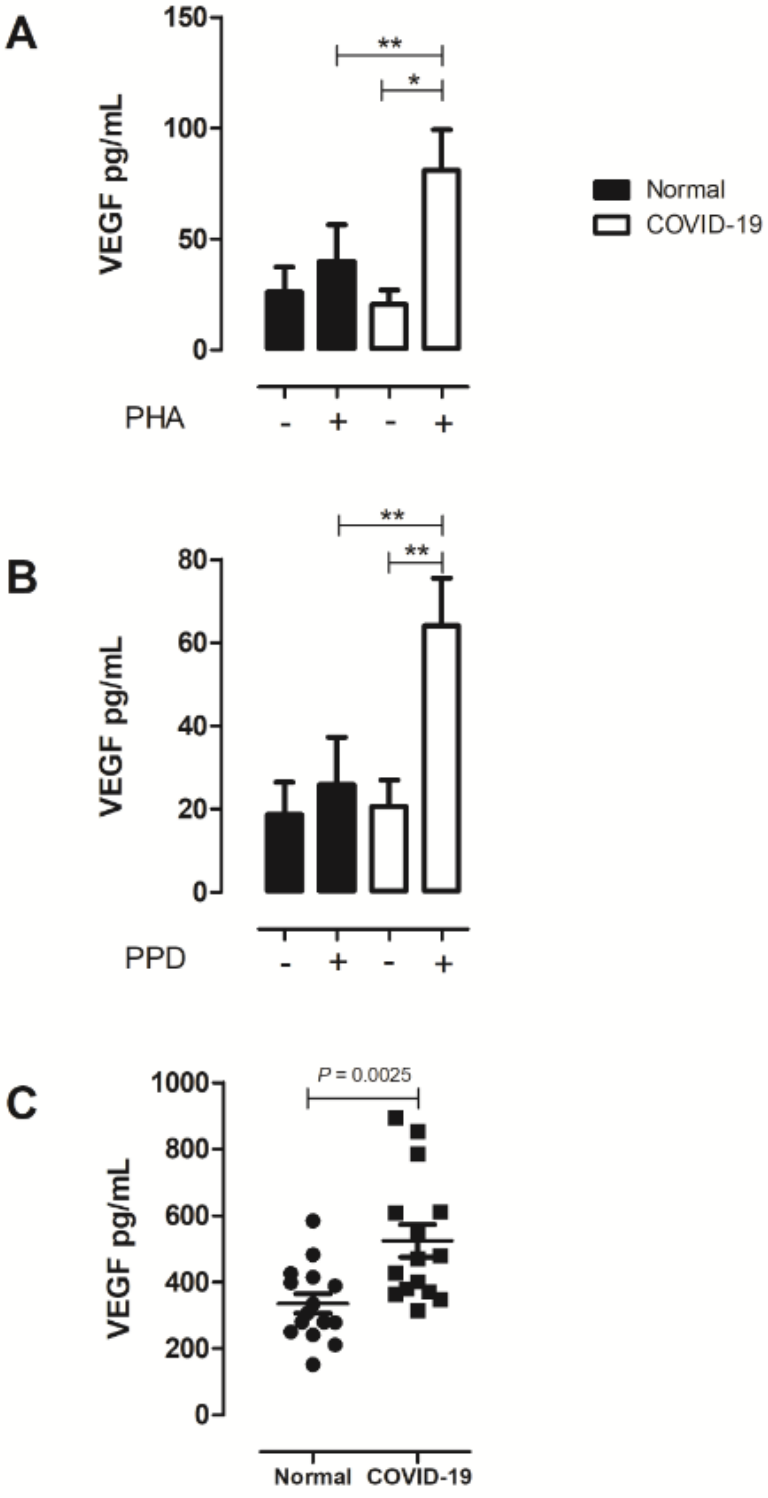

Figure 4

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 (2x105 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 squares) and non-infected healthy donors (solid squares) 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 ($p \leq 0.05$).

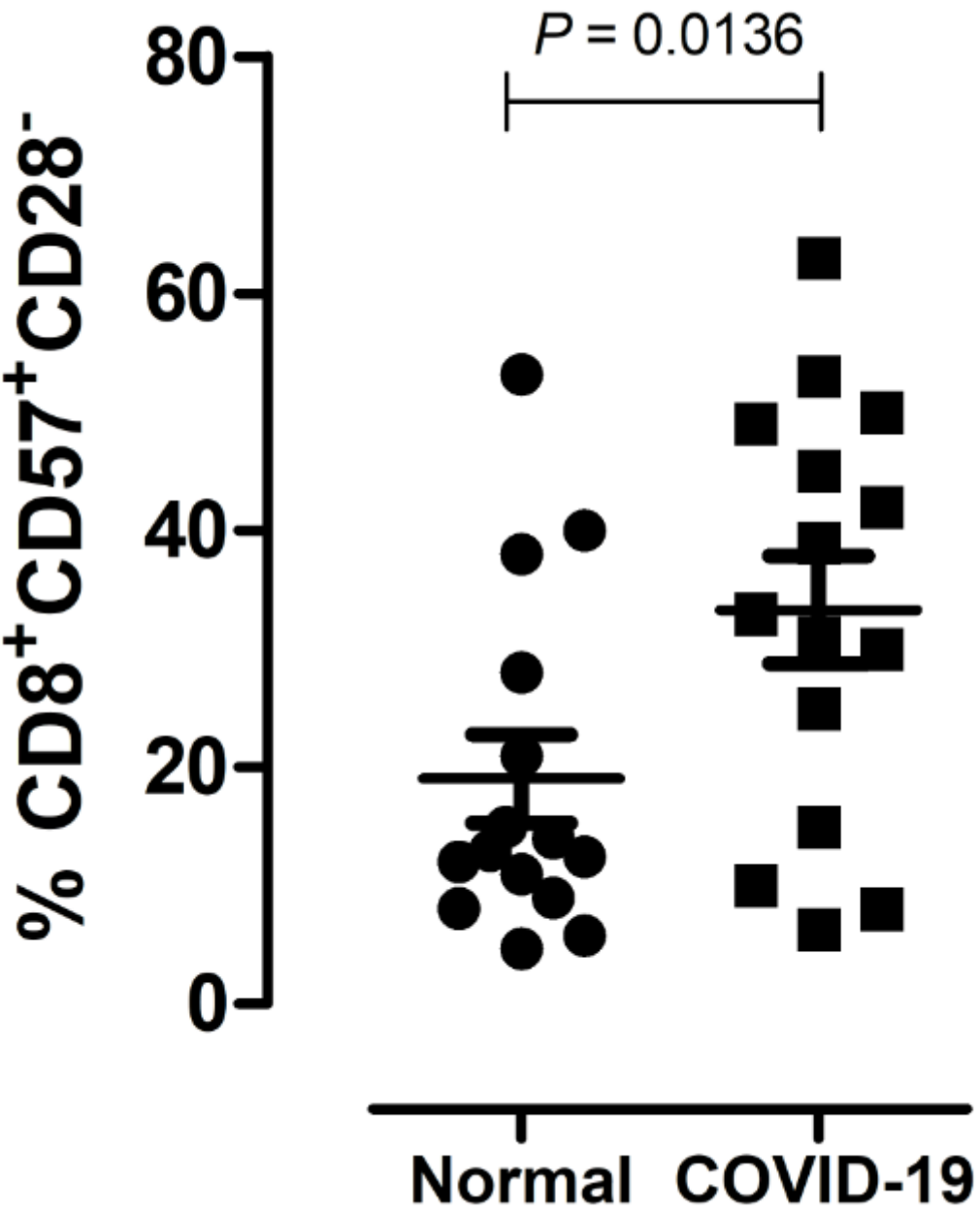

Figure 5

Human late-differentiated senescent (CD57+CD28−) CD8+ T lymphocytes are increased in SARS-CoV-2 – acutely infected severe patients. Statistical analysis compared the population of CD28−CD57+ T cells within the CD8+ T cell compartment in severe COVID-19 patients versus non-infected healthy control individuals in peripheral blood mononuclear cells. Data are expressed as mean ± SEM. Flow cytometry plots are representative of at least three independent experiments. Differences between groups are significant ($p \leq 0.05$).