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Article

Generation of Cytotoxic T cells and dysfunctional CD8 T cells in severe COVID19 patients

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Abstract: COVID-19, the infectious disease caused by SARS-CoV-2, has spread on a pandemic scale. The virus infection can evolve asymptomatically or generate severe symptoms, influenced by the presence of comorbidities. Lymphopenia in patients affected with COVID-19 according to the severity of symptoms is frequent. However, the profile of CD4+ and CD8+ T-cells regarding cytotoxicity and antiviral factor expression has not yet been completely elucidated in acute SARS-CoV-2 infections. The purpose of this study is to evaluate the phenotypic and functional profile of T-lymphocytes in patients with moderate and severe/critical COVID-19. During this pandemic period, we analyzed a cohort of 62 confirmed patients with SARS-CoV-2 (22 moderate cases and 40 severe/critical cases). Albeit lymphopenia, we observed an increase in the expression of CD28, co-stimulator molecule, and activation markers (CD38 and HLA-DR) in T-lymphocytes as well as an increase in the frequency of CD4+ T-cells, CD8+ T-cells, and NK cells that express the immunological checkpoint protein, PD-1, in patients with severe/critical condition compared to healthy controls. Regarding the cytotoxic profile of peripheral blood mononuclear cells, an increase in the response of CD4+ T-cells already at baseline level was observed, scarcely changed upon PMA and Ionomycin stimulation. Meanwhile, CD8+ T-lymphocytes decreased cytotoxic response, evidencing a profile of exhaustion in patients with severe COVID-19. As observed in the t-SNE technique CD4+ T-cytotoxic and CD8+ T with low granzyme production evidencing their dysfunctionality in severe/critical conditions. In addition, purified CD8+ T-lymphocytes from patients with severe COVID-19 showed an increased constitutive expression of differentially expressed genes associated with the caspase pathway, inflammasome, and antiviral factors, and curiously, reduced expression of TNF-α. The cytotoxic profile, by CD4+ T-cells, may compensate for the dysfunction/exhaustion of TCD8+ in acute SARS-CoV-2 infection. These findings may provide an understanding of the interplay of cytotoxicity between CD4+ T-cells and CD8+ T-cells in the severity of acute COVID-19 infection.

Keywords: SARS-CoV-2, COVID-19, T-lymphocytes, antiviral response, cytotoxic factors.

1. Introduction

COVID-19, a disease caused by the SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus 2), has spread on a pandemic scale since the first case was reported in Wuhan, China, in 2019 (C. Huang et al. 2020). According to data from the World Health

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Organization (WHO), as of June 17, 2022, more than 535 million individuals have been infected worldwide and 6,3 million have died (WHO, 2022). Most patients with the disease develop mild or moderate symptoms, however, a portion of patients progress to severe pneumonia and Severe Acute Respiratory Syndrome (SARS), septic shock, and/or multiple organ failure (Cao 2020; Shoaib et al. 2021). SARS-CoV-2 can infect a wide range of cells, including cardiocytes and endothelial, testicular cells, and the bile duct (Osuchowski et al. 2021). However, the expansion of vaccination protocols as well as booster doses is contributing to the reduction of symptoms, the severity of infection, and deaths (Y. Z. Huang and Kuan 2022).

An effective immune response against viral infections is mediated by the activation of cytotoxic T-cells that can eliminate infected cells. Both CD4 and CD8 T-cells have cytotoxic activities contributing to the elimination of virally infected cells, including innate cells such as Natural Killer cells (Phetsouphanh, Pillai, and Zaunders 2017). In this context, in the lung tissue of patients with severe disease, an intense infiltrate of CD4+ and CD8+ T-cells is observed, with strong expression of granzyme B(J. W. Song et al. 2020). In the context of COVID-19, a pro-inflammatory Th1-cytotoxic response against SARS-CoV-2 spike, membrane, and nucleocapsid proteins is also reported (Taus et al. 2022). CD8+ T-cells exhibit a controversial role in COVID-19, with reduced levels of CD107a, IFN-γ, IL-2, and granzyme B compared to healthy (Zheng et al. 2020); or by CD8+ T-cells with increased production of granzyme A and B and perforin during COVID-19 (Ahmadi et al. 2020).

A hallmark of the SARS-CoV-2 acute infection is the pronounced reduction in the numbers of CD4+ T-lymphocytes, CD8+ T-lymphocytes, B-lymphocytes, and NK cells, which are associated with a higher mortality rate (C. Huang et al. 2020; Jafarzadeh et al. 2021) (Qin et al. 2020).

Although T-cell responses are important in eliminating viral respiratory infections (Vasileiou et al. 2020), exacerbation or dysfunctional responses may contribute to the pathogenesis of COVID-19. Increased proinflammatory cytokine levels are of great importance for the recruitment of immune cells to the site of infection and the fight against the virus, but systemic immune hyperactivation due to SARS-CoV-2 infection can promote loss of negative feedback in the immune system, generating an overproduction of inflammatory cytokines (P. Song et al. 2020). The pro-inflammatory environment and constant cellular activation during the SARS-CoV-2 infection can also promote exhaustion, generating immune dysfunction with increased PD-1 expression. However, PD-1 expression has also been linked to avoiding exacerbated responses, not just suggesting cellular exhaustion in COVID-19 (Schönrich and Raftery 2019; Zenarruzabeitia et al. 2021).

Although the host's innate immune system has elaborated antiviral defense programs, viruses continually develop strategies to evade the host's immune response. SARS-CoV-2 proteins can antagonize type I IFN response and signaling (Yuen et al. 2020), by mechanisms such as suppression of STAT2 phosphorylation, and inhibition of STAT1 nuclear translocation, among others (Xia et al. 2020). However, the antagonistic mechanisms of these viral proteins and their contributions to the development and transmission of COVID-19 are poorly understood (Lopez et al. 2020). It is also shown in COVID-19 patients admitted to the ICU (Intensive Care Unit), higher levels of CD95 expression on T-cells as well as sFasL in plasma, both of which are associated with higher levels of caspase activation, as well as transcripts of pro-apoptotic members of the Bcl-2 family, Bax and Bak, are up-regulated. This indicates that CD4 and CD8 T-cells from COVID-19 patients are more likely to die from apoptosis (André et al. 2022).

This study aims to phenotypically and functionally evaluate T-lymphocytes in moderate and severe/critical cases of COVID-19. Data showed in severe COVID-19 patients, an enhancement of CD4+ and CD8+ T-cytotoxic profiles, whereas CD4+ T-cells are less activated than CD8+T-cells. Dysfunctional cytotoxicity function of CD8 T-cells was linked with the expression of genes associated with the caspase pathway as well as inflammasome showing to be more prone to death.

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2. Materials and Methods

Casuistic

Blood samples in EDTA and heparin from ward and ICU from the Central Laboratory Division (DLC) of the Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo (HCFMUSP) were used. Complete blood samples (in EDTA) were kept at 4ºC and used the next day. Heparin samples were used on the same day of collection. As inclusion criteria, it was necessary to confirm the diagnosis of COVID-19 through the detection of SARS-CoV-2 RNA by Polymerase Chain Reaction – Reverse Transcriptase (RT-PCR). Patients over the age of 75 years and who did not test positive for SARS-CoV-2 were excluded from the study. Also, 25 healthy individuals negative for SARS-CoV-2, checked by RT-PCR, were included as a control.

In the cohort of 62 patients infected with COVID-19, there were 34 males and 28 females, which were included in the study. Patients were categorized based on the WHO classification (WHO, 2020), hospitalized patients without oxygen therapy or under the use of oxygen by mask or nasal cannula were considered "moderate". Patients admitted under non-invasive ventilation or high-flow oxygen were considered "severe" and patients admitted under invasive ventilation without or with support from another organ [e.g., extracorporeal membrane oxygenation (ECMO) or replacement therapy] were considered "critical" cases. Severe and critical cases were evaluated together, and critical cases were flagged. The EDTA samples were obtained from May to July 2020 and the heparin samples between May and July 2021.

Phenotypic analysis of CD4+ and CD8+ T-lymphocytes in peripheral blood by flow cytometry

For the phenotypic characterization, 100µL of whole blood collected in EDTA tubes were incubated with a viability marker LIVE/DEAD Fixable Red Dead Cell Stain Kit (Invitrogen, Carlsbad, CA, USA), for 20 minutes and subsequently incubated with surface antibodies (CD3-BV506 clone SK7, CD4-Pe-Cy7 clone SK2, CD8-APC-Cy7 clone MEM-31, CD28-FITC clone CD28.2, CD38-PerCP-Cy5.5 clone HIT2, and HLA-DR-V500 clone G46-8) for 30 minutes at room temperature. After this period, the samples were washed and fixed with 4% formalin for 15 minutes; and red blood cells were lysed with the FACS Lysing Reagent (BD Biosciences, CA, USA) for 15 minutes at room temperature. Then the cells were washed and resuspended in phosphate buffer (PBS). To evaluate PD-1 expression in CD4+ and TCD8+ T-lymphocytes, cell staining was performed from PBMCs, incubated with surface antibodies (CD3-BV605 clone SK7, CD4-V500 clone RPA-T4, CD8-V450 clone RPA-T8, CD56-AL-100 clone B159, and PD-1-APC clone MIH4). Approximately 100,000 events were acquired per sample on a Fortessa LSR flow cytometer (BD Biosciences). The Fluorescence Minus One (FMO) labeling strategy was used, which refers to labeling the sample with all Antibodies (Ab) except the Ab to be analyzed. Data analysis was performed using the FlowJo™ software. The analysis strategies for T-lymphocytes and PD-1 expression are illustrated in Supplementary Figure 1 and Supplementary Figure 2.

Cytotoxic CD4+ and CD8+ T-cells profile

The volume of blood in a heparinized tube was diluted in saline solution and centrifuged in Ficoll-Hypaque solution (Amersham Pharmacia Biotech, NJ, USA) for 20 minutes at 2200 rpm. Afterward, the PBMC obtained were washed twice in saline solution for ten minutes at 1200 rpm.

To assess the cytotoxic profile of CD4+ and CD8+ T-lymphocytes, $1x10^6$ PBMC were distributed in a 48-well microplate (Costar, Cambridge, MA, USA) in RPMI culture medium (Gibco, Carlsbad, CA, USA) containing 5% AB human serum (Sigma-Aldrich, St. Louis, MO, USA). Cells were stimulated with 30ng/mL PMA and 500ng/mL Ionomycin (Sigma-

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Aldrich) in the presence of CD107a PE-Cy 5 antibody (BD Biosciences) and 10µg/mL of Brefeldin A (Sigma-Aldrich) and incubated at 37° at 5% CO2 for six hours. Subsequently, cells were collected, washed, and resuspended in PBS with the viability marker LIVE/DEAD Fixable Red Dead Cell Stain Kit (Invitrogen) and incubated for 20 minutes at room temperature. Then the cells were washed with PBS and incubated with antibodies for 30 minutes in the dark at room temperature (CD3-BV605 clone SK7, CD4-V500 clone RPA-T4, CD8-APC-Cy7 clone MEM-31, CD56-AL-700 clone B159, Granzyme B-V450 clone GB11, CD107a-Pe-Cy5 clone H4A3, TNF-Pe-Cy7 clone Mab11, Perforin-PE clone 27-35, and IFN-y-FITC clone B27). Subsequently, the cells were fixed for 15 minutes with 4% formalin and incubated for 30 minutes with the antibodies for intracellular labeling together with saponin. After this period, the cells were washed and resuspended in PBS, and after 18 hours, were acquired in a Fortessa LSR flow cytometer (BD Biosciences). Approximately 100,000 events were sampled. Data analysis was performed using the FlowJo™ software. The gating strategy used is illustrated in Supplementary Figure 3.

PCR array of the expression of antiviral factors in purified CD8+ T-lymphocytes

CD8+ T-lymphocytes were isolated from PBMCs using the EasySep™ Human CD8 Positive Selection Kit II, on a STEMCELL EasyEights magnet, following the manufacturer's instructions. After isolation, the CD8+ T-lymphocytes obtained were quantified, and the degree of purity was above 80% (CD3-BV605 clone SK7 and CD8-V450 clone RPA-T8). The RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA) was used to extract total RNA from the samples, following the manufacturer's recommendations. RNA levels were measured using the NanoDrop ND-1000 spectrophotometer (Thermo Scientific, MA, USA). For cDNA synthesis, the RT2 First Strand kit (Qiagen) was used.

For the real-time PCR reactions, the RT2 SYBR Green/ROX qPCR Master Mix (Qiagen) was used, which contains SYBR Green as a fluorophore and ROX as a passive reference. The PCR Array Kit used was PAHS122Z Antiviral Response (Qiagen) according to the manufacturer's instructions. Gene expression data from purified CD8+ T-lymphocytes were analyzed using the comparative Ct method. For normalization of the data, an average value of the reference genes was used, ACTβ (beta-actin), GAPDH (glyceraldehyde 3 phosphate dehydrogenase), HPRT1 (hypoxanthine phosphoribosyl transferase 1), and RPLP0 (ribosomal protein, large, P0).

Statistical analysis

To perform the statistical analysis and graphic representation of the data, the Graph Pad Prism 9 program (Graph Pad Software Inc., La Jolla, CA, USA) was used. Results were expressed as the median and interquartile range (IQR). Analysis of variance was performed using the One-Way ANOVA test, using the non-parametric Kruskal-Wallis test to compare the three groups of data. For comparative analyzes between two groups, the Mann-Whitney test was used and in the correlation analysis, the Pearson test was used. The level of significance was considered when $P \leq 0.05$.

3. Results

Decreased frequency of activated CD4+ and CD8+ T-lymphocytes and increased PD1 expression in patients with SARS-CoV-2 infection

In the cohort, there are 62 patients infected with COVID-19: 34 males and 28 females, with diagnosis confirmed by PCR, all of which were included in the study. We also included 25 uninfected patients. The demographic data of the individuals participating in the study are summarized in Figure 1. It is observed that 90.9% of patients with moderate infection were discharged and 9.09% died. As for patients with severe/critical infection, 62.5% were discharged and 32.5% died, 2.5% were transferred to another institute and for 2.5% we were unable to access their medical records. Both in individuals with moderate

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and severe/critical infection, the occurrence of Systemic Arterial Hypertension (SAH) and/or cardiovascular disorders was prevalent (Figure 1).

Figure 2A-C shows the percentage of total lymphocytes and CD4+ and CD8+ T-cells, respectively, in living total cells. It is possible to evidence a reduction in the frequency of total lymphocytes in patients with severe/critical disease in relation to individuals without the infection and patients with moderate disease (Figure 2A). As for CD4+ and CD8+ T-lymphocytes, a percentage reduction was observed in infected patients compared to uninfected individuals (Figures 2B and 2C). In the evaluation of the blood count, leukocytosis was evidenced in patients with severe/critical infection in relation to the uninfected and with moderate disease (Figure 2D). Lymphopenia was also evidenced in patients with severe/critical infection (Figure 2E). The neutrophil/lymphocyte ratio is increased in patients with severe disease compared to uninfected patients with moderate disease, indicating leukocytosis by neutrophilia and lymphopenia in severe cases of infection (Figure 2F).

	Uninfected (N=25)		Moderate (N=22)		Severe/Critical (N=40)	
Female / Male	14/11		11/11		17/23	
	MEAN	SEM	MEAN	SEM	MEAN	SEM
Age (general)	46.3	2.7	55.2	2.8	52.5	1.8
Age (F)	48.4	3.5	53.7	5.9	49.2	2.7
Age (M)	47.3	4.5	54.5	4.2	55.1	2.5
	Nº	%	Nº	%	Nº	%
Outcome						
Discharge	-	-	20	90.90	25	62.50
Death	-	-	2	9.09	13	32.50
Transfer to another institute	-	-	0	0.00	1	2.50
No access to medical records	-	-	0	0.00	1	2.50
Comorbidities						
SAH/Cardiovascular disorder	-	-	13	59.09	23	57.50
Diabetes Mellitus	-	-	5	22.73	12	30.00
Neoplasms	-	-	1	4.55	1	2.50
Coagulopathies	-	-	2	9.09	2	5.00
Use of alcohol/cigarette	-	-	4	18.18	4	10.00
Obesity	-	-	9	40.91	13	32.50
Transplants			1	4.55	2	5.00
Kidney disorders	-	-	6	27.27	6	15.00
Neurological desorders	-	-	1	4.55	0	0.00
Respiratory disorders	-	-	0	0.00	1	2.50
Metabolic disorders	-	-	2	9.09	4	10.00
Liver disorders	-	-	0	0.00	2	5.00
Autoimmune diseases	-	-	1	4.55	1	2.50
No comorbity	-	-	2	9.09	8	20.00
Number of associated comorbidities						
0	-	-	2	9.09	8	20.00
1	-	-	5	22.73	12	30.00
2	-	-	5	22.73	11	27.50
3	-	-	6	27.27	8	20.00
4	-	-	2	9.09	3	7.50
5	-	-	1	4.55	0	0.00

Figure 1. Demographic data of patients affected by SARS-CoV-2 and uninfected individuals. Data on age, outcome, and comorbidities of uninfected individuals and patients with COVID-19 (moderate and severe/critical). Caption: M – male; F – female; N – sample number; SAH – systemic arterial hypertension.

Subsequently, we evaluated the frequency of total lymphocytes, CD4+ and CD8+ T-cells in patients with moderate and severe/critical disease separated by SARS-CoV-2 positive PCR day intervals (from 1-7 days and from 8-20 days positive PCR). In Figures 2H and 2I, an increase in the frequency of CD4+ and CD8+ T-lymphocytes was detected, respectively, within total living cells, in patients with the moderate disease after the seventh day of positive PCR in relation to patients with positive PCR before this period. These data indicate a numerical recovery of CD4+ and CD8+ T-lymphocytes after the seventh day of positive PCR in moderate patients, not observed in individuals with severe/critical infection, indicating the persistence of lymphopenia with the severity of the infection.

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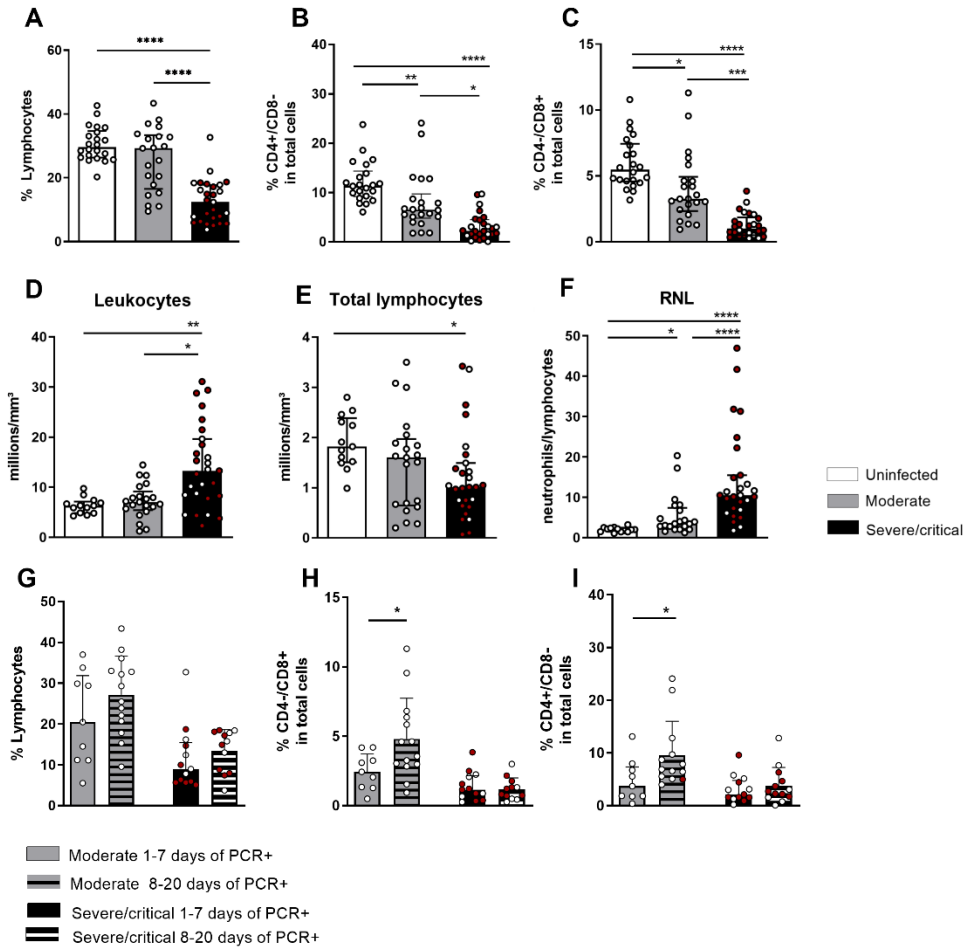

Figure 2. Decreased frequency of CD4+ and CD8+ T-lymphocytes in patients with severe/critical disease from SARS-CoV-2 infection. The graphs show the frequency of cells within living cells (A) total T-lymphocytes, (B) CD4+ T-lymphocytes and (C) CD8+ T-lymphocytes, (D) leukocytes number (millions/mm3), (E) total lymphocytes number (millions/mm3), (F) neutrophil/lymphocyte ratio, and (G-I) frequency of CD4 and TCD8 T-lymphocytes from SARS-CoV-2 infected patients with moderate or severe critical disease between 1-7 days positive and 8-20 days positive PCR. Red dots represent patients with the critical infection. The bars represent the median and interquartile range. * $P < 0.05$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

After evaluating the frequency of CD4+ and CD8+ T-lymphocytes, we analyzed the expression of activation markers in these cell populations of patients infected with SARS-CoV-2. Figures 3A and 3D show the expression of the CD38+ marker on CD4+ and CD8+ T-lymphocytes, respectively. We observed a reduction in the percentage of CD4+ CD38+ T-lymphocytes (Figure 3A). In contrast, an increase in CD38+ expression in CD8+ T-lymphocytes was observed in patients compared to uninfected individuals (Figure 3D). The increase of CD38+ in CD8 T-lymphocytes was also observed in the evaluation of MIF (Supplementary Figure 5D). We observed an increase in the percentage of CD4+ T-lymphocytes (Figure 3B) and CD8+ T-lymphocytes (Figure 3E) that express HLA-DR in COVID-19 patients, regardless of the severity of symptoms compared to uninfected individuals. In Figures 3C and 3F,it is represented the expression of CD28+ on CD4+ and CD8+ T-lymphocytes. We observed an increase in the frequency of CD4+ CD28+ T-lymphocytes in patients with severe/critical disease compared to their controls (Figure 3C).

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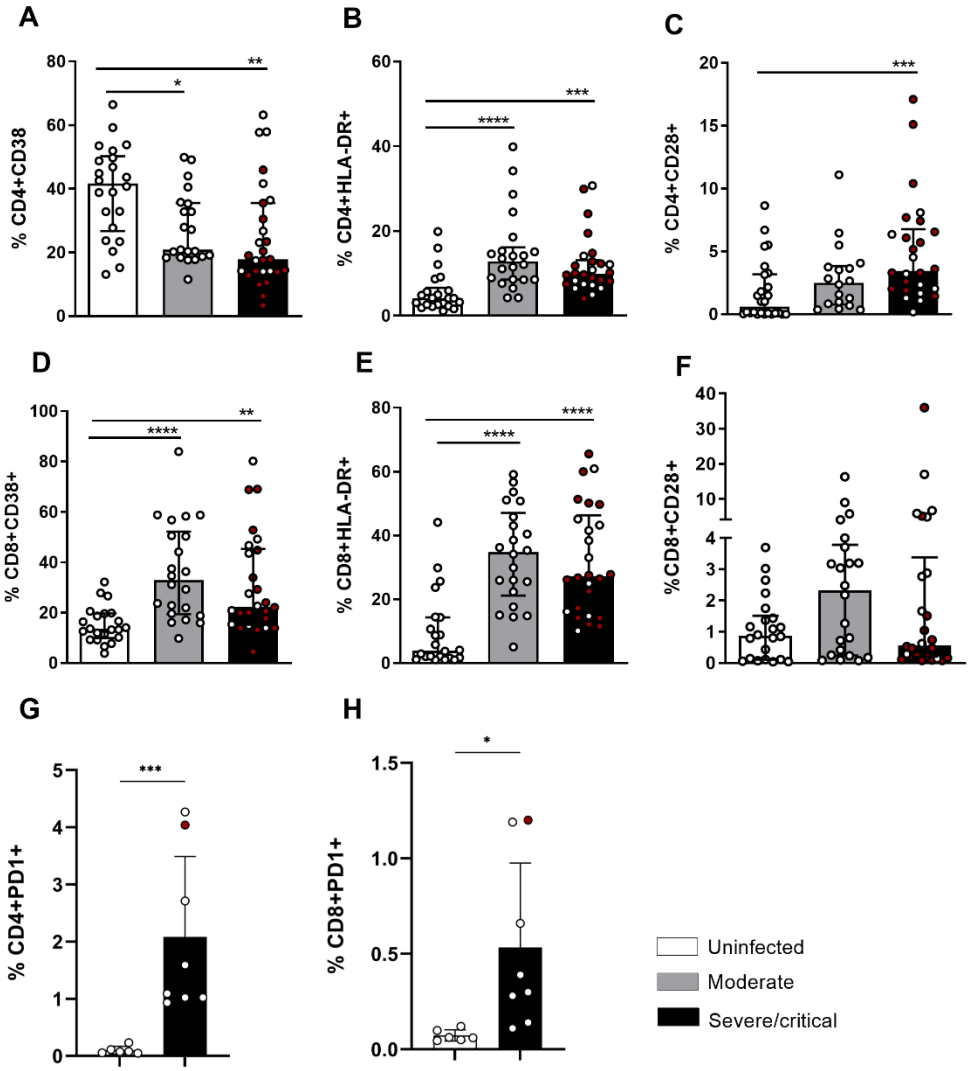

Figure 3. Increased frequency of activation markers and PD-1 expression of CD4+ and CD8+ T-lymphocytes of patients infected with SARS-CoV-2. Frequency in CD4+ T-lymphocytes: (A) CD38+, (B) HLA-DR+, (C) CD28+ frequencies in CD8+ T-lymphocytes of (D) CD38+, (E) HLA-DR+, and (F) CD28+, (G) frequency of PD-1 expression of CD4+ T-lymphocytes, (H) frequency of PD-1 expression of CD8+ T-lymphocytes. The red dots represent patients with the critical infection. The bars represent the median and interquartile range. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

To assess the exhaustion phenotype in critically ill patients with COVID-19, we verified an increase in the frequency of CD4+ and CD8+ T -that express PD-1 in severe/critical patients compared to uninfected individuals. In the MFI analysis, we did not observe any change in the expression of PD-1 (Supplementary Figures 5G and 5H).

Taken together, these results show that lymphopenia worsens with the severity of symptoms. CD4+ T -are less activated while CD8+ T-cells are more activated, as well as lymphocytes show an exhaustion phenotype in COVID-19, which may contribute to the pathogenesis of the infection.

Dysfunctional TCD8+ lymphocytes and generation of cytotoxic TCD4+ lymphocytes in COVID-19

The functionality of T-cells and NK cells, as well as the production of granzyme, perforin, CD107a, IFN-γ, and TNF in PBMCs from uninfected individuals and patients with

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severe/critical COVID-19, was evaluated by flow cytometry, at baseline or stimulated with PMA and Ionomycin.

It is noteworthy that in the basal condition (not stimulated), higher production of granzyme, IFN-γ, and CD107a was observed in CD4+ T-lymphocytes of patients with severe infection compared to uninfected individuals (Figure 4A, Supplementary Figure 6A). After stimulation, no difference was observed between the groups (Figure 4B, Supplementary Figure 6B). The data show that CD4+ T-cells already have an ex vivo alteration and were little affected by the stimulus. The results show that SARS-CoV-2 infection can induce the generation of cytotoxic CD4+ T-cells, with the presence of IFN-γ, granzyme, and CD107a.

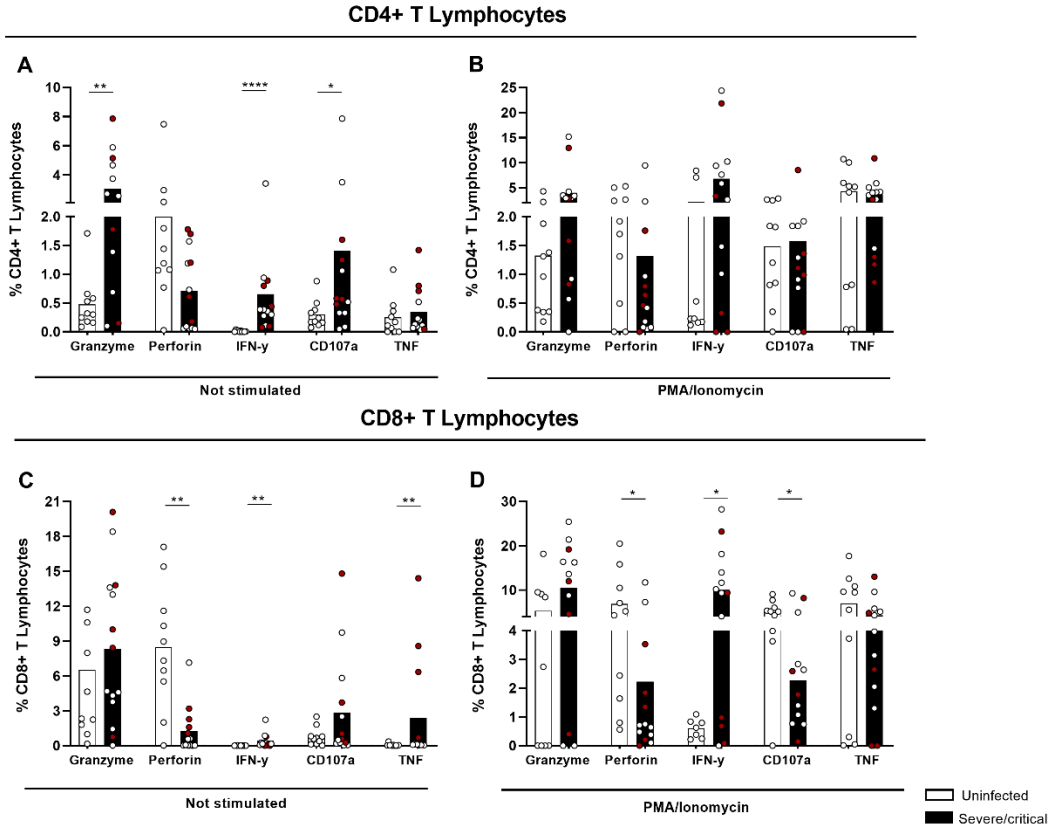

Figure 4. Cytotoxic profile of CD4+ and CD8+ T-lymphocytes in SARS-CoV-2 infection. The graphs represent the cytotoxic profile (granzyme A, perforin, CD107a, IFN-γ, TNF) of CD4+ and CD8+ T-lymphocytes from PBMCs from uninfected and patients with severe/critical COVID-19. Basal levels of the cytotoxic profile of (A) CD4+ T-lymphocytes and (D) CD8+T-lymphocytes and upon stimulation with PMA and Ionomycin of (B) CD4+ T-lymphocytes and (C) CD8+ lymphocytes. The red dots represent patients with critical infections. The bars represent the median and interquartile range. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

As for CD8+ T-lymphocytes, at basal condition, there was a decrease in perforin production, but an increase in IFN-γ and TNF in severe/critical cases compared to uninfected individuals (Figure 4C). When cells were stimulated with PMA and Ionomycin, increased production of IFN-γ was observed, with a drop in production of perforin and CD107a in critically ill patients (Figure 4D). The data emphasize that CD8+ T-cells, despite the expression of IFN-γ, show an altered expression of degranulation and cytotoxic markers in COVID-19.

To better understand the ability of lymphocyte subtypes to produce cytotoxic factors in severe/critical COVID-19 infection, we performed a dimensionality reduction assessment allowing us to explore populations by the t-SNE technique. To perform the t-SNE, singlets, single cells, and live cells were selected and subsequently, the lymphocyte gate was performed. A total of 1,000 events were selected to analyze the generation of population clustering. In the t-SNE evaluation, a total of seven clusters were identified, which

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share common characteristics (Figure 5A and 5B). With the t-SNE data, we classified the populations into four groups: one (basal uninfected), two (basal severe/critical COVID-19), three (stimulated uninfected), and four (stimulated severe/critical COVID-19). Figure 5D shows the overlap of these populations. Figure 5E shows the clusters corresponding to the basal situation of the uninfected patients (red) and the patients with severe COVID-19 (blue), while Figure 5F shows the clusters corresponding to the stimulated cells of the uninfected patients and patients with severe COVID-19.

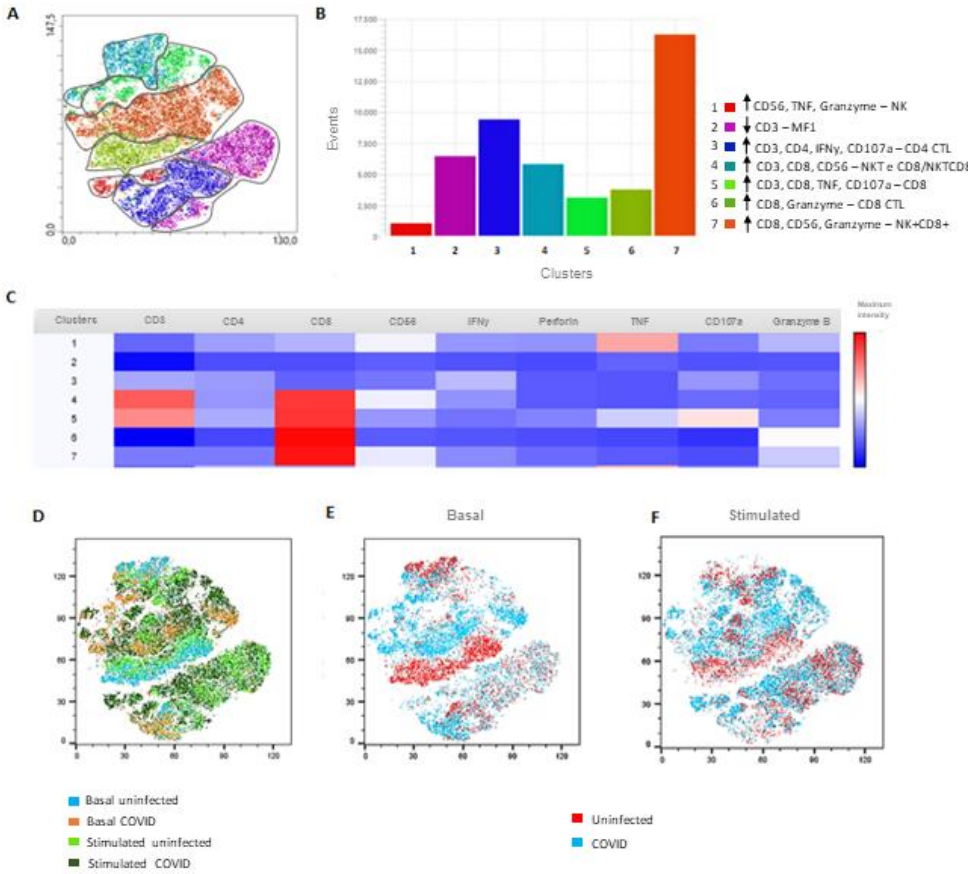

Figure 5. T-SNE-guided population definition of lymphocyte subtypes from uninfected individuals affected by severe/critical SARS-CoV-2. (A) Merged PBMC samples creating a single t-SNE map with the signal strength of lymphocyte phenotypic markers and cytotoxic factors. (B) Column graphic representation of the seven clusters identified in the analysis and the corresponding value of events for each of them. (C) Heat map of the MFI of the markers used, identified in each of the clusters. (D) Overlay of baseline cell populations from uninfected individuals (1), basal severe/critical COVID-19 (2), stimulated uninfected (3), and stimulated severe/critical COVID-19 (4). (E) Overlapping cell populations from uninfected (1 red) or infected (2 blue) patients at baseline. (F) Overlapping cell populations from uninfected (3 red) or infected (4 blue) patients in a stimulated situation.

We observed that both in basal and stimulated situations, there is a lower intensity of Cluster 6 (CD8 and granzyme expression more prominent) in patients with severe symptoms (Figure 5C, Supplementary Figure 7). This data relates to the reduction in the production of cytotoxic factors in patients with severe/critical conditions of COVID-19 observed in conventional cytometry, evidencing dysfunction of CD8+ T-lymphocytes.

With the stimulus, especially in COVID-19 patients with severe/critical condition, we also observed a greater intensity of Cluster 3 (cytotoxic CD4+ T-lymphocyte), as evidenced in conventional cytometry data (Figure 4, Supplementary Figure 7), indicating the cytotoxic role of this population in the severe/critical COVID-19 immune response.

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Taken together, these results show that in COVID-19, there is an induction of the CD4+ T-lymphocyte cytotoxic response in severe/critical cases. Interestingly, the production of perforin and CD107a are altered in CD8+ T-lymphocytes after stimulation, showing a cytotoxic dysfunction. On the other hand, CD4+ T-lymphocytes show that, despite the increase in the baseline condition, they are responsive to stimulation via Protein Kinase C (PKC) and balanced at the levels of the uninfected group.

Increased differential gene expression associated with the caspase and inflammasome pathway in TCD8 lymphocytes from patients with severe COVID-19

As observed in the previous results, CD8+ T lymphocytes from individuals affected with severe COVID-19 are found to have increased PD-1 expression as well as reduced production of cytotoxic factors. There is also an increase in the production of IFN-γ and TNF, indicating an exhausted and inflammatory profile. To evaluate whether this observed inflammatory and exhausted profile can affect the expression of antiviral factors and the expression of signaling molecules, an array PCR of 84 genes associated with these factors was performed.

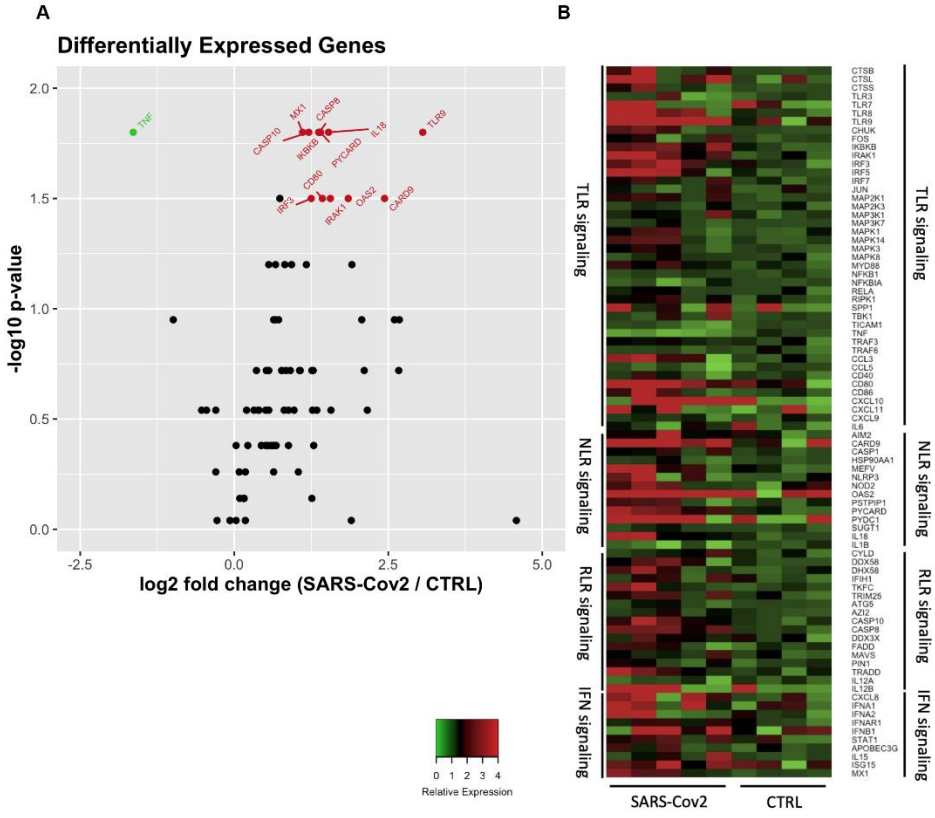

Figure 6. Differentially expressed genes of the signaling pathway of innate immunity and anti-viral factors in CD8+ T-lymphocytes in severe COVID-19. (A) Expression of antiviral factors by PCR array of 84 genes of CD8+ T-lymphocytes from individuals with severe COVID-19 compared to uninfected. The red dots represent the up-regulated genes in severe SARS-CoV-2 infection, the green dot represents the down-regulated gene, black dots represent genes that do not change. (B) Heat map of expression of antiviral factors of CD8+ T-lymphocytes from uninfected (n = 6) and infected individuals with severe COVID-19. Genes are divided into groups according to the associated signaling pathway.

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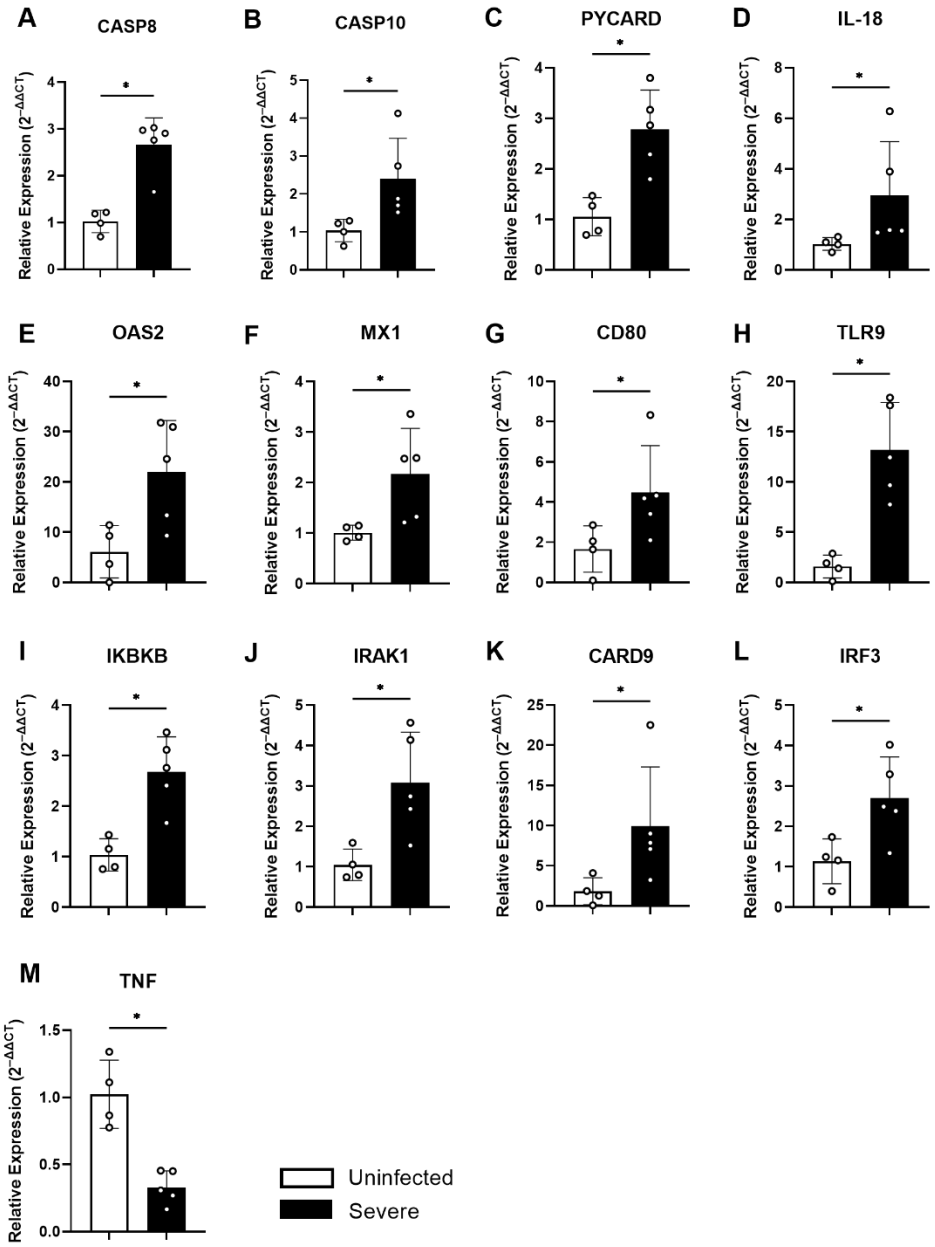

Figure 7. Differentially expressed genes in CD8+ T-lymphocytes of severe COVID-19 patients. Expression of anti-viral factors by array PCR of CD8+ T-lymphocyte genes from individuals with severe COVID-19 compared to uninfected individuals who show a difference. The bars represent the median and interquartile range. * P < 0.05.

In Figure 6, a heat map of the relative gene expression of antiviral factors and constitutive CD8+ T-lymphocyte signaling pathways from five patients with severe COVID-19 and four uninfected patients is illustrated by a heatmap. Overall, we observed greater intensity of gene expression in COVID-19 positive patients compared to controls (Figure 6B). Figure 6A and Figure 6B show the Volcano plot and column graph of these data, respectively, highlighting the up and down-regulated genes in severe SARS-CoV-2 infection relative to uninfected individuals. We observed that *CASP8, CASP10, PYCARD, CARD9, IL-18, CD80, TLR9, IRF3, IRAK1, IKBKB, OAS2,* and *MX*1 genes were up-regulated and *TNF* was down-regulated. The expression of genes CASP8, CASP10 are related to the caspase and apoptosis pathway while PYCARD, CARD9 and IL-18 are related to the inflammatory responses to infection. Thus, the increased expression of these genes is associated with an increase in inflammatory markers observed in the phenotypic analysis,

 doi:10.20944/preprints202208.0496.v1

as well as a reduction in the number of lymphocytes evidenced in SARS-CoV-2 infection. Overall, severe COVID-19 is shown to induce genes associated with caspase pathways and apoptotic processes, especially involving the extrinsic pathway and inflammasome-associated factors. Severe COVID-19 is also shown to up-regulate the *CD80* gene (also called B7.1) (Figure 7G) and *TLR9* (Figure 7H). It up-regulated some genes associated with cell signaling pathways after pathogen recognition such as *IRAK1, IKBKB,* and *IRF3* (Figure 7I, 7J, and 7L). We also evidenced increased expression of viral *OAS* (Figure 6E) and *MX1* (Figure 6F) in patients with severe COVID-19. Finally, we showed that *TNF* was down-regulated in TCD8 lymphocytes in severe SARS-CoV-2 infection (Figure 7M).

Overall, the data show increased expression of genes associated with signaling in response to viral stimulus and inflammatory and apoptotic pathways in severe COVID-19, being related to the pathogenesis of the SARS-CoV-2 infection.

4. Discussion

There are gaps in knowledge about the pathogenesis of acute SARS-CoV-2 infection. T-cells play an important role in the elimination of viruses and the long-term protection against infections, however, they may exhibit a dysfunctional profile, and/or collaborate with tissue damage in target organs. In this context, the functional assessment of CD4+ and CD8+ T-cells, as well as their implications, becomes relevant in SARS-CoV-2 infection.

In the cohort of patients infected with SARS-CoV-2 at Hospital das Clínicas – FMUSP, we classified patients with moderate and severe/critical symptoms. It was observed that 90.9% of patients with moderate symptoms were discharged and 9.09% died. In severe/critical cases, 62.5% were discharged and 32.5% died. Patients with severe manifestations such as pneumonia, hypoxemia, SARS, sepsis and septic shock, cardiomyopathy, arrhythmia, and acute kidney injury required hospitalization, and supportive care and are more likely to die as a result of the disease (Gandhi, Lynch, and del Rio 2020). The most frequent comorbidities observed in our cohort were sequentially systemic arterial hypertension and cardiovascular disorders, obesity and diabetes mellitus, and similarly between clinical conditions, moderate or severe/critical. In chronic comorbidities, prolonged pro-inflammatory state and dysfunction of innate and adaptive immunity are the drivers of worse clinical outcomes in patients infected with SARS-CoV-2 (Sapkal et al. 2020). In patients with obesity and diabetes, ACE2 expression was found to be up-regulated, thus increasing susceptibility to SARS-CoV-2 infection (Herman-Edelstein et al. 2021). These data may help to understand the relationship between comorbidities and the severity of SARS-CoV-2 infection.

Leukocytosis was more pronounced in patients with a severe/critical condition, which was associated with neutrophilia, and with an increase in RNL, which highlights lymphopenia in these patients. The RNL has been indicated as a predictor of severity of COVID-19 since the infiltration of neutrophils in the lung and neutrophilia correlates with the histopathological lesion (Narasaraju et al. 2020). After the first week of infection, we observed an increase in the frequency of T-lymphocytes in patients with moderate infection, but not in severe/critical cases. It is described that patients with prolonged hospitalization due to COVID-19 did not show recovery of B-lymphocyte and CD4 T-lymphocyte counts (Mitsuyama et al. 2021). These data indicate a slower restoration capacity of lymphocyte frequency in the most severe cases of infection.

We observed a reduction in the frequency of total lymphocytes, T-CD4+ and T-CD8+, more markedly in patients with severe/critical conditions. In fact, reduced lymphocyte frequency is a recurrent feature in SARS-CoV-2 infection with reduced numbers of CD4+ T-lymphocytes, CD8+ T-lymphocytes, B-lymphocytes, and NK cells; it has been described to have a strong association with the mortality rate and gravity (C. Huang et al. 2020;

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Jafarzadeh et al. 2021). Several mechanisms may be associated with the occurrence of lymphopenia in SARS-CoV-2 infection, such as the attraction of T and NK cells to sites of infection and sequestration of lymphocytes in target organs (Ahmed, Jo, and Lee 2020; Li et al. 2004; Lin et al. 2020); SARS-CoV-2 infection in human T (Wang et al. 2020); higher expression of p53 in PBMCs of patients with COVID-19, which leads to apoptosis (Xiong et al. 2020), among others. In this context, higher levels of CD95 expression on T-cells and sFasL in plasma, both associated with higher levels of caspase activation, are described in patients admitted to the ICU due to COVID-19. Genes such as Bax, and Bak are up-regulated, indicating that CD4 and CD8 T-cells from COVID-19 patients are more likely to die from apoptosis (André et al. 2022).

In the evaluation of CD4+ T-lymphocytes, we observed an increase in the frequency of HLA-DR and CD28 expression and a reduction of CD38+ expression. It seems that CD4+ T-cells in severe COVID-19 patients are in an activate status but not chronically, whereas they showed up-regulation of PD1 expression. PD-1 expression has been linked to avoiding exacerbated responses, not just suggesting cellular exhaustion (Schönrich and Raftery 2019; Zenarruzabeitia et al. 2021). This may emphasize the activated but exhausted profile in COVID-19, according to the severity of the symptoms. At baseline, we detected an increase in the frequency of granzyme, IFN-γ, and CD107a of CD4+ T-lymphocytes in patients with severe/critical COVID-19. In t-SNE, we also observed qualitatively greater intensity of the cluster that characterizes CD4+ T-lymphocytes with a cytotoxic profile. Our data show cytotoxic factors such as CD107a, granzyme, and IFN-γ, representative of the T-CD4+ CTL function, and which are generated in COVID-19, as already described for other viral infections (Phetsouphanh, Pillai, and Zaunders 2017). In the context of COVID-19, a Th1-cytotoxic pro-inflammatory response against the spike, membrane, and nucleocapsid proteins of SARS-CoV-2 is also reported (Taus et al. 2022). However, the differences between patients and unexposed individuals were mainly quantitative rather than qualitative, suggesting that the cells identified are not unique to COVID-19, but may represent a common cellular phenotype of antiviral T-cells (Bacher et al. 2020). On the other hand, a reduction in the production of IFN-γ and granzyme A in CD4+ T-lymphocytes in patients with COVID-19 is also reported in another study (Mazzoni et al. 2020), indicating a functional change.

In our cohort of patients, CD8+ T-cells showed increased expression of CD38, HLA-DR+, and CD28+. Moreover, an increase of IFN-γ in basal and stimulated levels of CD8+ T-lymphocytes in patients with severe/critical condition in relation to controls stands out, with a decrease in CD107a, perforin, and an increased expression of PD1. This finding was also verified in the t-SNE evaluation, with the reduced cluster of cells expressing a greater intensity of CD8 and granzyme. This shows that CD8+ T-cells are dysfunctional in severe/critical conditions of infection. Decreased production of CD107a+, IFN-γ+, IL-2+, and granzyme B+ is also described in CD8+ T-lymphocytes in COVID-19 (Zheng et al. 2020). In contrast, is also described that SARS-CoV-2 infection induces a cytotoxic response of CD8+ T-cells, characterized by the simultaneous production of granzyme A and B and perforin (Ahmadi et al. 2020).

Albeit the IFN-γ production by CD8+ T-lymphocytes with disease severity, it was unknown about the antiviral factors and apoptotic molecules expression. We find an equilibrated expression of ISGs such as *ISG15*, *IRF7*, *STAT1*, *IFNA1*, *IFNA2*, and *IFNAR1* by PCR array in CD8+ T-lymphocytes. We also evidenced an increase in the expression of *OAS2* and *Mx1*, which play an important role in the defense against viral infections, catalyzing the synthesis of 2'-5'-oligoadenylate for the activation of RNase L (Koul et al. 2020) or inhibiting the infection by blocking the viral transcription and replication, respectively (Bizzotto et al. 2020; Haller et al. 2015). It is reported in SARS-CoV-2 infection, that the virus can suppress the type I IFN response by evasion mechanisms such as ubiquitination of cytosolic sensors, inhibition of translocation of nuclear factors, by decreasing *STAT1* phosphorylation, among other mechanisms that are still not fully understood (Kindler, Thiel, and Weber 2016; Lei et al. 2020; Xia et al. 2020). Despite the presence of antiviral

 doi:10.20944/preprints202208.0496.v1

expression by CD8 T-cells, in severe cases of COVID-19, which is induced by IFN-g, the dysregulation in cytotoxic components contents disables them from effective cytotoxic action.

It was noticeable that the increased DEGS expression was associated with caspase pathways and apoptotic processes such as *CASP8*, *CASP10*, *PYCARD*, *CARD9*, and *IL-18* genes. These components evidenced a pro-apoptotic, which in turn may contribute to the lymphopenia. It has been described that besides CD4+ T-cells, CD8 T-cells are also dying in severely-affected COVID-19 patients compared to uninfected (André et al. 2022). Other studies also showed increased expression of genes associated with inflammation in macrophages challenged with SARS-CoV-2 (Abdelmoaty et al. 2021) and associated with the caspase pathway and apoptosis (Cardinez et al. 2018). It is reported that the inflammasome (NLRP3, associated with IL-18 activation) is activated during SARS-CoV-2 infection, being proposed as an indicator of COVID-19 disease severity, predicting the release of pro-inflammatory cytokines that lead to dysregulated immune responses and tissue damage (Bader et al. 2022).

Interestingly, we showed that TNF was down-regulated in CD8+ T-lymphocytes in severe SARS-CoV-2 infection. TNF is associated with the effector function of cytotoxic cells such as NK and CD8+ T-lymphocytes but also displays an immunosuppressive role, facilitating the biological activity of Tregs and myeloid-derived suppressor cells (Bertrand et al. 2016). The presence of TNF in CD8+ T-cells has a negative effect inducing the death of these cells, thus, the reduction of gene expression may indicate an attempt by CD8+ T-lymphocytes to maintain their effector function and prevent cell death. In addition, CD8+ T-cells with an exhausted profile can be modulating cellular apoptosis.

5. Conclusions

Taken together, these findings highlight the involvement of T-cells in the immunopathogenesis of COVID-19. T-lymphocytes show an activated and exhaustion phenotype, according to the severity of symptoms. In the cytotoxic profile, an increase in the response of CD4 cells was evidenced already in the ex vivo condition. CD8+ T-lymphocytes show a more dysfunctional and exhausted profile in the cytotoxic response, whereas induction of the antiviral genes expression. These findings may provide a better understanding of factors associated with infection severity. More studies are needed to assess the involvement of these cells in the course of COVID-19 disease.

Author Contributions: Overall study conceptualization: SCG-S, MNS, and LMO. Study design: SCG-S, MNS, LMO, and RWA. Conducting experiments: SCG-S, ANJ, LMO, and NZP. Data analysis: SCG-S, FSYY, LMO, and NZP. Providing reagents: MNS, CAB, and AJS. Primary writing of the manuscript: SCG-S, RWA, and MNS. Reviewing and revising the manuscript: SCG-S, LMO, RWA, NZP, FSYY, AJP, TMY, MMSA, YALR, CAB, EAO, AJSD, and MNS. All authors contributed to the article and approved the submitted version.

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Institutional Review Board Statement: This study was approved by the local ethics committee (HCFMUSP nº 30800520.7.0000.0068-2020) and was carried out in accordance with the 2013 revision of the Declaration of Helsinki.

Informed Consent Statement: The studies involving human participants were reviewed and approved by HOSPITAL DAS CLÍNICAS DA FACULDADE DE MEDICINA DA UNIVERSIDADE DE SÃO PAULO. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Conflicts of Interest: The authors declare no conflict of interest

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