








ORIGINAL ARTICLE

The relationship between peripheral immune response and disease severity in SARS-CoV-2-infected subjects: A cross-sectional study

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Abstract

Coronavirus disease 2019 (COVID-19) is a respiratory infection caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and marked by an intense inflammatory response and immune dysregulation in the most severe cases. In order to better clarify the relationship between peripheral immune system changes and the severity of COVID-19, this study aimed to evaluate the frequencies and absolute numbers of peripheral subsets of neutrophils, monocytes, and dendritic cells (DCs), in addition to quantifying the levels of inflammatory mediators. One hundred fifty-seven COVID-19 patients were stratified into mild, moderate, severe, and critical disease categories. The cellular components and circulating cytokines were assessed by flow cytometry. Nitric oxide (NOx) and myeloperoxidase (MPO) levels were measured by colourimetric tests. COVID-19 patients presented neutrophilia, with signs of emergency myelopoiesis. Alterations in the monocytic component were observed in patients with moderate to critical illness, with an increase in classical monocytes and a reduction in nonclassical monocytes, in addition to a reduction in the expression of HLA-DR in all subtypes of monocytes, indicating immunosuppression. DCs, especially plasmacytoid DCs, also showed a large reduction in moderate to critical patients. COVID-19 patients showed an increase in MPO, interleukin (IL)-12, IL-6, IL-10, and IL-8, accompanied by a reduction in IL-17A and NOx. IL-10 levels ≥ 14 pg/ml were strongly related to the worst outcome, with a sensitivity of 78.3% and a specificity of 79.1%. The results of this study indicate the presence of systemic

effects induced by COVID-19, which appear to be related to the pathophysiology of the disease, highlighting the potential of IL-10 as a possible prognostic biomarker for COVID-19.

KEYWORDS

COVID-19, IL-10, immune system, inflammation, SARS-CoV-2

INTRODUCTION

Coronavirus disease 2019 (COVID-19) is a respiratory infection caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [1], and the first record of the disease was registered in 2019. The virus rapidly spread around the world, triggering a pandemic that caused a collapse of health systems, and already accounts for more than 244 million cases and 4 million deaths worldwide [2]. So far, Brazil is one of the most affected countries. Until the beginning of July 2021, there were about 20.9 million cases and more than 600 000 deaths from the disease in the country [3].

Although most individuals with COVID-19 develop only the mild form of the disease, some patients progress to more severe forms and develop complications such as acute respiratory distress syndrome, thromboembolic events, hyperinflammation and multiple organ failure [4]. In this context, studies have demonstrated a link between host immune response and disease severity [5, 6].

An effective immune response against SARS-CoV-2 requires the involvement of cells from both the innate and the adaptive immune systems. However, in contrast to what is observed in most antiviral responses, patients with COVID-19 have some variations in their immune response, such as a reduction in lymphocyte count and an increase in the neutrophil-to-lymphocyte ratio, which are considered hallmarks of this disease [4, 7–9]. Moreover, there is evidence that COVID-19 is marked by dysregulation in the myeloid cell compartment [9]. Furthermore, critically ill patients exhibit high serum concentrations of pro-inflammatory cytokines such as interleukin (IL)-6, IL-1 β , IL-2, IL-8, IL-17, and tumour necrosis factor- α (TNF- α) [10, 11]. The mononuclear phagocyte system (MPS) plays a central role in this dysregulated immune response and is a major contributor to the hyperinflammatory syndrome [12, 13]. In peripheral blood, the MPS is composed of dendritic cells (DCs) and monocytes, which are efficient antigen-presenting cells (APCs), in addition to their role in the production of cytokines and the regulation of immune responses [14, 15].

Circulating monocytes have both pro-inflammatory and resolution functions and are subdivided into three

types: classical monocytes (cMo, CD14⁺⁺ CD16⁻), intermediate monocytes (iMos, CD14⁺ CD16⁺), and nonclassical monocytes (ncMo, CD14^{-/low} CD16⁺). iMo and ncMo are also called tissue macrophages (TiMas) [16]. In turn, DCs are potent APCs, leading to naive T-cell activation and effector differentiation [17]. In blood, DCs can be divided into two subtypes: classical DCs (cDCs, CD11c⁺⁺ CD123^{dim}) and plasmacytoid DCs (pDCs, CD11c^{dim} CD123⁺⁺), which secrete IFN- α [18].

Although the MPS is essential for host defence, several lines of evidence indicate that this system also plays an important role in the immunopathogenesis of COVID-19, since the hyper-inflammatory response induced by SARS-CoV-2 seems to be a major cause of disease severity and death in infected patients [19, 20]. Thus, this study aimed to evaluate the frequencies and absolute numbers of peripheral subsets of neutrophils, monocytes, and DCs and, in parallel, to evaluate the levels of important inflammatory mediators in the blood of healthy people and subjects at different clinical stages of COVID-19, in order to better clarify their relationship with peripheral immune system changes and the severity of COVID-19.

METHODS

Patient selection and controls

This study included a total of 30 healthy individuals and 157 patients with early and late COVID-19, from August 2020 to June 2021. Hospitalized patients were accommodated at the University Hospital of the Federal University of Santa Catarina and Nereu Ramos Hospital, both in Florianópolis, Brazil. All study participants had a COVID-19 diagnosis confirmed by RT-PCR or an antigen test. Demographic data, age, sex, medical history, symptoms, computed tomography of the chest, and laboratory findings were recorded from each patient. Disease severity was classified as mild, moderate, severe, or critical according to the guidelines released by the World Health Organization (WHO) [21].

Patients with cancer, individuals seropositive for human immunodeficiency virus (HIV), pregnant women,

and people who received vaccines against SARS-CoV-2 were excluded from the study. Exclusion criteria for healthy individuals included a history of neoplasm, infections in the previous seven days, heavy alcohol consumption, diabetes mellitus, autoimmune disease, and chronic inflammatory disease.

Written informed consent was obtained from all participants. The study was approved by the Ethics Committee of the Federal University of Santa Catarina (CEPSH/UFSC protocol no. CAAE 31124820.1.0000.0121 May/2020). Blood from all individuals was processed within 24 h of collection for flow cytometric analysis and plasma separation. Plasma was stored at -80°C for further analysis of cytokine profile, myeloperoxidase activity (MPO), and nitric oxide levels (NOx).

Immunophenotyping by flow cytometry

For flow cytometry immunophenotyping, the monoclonal antibodies CD62L (FITC, LT-TD180), CD4 (PerCP, MEM-241), and CD11c (PerCP Cy5-5, BU15) from Exbio; CD10 (PECy7, HI10A), CD3 (APCH7, SK7), CD16 (FITC, 3G8), CD19 (APCH7, HIB19), CD20 (APCH7, 2H7), CD45 (PacO, HI30) and HLA-DR (PacB, L243) from BD Biosciences; and CD56 (PE, N901 NKH-1), CD14 (APC, RMO52), and CD123 (PE, SSDCLY107D2) from Beckman Coulter were used. Staining was based on an eight-colour panel, as previously described by Cardoso and Santos-Silva (2019) for the identification of monocytes, neutrophils and DCs [22, 23]. The expression of CD56 on monocytes and CD62L on monocytes and neutrophils was determined using different panels, which also had markers for these cell lines (HLA-DR, CD4 and CD45) [23]. More information about the antibodies is presented in Table S1.

For antibody labelling, 300 μl of peripheral blood-EDTA/K3 was used. The staining protocol was lyse-wash. Detailed information on staining procedures and acquisition parameters is described in our previous study [23]. Tubes were acquired from the flow cytometer immediately after preparation, using a three-laser FACSCanto II flow cytometer equipped with FACSDiva version 8 software (BD Biosciences). 500 000 to 1 000 000 gated CD45+ events were recorded for each individual. All antibodies used were previously titrated. Cut-off points and interferences between fluorochromes were evaluated by fluorescence-minus-one (FMO) controls and internal negative and positive controls. An automatic standard compensation was applied to each acquisition. The performance of the flow cytometer

was checked daily using calibration beads (BD™ CS&T beads; BD Biosciences). The gating strategy was performed as proposed by Cardoso and Santos-Silva and other studies in the literature [23–26]. Data analysis was performed by two different operators using Infinicyt 2.0 analytics software (Cytognos, Spain).

HLA-DR, CD56, and CD62L expression

To analyse the HLA-DR expression on monocyte subtypes, the mean fluorescence intensity (MFI) of the HLA-DR marker for cMo, iMo, ncMo and TiMas (iMo and ncMo together) subtypes were evaluated. Then, the MFI values were compared between groups, and the iMo/cMo, ncMo/cM and TiMas/cMo HLA-DR ratios were calculated, in order to reduce the variability associated with antibody lots, staining and daily equipment conditions. The CD56 expression on monocytes and the CD62L expression on monocytes and neutrophils were evaluated by the percentage of cells that express these markers on their surface [23].

Quantification of cytokine plasma levels

Quantification of plasma levels of IL-12p70, IL-10, IL-6, IL-1 β , IL-17A, IL-4, IL-2, TNF- α , and interferon- γ (IFN- γ) was performed by the Human Inflammatory Cytokine Cytometric Bead Array (CBA) kit, and the BD™ Human Th1/Th2/Th17 kit (BD Biosciences) according to the manufacturer's instructions. Measurements were realized in a BD FACSVerser™ flow cytometer (BD Biosciences), and analysis was performed using FCAP Array™ software (BD Biosciences).

Quantification of MPO activity

Myeloperoxidase activity was determined according to the methodology described by Rao et al., after incubation of the samples with reagent solution (0.167 mg/ml of *o*-dianisidine, 2HCl, and 0.0005% of H₂O₂) and spectrophotometric reading [27]. The enzymatic activity was determined by interpolation from a standard MPO curve (0.7–140 mU/ml) by colourimetric measurements (450 nm) in an ELISA plate reader (HEALES).

Dosage of NOx metabolite levels

Nitric oxide plasma levels were quantified according to the Griess colourimetric reaction [28] by the formation

of its metabolites nitrite (NO_2^-) and nitrate (NO_3^-). Levels of nitrite were determined by interpolation from a standard sodium nitrite curve (0–150 μM) by colourimetric measurements (540 nm) in an ELISA plate reader (HEALES).

Statistical analysis

The normality of the data distribution was assessed using the Shapiro–Wilk test. Continuous variables were compared using analysis of variance with a post hoc Bonferroni test in the case of a parametric distribution and were expressed as mean \pm standard deviation (SD). Non-parametric variables were compared using the Kruskal–Wallis test with a post hoc Dunn–Bonferroni test and expressed as median (range). Categorical variables were evaluated using a chi-square test. Variables were compared with the outcome (death/survival) using the Mann–Whitney U test, and significant results were subjected to multivariate logistic regression. Sensitivity, specificity and cut-off of the variables in relation to the outcome were evaluated using the receiver operating characteristic (ROC) curve. The survival curve with established cut-offs was assessed using the Kaplan–Meier curve with a log-rank test. A P -value ≤ 0.05 was considered significant. Statistical analysis was performed using MedCalc version 20.015 (Belgium).

RESULTS

Patient characteristics

In this study, 187 subjects were included: 30 healthy controls and 157 patients diagnosed with COVID-19, who were classified according to the clinical presentation of the disease as mild ($n = 36$), moderate ($n = 30$), severe ($n = 32$), and critical ($n = 59$). The mean age of the control group, with male predominance (66%), was 50.9 ± 10.2 years. The clinical and demographic characteristics of patients with COVID-19 are described in Table 1. The most-reported comorbidities among patients were arterial hypertension (47.1%, $P < 0.001$) and diabetes mellitus (28.7%, $P = 0.003$), observed mainly in patients with higher-risk clinical conditions. Blood counts were performed for all patients in the study, as well as additional tests for patients who required hospitalization. Laboratory findings of COVID-19 patients are shown in Table 2 and Table S2. As expected, a gradual increase in the levels of c-reactive protein ($P = 0.008$) and lactate dehydrogenase ($P = 0.033$) was observed according to the severity of the disease. Regarding D-dimer, results above the reference value (500 ng/ml) were observed in all groups. However, no difference was found in the D-dimer value when the moderate to critical groups were compared. An increase in white blood cell count ($P < 0.001$), accompanied by neutrophilia ($P = 0.012$)

TABLE 1 Characteristics of the COVID-19 patients

Variable	All ($n = 157$)	Mild ($n = 36$)	Moderate ($n = 30$)	Severe ($n = 32$)	Critical ($n = 59$)	P
Age (years), median (range)	55.0 (22.0–98.0)	37.50 (22.0–71.0) a	52.0 (32.0–74.0) b	56.5 (25.0–93.0) b	60.0 (39.0–98.0) b	<0.001
Sex						
Women, n (%)	72 (45.9%)	22 (61.1%)	9 (30.0%)	15 (46.9%)	26 (44.1%)	0.090
Men, n (%)	85 (54.1%)	14 (38.9%)	21 (70.0%)	17 (53.1%)	33 (55.9%)	
Comorbidities						
Arterial hypertension, n (%)	74 (47.1%)	2 (5.6%)	12 (40.0%)	21 (65.6%)	39 (66.1%)	<0.001
Diabetes mellitus, n (%)	45 (28.7%)	3 (8.3%)	6 (20.0%)	12 (37.5%)	24 (40.7%)	0.003
Obesity (BMI ≥ 30 kg/m ²), n (%)	56 (35.7%)	4 (11.1%)	8 (26.7%)	8 (25.0%)	36 (22.9%)	0.290
Cardiopathy, n (%)	12 (7.6%)	3 (8.3%)	2 (6.7%)	3 (9.4%)	4 (6.8%)	0.967
Autoimmune disease, n (%)	6 (3.8%)	1 (2.8%)	0	1 (3.1%)	4 (6.8%)	0.430
Kidney disease, n (%)	5 (3.2%)	0	1 (3.3%)	1 (3.1%)	3 (5.1%)	0.598
Lung disease, n (%)	16 (10.2%)	1 (2.8%)	1 (3.3%)	5 (15.6%)	9 (15.3%)	0.094
Other, ^a n (%)	16 (10.2%)	0	8 (26.7%)	3 (9.4%)	5 (8.5%)	0.004

Note: $P \leq 0.05$ was considered significant.

Abbreviation: BMI, body mass index.

^aOther comorbidities included liver disease, stroke, and acute myocardial infarction.

TABLE 2 Laboratory findings of COVID-19 patients

Variable	n	All	n	Mild	n	Moderate	n	Severe	n	Critical	P
Urea (mg/dl), median (range)	116	40.0 (14.0–391.0)	-	-	26	38.5 (22.0–92.0)	32	35.5 (18.0–127.0)	58	44.0 (15.0–391.0)	0.109
Creatinine (mg/dl), median (range)	117	0.94 (0.34–2.76)	-	-	27	1.01 (0.60–2.14)	32	0.90 (0.51–1.95)	58	0.90 (0.34–2.76)	0.322
D-dimer (ng/ml), median (range)	93	1073.0 (190.0–35 000.0)	-	-	18	1031.5 (210.0–35 000.0)	24	794.0 (190.0–4163.0)	51	1311.0 (313.0–35 000.0)	0.113
CRP (mg/L), median (range)	117	87.30 (0.86–352.10)	-	-	27	51.00 (4.20–302.60)	32	94.25 (1.70–271.40) a, b	58	95.20 (0.86–352.10)	0.008
LDH (U/L), median (range)	67	340 (151–645)	-	-	14	273 (151–506)	19	303 (162–645)	34	383 (177–612)	0.033
RBC million/mm ³ , median (range)	157	4.37 (2.44–7.85)	36	4.52 (3.87–5.79)	30	4.48 (2.82–7.85)	32	4.54 (3.19–5.55)	59	4.12 (2.44–5.90)	0.002
Hemoglobin (g/dl), median (range)	157	12.80 (6.90–21.70)	36	13.65 11.40–16.80)	30	13.35 (9.10–21.70)	32	13.50 (10.00–16.40)	59	12.10 (6.90–15.90)	<0.001
Hematocrit (%), median (range)	157	39.10 (21.40–67.10)	36	40.75 (34.10–53.70)	30	39.15 (25.30–67.10) a, b	32	40.55 (31.10–49.10) a, b	59	36.70 (21.40–49.30)	0.002
Platelets/mm ³ , median (range)	157	261 000 (20 000–740 000)	36	260 500 (20 000–459 000)	30	249 500 (91 000–604 000)	32	259 500 (72 000–547 000)	59	267 000 (107 000–740 000)	0.584
Leukocytes/mm ³	157	8130 (1046–21 070)	36	7195 (3690–10 840)	30	7870 (2600–15 250)	32	8660 (1046–15 240) a, b	59	10 830 (1560–21 070) b	<0.001
Lymphocytes/mm ³ , median (range)	157	1150 (300–7000)	36	2395 (990–3870)	30	1150 (380–3430)	32	940 (300–7000)	59	900 (330–6000)	<0.001
Neutrophils/mm ³ , median (range)	157	6180 (1110–17 880)	36	7015 (1310–16 585)	30	7450 (2830–13 110)	32	6355 (1110–17 880) a, b	59	5040 (1740–11 660)	0.012
Monocytes/mm ³ , median (range)	157	480 (120–1739)	36	480 (150–1739) a, b	30	400 (160–1630)	32	590 (120–1690)	59	450 (220–900)	0.036
Eosinophils/mm ³ , median (range)	157	20 (0–947)	36	5 (0–947)	30	5 (0–220)	32	35 (0–310)	59	40 (0–760)	0.040
Basophils/mm ³ , median (range)	157	20 (0–103)	36	20 (0–103)	30	10 (0–80)	32	20 (0–70)	59	20 (0–60)	0.062

Note: $P \leq 0.05$ was considered significant. Differences were tested using the Kruskal–Wallis test with a post hoc Dunn–Bonferroni test. $P \leq 0.05$ was considered significant. Different letters indicate values that are significantly different from each other.

Abbreviations: CRP, c-reactive protein; LDH, lactate dehydrogenase; RBC, red blood cells.

and lymphopenia ($P < 0.001$), was observed, especially in the most severe cases. This leads to an increase in the neutrophil-to-lymphocyte ratio, which has previously been reported as a worse prognostic factor in patients with COVID-19 [29].

COVID-19 causes an increase in peripheral blood neutrophils with increased expression of CD62L

In Figure 1a, it is possible to observe a significant increase in mature neutrophils in patients with a clinical condition from moderate to critical ($P < 0.001$) when compared with the control group, especially in patients with severe and critical conditions, in whom the percentage of mature neutrophils exceeds 80% of total leukocytes (Table S3; Figure 1a,c). Similar results were observed in the absolute and relative counts of immature neutrophils ($P < 0.001$; Figure 1b,d). Patients with mild COVID-19 showed results similar to those of the control group. Neutrophil expression of the adhesion molecule CD62L was also assessed (Figure 1e,f). Thus, it was possible to observe that in groups with a clinical presentation from moderate to

critical, in addition to an increase in mature and immature neutrophils, more cells also began to express CD62L ($P < 0.001$).

Monocyte subsets are altered in COVID-19 patients compared with healthy controls

Patients with COVID-19 presented no significant difference in absolute monocyte counts when compared with healthy controls (Figure 2a). Regarding the percentage of leukocytes (Figure 2b), a reduction in monocytes was observed in severe patients compared with the control group ($P = 0.002$). When the groups of patients with COVID-19 were compared, an increase in the percentage of monocytes in mild patients was found, which caused a significant difference when compared with severe ($P < 0.001$) and critical ($P < 0.001$) patients. Regarding monocyte subtypes (cMo, iMo and ncMo), significant differences were observed within the monocytic compartment. As expected, the healthy control group had cMo as the main subtype, followed by ncMo and iMo (Figure 2c–e). However, COVID-19 patients had a higher frequency of iMo than ncMo. Although a

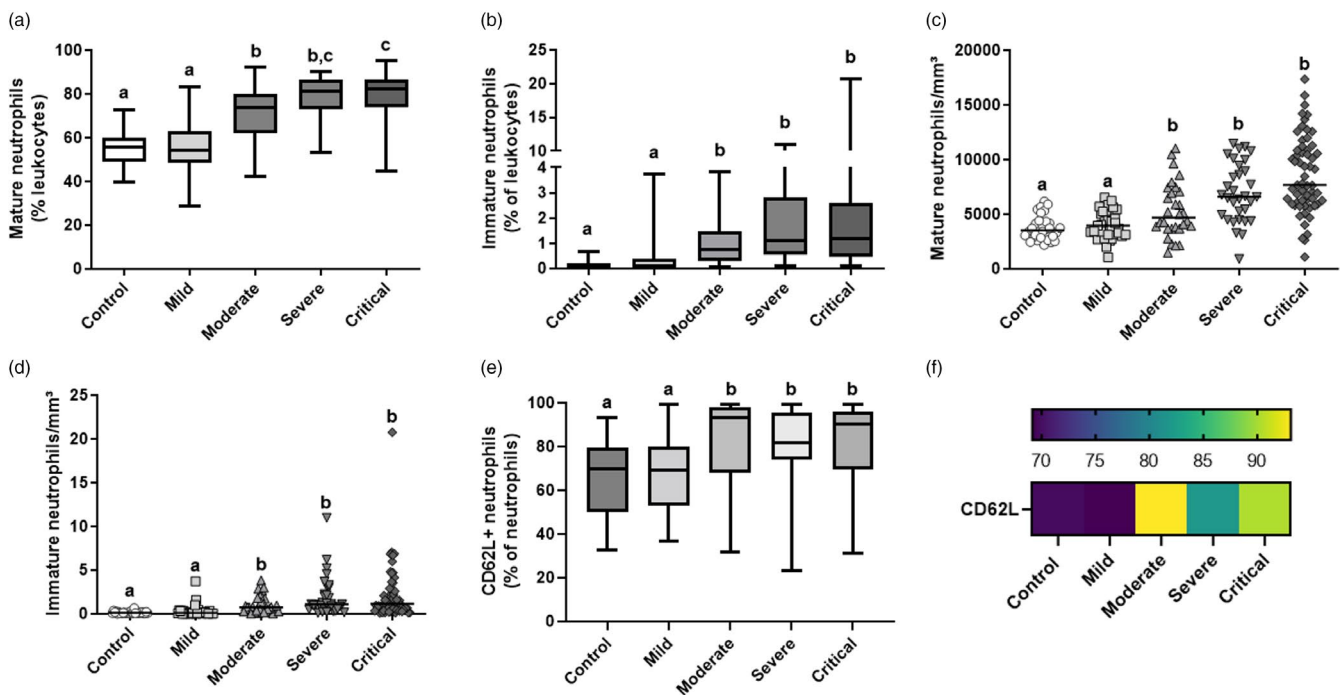


FIGURE 1 Absolute and relative numbers of neutrophils and CD62L expression on neutrophils in COVID-19 patients and controls. (a–e) Absolute and relative numbers of mature and immature neutrophils, obtained by manual gating. (e, f) CD62L expression on neutrophils of healthy controls and COVID-19 patients. Values are presented as a percentage of CD62L+ neutrophils. The colours in the heatmap (f) represent the median of the percentage of CD62L expression on neutrophils, varying from dark blue for lower expression to yellow for higher expression. Different letters indicate values that are significantly different from each other ($P \leq 0.05$). Boxplot representation (centreline: median; box limits: upper and lower quartiles; whiskers: range). Differences were tested using the Kruskal–Wallis test with a post hoc Dunn–Bonferroni test

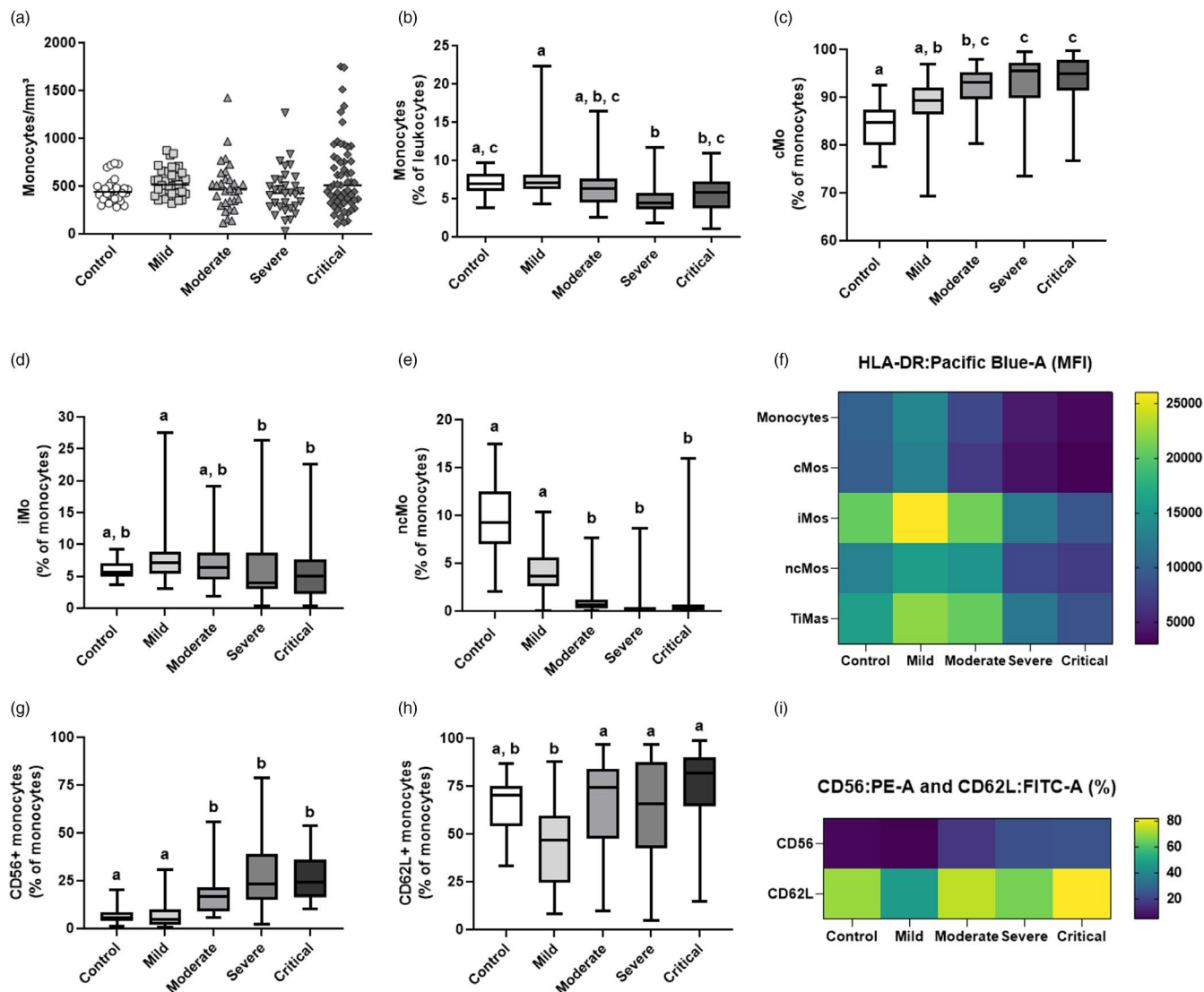


FIGURE 2 Changes in the monocytic compartment in the peripheral blood of COVID-19 patients. (a–e) Absolute and relative numbers of monocytes and their subtypes (cMo, iMo, ncMo), obtained by manual gating. (f) Monocytes' HLA-DR expression presented as MFI. (g–i) CD56 and CD62L expression in monocytes. Values are presented as a percentage of CD56+ and CD62L+ monocytes. The colours in the heatmap (f, i) represent the median of the intensity of HLA-DR and the percentage of CD62L expression on monocytes, varying from dark blue for lower expression to yellow for higher expression. Different letters indicate values that are significantly different from each other ($P \leq 0.05$). Boxplot representation (centreline: median; box limits: upper and lower quartiles; whiskers: range). Differences were tested using the Kruskal–Wallis test with a post hoc Dunn–Bonferroni test. cMo, classical monocytes; iMo, intermediate monocytes; MFI, mean fluorescence intensity; ncMo, nonclassical monocytes

significant increase in cMo was observed, especially in moderate to critical cases ($P \leq 0.001$; Figure 2c), there was also a large reduction in the amount of ncMo, up to 30-fold in critical patients when compared with healthy controls ($P < 0.001$; Figure 2e). iMo frequencies did not differ from the frequencies in the control group (Figure 2d). Regarding the COVID-19 groups, patients with mild disease presented higher percentages of iMo than patients with severe ($P = 0.046$) or critical ($P = 0.026$) disease. Complete information regarding the absolute and relative values of monocytes and their subtypes is given in Table S3.

Within the monocytic compartment, surface expression of HLA-DR was also evaluated (Figure 2f, Table 3). Among the groups of patients with COVID-19, in patients with a mild clinical status, high values of HLA-DR expression by monocytes were observed; these values decreased in moderate patients ($P = 0.030$) and reduced even more significantly in critical patients ($P < 0.001$; Figure 2f). Compared with controls, severely and critically ill patients showed a significant reduction in HLA-DR expression on monocytes ($P < 0.001$). Regarding monocyte subtypes, similar results were observed, with an increase in HLA-DR expression on cMo,

TABLE 3 Surface expression of HLA-DR on monocytes

Variable	Control	Mild
HLA-DR Monocytes (MFI)	10 137.13 (3099.94–27 788.00) a, b	13 430.71 (3296.89–61 158.30) a
HLA-DR cMo (MFI)	9852.00 (2701.23–18 734.22) a, b	12 843.70 (3073.18–54 038.00) a
HLA-DR iMo (MFI)	20 547.49 (4095.82–42 651.44) a, b	26 011.35 (4722.27–128 723.13) a
HLA-DR ncMo (MFI)	13 054.77 (5597.31–29 060.58) a	15 649.56 (3521.43–91 910.08) a
HLA-DR TiMas (MFI)	15 824.14 (6289.47–29 529.78) a, b	22 128.84 (4363.37–109 464.50) a
HLA-DR iMo/cMo (ratio)	2.12 (0.71–3.76) a, b	2.00 (1.13–4.55) a
HLA-DR ncMo/cMo (ratio)	1.44 (0.91–2.53)	1.29 (0.69–3.73)
HLA-DR TiMas/cMo (ratio)	1.67 (1.07–2.78) a	1.75 (1.07–4.35) a

Note: Variables are presented as median and range. Differences were tested using the Kruskal–Wallis test with a post hoc Dunn–Bonferroni test. $P \leq 0.05$ was considered significant. Different letters indicate values that are significantly different from each other.

Abbreviations: cMo, classical monocytes; iMo, intermediate monocytes; MFI, MFI, mean fluorescence intensity; ncMo, nonclassical monocytes; TiMas, tissular macrophages (iMo + ncMo).

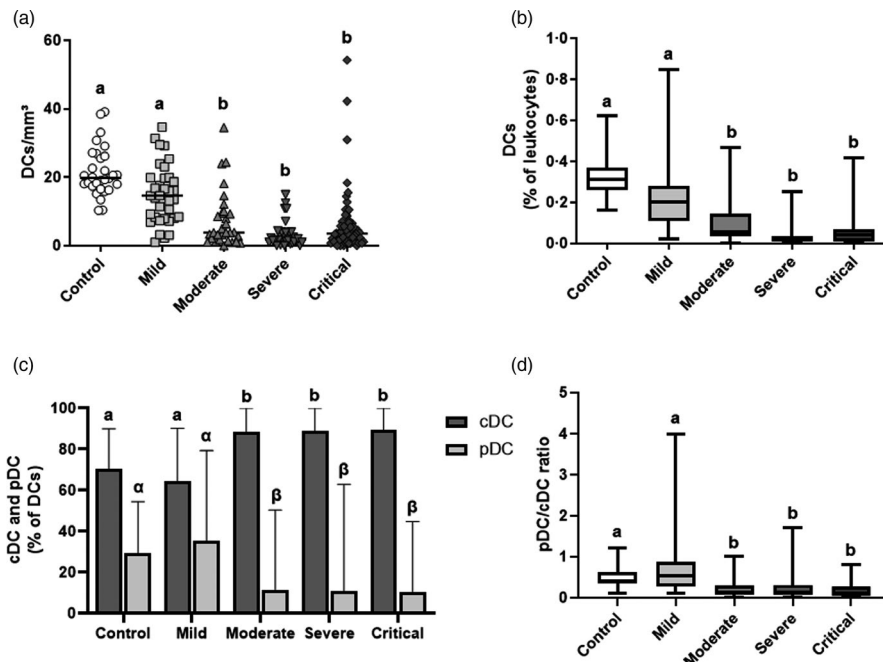


FIGURE 3 Changes in the DC compartment in the peripheral blood of COVID-19 patients. (a, b) Absolute and relative numbers of DCs, obtained by manual gating. (c, d) DC subsets in COVID-19 patients. Boxplot representation (centreline: median; box limits: upper and lower quartiles; whiskers: range). Different letters indicate values that are significantly different from each other ($P \leq 0.05$). Differences were tested using the Kruskal–Wallis test with a post hoc Dunn–Bonferroni test. cDC, classical dendritic cell; DC, dendritic cell; pDC, plasmacytoid dendritic cell

iMo and ncMo of mild patients when compared with severe and critical patients, who showed a significant reduction in the expression of this protein ($P < 0.001$). This reduction occurs mainly in cMo, leading to an increase in the HLA-DR TiMas/cMo ratio in moderate to critical patients ($P < 0.001$).

Monocyte expression of the adhesion molecules CD56 and CD62L was also assessed (Figure 2g–i). In patients with moderate to critical COVID-19, an increase in CD56 expression was observed when compared with control and mild patients ($P < 0.001$). Mild patients showed no difference in the expression of CD56 on monocytes when compared with controls. Regarding CD62L expression on monocytes, mild patients showed a reduction in this adhesion molecule when compared with the moderate to critical groups ($P < 0.001$). Nevertheless, no difference

was observed between COVID-19 patients and the control group.

COVID-19 leads to changes in the DC compartment

Patients with moderate to critical COVID-19 presented a reduction in the absolute and relative values of DCs when compared with the control group and mild patients ($P < 0.001$; Figure 3). Then, DCs were subclassified into cDC and pDC, and their relationship with disease severity was evaluated. Interestingly, when considering only the DC compartment, an increase in the percentage of cDC was observed in patients with moderate to critical COVID-19 compared with the control group and mild

Moderate	Severe	Critical	P
7834·26 (2566·30–21 453·32) b, c	4624·53 (1205·99–16 104·05) c, d	3448·52 (800·93–12 059·95) d	<0·001
6727·81 (2406·28–20 213·15) b, c	4017·48 (1028·70–13 844·52) c, d	3017·52 (776·03–10 545·47) d	<0·001
20 972·72 (5025·08–41 716·97) a, b	12 319·16 (3096·65–55 557·77) b, c	9131·65 (1190·56–43 963·02) c	<0·001
14 847·65 (3697·07–40 548·87) a	7980·51 (1424·24–29 335·06) b	7016·01 (7016·01–28 984·43) b	<0·001
20 583·81 (5025·08–39 684·46) a, b	12 041·37 (2937·77–54 376·65) b	8890·53 (1190·56–41 129·34) c	<0·001
3·02 (0·81–7·99) c	3·30 (1·33–12·05) c	3·28 (0·92–12·34) b, c	<0·001
1·92 (0·00–5·21)	1·37 (0·00–7·09)	1·71 (0·00–9·24)	0·409
2·88 (0·82–7·55) b	3·15 (1·22–12·05) b	3·11 (0·91–11·90) b	<0·001

patients ($P < 0.001$). Thus, a decrease in the amount of pDC within the DC compartment was also observed, leading to a significant reduction in the pDC/cDC ratio of patients with moderate to critical condition, when compared with the control group and patients with a milder form of the disease ($P < 0.001$).

MPO, NO_x, and cytokine measurements in COVID-19 patients

Plasma levels of cytokines, NO_x and MPO in patients infected with SARS-CoV-2 and in healthy controls were also evaluated. As expected, patients with COVID-19 had an increase in MPO levels compared with healthy controls and patients with moderate to critical condition ($P < 0.001$; Figure 4a). When groups of patients with COVID-19 were compared, those in a critical condition differed only from mild patients ($P < 0.001$). Regarding NO_x levels, although all COVID-19 patients presented a reduction in NO_x levels when compared with the control group ($P < 0.001$ Figure 4b), no difference was observed between the infected groups.

Plasma levels of cytokines also showed significant changes (Figure 4c–j). The assessment of IL-12p70 levels showed an increase in this cytokine when SARS-CoV-2-infected patients were compared with the control group ($P < 0.001$), but there was no difference associated with disease severity. Regarding the levels of IL-10 and IL-6, there was no difference between patients with mild COVID-19 and controls, but a significant increase was observed in patients with moderate to critical disease ($P < 0.001$). IL-8 also showed an increase in patients with COVID-19, but only patients in the critical group differed significantly from the controls ($P < 0.001$). Although many cytokines had increased levels, IL-17A levels were shown

to be reduced in COVID-19 patients, especially in patients in the severe and critical groups, when compared with the control group ($P < 0.001$). IFN- γ levels were variable in relation to the groups and were shown to be increased in patients with moderate and critical COVID-19, when compared with the control group ($P < 0.002$). Finally, regarding IL-4 and IL-2 plasma levels, mild patients showed increased levels of these interleukins when compared with controls ($P < 0.001$), but the other groups did not show any significant difference when compared with the control group. TNF- α and IL-1 β levels did not show significant changes. Complete information regarding MPO, NO_x, and cytokine levels is given in Table S4.

Association between alterations in the immune system and mortality

After the alterations in the peripheral immune system of COVID-19 patients were evaluated, the relationship of these alterations with the outcome was tested. For this, all parameters were grouped and compared according to the outcome (death or survival) using the Mann–Whitney test. Twenty-three patients (14.5%) died within the first 30 days, and several parameters were significant when compared with outcome: NO_x (μ M), monocyte CD62L+ (% of monocytes), monocyte CD56+ (% of monocytes), monocytes (% of leukocytes), cMo (% of leukocytes), HLA-DR expression in monocytes, cMo, iMo, ncMo, DC (% of leukocytes), DC/mm³, mDC (% of leukocytes), mDC (% of DCs), mDC/mm³, pDC (% of leukocytes), pDC (% of DCs), pDC/mDC ratio, IL-10 (pg/ml), IL-6 (pg/ml) and IL-8 (pg/ml) (Table S5). Then, these parameters were subjected to multivariate logistic regression. After these analyses, only IL-10 was related to the outcome ($P = 0.014$; odds ratio = 1.13, CI95%

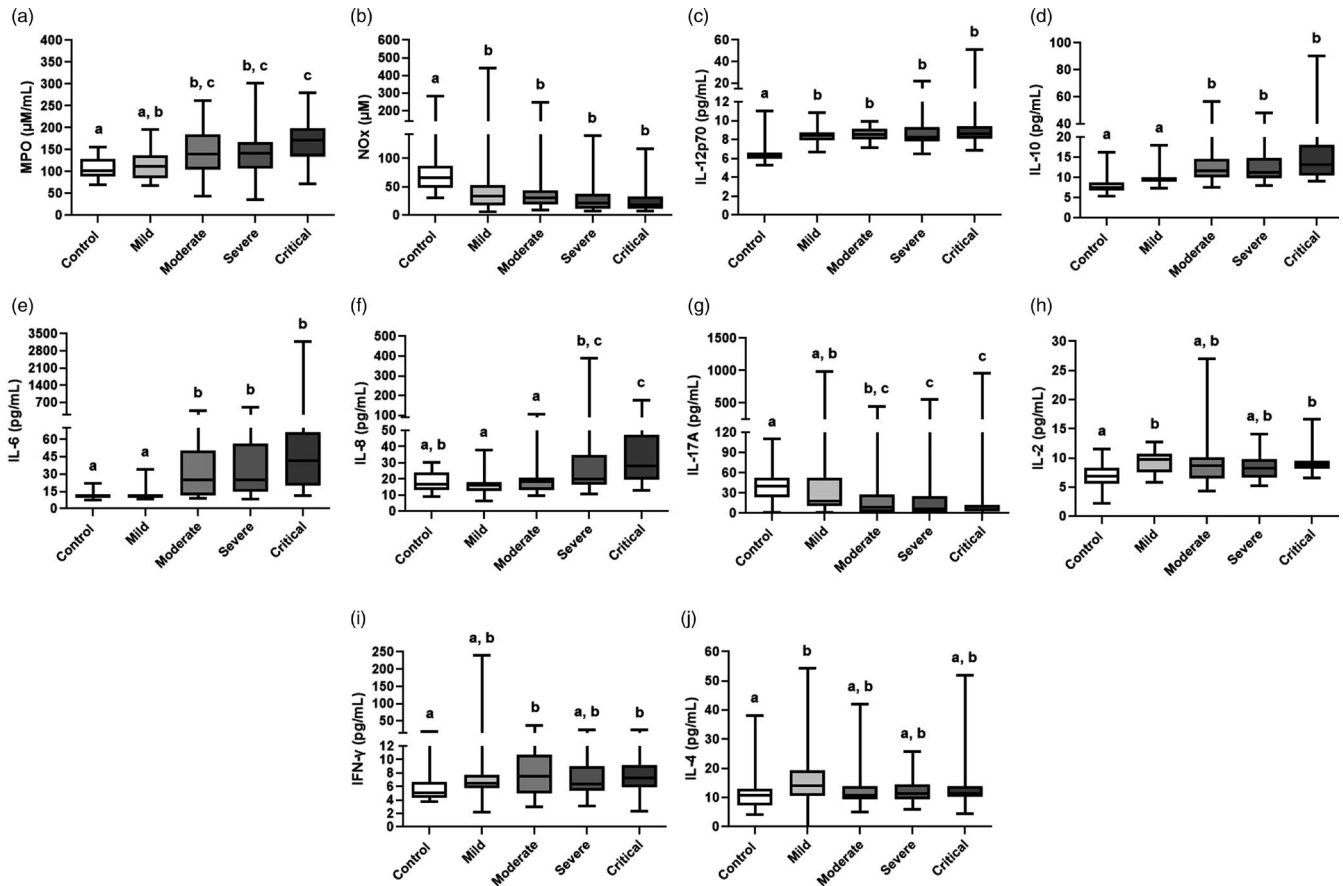


FIGURE 4 Changes in MPO, NOx, and cytokine levels in the peripheral blood of COVID-19 patients. Plasma levels of MPO (a), NOx (b), IL-12p70 (c), IL-10 (d), IL-6 (e), IL-8 (f), IL-17A (g), IL-2 (h), IFN- γ (i) and IL-4 (j) in healthy controls and mild, moderate, severe and critical COVID-19 patients. Boxplot representation (centreline: median; box limits: upper and lower quartiles; whiskers: range). Different letters indicate values that are significantly different from each other ($P \leq 0.05$). Differences were tested using the Kruskal–Wallis test with a post hoc Dunn–Bonferroni test (non-parametric variables) and ANOVA with a post hoc Bonferroni test (parametric variables). ANOVA, analysis of variance; IL, interleukin; IFN, interferon MPO, myeloperoxidase; NOx, nitric oxide

=1.02–1.25). Thus, to assess the sensitivity and specificity of IL-10 as a prognostic biomarker, a ROC curve (AUC = 0.839; $P < 0.001$) was performed. The established cut-off for IL-10 was ≥ 14 pg/ml, with a sensitivity of 78.3% and a specificity of 79.1% (Figure 5a). Figure 5b shows the Kaplan–Meier curve for 30-day mortality according to IL-10 levels at a cutoff of ≥ 14 pg/ml. The Kaplan–Meier survival probability was $95.5 \pm 2.0\%$ in subjects with IL-10 levels < 14 pg/ml and $61.7 \pm 7.1\%$ in those with values ≥ 14 pg/ml ($P < 0.001$).

DISCUSSION

Since the beginning of 2020, COVID-19 has become a research topic in laboratories around the world because of the serious consequences that this highly transmissible and occasionally lethal disease has for the world population. The first studies published at the start of the pandemic demonstrated that COVID-19 behaved

differently from other viral infections, with a high number of neutrophils and a reduction in lymphocyte counts [4, 30–32]. Because of this, the immune response has been related to the immunopathogenesis of the disease [4, 7–13].

In this regard, neutrophils have been highlighted as the essential effector cells in the development of COVID-19 [29, 33–35]. In our cross-sectional study, 157 patients with varying degrees of COVID-19 severity and 30 healthy controls were evaluated. Regarding the population of neutrophils in peripheral blood (PB), an increase in the count of these cells was observed in patients infected with SARS-CoV-2, particularly in patients with moderate to critical conditions. Furthermore, in these patients, an increase in immature neutrophils was observed, indicating emergency myelopoiesis.

Granulocytes are the most abundant cells in PB and are among the first cells to be recruited to fight infections, mediating both the innate and the adaptive immune response [36, 37]. Like our study, other studies also reported an

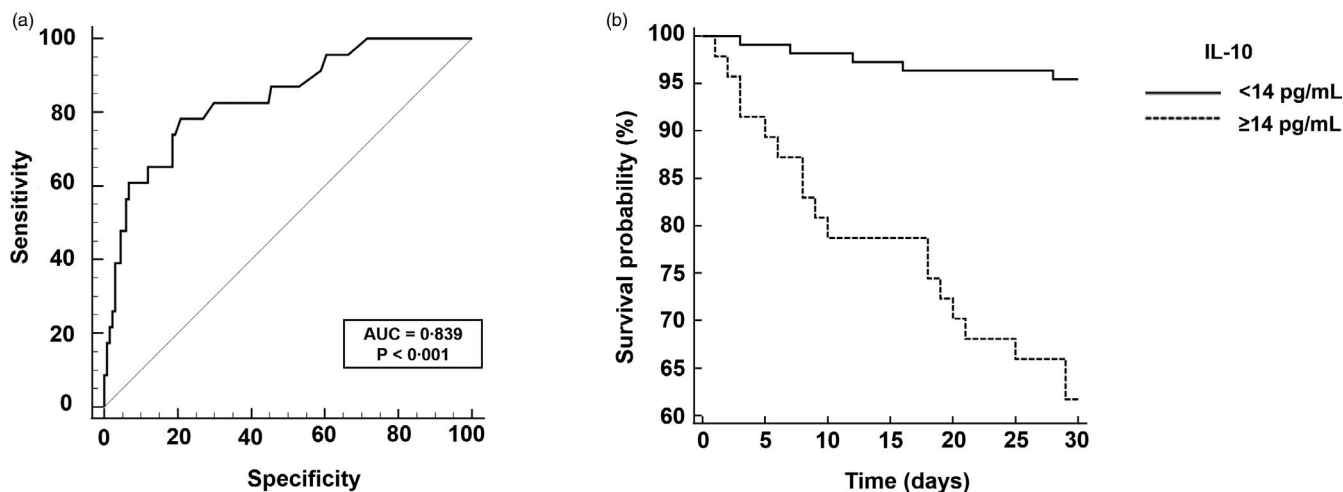


FIGURE 5 ROC curve and cumulative 30-day survival of COVID-19 patients. (a) The established cut-off for IL-10 was ≥ 14 pg/ml, with a sensitivity of 78.3% and a specificity of 79.1%. (b) The Kaplan–Meier survival probability was $95.5 \pm 2.0\%$ in subjects with IL-10 levels < 14 pg/ml and $61.7 \pm 7.1\%$ in those with values ≥ 14 pg/ml ($P < 0.001$). Differences were tested using a log-rank test. ROC, receiver operating characteristic

increase in neutrophil counts in patients with COVID-19 and emergency myelopoiesis with a release of immature neutrophils [9, 38]. Furthermore, there is evidence that these cells are more activated in the most severe cases and contribute to immunothrombosis by the formation of neutrophil extracellular traps (NETs) [33–40]. All these features are regarded as worse prognostic factors in patients with COVID-19 [8, 29, 41].

The results of this study also highlighted the increased expression of CD62L in neutrophils from patients with moderate to critical conditions. CD62L (L-selectin) is an adhesion molecule expressed in several leukocytes, and it is involved in the attachment of leukocytes to the endothelium and the “rolling” of granulocytes, facilitating migration to inflammatory sites [42, 43]. It is supposed that L-selectin is also involved in the amplification of the inflammatory process, by allowing adherent neutrophils to recruit additional neutrophils [44–46]. A study conducted by Schulte-Schrepping et al. evaluated the immunophenotype of neutrophils in the PB of patients with COVID-19 and reported an increase in neutrophils with a suppressor profile (elevated PD-L1 surface expression and CD62L downregulation) in patients infected with SARS-CoV-2 [9]. However, in our study, in patients with advanced disease progression, an increase in neutrophils with a predominantly mature profile and increased expression of CD62L was observed, suggesting that these are cells with migratory stimulation to inflammatory sites, such as the lungs, which have a high increase in neutrophil count in patients with COVID-19, in agreement with the disease progression and related tissue damage [40, 47].

In addition to an increase in PB neutrophils, this study also found an increase in MPO levels in patients with

moderate to critical COVID-19. When exposed to infectious agents, neutrophils are able to release (intra- or extracellularly) MPO from azurophil granules [48, 49]. Once released, MPO uses H_2O_2 to produce HOCl, which has a high viricidal capacity and competes with O_2 at heme-binding sites, decreasing O_2 saturation. In addition, MPO contributes to the formation of several other reactive oxygen species (ROS) and consumes NO for the formation of radical peroxynitrite ($ONOO\bullet$), contributing to the respiratory burst and increased inflammation [50, 51]. In this study, together with an increase in MPO, patients with COVID-19 presented a reduction in NOx, which was more profound in critically ill patients. One hypothesis for the reduction in NO levels in patients with COVID-19 is NO consumption for the generation of ROS, which, once produced, also reduce NO levels [52, 53]. Another hypothesis is the reduction in ACE2 expression after cells are infected by SARS-CoV-2, which reduces the production of NO via the renin–angiotensin system [54, 55]. The reduction in NO favours thrombotic events, due to its importance as a vasodilator and anticoagulant [49, 53, 56]. Other studies have also found a reduction in plasma NO in COVID-19 patients, but with an increase in NO in critically ill patients and with reduced activity of the MPO enzyme [57, 58].

While granulocytes make the first contact with pathogens, APCs make the necessary link between the innate and the adaptive immune response, presenting antigens to effector and antibody-producing cells [17]. Monocytes and DCs are known as professional APCs and play an essential role in an effective response to an infectious agent [59]. In our study, the frequencies of monocytes and their subtypes, cMo, iMo and ncMo, were evaluated. Although the

absolute values of monocytes did not differ between the groups evaluated, relevant alterations were observed in the monocytic compartment, which showed an increase in cMo and a reduction in ncMo, particularly in severe and critical patients.

Of all monocyte subsets, cMo are the most abundant in the circulation and actively participate in the response against pathogens, with a pro-inflammatory role and a high capacity for transendothelial migration, where they can differentiate into macrophages and monoDCs, limiting inflammatory damage and initiating tissue repair. The ncMo, in turn, are specialized in complement and FcR-mediated phagocytosis, transendothelial migration, and anti-viral responses. However, in general, ncMo have less inflammatory functions, such as the removal of cell debris and endothelial repair during homeostasis [60, 61]. Some studies assessed the monocytic compartment in the PB of patients with COVID-19 and reported an increase in cMo and a reduction in ncMo, considered to be key determinants of severe COVID-19 [62–64]. In addition, these studies suggested that cMo show signs of activation in the circulation, becoming the major source of inflammatory cytokines within PBMCs in patients with COVID-19 [62, 63, 65, 66]. However, a transcriptomic study carried out in PB reported that the major source of cytokine production does not seem to come from cells present in the circulation, due to the low expression of these genes in PB, but rather from monocytes and macrophages migrating to the lungs and epithelial cells [67].

One of the reasons that monocytes are considered professional APCs is the expression of MHC-II on their surface [59]. Thus, in addition to evaluating the frequency of monocytes and their subtypes in COVID-19 patients, our study also evaluated the expression of HLA-DR (MHC-II) on the surface of these cells. Interestingly, it was observed that patients with moderate to critical COVID-19 showed a significant decrease in the expression of HLA-DR, indicating immunosuppression, particularly in severe and critical patients. This reduction occurred mainly in cMo, leading to a significant increase in the TiMas/cMo ratio. Thus, despite being increased in quantity, cMo is not performing one of their primary functions, which is the presentation of antigens to helper T cells [59]. Monocytes are extremely plastic cells and can exert pro- and anti-inflammatory activities. The result of these two forces is reflected in the level of HLA-DR expression on the surface of these cells, making HLA-DR expression in circulating monocytes (mHLA-DR) a marker of sepsis-induced immunosuppression [68, 69]. Studies revealed that the loss of mHLA-DR is also accompanied by a reduction in ncMo in patients with severe or critical COVID-19, correlating this marker with the severity of the disease [31, 70, 71]. Furthermore, in our study, no changes were found

in IFN- γ levels among patients with COVID-19. IFN- γ is a key factor for antigen presentation via MHC-II, and it is strongly increased in other conditions of hypercytokinemia, such as the macrophage activation syndrome [62]. Despite the reduction in the ability to present antigens, in our study, a large increase in CD56+ monocytes was observed in patients with a moderate to critical condition. CD56+ monocytes were first reported in mice and have NK-like characteristics [72, 73]. In humans, these cells have a high phagocytic capacity [72, 74].

Dendritic cells are also considered professional APCs, in addition to their important role in sensing microbial pathogens and in the secretion of pro- and anti-inflammatory cytokines [71, 75]. In our study, patients with moderate to critical COVID-19 showed a marked reduction in the number of circulating DCs, particularly pDCs. This reduction was also found in other studies [64–67]. Furthermore, other studies have also observed a reduction in the expression of innate sensor genes in DCs in patients infected with SARS-CoV-2 and a reduction in the expression of MHC-II-related genes, while an *in vitro* study noted that exposure to SARS-CoV-2 caused an increase in pro-apoptotic pathways in pDCs [33, 64, 66, 67]. These findings demonstrate not only a reduction in DCs but also an impaired function of DCs in COVID-19 patients.

Finally, this study also assessed the levels of soluble mediators involved in inflammation. One of the cytokines most related to a worse prognosis in patients with COVID-19 is IL-6, which was increased in most patients, especially in those with more severe disease [62, 76, 77]. The results of our study show an increase in IL-6 levels in patients with moderate to critical COVID-19. This generates a systemic pro-inflammatory response since IL-6 is involved in several mechanisms, such as acute phase response, inflammation, B and T cell proliferation, hematopoiesis and neutrophil chemotaxis [78, 79]. The increase in IL-6 levels is correlated with an increase in IL-10, which has also been shown to be increased in patients with moderate to critical COVID-19. IL-10 levels were the only parameter related to the reduced survival of COVID-19 patients, with IL-10 levels ≥ 14 pg/ml indicating a worse prognosis. This is an interesting result, since IL-10 is often associated with immunosuppression and anti-inflammatory activity. However, IL-10 can also be an immune-activating and proinflammatory cytokine [80, 81], meaning that in COVID-19 patients, IL-10 may fail to suppress inflammation or act in a pro-inflammatory manner. Thus, IL-10 may play an important role in the pathophysiology of the disease, which makes this cytokine a possible prognostic biomarker [80–84]. Although, more clinical studies focusing on this specific inflammatory marker and the correlation with the survival of the

patients with COVID-19 must be conducted to clarify and reinforce this important laboratory finding.

This study also found increased IL-8 plasma levels in patients with severe and critical COVID-19. IL-8 acts as a chemoattractant and can activate monocytes, T cells, neutrophils, and other immune cells, in addition to its role in the formation of NETs, which yields a prothrombotic phenotype [85, 86]. Therefore, several studies have reported the importance of IL-8 in the cytokine release syndrome and found an association of IL-8 levels with the duration of the disease [87, 88]. All these findings corroborate studies in the literature that found that these cytokines are increased in patients with COVID-19 [62, 76, 77]. However, unlike other studies, our study observed a reduction in IL-17A in patients with moderate to critical COVID-19. Nevertheless, this result is not so discrepant, since SARS-CoV-2 also seems to stimulate Th-2 cytokine production and suppress Th17-mediated inflammation [76, 89].

In conclusion, the results of this study indicate the presence of systemic effects induced by COVID-19, which appear to be related to the pathophysiology of the disease. Patients with COVID-19 had a pro-inflammatory phenotype and signs of immunosuppression, with overstimulation of innate immunity and an impaired adaptive response. Many alterations have already started to appear in patients with moderate conditions, which highlights the need to monitor all patients, even those at less severe stages of the disease. A better understanding of the immunological aspects of COVID-19 can contribute to the early detection of more serious conditions and to the development of new options for treating the disease. This study has some limitations. The patients were at different stages of the disease since the samples were not collected with the same count of disease days. In addition, some patients, especially those that were hospitalized, used various medications, which can affect their immune and inflammatory profile.

INFORMED CONSENT

Informed consent was obtained from all individual participants included in this study.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

ETHICS APPROVAL

This study was conducted in accordance with the 1964 Helsinki Declaration and Brazilian National Health

Council Resolution No. 196/1996. Experimental protocols were approved by the Human Research Ethics Committee of the Federal University of Santa Catarina, Brazil.


DATA AVAILABILITY STATEMENT

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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