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RESEARCH ARTICLE

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Alterations in CD39/CD73 axis of T cells associated with COVID-19 severity

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Abstract

Purinergic signaling modulates immune function and is involved in the immunopathogenesis of several viral infections. This study aimed to investigate alterations in purinergic pathways in coronavirus disease 2019 (COVID-19) patients. Mild and severe COVID-19 patients had lower extracellular adenosine triphosphate and adenosine levels, and higher cytokines than healthy controls. Mild COVID-19 patients presented lower frequencies of $CD4^+CD25^+CD39^+$ (activated/memory regulatory T cell [mTreg]) and increased frequencies of high-differentiated ($CD27^-CD28^-$) $CD8^+$ T cells compared with healthy controls. Severe COVID-19 patients also showed higher frequencies of $CD4^+CD39^+$, $CD4^+CD25^-CD39^+$ (memory T effector cell), and high-differentiated $CD8^+$ T cells ($CD27^-CD28^-$), and diminished frequencies of $CD4^+CD73^+$, $CD4^+CD25^+CD39^+$ mTreg cell, $CD8^+CD73^+$, and low-differentiated $CD8^+$ T cells ($CD27^+CD28^+$) in the blood in relation to mild COVID-19 patients and controls. Moreover, severe COVID-19 patients presented higher expression of PD-1 on low-differentiated $CD8^+$ T cells. Both severe and mild COVID-19 patients presented higher frequencies of $CD4^+$Annexin-V^+ and $CD8^+$Annexin-V^+ T cells, indicating increased T-cell apoptosis. Plasma samples collected from severe COVID-19 patients were able to decrease the expression of CD73 on $CD4^+$ and $CD8^+$ T cells of a healthy donor. Interestingly, the in vitro incubation of peripheral blood mononuclear cell from severe COVID-19 patients with adenosine

reduced the nuclear factor-κB activation in T cells and monocytes. Together, these data add new knowledge to the COVID-19 immunopathology through purinergic regulation.

KEYWORDS

adenosine, ATP, inflammation, purines, SARS-CoV-2, T lymphocytes

1 | INTRODUCTION

The coronavirus disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2; coronavirus disease 2019 [COVID-19]) is a hyperinflammatory condition that can be asymptomatic or manifested as a broad spectrum of disease ranging from few symptoms (mild COVID-19 cases) to severe pneumonia that may evolve to SARS and death (severe COVID-19 cases; Chen et al., 2020). Although several SARS-CoV-2 infections generate asymptomatic or mild COVID-19, a considerable proportion of the remaining cases show severe and critical pneumonia requiring oxygen support and mechanical ventilation (Pascarella et al., 2020).

Host factors and the physiological environment determine the type and the strength of the immune response during the viral infection, as well as the disease outcomes. Thus, the biology of several immune cells, including lymphocytes, can be influenced by blood extracellular adenine nucleotides—adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP), and nucleoside (adenosine; Cekic & Linden, 2016). Purinergic signaling regulates a number of immune cell functions, such as cell-to-cell interactions, cell death, cytokine and chemokine secretion, surface antigen shedding, and cell proliferation (Antonioli et al., 2013). During infections, once released by damaged cells, ATP acts as a damage-associated molecular pattern (DAMP) through P2 receptors (P2X and P2Y receptors) activating immune cells and inducing strong proinflammatory effects (Idzko et al., 2014). Interestingly, severe COVID-19 patients had higher ATP content in the bronchoalveolar lavage supernatant associated with P2RX7-inflammasome activation in macrophage compared with non-COVID-19 patients (Wauters et al., 2021). Furthermore, ATP and ADP levels had positive correlations with markers of blood coagulation and leukocytes showed an upregulation of the several purinergic receptors and ectonucleotidases activities (Schultz et al., 2022). Thus, alterations in adenine-based purine molecules may contribute to the hyperinflammation and immune dysfunction observed in COVID-19 pathophysiology.

The ectoenzyme CD39 (ecto-nucleoside triphosphate diphosphohydrolase 1) converts extracellular ATP into AMP and then CD73 (ecto-5′-nucleotidase) dephosphorylates AMP into adenosine (Antonioli et al., 2013; Deaglio et al., 2007). Adenosine signaling is mediated by G-protein-coupled adenosine receptors, which act as a mechanism for regulating intracellular cyclic AMP levels to induce immunosuppressive events (Mandapathil et al., 2010).

The expression and activity of both CD39 and CD73 change under the pathological context of acute and chronic viral infections (Aeffner et al., 2015; He et al., 2015; Leal et al., 2005). In addition, CD39 expression in $CD8^+$ T cells has also been described as a marker of exhaustion in virus infection (Gupta et al., 2015). There are interconnected changes in the immune system and in the purinergic signaling. Those changes are mediated by the dynamics of adenine-based purine molecules and CD39/CD73 axis that directly impact the host immune response to viral infection (Hasan et al., 2022). During SARS-CoV-2 infection, the active replication and release of the virus can cause the host cells to undergo pyroptosis, promoting the outpour of ATP, which acts as a DAMP in the extracellular environment activating several purinergic components. On the other hand, the dephosphorylation of ATP to adenosine activates immunosuppressive actions mediated mainly by regulatory T cells (Treg cells). In this sense, the modulation of purinergic signaling of T cells expressing CD39/CD73 ectonucleotidases and the extracellular levels of adenine-based purine molecules may contribute to the impaired control of acute hyperinflammatory response through a failure of immunosuppressive axis in COVID-19 (Zarei et al., 2021). The understanding of how purinergic signaling acts during acute SARS-CoV-2 infection could contribute to the development of potential pharmacological targets in the treatment of the disease and may attenuate the progression of severe COVID-19 cases and bad outcomes.

Ahmadi et al. (2020) found lower proportions of $CD8^+CD73^-$ T cells in the peripheral blood of COVID-19 patients and demonstrated that these cells possess a significantly higher cytotoxic effector phenotype. To better understand the role of the CD39/CD73 pathway in the immunopathology of SARS-CoV-2 infection, this study evaluated the systemic levels of ATP, ADP, AMP, and adenosine and the frequencies of $CD4^+$ and $CD8^+$ T cells expressing CD39 and CD73 ectonucleotidases, the proportions of $CD8^+CD27^{-/+}CD28^{-/+}$ expressing PD-1, as well as the rates of lymphocyte apoptosis in the peripheral blood from mild and severe COVID-19 patients.

2 | METHODS

2.1 | Study patients and ethics

We prospectively evaluated a convenience sample of mild and severe COVID-19-positive patients admitted at the Hospital Moinhos de

Vento between July 2020 and November 2020, and volunteering uninfected healthy individuals. Infection with SARS-CoV-2 was confirmed by reverse-transcription polymerase chain reaction (RT-PCR) with nasopharyngeal and oropharyngeal swab samples. This study was approved by the Moinhos de Vento Ethics Committee N 3977144 (Porto Alegre/Brazil) and informed consent was obtained from all participants. Blood samples (5 ml) were obtained from patients with mild or severe COVID-19 into K2EDTA tubes (Becton & Dickinson) within 6 h from hospital admission and were kept refrigerated (2–4°C) until the moment of processing (2 h after the collection). Flow cytometry experiments (described below) were conducted with fresh blood. The remaining blood was centrifuged (1500*g*, 10 min), plasma was aliquoted, and was kept at −80°C until plasma analysis.

Disease severity was classified according to the World Health Organization classification after completing the follow-up questionnaire (WHO, 2021). Clinical and sociodemographic data were collected from the patient's electronic medical records upon admission to the unit. Body mass index was calculated from weight and height data.

2.2 | Analysis of adenine-based purine concentrations and lipopolysaccharide (LPS) levels by liquid chromatography-tandem mass spectrometry (LC-MS/MS)

An aliquot of 150 µl of acetonitrile was added to the plasma samples (50 µl) and the mixture was shaken for 60 s. After centrifugation for 6 min at 9000*g*, the supernatant was collected and a 25 µl aliquot was directly injected into the LC-MS/MS system. The analytical system consisted of a Nexera UFLC system coupled to a LCMS-8040 triple quadrupole mass spectrometer (Shimadzu). The electrospray ionization-MS/MS parameters were set in positive and negative ion modes (polarity switch, 25 ms) as follows: capillary voltage, positive 4500 V, and negative 3000 V; desolvation line temperature, 200°C; heating block temperature, 500°C; drying gas, 18 L/min; and nebulizing gas, 2 L/min. Analyses were carried out with multiple reaction monitoring (MRM) by using the following fragmentations: m/z 268.0 → m/z 136.0 for detection of adenosine ($[M + H]^+$); m/z 346.0 → m/z 210.1 for detection of AMP ($[M - H]^-$); m/z 426.0 → m/z 327.4 for detection of ADP ($[M - H]^-$); and m/z 506.0 → m/z 207.5 for detection of ATP ($[M - H]^-$). The chromatographic separation was conducted with a Shim-pack GISS column (2.1 × 100 mm, 1.9 µm particle size; Shimadzu) eluted with flow rate of 0.3 ml/min. The gradient mobile phase system consisted of water (Solvent A) and acetonitrile (Solvent B) both fortified with 0.2% acetic acid and 0.1% tributylamine as follow: 0–4.5 min, 10–90% of B; 4.5–5.5 min, 90% of B; 5.5–5.6 min, 90–10% of B; 5.6–10 min, 10% B. The column oven was kept at 30°C. The data were processed using LabSolutions software (Shimadzu). In addition, LPS concentration was determined by quantification of 3-hydroxytetradecanoic acid as described by Teixeira et al. (2021).

2.3 | Cytokine levels

The plasma concentrations of interleukin-1A (IL-1A; from PeproTech) and IL-6, IL-10, and transforming growth factor-β (TGF-β; all from eBioscience, ThermoFisher) were quantified by enzyme-linked immunosorbent assay (ELISA) in a microplate reader (EzReader). The detection limits of each cytokine were IL-6, 2–200 pg/ml; IL-10, 2–300 pg/ml; IL-1A, 2–1000 pg/ml; TGF-β, 20–500 pg/ml.

2.4 | Immunophenotyping

Whole blood samples (100 µl) were incubated with monoclonal surface antibodies (all antihuman) at 4°C for 20 min in accordance with the following combination: (a) Fluorescein isothiocyanate (FITC)-conjugated anti-CD4, Pe-conjugated anti-CD25, PerCP-Cy5.5-conjugated anti-CD39, allophycocyanin (-conjugated anti-CD73; (b) FITC-conjugated anti-CD8, Pe-conjugated anti-CD39, APC-conjugated anti-CD73; and (c) FITC-conjugated anti-CD8, Pe-conjugated anti-CD28, PerCP-Cy5.5-conjugated anti-CD27, APC-conjugated anti-PD-1. Then, samples were incubated for 10 min with lysing buffer (BD Biosciences) and then centrifuged at 500*g* for 5 min. The supernatant was discarded and samples were resuspended in phosphate buffered saline (PBS; 1ml, pH 7.2) and then centrifuged at 500*g* for 5 min. Finally, the samples were resuspended in 0.5 ml PBS and analyzed in flow cytometry. Cell phenotype was acquired using CELLQuest Pro Software (BD Bioscience) on a FACSCalibur flow cytometer (BD Bioscience). A minimal of 50,000 events/tubes were acquired, and granulocytes, lymphocytes, and monocytes were identified and gated according to each forward scatter and side scatter profiles. In the $CD8^+$ T cells gate, cells were further characterized by CD27/CD28 expression: $CD27^+CD28^+$ were defined as low-differentiated cells and $CD27^-CD28^-$ were defined as high-differentiated cells (van Aalderen et al., 2015). PD-1 expression was evaluated in both $CD8^+CD27^-CD28^-$ and $CD8^+CD27^+CD28^+$ T cells subsets and presented as mean fluorescence intensity.

2.5 | Mitochondrial membrane polarization and apoptosis analysis

The mitochondrial membrane potential (ΔΨm) was quantified according to a method previously described (Ferlini & Scambia, 2007), using the fluorescent dye rhodamine 123 (Rh 123, Sigma-Aldrich). The detection of apoptosis was performed by using additional labeling with Annexin V–FITC following manufacturer's guidelines (Invitrogen, ThermoFisher). Analyses were performed by using CELLQuest Pro Software (BD Bioscience) on a FACSCalibur flow cytometer (BD Bioscience).

2.6 | Cell culture and in vitro experiments

Peripheral blood mononuclear cells (PBMCs) of a healthy noninfected donor were isolated from peripheral blood using histopaque gradient

solution, Histopaque 1077 (Sigma-Aldrich) as previously described (Dorneles et al., 2020). Then, the cells were washed and suspended in Roswell Park Memorial Institute-1640 medium (Sigma-Aldrich) supplemented with 2 g/L sodium bicarbonate, 2% glutamine, 100 U/ml penicillin–0.1 mg/ml streptomycin (Sigma-Aldrich), and 10% of plasma acquired from mild COVID-19 ($n = 9$), severe COVID-19 ($n = 9$), or healthy controls ($n = 9$) for 15 h (37°C, 5% CO_2). PBMCs were collected, washed with PBS 1×, and stained with anti-CD4 Pe, anti-CD8 FITC, anti-CD39 Percp-Cy5.5, and anti-CD73 APC (all antihuman antibodies from BD Bioscience) for Flow Cytometry analysis in BD FacsCalibur (BD).

To test the effect of in vitro adenosine treatment on COVID-19-related hyperinflammation, PBMC (1×10^6 cells/well) of severe ($n = 3$) COVID-19 patients were seeded in 12-well plates and treated with adenosine (100 µM) or PBS as control for 24 h (37°C, 5% CO_2). The supernatants were frozen at −80°C until cytokine production analysis. Then, cells were stained with 5 µl monoclonal antibodies (all antihuman) conjugated with specific fluorochromes: CD3 FITC (EbioScience) or CD14 FITC (BIOGEMS). Thereafter, cells were washed with 1.5 ml PBS. For intracellular analysis, cells stained with anti-CD3 or anti-CD14 antibodies were incubated with fixation and permeabilization buffers following the manufacturer's recommendation (Ebioscience). After the period, samples were incubated with Phospo-NF-κBp65 (Ser529) Pe (Ebioscience) monoclonal antibody during 30 min. Then, cells were washed and resuspended in Flow Cytometry Staining Buffer (Ebioscience) and analyzed by flow cytometry as described above. The levels of tumor necrosis factor-α (TNF-α), IL-1β, IL-17a, and IL-10 were evaluated in the culture supernatant by ELISA using commercial kits (all from Ebioscience, ThermoFisher). The detection limits of each cytokine were IL-1β, 2–400 pg/ml; IL-10, 2–300 pg/ml; IL-17A, 2–1000 pg/ml; TNF-α, 2–500 pg/ml.

2.7 | Statistical analysis

Normality of data was checked by Kolmogorov–Smirnov test and the values were presented as mean ± SD. Categorical variables were presented as relative frequency and analyzed by χ^2 test. To identify associations between adenine-based purine levels and symptoms, we used Mann–Whitney *U*-test to compare SARS-CoV-2-infected patients with and without specific disease symptoms. A one-way analysis of variance followed by Bonferroni's post hoc test for multiple comparisons was used to verify between-groups differences. Pearson's coefficient test was performed to check correlations between the variables. $p \leq 0.05$ was considered statistically significant. The SPSS 20.0 (IBM Inc.) software was used in all analysis.

3 | RESULTS

3.1 | Participant's characteristics

The characteristics of severe ($n = 22$), mild ($n = 24$) COVID-19 patients, and healthy control ($n = 13$) participants are presented in Table S1. All patients had COVID-19 confirmed by RT-PCR. Severe COVID-19 patients were older ($p < 0.001$), presented more days from symptom onset ($p < 0.001$), and required oxygen use during hospitalization ($p < 0.001$) compared with mild COVID-19 patients. Furthermore, several symptoms were reported by severe COVID-19 patients, including cough ($p = 0.007$), dysgeusia ($p = 0.04$), anosmia ($p = 0.001$), vomiting ($p = 0.007$), diarrhea ($p = 0.004$), skin rash ($p = 0.01$), fatigue ($p = 0.01$), and stuffy nose ($p = 0.005$). Regarding associated medical condition, severe COVID-19 patients reported hypertension ($p = 0.002$), diabetes mellitus ($p = 0.001$), cardiovascular diseases ($p = 0.001$), heart failure ($p = 0.001$), chronic obstructive pulmonary disease ($p = 0.001$), asthma ($p = 0.001$), and dyslipidemia ($p = 0.001$).

3.2 | COVID-19 impacts on systemic purinergic molecules and cytokine levels

The plasma concentrations of adenine-based purines ATP, ADP, AMP, adenosine, and cytokines are shown in Figure 1. ATP levels were decreased in mild ($p = 0.03$) and severe ($p = 0.03$) COVID-19 patients compared with that in healthy controls. Systemic adenosine levels were higher in healthy controls compared with mild ($p = 0.03$) and severe ($p = 0.02$) COVID-19 patients. Severe COVID-19 presented higher systemic IL-6 and IL-10 levels compared with that in healthy controls and mild COVID-19 patients ($p < 0.01$ for all comparisons). In addition, lower IL-17A levels were identified in the peripheral blood of healthy controls compared with that in mild ($p = 0.03$) and severe COVID-19 ($p = 0.02$) patients.

Then, we compared the systemic levels of adenine-based purines in patients with or without clinical symptoms of COVID-19. COVID-19 patients with nausea symptom ($n = 13$) presented lower adenosine levels ($p = 0.04$) and a tendency to decreased AMP levels ($p = 0.07$). Furthermore, higher ADP levels were found in patients reporting stuffy nose symptom compared to patients without stuffy nose ($p = 0.02$, $n = 19$). Skin rash was reported by two patients and presented lower ATP ($p = 0.01$), ADP ($p = 0.01$), and AMP ($p = 0.01$) levels (Figure 2). Interestingly, LPS levels, a microbial translocation marker who is elevated in COVID-19 patients, were higher in patients with nausea symptom ($p = 0.02$) and inversely correlated with the systemic adenosine levels ($r = -0.54$; $p = 0.03$).

3.3 | Altered CD39 and CD73 expression in T lymphocytes of COVID-19 patients

The T-cell subsets was evaluated in healthy controls ($n = 8$), mild COVID-19 ($n = 12$), and severe COVID-19 patients ($n = 13$; Figure S1). Lower frequencies of $CD4^+$ and $CD8^+$ T cells ($p < 0.001$ vs. healthy controls; $p = 0.03$ vs. mild COVID-19) were found in mild and severe COVID-19 patients. Reduced peripheral frequency of $CD4^+CD25^-$ T cells were also found in mild COVID-19 ($p = 0.01$) and severe COVID-19 ($p = 0.001$) groups compared with healthy controls.

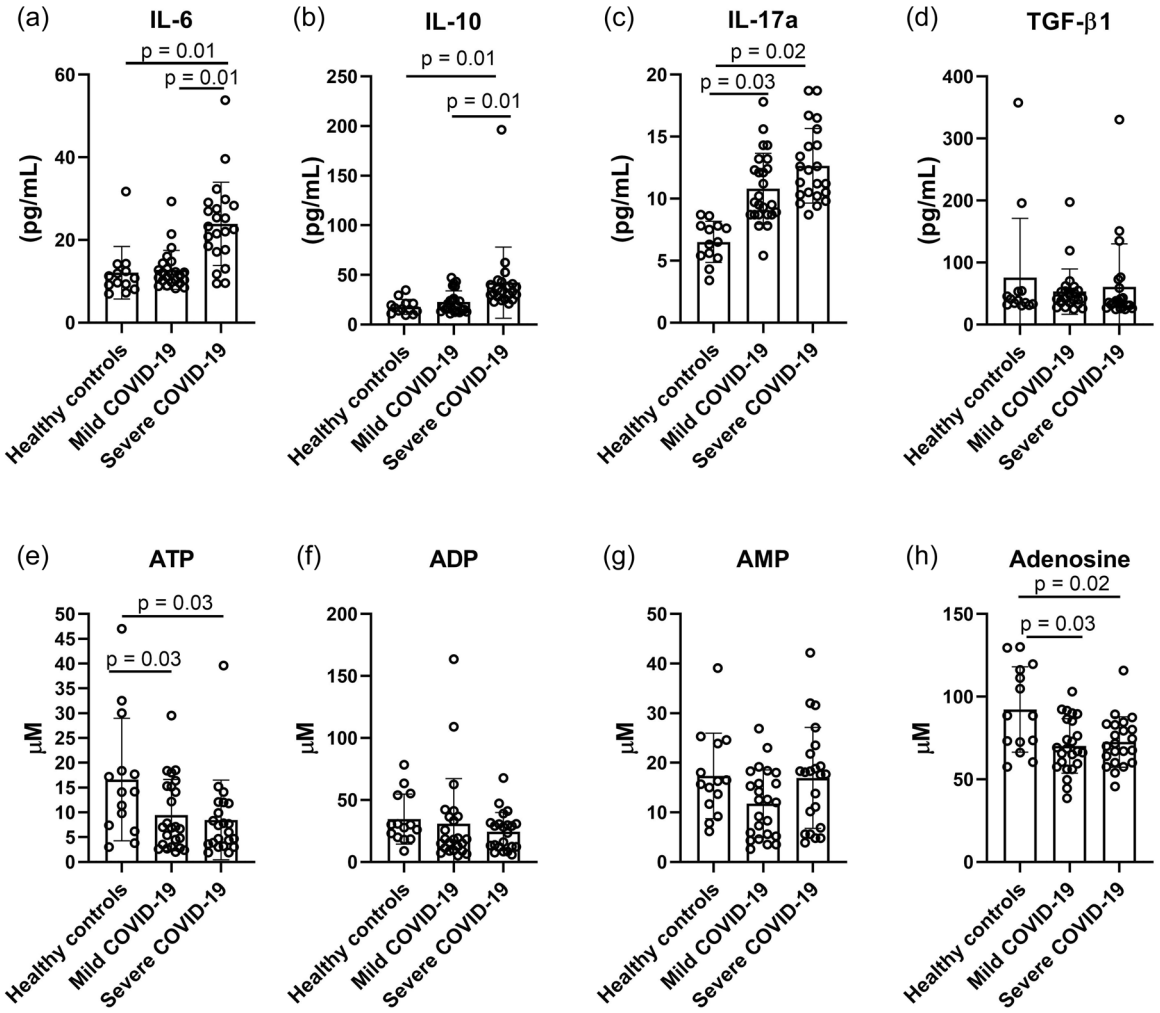

FIGURE 1 The systemic levels of systemic cytokines (a, interleukin-6 [IL-6]; b, IL-10; c, IL-17A; d, transforming growth factor-β1 [TGF-β1]) and adenine-based purines (e, adenosine triphosphate [ATP]; f, adenosine diphosphate [ADP]; g, adenosine monophosphate [AMP]; h, Adenosine) of healthy controls, mild and severe coronavirus disease 2019 (COVID-19) patients. Data are presented as mean ± SD. Group comparisons were performed by one-way analysis of variance with Bonferroni's post hoc test ($p \leq 0.05$).

Figure 3 shows the frequency of CD4, $CD8^+$, $CD4^+CD25^-$, $CD4^+CD25^+$ T cells expressing $CD39^+$ and $CD73^+$, and the proportions of $CD8^+CD27^{-/+}CD28^{-/+}$ T cells expressing PD-1. The frequency of $CD4^+$ ($p = 0.01$ vs. controls; $p = 0.03$ vs. mild COVID-19) and $CD4^+CD25^-$ ($p = 0.002$ vs. controls; $p = 0.004$ vs. mild COVID-19) T cells expressing $CD39^+$ were higher in severe COVID-19. On the other hand, the proportions of $CD4^+CD73^+$ ($p = 0.04$ vs. controls), $CD4^+CD25^+CD39^+$ ($p = 0.001$ vs. controls; $p = 0.002$ vs. mild COVID-19) and $CD8^+CD73^+$ ($p = 0.001$ vs. controls; $p = 0.002$ vs. mild COVID-19) T cells were lower in severe COVID-19 patients. Higher frequency of high-differentiated ($CD27^-CD28^-$) $CD8^+$ T cells was identified in the peripheral blood of mild COVID-19 patients ($p = 0.001$ vs. healthy control) and severe COVID-19 subjects ($p = 0.001$ vs. healthy control), and lower proportions of low-differentiated ($CD27^+CD28^+$) $CD8^+$ T cells in the severe COVID-19 group ($p = 0.05$ vs. healthy controls). The expression of PD-1 was higher in low-differentiated $CD8^+$ T cells of severe COVID-19 patients ($p = 0.008$ vs. healthy controls).

3.4 | Higher apoptosis and mitochondrial dysfunction in lymphocytes of COVID-19 patients

Next, we evaluated the apoptosis and mitochondrial membrane polarization in healthy controls ($n = 6$), mild COVID-19 ($n = 6$) and severe COVID-19 ($n = 6$) individuals (Figure 4). COVID-19 patients presented decreased mitochondrial membrane polarization (severe COVID-19 vs. healthy control, $p < 0.001$; mild vs. healthy control, $p = 0.03$; Figure 4a), indicating mitochondrial membrane depolarization. COVID-19 patients presented higher $CD4^+$Annexin V^+ (severe COVID-19 vs. healthy controls, $p = 0.001$; mild COVID-19 vs. healthy controls, $p = 0.01$) and $CD8^+$Annexin V^+ (severe COVID-19 vs. healthy controls, $p = 0.001$; mild COVID-19 vs. healthy controls, $p = 0.01$). Severe COVID-19 patients also presented higher $CD8^+$Annexin V^+ T cells compared with mild COVID-19 subjects ($p = 0.03$; Figure 4b).

Figure 4c shows the heat map of Pearson's coefficient correlation test. ATP levels inversely correlated with $CD8^+CD27^-CD28^-$

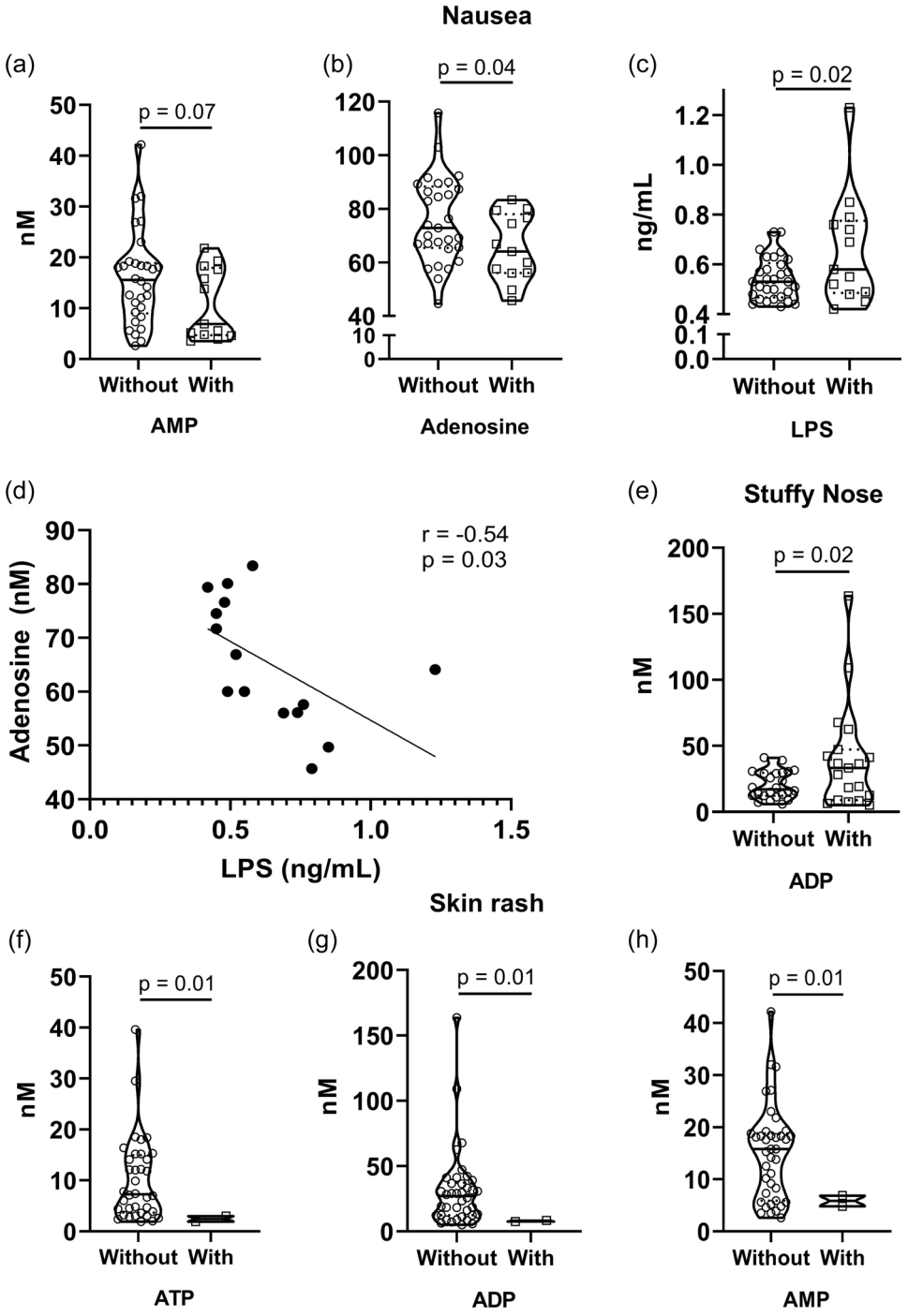

FIGURE 2 The association of adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), and adenosine levels, and lipopolysaccharide (LPS) concentrations with clinical symptoms of acute severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. Significant differences of AMP (a), Adenosine (b), and LPS (c) were found in individuals reporting nausea. Adenosine inversely correlated with LPS (d) in coronavirus disease 2019 (COVID-19) patients. Higher ADP levels were found in patients reporting stuffy nose symptom compared to patients without stuffy nose $p = 0.02$, $n = 19$ (e). COVID-19 individuals reporting stuffy nose presented higher ADP levels (f). Lower ATP (g), ADP (h), and AMP (i) were found in COVID-19 patients reporting skin rash. The levels of adenine-based purine molecules and endotoxin were compared between patients with and without clinical symptoms through Mann–Whitney *U* test and the significant results were presented ($p < 0.05$). The correlation between adenosine levels and LPS concentrations in patients reporting nausea symptom were performed by Pearson's coefficient correlation test ($p < 0.05$).

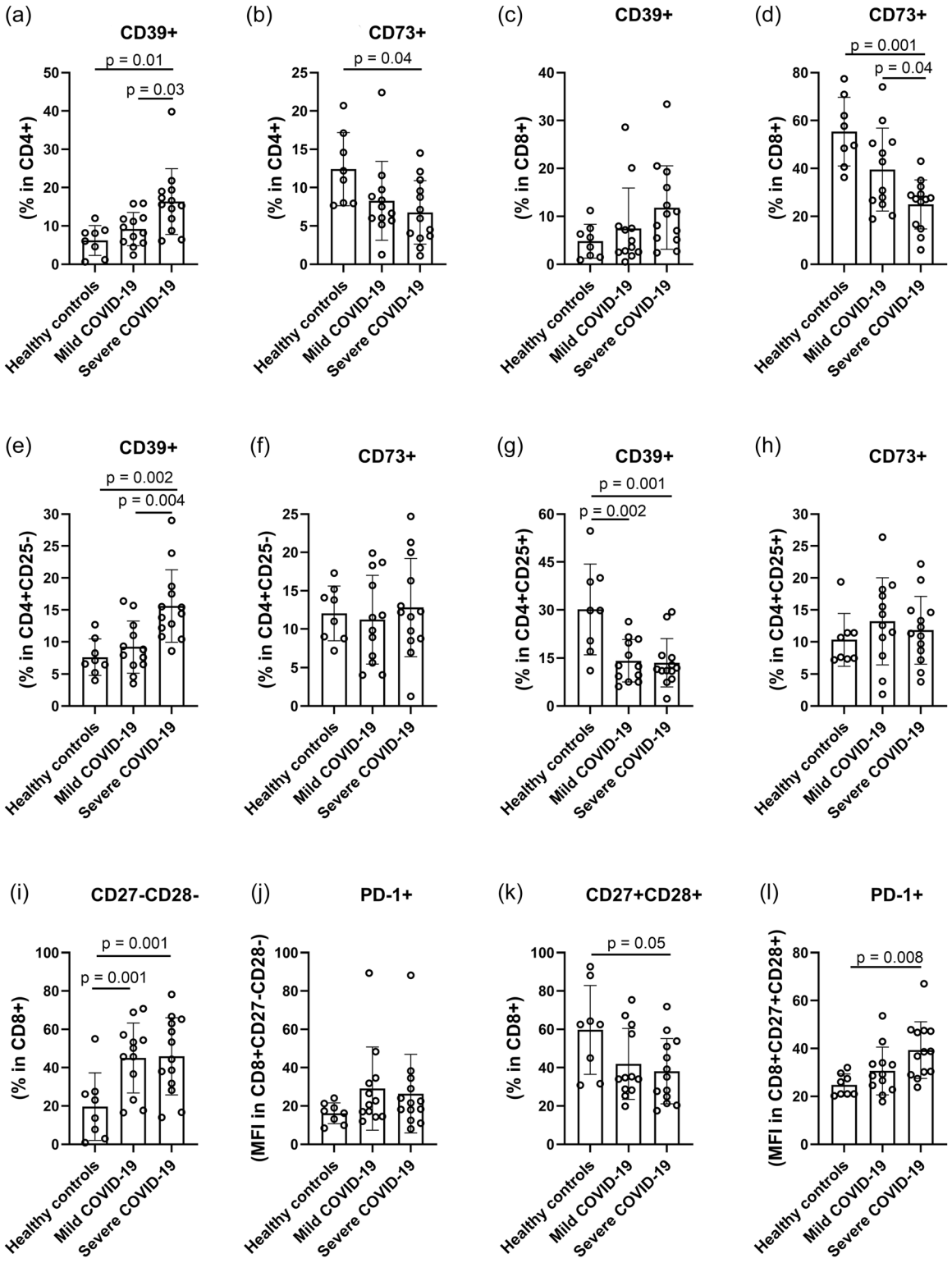

FIGURE 3 The frequency of CD4, $CD8^+$, $CD4^+CD25^-$, $CD4^+CD25^+$ T cells expressing $CD39^+$ and $CD73^+$, and the proportions of $CD8^+CD27^{-/+}CD28^{-/+}$ T cells expressing PD-1 in the peripheral blood of healthy controls, mild, and severe COVID-19 patients. Data are presented as mean ± SD. Group comparisons were performed by one-way analysis of variance with Bonferroni's post hoc test ($p \leq 0.05$).

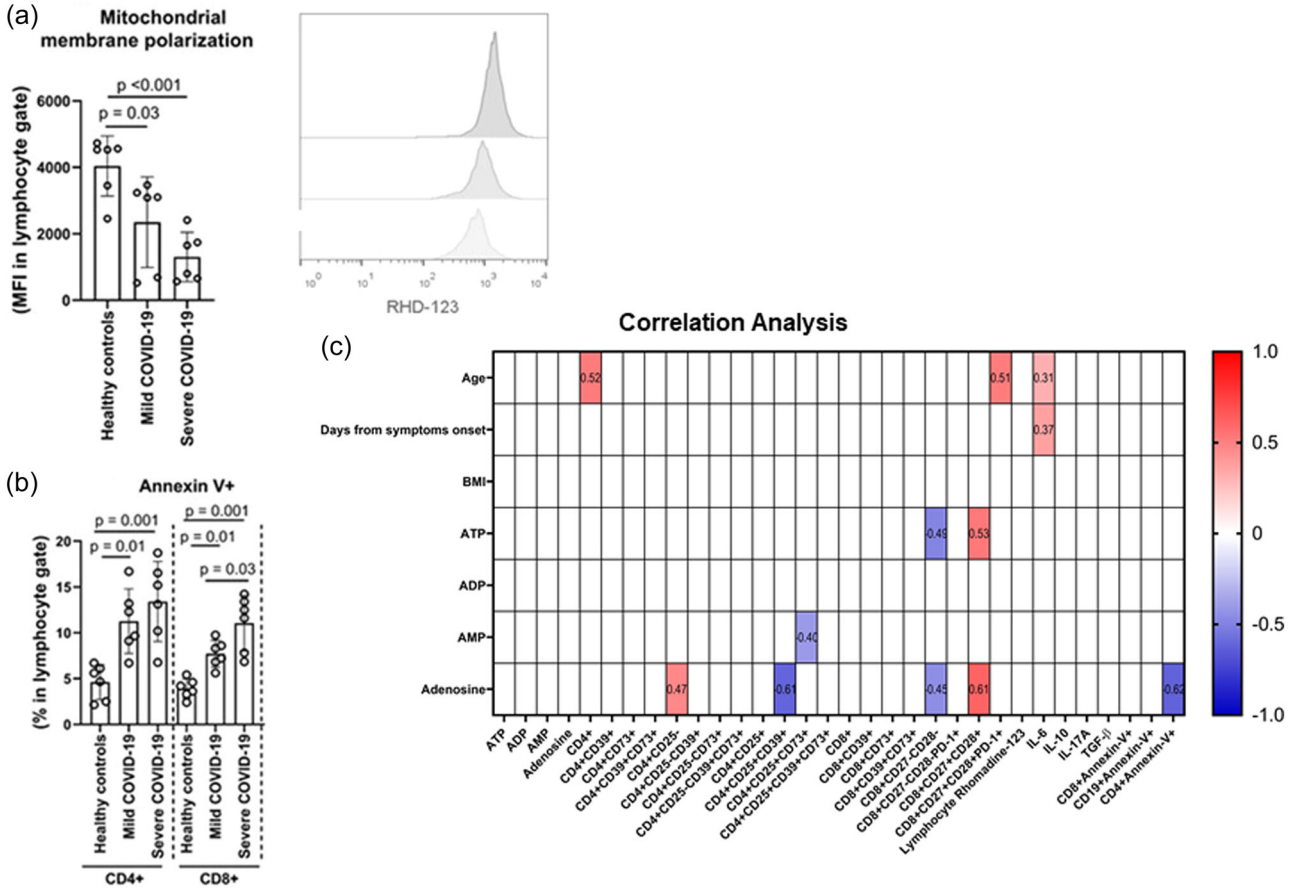

FIGURE 4 Mitochondrial membrane polarization (a) and apoptosis (b) of lymphocytes in coronavirus disease 2019 (COVID-19) patients and healthy controls. Data are presented as mean ± SD. Group comparisons were performed by one-way analysis of variance with Bonferroni's post hoc test ($p \leq 0.05$). The Correlation heat map including adenine-based purines and immune variables at admission to the Hospital (c). Pearson's correlation coefficients are plotted. Cells were colored according to the strength and trend of correlations (shades of red = positive, shades of blue = negative correlations). Statistical analysis performed through Pearson's coefficient correlation.

($r = -0.49$; $p = 0.01$) and positively correlated with $CD8^+CD27^+CD28^+$ T-cell proportions ($r = 0.53$; $p = 0.03$). AMP levels inversely correlated with $CD4^+CD25^+CD73^+$ ($r = -0.40$; $p = 0.04$). Adenosine levels positively correlated with the peripheral frequency of $CD4^+CD25^-$ ($r = 0.47$; $p = 0.01$) and $CD8^+CD27^+CD28^+$ ($r = 0.61$; $p = 0.001$), but negatively correlated with $CD4^+CD25^-CD39^+$ ($r = -0.61$; $p = 0.001$), $CD4^+CDCD27^+CD28^+$ ($r = -0.45$; $p = 0.02$) and $CD4^+$Annexin-V^+ ($r = -0.62$; $p = 0.03$) proportions. The age of the COVID-19 patients correlated with the frequency of $CD4^+$ T cells ($r = 0.52$; $p = 0.03$), the expression of PD-1^+ on $CD8^+CD27^+CD28^+$ T cells ($r = 0.51$; $p = 0.02$) and the IL-6 levels ($r = 0.31$; $p = 0.04$). Days from symptoms onset correlated only with the plasma IL-6 levels ($r = 0.37$; $p = 0.02$).

3.5 | In vitro adenosine treatment reduces nuclear factor-κB (NF-κB) expression in PBMC

The incubation of PBMC from a healthy noninfected control donor with the plasma of severe COVID-19 patients decreased the expression of CD73 on the cell surface of $CD4^+$ (Figure 5a; $p = 0.01$ vs. healthy controls; $p = 0.02$ vs. mild COVID-19) and $CD8^+$ (Figure 5b; $p = 0.01$ vs. healthy controls; $p = 0.02$ vs. mild COVID-19) T cells, without changes in CD39 expression. Interestingly, PBMC from severe COVID-19 patients in vitro treated with adenosine (100 μM) reduced the NF-κB activation in both $CD3^+$ T cells (Figure 5c, $p = 0.04$) and $CD14^+$ monocytes (Figure 5d, $p = 0.04$). Moreover, lower levels of IL-1β and IL-17a ($p < 0.05$ for both) were found in the culture supernatant of PBMC treated with adenosine, despite no changes in IL-10 and TNF-α production.

4 | DISCUSSION

This study identified a relationship between COVID-19 severity and lower systemic adenine-based purines and the frequency of circulating lymphocytes expressing CD39 and CD73 ectonucleotidases. We found decreased plasma levels of ATP and adenosine in COVID-19 patients, concomitant with markedly altered frequencies of $CD4^+$ and $CD8^+$ T cells expressing CD39 and CD73 enzymes on the cell surface. In addition, plasma samples collected from severe COVID-19 patients

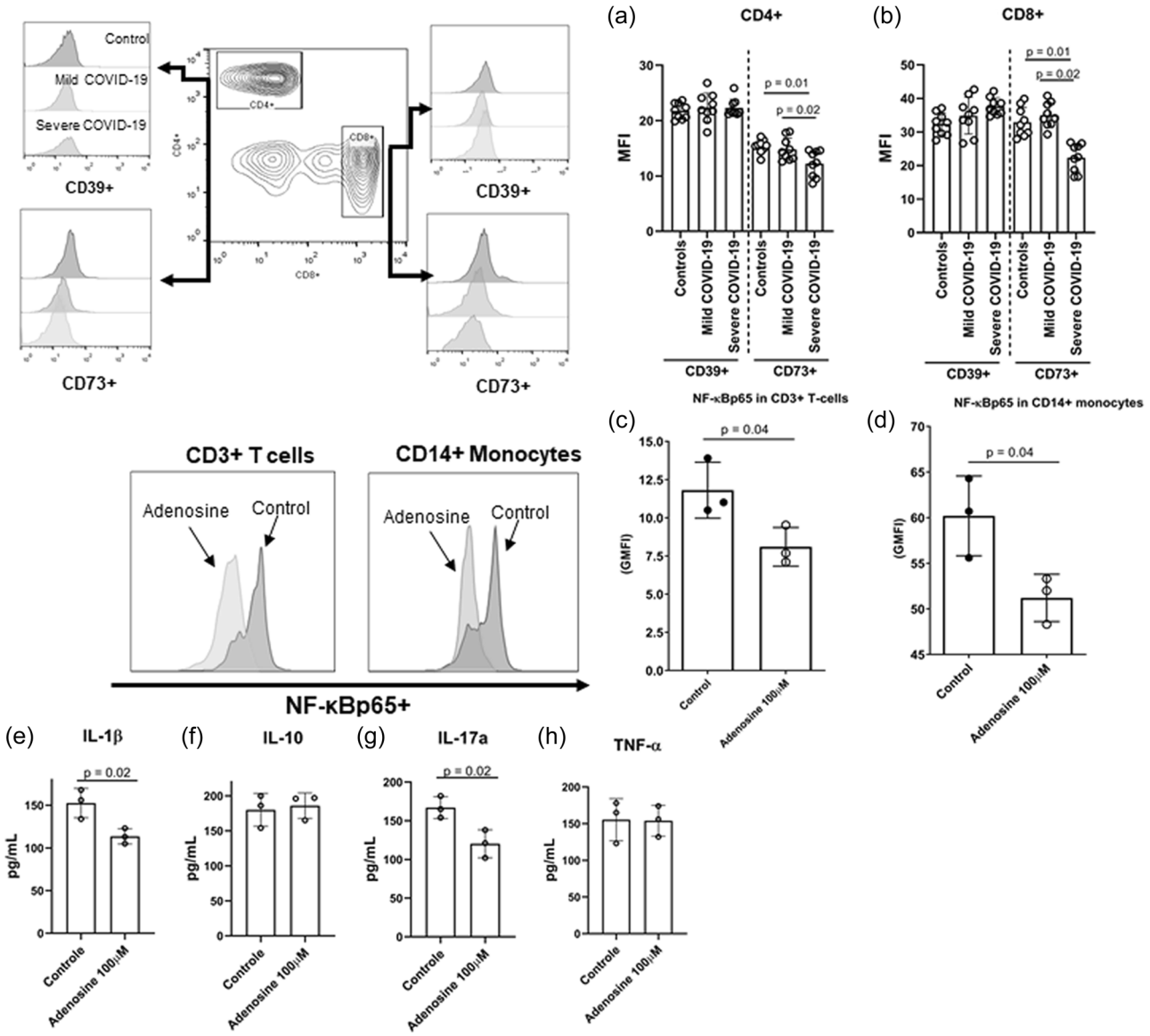

FIGURE 5 CD39 and CD73 expression in $CD4^+$ (a) and $CD8^+$ (b) T cells of a healthy noninfected donor after the incubation with plasma obtained from controls, mild coronavirus disease 2019 (COVID-19) or severe COVID-19 patients. In addition, peripheral blood mononuclear cell (PBMC) of severe COVID-19 ($n = 3$) were treated in vitro with adenosine and the activation of NF-κBp65 were evaluated in $CD3^+$ T cells (c) and $CD14^+$ monocytes (d), as well as the production of interleukin (IL)-1β (e), IL-10 (f), IL-17a (g), and tumor necrosis factor-α (TNF-α) (h) were evaluated. Data are presented as mean ± SD. Group comparisons were performed by one-way analysis of variance with Bonferroni's post hoc test ($p \leq 0.05$).

were able to decrease the expression of CD73 on $CD4^+$ and $CD8^+$ T cells of a healthy donor and the in vitro incubation of PBMC from severe COVID-19 patients with adenosine reduced the NF-κB activation in T cells and monocytes, and lower levels of IL-1β and IL-17a in the supernatant of cell culture. Furthermore, COVID-19 severity was associated with an accumulation of highly differentiated $CD27^-CD28^-$ $CD8^+$ T cells proportions and decreased low-differentiated $CD27^+CD28^+CD8^+$ T-cell frequency in the peripheral blood concomitant with higher expression of the exhaustion marker PD-1 in this population. COVID-19 patients also presented higher rates of lymphocyte mitochondrial membrane depolarization and increased apoptosis rates in $CD4^+$ and $CD8^+$ T cells.

Lower ATP plasma levels are a surprising new result in SARS-CoV-2 infection. Extracellular ATP was found at high concentrations in bronchoalveolar lavage fluid from patients with acute respiratory distress syndrome and in the blood of septic patients (Cicko et al., 2018). On the other hand, extracellular ATP facilitates interferon (IFN) type I secretion through p38/JNK/ATF-2 signaling pathway in vesicular stomatitis virus-infected mice (Zhang et al., 2017). Thus, limiting the extracellular levels of ATP in SARS-CoV-2 infection may contribute to the delay in IFN type I, turning off the initial alarm of the purinergic system. SARS-CoV-2 infection impairs the rapid IFN-I pathway activation in mild and severe patients, leading to lower antiviral response and worse outcomes in critically ill COVID-19

patients (Arunachalam et al., 2020). Furthermore, we identified lower levels of AMP and adenosine in COVID-19 patients reporting nausea, higher ADP levels in COVID-19 patients reporting stuffy noses, and diminished levels of ATP, ADP, and AMP in individuals reporting skin rash. Although the biological and clinical significance is not clear, these results suggest that the modulation of extracellular adenine-based purine molecules may be involved in the clinical symptoms of COVID-19. In this sense, the deregulation in purinergic signaling is associated with the hyperinflammatory and coagulation status of COVID-19, as well as causing exacerbations in clinical symptoms. Interestingly, a pilot clinical trial demonstrated that elderly COVID-19 patients taking between 1.2 and 1.6 g of oral ATP daily had improved COVID-19 survival and fewer clinical symptoms compared to the control infected group (Abraham et al., 2021). Thus, the association between adenine-based purine molecules and clinical symptoms may indicate that purinergic signaling could be a target for pharmacologic therapies as previously suggested by hypotheses studies (Berger et al., 2022; Hasan et al., 2022; Simões et al., 2021). Also, the correlation analysis revealed a positive correlation between extracellular ATP levels and the peripheral frequency of low differentiated $CD8^+$ T cells. This result may suggest that decreased extracellular ATP during COVID-19 disease may impact the regulation of total $CD8^+$ T-cell differentiation.

Here we showed that COVID-19 patients present lower adenosine levels in the blood, which may contribute to the uncontrolled inflammation, and severity of the disease. Recent data highlighted the increased inosine levels and upregulated adenosine deaminase activity in the blood of COVID-19 patients, suggesting increased adenosine metabolization during active SARS-CoV-2 infection (Schultz et al., 2022). On the other hand, the hydrolysis of ATP seems to be reduced by PBMC, while platelets showed the highest nucleotide hydrolysis in severe and moderate COVID-19 patients (da Silva et al., 2022). Adenosine binds the P1 receptors, mainly the AR2A subtype, of immune cells (i.e., lymphocytes, monocytes, and macrophages) to induce several events to suppress the inflammatory response, such as the downregulation of the master inflammatory transcription factor NF-κB (Linden & Cekic, 2012; Mandapathil et al., 2010; Sitkovsky et al., 2004). Furthermore, our data revealed higher levels of IL-6, IL-10, and IL-17A plasma concentrations in the severe COVID-19 group, indicating a Th1/Th17 inflammatory polarization. Reinforcing the regulatory effect of adenosine in the inflammation and cytokine storm, the use of inhaled adenosine in COVID-19 patients has successfully decreased the length of hospitalization and improved the prognosis of patients (M et al., 2021; Spiess et al., 2021). We found that in vitro adenosine treatment lowers the NF-κB activation in both T cells and monocytes, suggesting a role for the therapeutic adenosine treatment in the reduction of hyperinflammation in COVID-19. Collectively, our data indicate alterations in adenine-based purinergic molecules, mainly ATP and adenosine levels, during the initial phase of SARS-CoV-2 infection.

Here we also described a negative correlation between plasma adenosine levels and the $CD4^+$ T-cell apoptosis rate. Previous studies have suggested that adenosine and its analog are related to antiapoptotic events through A2A receptor activation in $CD4^+$ T cells (Himer et al., 2010). On the other hand, mitochondrial dysfunction is involved in the induction of apoptosis, thus increasing the depolarization of transmembrane potential (Wang & Youle, 2009). Here, lymphocytes of COVID-19 patients presented a state of mitochondrial membrane depolarization and higher rates of apoptosis. Interesting, mitochondria have a central role in T-cell activation by producing ATP and higher mitochondrial dysfunction may lead to failure in the purinergic regulation and cell homeostasis (Ledderose et al., 2014).

The increased expression of CD39 and CD73 in lymphocytes rapidly converts extracellular ATP to adenosine to induce anti-inflammatory effects (Antonioli et al., 2013). Here we described increased proportions of $CD4^+CD39^+$ T cells and lower frequencies of $CD4^+CD73^+$ and $CD8^+CD73^+$ in the peripheral blood of severe COVID-19 patients. Our results contrast, at least in part, with previous data from da Silva et al. (2022) who found increased expression of both CD39 and CD73 ectonucleotidases in $CD45^+$ leukocytes. In this sense, these results indicate that purinergic signaling may be compromised in several blood immune cells, including monocytes and neutrophils. The higher expression of CD39 together with lower CD73 on T cells may explain the diminished extracellular levels of both ATP and adenosine concomitant to an exacerbated inflammatory state in COVID-19 patients.

Schultz et al. (2022) found increased CD39 expression in leukocytes of COVID-19 patients by using a bioinformatics approach. In COVID-19 patients, higher $CD39^+$ T-cell frequency was previously associated with the expression of PD-1, confirming the hypotheses that SARS-CoV-2 infection induces a hyperactivated exhausted lymphocyte profile (Mathew, Giles, Baxter, Greenplate, et al., 2020), contributing to the T-cell dysfunction in COVID-19. Interestingly, Wang, Veurich, Kalbasi, Graham, et al. (2021) identified increased CD39 expression in the lung, liver, spleen, and PBMCs from severe COVID-19 patients, which correlated with days in the hospital, days in intensive care unit (ICU), and markers of coagulation, indicating associations between ectonucleotidases and disease progression. Similarly, CD39 messenger RNA (mRNA) as upregulated in peripheral blood leukocytes in association with higher soluble CD39 levels in severe COVID-19 patients, which were related to the length of hospital day and ICU admission (Díaz-García et al., 2022). Furthermore, upregulation in CD39 expression in late-stage COVID-19 was associated with heightened levels of STAT-3 and HIF-1α, but decreased levels of CD39-antisense RNA, possibly a purinergic imbalance that contributes to metabolic changes and T-cell dysfunction (Wang, Veurich, Kalbasi, Shaefi, et al., 2021). $CD4^+CD39^+$ T cells identify an effector lymphocyte that is prone to apoptosis in older adults (Fang et al., 2016), corroborating with our data regarding higher apoptosis rate in the $CD4^+$ T cells of COVID-19 patients. On the other hand, lower frequencies of $CD4^+CD73^+$ and $CD8^+CD73^+$ T cells were identified in severe COVID-19 patients. These results are in line with some recent data that demonstrated an association between skewing of T cell receptor repertoire with early $CD4^+$ and

$CD8^+$ activation, and altered expression of several immune checkpoints, such as Tim-3, PD-1, and CD73 (Schultheiß et al., 2020).

Similar to our study, Shahbaz et al. (2021) showed that patients with COVID-19 infection presented higher expression of CD39 but lower CD73 expression in $CD4^+$ T cells, which may contribute to the inflammatory exacerbation. The lack of CD73 expression in T cells may impact lymphocyte homing to draining lymph nodes, as adenosine generated by CD73 regulates lymphocyte migration through $A_{2B}A2$ signaling (Takedachi et al., 2008). The downregulation of CD73 impairs not only the extracellular adenosine generation and anti-inflammatory response but also the modulation of innate immune activation during the viral immune response (Aeffner et al., 2015). In severe COVID-19 patients, $CD8^+$ T cells lacking CD73 expression possess a significantly cytotoxic effector functionality compared with $CD8^+CD73^+$ T cells and correlate with plasma ferritin level, a surrounding clinical marker of uncontrolled systemic inflammation (Ahmadi et al., 2020). Here, the diminished proportions of $CD8^+CD73^+$ T cells were accompanied by low frequencies of low differentiated T cells and higher proportions of $CD8^+CD27^-CD28^-$ T cells in the peripheral blood of COVID-19 patients. Highly differentiated $CD27^-CD28^-$ cytotoxic T cells presents low telomerase activity and decreased Akt phosphorylation, which limits the ability of these cells to be maintained in continuous proliferation after antigen recognition (Plunkett et al., 2007). Although we were unable to determine the memory phenotype of T cells due to methodological reasons, our results indicate that COVID-19 patients display a complex alteration in differentiation status and memory acquisition in adaptive immunity-related to disturbances in purinergic signaling. Moreover, we found increased PD-1 expression in low differentiated $CD8^+CD27^+CD28^+$ T cells of the severe COVID-19 group, indicating an exhaustion profile. Similarly, COVID-19 patients presented $CD8^+$ T cells enriched for expression of CD38, HLA-DR, KI67, CD39, and PD-1, highlighting the co-expression of activation markers with features of inhibitory/exhausted phenotype (Mathew, Giles, Baxter, Oldridge, et al., 2020).

The combining analysis of CD25 and CD39 expression in $CD4^+$ T cells reveals distinct phenotypes: $CD4^+CD25^+CD39^+$ (activated/memory Treg, mTreg) and $CD4^+CD25^-CD39^+$ (memory T effector cell; mTeff; Dorneles et al., 2019; Dwyer et al., 2010). These populations dynamically change during the acute inflammatory response in response to extracellular purine molecules accumulation (Mandapathil et al., 2010). We found an accumulation of mTeff cells in severe COVID-19 patients. $CD39^+$ mTeff cells are nonsuppressive and have a memory phenotype that expresses higher levels of mRNA Th-lineage-specific cytokine profile, mainly Th1 and Th17 subsets (Zhou et al., 2009). In this sense, the accumulation of $CD4^+CD25^-CD39^+$ T cells in the peripheral blood of severe COVID-19 patients may contribute to the skewing toward the Th17 profile. In humans, $CD4^+CD25^-CD39^+$ T cells are $CD45RO^+$, $Foxp3^-$, secrete higher amounts of IFN-γ and IL-17, and are increased following autoantigen recognition (Moncrieffe et al., 2010).

$CD4^+CD25^+CD39^+$ mTreg cells were decreased in mild and severe COVID-19 patients. Furthermore, the proportions of $CD4^+CD25^+$ T cells co-expressing CD39 and CD73 were also reduced in COVID-19 patients. These data indicate a failure in the immunosuppressive axis of adenosine generation by Treg cells during SARS-CoV-2 infection. Contrary to us, an upregulation in ENTPD1 (CD39) gene expression within the Treg pathway in peripheral tissues of severe COVID-19 patients was previously reported, suggesting higher Treg suppression mediated by the CD39 axis may occur in the infected tissues during acute SARS-CoV-2 infection that may contribute to secondary infections by others pathogens (Wang, Veurich, Kalbasi, Shaefi, et al., 2021). On the other hand, Meckiff et al. (2020) described an imbalance of Tregs cells and cytotoxic reactive $CD4^+$ T cells in severe COVID-19. Considering that severe COVID-19 patients presented lower extracellular adenosine levels in the peripheral blood, we postulated that there a distinct Treg-expressing CD39/CD73 ectonucleotidases in the peripheral blood compared with infected tissues during COVID-19. As Treg cells are known to play an important role in limiting the host antiviral response and the consequent tissue immunopathology, the present data shows decreased CD39/CD73 axis in Treg that may be related to the decreased Treg response to SARS-CoV-2 and have a relevant impact on fueling systemic inflammation in COVID-19 patients (Belkaid & Tarbell, 2009).

Considering the COVID-19 pandemic status, efforts to understand the immunopathological role of SARS-CoV-2 infection have been made by several research groups. Here we found significant findings that indicate the alterations in adenine-based purine molecules and T-cell phenotype in COVID-19 and both seem to be correlated. The range of disease severity directly impacts in the alterations of $CD4^+$ and $CD8^+$ T cells expressing CD39/CD73 ectonucleotidases, which suggests that disturbances in purinergic signaling may result in poor clinical outcomes.

5 | CONCLUSION

In summary, we describe for the first time an imbalance in the levels of extracellular adenine-based purine molecules and alterations in CD39/CD73 ectonucleotidases axis in $CD4^+$ and $CD8^+$ T cells of COVID-19 patients with different degrees of disease severity. The reduced adenosine extracellular levels and alterations in T cells phenotype may impact on COVID-19 severity. Collectively, these data add new knowledge regarding the immunopathology of COVID-19 through purinergic regulation.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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