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Increased LPS levels coexist with systemic inflammation and result in monocyte activation in severe COVID-19 patients

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ABSTRACT

Mucosal barrier alterations may play a role in the pathogenesis of several diseases, including COVID-19. In this study we evaluate the association between bacterial translocation markers and systemic inflammation at the earliest time-point after hospitalization and at the last 72 h of hospitalization in survivors and non-survivors COVID-19 patients. Sixty-six SARS-CoV-2 RT-PCR positive patients and nine non-COVID-19 pneumonia controls were admitted in this study. Blood samples were collected at hospital admission (T1) (Controls and COVID-19 patients) and 0–72 h before hospital discharge (T2, alive or dead) to analyze systemic cytokines and chemokines, lipopolysaccharide (LPS) concentrations and soluble CD14 (sCD14) levels. THP-1 human monocyctic cell line was incubated with plasma from survivors and non-survivors COVID-19 patients and their phenotype, activation status, TLR4, and chemokine receptors were analyzed by flow cytometry. COVID-19 patients presented higher IL-6, IFN-γ, TNF-α, TGF-β1, CCL2/MCP-1, CCL4/MIP-1β, and CCL5/RANTES levels than controls. Moreover, LPS and sCD14 were higher at hospital admission in SARS-CoV-2-infected patients. Non-survivors COVID-19 patients had increased LPS levels concomitant with higher IL-6, TNF-α, CCL2/MCP-1, and CCL5/RANTES levels at T2. Increased expression of CD16 and CCR5 were identified in THP-1 cells incubated with the plasma of survivor patients obtained at T2. The incubation of THP-1 with T2 plasma of non-survivors COVID-19 leads to higher TLR4, CCR2, CCR5, CD69 and CCR7 expression. In conclusion, the coexistence of increased microbial translocation and hyperinflammation in patients with severe COVID-19 may lead to higher monocyte activation, which may be associated with worsening outcomes, such as death.

1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection causes Coronavirus disease 2019 (COVID-19), a disease with diverse clinical manifestations [1]. While most COVID-19 cases are asymptomatic or mild, the severe form of COVID-19 can occur with detrimental manifestations such as acute respiratory distress syndrome (ARDS), multi-organ failure, and death [2]. The COVID-19 immunopathology is mainly characterized by a hyperinflammatory state, strong innate cells response, and lymphocyte activation with an exhausted profile [3,4].

The mechanism underlying the inflammatory state of COVID-19 is
not fully understood at this time. Although the respiratory tract is the main site of infection for COVID-19, the pathogenesis of the disease can also involve different organs and systems, such as the heart, kidneys, intestines, vasculature, and liver [3]. In the gastrointestinal (GI) tract, symptoms such as diarrhea and abdominal distention have been reported in several patients [5] and enterocytes in the ileum and colon express the angiotensin-converting enzyme 2 (ACE-2) receptor and may act as a site for SARS-CoV-2 entrance and predispose to enteric infection [6]. Furthermore, the transmembrane serine protease 2 (TMPRSS2), also highly expressed in enterocytes, is able to prime the spike protein of SARS-CoV-2 and mediate the virus entry into cells [7]. Additionally, RNA viral particles were detected in stool samples, raising the possibility that bacterial translocation and microbial products from the GI tract to the peripheral blood might contribute to the hyperinflammatory state and COVID-19 severity [8]. The gut and lungs are anatomically distinct but the proximal communication and complex pathways involving their respective microbiota indicate the existence of a gut-lung axis. In this sense, airways infections by viruses and bacteria are accompanied by alterations in the composition of intestinal microbiota, increased GI symptoms, gut dysfunction and barrier disruption [8]. Ahlawat and Sharma [9] and others [10–12] proposed a gut-lung axis during the SARS-CoV-2 infection through the hyperactive host immune system releasing higher levels of inflammatory cytokines with consequent lung hyper-permeability, gut infection, and disruption of the intestinal permeability that leads to the leakage of gut microbes and associated metabolites into circulation. Interestingly, disruption in gut barriers and microbial translocation is more likely to occur in older individuals and those with chronic diseases, which contribute to the exacerbation of systemic inflammation by activating innate immune cells, mainly monocytes and neutrophils [10].

Lipopolysaccharide (LPS) is a product derived from the membrane of gram-negative bacteria that acts as a potent immune-activating stimulus when recognized by innate immune cells expressing toll-like receptor 4 (TLR4) [13]. Then, innate immune cells become activated and produce high amounts of proinflammatory mediators including cytokines, such as interleukin (IL)-6 and tumor necrosis factor-alpha (TNF-α) and chemokines (i.e., CCL2/MCP-1 and CCL5/RANTES) [14]. In this sense, peripheral blood LPS is a well-recognized marker of translocation of bacterial products after events that compromise the integrity of the intestinal mucosa [15,16]. Moreover, soluble CD14 (sCD14) is released after cleavage from the membrane form (mCD14) on the surface of monocytes through exposure to the LPS stimulation and is considered a marker of microbial translocation [16,17].

There is emerging evidence indicates that severe SARS-CoV-2 infection induces disruption in the gut barrier contributing to the systemic spread of bacteria and microbial products which affect the host's response to the infection [11,18,19]. Mechanistically, it was proposed that SARS-CoV-2 infects enterocytes and causes microbial translocation through transcellular permeability and paracellular pathway using the tight junctions. Additionally, viral infection and replication culminate in the activation of innate and adaptive immunity that induces intestinal damage and increase the intestinal permeability precipitating intestinal barrier failure and bacterial translocation, which in turn auto-fuel vicious cycles of systemic inflammation and tissue damage [18,19]. However, the time course of the presence of microbial product in the peripheral blood of COVID-19 patients during hospitalization remains unknown. Here, we investigated the association between microbial translocation markers and systemic inflammation in the hospital admission and at the discharge time from the hospital in survivors and non-survivors COVID-19 patients. We hypothesized that microbial translocation markers are associated with systemic inflammation and could contribute to the death of hospitalized COVID-19 patients.

2. Methods

2.1. Study population

Hospitalized COVID-19 patients with confirmed SARS-CoV-2 RT-PCR positive test on nasopharyngeal specimens (n = 66) were consecutively recruited after admission in the COVID-19 Unit of Hospital São Camilo (Esteio/RS, Brazil) between June/2020 and December/2020 to a clinical cohort study. The study was approved by the UCFSPA Ethics Committee (CAAE: 38886220.0.0000). Informed consent was obtained from those legally responsible for the patients after the research objectives and procedures were explained. The authors signed an agreement to preserve patient and staff anonymity related to the use of this data. All patients were diagnosed with COVID-19 infection according to the World Health Organization interim guidance and Diagnosis and Treatment Guideline for Novel Coronavirus Pneumonia and were positive for at least two nucleic acid tests for SARS-CoV-2 [20].

Clinical and socio-demographic data were collected from the patient’s electronic medical records upon admission to the unit. Blood samples from 9 age- and sex-matched SARS-CoV-2 RT-PCR negative controls admitted to hospital with pneumonia were also obtained.

2.2. Blood collection and laboratory analysis

Blood samples were collected from the controls (n = 9) and all COVID-19 patients (n = 66) from the antecubital vein into 4 mL tubes with EDTA as anti-coagulant at the earliest time-point after hospital admission (T1). Additional blood samples were collected from 14 survivors and 12 non-survivors COVID-19 patients at the discharge time from the hospital (T2: 0 to 72 h before leaving hospital or death). Plasma samples were obtained by centrifugation (2000g, 10 min), aliquoted and immediately kept at −80 °C until analysis.

Plasma levels of IL-6, IL-10, TNF-α, TGF-β1 (all from Invitrogen Life Sciences, USA), IFN-γ, CCL2/MCP-1, CCL4/MIP-1, and CCL5/RANTES (all from Peprotech, USA) were analyzed by Enzyme Linked Immunosorbent Assay (ELISA) following the manufacturer’s procedure using a microplate reader (EzBiochrom, USA). The intra-assay coefficient of variability was <7.5%. The detection limits of each cytokine were: IL-6, 2–200 pg/mL; IL-10, 4–200 pg/mL; IFN-γ, 10–300 pg/mL; TNF-α, 2–200 pg/mL; TGF-β1, 2–500 pg/mL; CCL5/RANTES, 20–1000 pg/mL; CCL4/MIP-1, 50–1500 pg/mL; CCL2/MCP-1, 50–1000 pg/mL. The soluble form of CD14 was analyzed using Human CD14 ELISA Kit from Invitrogen (USA), with detection limits ranging 8.23–8000 ng/mL.

2.3. Liquid chromatography-tandem mass spectrometry determination of LPS

LPS concentration was determined by quantitation of 3-hydroxytetradecanoic acid as described by Pais de Barros and colleagues [21]. Briefly, 150 µL of plasma was hydrolyzed with 75 µL of NaCl 150 mM, 300 µL of HCl 8 mol for 4 h at 90 °C and extracted with 4 mL of a hexane. Samples were dried under nitrogen stream and resuspended in 50 µL of methanol immediately prior to the injection in the analytical system. A Nexera UFLC system coupled to a LC-MS-8040 triple quadrupole mass spectrometer (Shimadzu, Kyoto, Japan) was used for the analysis. The electrospray parameters were set in the negative ion mode ([M–H]−) as follows: capillary voltage, 3000 V; desolvation line temperature, 250 °C; heating block temperature, 500 °C; drying gas, 18 L/min; and nebulizing gas, 2 L/min. Collision-induced dissociation was obtained with 230 kPa argon pressure. Analyses were carried out with multiple reaction monitoring (MRM) by using the following fragmentation: m/z 243.1 → m/z 59.0. The chromatographic separation was conducted with an Acquity UPLC® C18 column (2.1 × 50 mm, 1.7 µm particle size) (Waters Corporation, Ireland). The analyses were performed in gradient elution mode with a flow rate of 0.4 mL/min and the gradient mobile phase system consisted of water (solvent A) and acetonitrile (solvent B) both
fortified with 0.2% acetic acid as follow: 0–1.5 min, 75–100% of B; 1.5–2.0 min, 100% of B; 2.0–2.1 min, 100–75% of B; 2.1–5.5 min, 75% B. The column oven was kept at 50 °C. The data were processed using LabSolutions software (Shimadzu, Kyoto, Japan). Calibration curves were constructed at intervals of 0.2–1000 ng/mL.

2.4. Cell culture and flow cytometry

THP-1 human monocyte cells (ATCC TIB-202) (2 × 10^5 cells/well) were cultured in RPMI media (Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin and streptomycin (both from Sigma-Aldrich) in 96 well plates for 24 h. Afterwards, the media plus FBS were removed; cells were washed with phosphate-saline buffer and incubated with RPMI supplemented with 10% serum of survivors and non-survivors COVID-19 patients for 15 h. Then, cells were labelled with monoclonal antibodies (all anti-human) conjugated with specific fluorochromes: CD14 FITC, CD16 PE, TLR4 PE, CD69 PERCP-CY5.5, CD192 (CCR2) PE, CD195 (CCR5), CD197 (CCR7) and HLA-DR PERCP-CY5.5 (all from EbioScience, ThermoFisher, USA). 10.000 events were acquired using CELLQuest Pro Software (BD Bioscience, USA) on a FACSCalibur flow cytometer (BD Bioscience, USA) to determine cell phenotype. THP-1 were identified and gated according to each forward scatter (FSC) and side scatter (SSC) profile, and the mean fluorescence intensity (MFI) was evaluated.

2.5. Statistical analysis

Normality of data was checked by Kolmogorov-Smirnov, with values presented as mean ± 95% CI for demographic and clinical characteristics and mean ± standard deviation (SD) for inflammatory mediators. Participant’s characteristics and inflammatory analysis were between-groups compared through a one-way ANOVA followed by Bonferroni’s post-hoc for multiple comparisons. The time-course of inflammatory response and immune variables were analyzed by a generalized linear mixed model. Correlation analyses between microbial translocation markers and inflammatory mediators were performed using Pearson’s Coefficient of Correlation. P values ≤ 0.05 were considered statistically significant. The SPSS 22.0 (IBM Inc, EUA) software was used in all analyses.

| Table 1 | Clinical and demographic subject’s characteristics. |
|---------|-----------------------------------------------------|
|         | Controls                                                | All                                      | Survivors                               | Non-survivors                           |
|         | (n = 9)                                                | (n = 66)                                 | (n = 43)                                | (n = 23)                                 |
| Age (years) | 73.7 (65.2–82.2)                                    | 64.8 (60.3–69.3)                       | 65.8 (59.5–72.1)                       | 63.6 (56.6–70.6)                       |
| Gender (female/male, %) | 33.3/66.6                                       | 47/53                                    | 46.5/53.5                              | 47.8/52.2                              |
| Body mass (kg) | 61.7 (38.2–85.2)                                    | 86.7 (79.5–94.0)^A                     | 79.7 (72.5–87.0)^A                     | 95.8 (82.4–109.2)^A                      |
| BMI (kg/m²) | 23.6 (12.4–34.8)                                    | 30.4 (27.8–33.1)^A                     | 28.7 (25.8–31.5)^A                     | 32.7 (27.7–37.8)^A                      |
| Nutritional Status (%) | Lean 50                                              | 17.9                                    | 22.7                                    | 11.8                                    |
| Symptoms (%) | Fever 74.2                                           | 80                                      | 72.7                                    |                                         |
|           | Dyspnea 62.8                                          | 80                                      | 80                                      |                                         |
|           | Cough 58.5                                            | 46.6                                    | 61.8                                    |                                         |
|           | Body aches 22.8                                       | 60                                      | 16.3                                    |                                         |
|           | Sore throat 22.8                                      | 26.6                                    | 21.8                                    |                                         |
|           | Flu-like syndrome 15.7                               | 40                                      | 18.8                                    |                                         |
|           | Fatigue 11.4                                          | 13.3                                    | 16.3                                    |                                         |
|           | Myalgia 8.5                                           | 20                                      | 9.0                                     |                                         |
|           | Nausea or vomiting 8.5                               | 20                                      | 5.4                                     |                                         |
|           | Headache 8.5                                          | 20                                      | 5.4                                     |                                         |
|           | Coryza 8.5                                            | 13.3                                    | 7.2                                     |                                         |
|           | Diarrhea 7.1                                          | 13.3                                    | 7.2                                     |                                         |
| Underlying medical condition (%) | Hypertension 21.3                                    | 19                                      | 22.7                                    |                                         |
|           | Diabetes Mellitus 18.7                                | 16.7                                    | 18.2                                    |                                         |
|           | Neurological diseases 14.7                            | 19                                      | 4.5                                     |                                         |
|           | Cardiovascular diseases 12                            | 4.8                                     | 22.7^B                                  |                                         |
|           | Rheumatic diseases 1.3                                | 2.4                                     | –                                       |                                         |
|           | Cancer 1.3                                            | 2.4                                     | –                                       |                                         |
|           | Chronic kidney disease 1.3                           | 2.4                                     | –                                       |                                         |
|           | Asthma 1.3                                            | 2.4                                     | –                                       |                                         |
| Pharmacological treatment (%) | Azithromycin 24.3                                    | 26.7                                    | 21.5                                    |                                         |
|           | Corticosteroids 23.7                                  | 23.3                                    | 24.1                                    |                                         |
|           | Ceftriaxone 7.7                                       | 5.8                                     | 10.1                                    |                                         |
|           | Levofloxacin 5.9                                     | 5.8                                     | 6.3                                     |                                         |
|           | Cefepime 5.9                                          | 7                                      | 5.1                                     |                                         |
|           | Vancomycin 4.7                                       | 2.3                                     | 5.1                                     |                                         |

Data are presented as mean ± standard deviation.
A Denotes statistical difference compared to Controls (p < 0.05).
B Denotes statistical difference compared to COVID-19 Survivors (p < 0.05).
3. Results

3.1. Characteristics of the study population

Firstly, we analyzed the baseline characteristics and inflammatory mediators in controls and COVID-19 hospitalized patients. To this, 66 patients admitted to COVID-19 care unit due to COVID-19 complications were enrolled in this study. Additionally, 9 COVID-19 negative controls were analyzed. COVID-19 patients presented higher body mass and body mass index than controls (p < 0.01), and 53.8% of patients were classified as obese. The mean length of stay in the hospital was 21.1 (15.6–26.6) days for COVID-19 patients, 84.8% of them were admitted to the intensive care unit (ICU) due to COVID-19 complications, and 34.8% patients died due to complications of SARS-CoV-2 infection. Forty-three (43) patients survived to SARS-CoV-2 infection and were classified as COVID-19 survivors. Patients and control data are shown in Table 1.

Interestingly, COVID-19 non-survivors (n = 23, 30.7% of COVID-19 patients) were heavier than controls (body mass, p = 0.01; BMI, p = 0.01) and COVID-19 survivors (n = 43, 57.3% COVID-19 patients) (body mass, p = 0.03; BMI, p = 0.02), but no difference between groups was found in the length of stay in the hospital (p > 0.05). COVID-19 non-survivors required more oxygen use during hospitalization than COVID-19 survivors (p = 0.002), but similar rates of ICU admission were observed (p > 0.05). The most reported symptoms by COVID-19 patients were fever (74.2%), dyspnea (62.8%), and cough (58.5%). Furthermore, 21.7% of COVID-19 patients were diagnosed with hypertension, 18.7% with diabetes mellitus 2, 14.7% with some neurologic disease, and 12% with some cardiovascular disease. Regarding pharmacological treatment, azithromycin (24.3%) and corticosteroids therapy (23.7%) were the predominant medicine adopted by medical staff during hospitalization. There was no difference between groups regarding symptoms, comorbidities, or pharmacological therapy during hospitalization (p < 0.05), unless the prevalence of cardiovascular diseases (COVID-19 survivors, 4.8%; COVID-19 non-survivors, 22.7%; p = 0.04).

Table 2

| Systemic inflammatory mediators in survivors and non-survivors COVID-19 hospitalized patients. |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| | Controls (n = 9) | All (n = 66) | Survivors (n = 43) | Non-survivors (n = 23) |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| Monocytes (cells/mm³) | 640.7 ± 308.3 | 589.7 ± 380.7 | 365.3 ± 396.3 | 282.2 ± 292.0 |
| IL-6, pg/mL | 18.7 ± 6.7 | 36.5 ± 18.7 | 36.3 ± 22.0 A | 36.9 ± 10.7 A |
| IL-10, pg/mL | 42.8 ± 22.4 | 37.8 ± 16.5 | 38.2 ± 17.8 A | 35.0 ± 13.6 A |
| IFN-γ, pg/mL | 29.5 ± 13.2 | 38.3 ± 12.5 | 36.7 ± 10.5 A | 41.3 ± 15.2 A |
| TNF-α, pg/mL | 11.2 ± 5.1 | 16.3 ± 7.3 A | 14.7 ± 6.4 A | 19.5 ± 8.0 A B |
| TGF-β1, pg/mL | 137.7 ± 94.7 A | 80.8 ± 48.0 | 78.6 ± 53.1 A | 84.8 ± 37.2 A |
| CCL2, pg/mL | 438.7 ± 175.7 A | 538.4 ± 118.3 A | 548.6 ± 116.5 A | 519.9 ± 121.8 A |
| CCL4, pg/mL | 359.7 ± 160.4 A | 556.0 ± 197.0 A | 597.7 ± 215.9 A | 477.8 ± 215.9 A |
| CCL5, pg/mL | 231.9 ± 68.0 | 378.2 ± 290.2 A | 448.5 ± 336.7 A | 246.6 ± 67.9 A |

Data are presented as mean ± standard deviation. Statistical significance was determined using the one-way ANOVA test followed by Bonferroni’s post-hoc. A Denotes statistical difference compared to controls (p < 0.05). B Denotes statistical difference compared to COVID-19 survivors (p < 0.05).

3.2. Systemic inflammatory mediators in survivors and non-survivors COVID-19 patients

COVID-19 patients presented a hyperinflammatory profile at hospital admission compared to controls as presented in Table 2. COVID-19 patients had increased systemic levels of IL-6 (p < 0.01), IFN-γ (p < 0.01), TNF-α (p < 0.01), CCL5/RANTES (p < 0.01), CCL4/MIP-1β (p < 0.01), and CCL2/MCP-1 (p < 0.01) but diminished circulating levels of TGF-β1 (p < 0.05). When COVID-19 patients were stratified in survivors and non-survivors, both groups presented higher IL-6 (p < 0.01), CCL2/ MCP-1 (p < 0.01), and CCL4/MIP-1β (p < 0.01) levels at hospital admission compared to controls.

However, only non-survivors had increased IFN-γ (p < 0.01) and TNF-α (p < 0.05) compared to controls, and higher TNF-α levels (p < 0.05) than survivors. Conversely, survivors had higher levels of CCL5/ RANTES (p < 0.01) and CCL4/MIP-1β (p < 0.05) compared to control and non-survivors groups, respectively. Furthermore, reduced TGF-β1 levels were found in both survivors and non-survivors COVID-19 patients compared to controls (p < 0.01). Finally, a strong monocytopenia was observed in COVID-19 non-survivors compared to COVID-19 survivors (p = 0.01). These data indicate that COVID-19 patients have higher levels of inflammatory cytokines/chemokines at the hospital admission and higher TNF-α plasma levels that may be associated with death.

3.3. Increased markers of microbial translocation in plasma of COVID patients are associated with systemic inflammation

Markers of microbial translocation were found in the peripheral blood of COVID-19 hospitalized patients. In fact, COVID-19 subjects, independently of the outcome group, presented higher sCD14s (p < 0.001 for all comparisons) and LPS (controls vs. COVID-19 patients, p < 0.001; controls vs. COVID-19 survivors, p < 0.001; controls vs. COVID-19 non-survivors, p < 0.005) levels at time of hospital admission (Fig. 1). We examined the Pearson’s Coefficient Correlation between inflammatory mediators, sCD14 and LPS for each participant. We highlight the significant correlations of sCD14 with TGF-β (r = −0.46; p < 0.001), CCL4/MIP-1β (r = 0.46; p < 0.001) and CCL2/MCP-1 (r = 0.29; p = 0.03). LPS was correlated with TNF-α (r = −0.32; p < 0.004), CCL5/ RANTES (r = 0.49; p < 0.001), and CCL4/MIP-1β (r = 0.27; p = 0.01) (Fig. 2).

3.4. Systemic inflammatory cytokines and chemokines and microbial translocation markers in COVID-19 survivors and non-survivors at T1 and T2

The time course of systemic inflammatory markers and variables related to microbial translocation in 14 survivors and 12 non-survivors COVID-19 patients were evaluated in two times. Peripheral blood of patients was collected at hospital admission (T1) and at the discharge time from the hospital (T2: 0 to 72 h before leaving hospital or death). The time course of systemic inflammatory mediators was presented in Fig. 3. Increases in IFN-γ (p < 0.01) and TNF-α (p < 0.05) concentrations were identified in survivors COVID-19 patients at T2. Non-survivors COVID-19 patients presented increased levels of IL-6 (p < 0.01), TNF- α (p < 0.05), CCL5/RANTES (p < 0.05), and CCL2/MCP-1 (p < 0.01) at T2.

Non-survivor patients presented higher LPS levels in T2 compared to T1 (p < 0.05), but sCD14 only tended to increase in T2 (p = 0.055). On the other hand, no changes were identified in sCD14 and LPS at the end of hospitalization in COVID-19 survivors. Furthermore, the delta value (Δ, T2 - T1) of LPS levels positively correlated with Δ CCL2/MCP-1 levels (r = 0.54; p = 0.008) (Fig. 4). Together, these data indicate that the increase in LPS levels may be associated with death.
3.5. Effect of plasma from COVID survivors and non-survivors on THP-1 cell phenotype

As hospitalized non-survivors COVID-19 patients presented higher levels of microbial translocation markers with concomitant increase in some plasmatic inflammatory mediators, we hypothesized if the plasma from COVID-19 patients alters the phenotype of monocytes including subpopulation, and activation markers and TLR4 and chemokine receptors expression. To evaluate this hypothesis, we incubated THP-1 human monocyte lineage with plasma of survivors (n = 8) or non-survivors (n = 8) COVID-19 patients collected at hospital admission (T1) and at the end of hospitalization (T2) to evaluate the expression of TLR4, CD14, CD16, CD69, HLA-DR, CCR2, CCR5, and CCR7 (Fig. 5).

Interestingly, plasma from COVID-19 non-survivor patients obtained at T2 increased the expression of TLR4 (p = 0.03), CCR2 (p = 0.05), CCR5 (p = 0.02) and CCR7 (p = 0.02) and the activation marker CD69 (p = 0.02) on the cell surface of THP-1 cells compared to T1 plasma from the same group. The expression of CD16 (p = 0.04) and CCR5 (p = 0.03) were higher in THP-1 cells incubated with plasma from COVID-19 survivor patients collected at T2. No statistical differences were found in CD14 and HLA-DR expression (p < 0.05). These data indicate that non-survival COVID-19 patients presented higher systemic levels of microbial translocation products and inflammatory mediators in the plasma that can increase activation/migration status of monocytes.

4. Discussion

Hospitalized COVID-19 patients display a broad spectrum of inflammatory responses that contributes to immune activation, disease severity, and the outcome of the disease [22]. In the present study, we hypothesized that microbial translocation markers could impact on inflammatory response in COVID-19 patients. Our main findings can be summarized as follows: a) higher levels of microbial translocation markers were found in COVID-19 patients on hospital admission; b) COVID-19 patients exhibited a proinflammatory status as shown by higher levels of IL-6, IFN-γ, TNF-α, CCL5/RANTES, CCL4/MIP-1β and CCL2/MCP-1; c) non-survivors COVID-19 patients showed significant increase in the amount of LPS and sCD14 during hospitalization associated with increased systemic levels of IL-6, TNF-α, CCL5/RANTES, CCL4/MIP-1β and CCL2/MCP-1; d) survivors COVID-19 patients maintained LPS and sCD14 levels at stable values concomitant with increases in IFN-γ levels; e) the incubation of THP-1 cells with plasma of COVID-19 patients obtained at T2 increased the expression of TLR4, CCR2, CCR5, and CD69 expression on the cell surface; while THP-1 cells incubated with plasma of survivors at T2 presented higher CCR5 and CD16 expression. Together, our data indicate that increased microbial translocation coexists with the hyper-inflammatory condition induced by SARS-CoV-2 infection and may lead to increased monocyte activation that may be associated with worse outcomes in severe COVID-19, such as death (Fig. 6).

Angiotensin-Converting Enzyme 2 (ACE-2), which is the most recognized receptor that binds to the Spike protein of SARS-CoV-2 and allows the host infection, is widely expressed in epithelial cells along
with the gastrointestinal (GI) tract [10,23]. Thus, SARS-CoV-2 can invade the intestinal tract causing GI dysfunction that disrupt intestinal barriers, increasing the translocation of microbial products [11,23]. Increased endotoxemia has been previously described in several pathological conditions associated with hospitalization, becoming a recognized marker of critical ill suffering from sepsis [24–26]. Furthermore, recent studies described disrupted gut barrier integrity and increased circulating bacteriome in moderate and severe COVID-19 patients [11,18,19].

In fact, probiotics, fecal microbiota transplantation and dietary therapies have been proposed to ameliorate barrier dysfunctions in patients with COVID-19 [7]. Here, we found increased LPS and sCD14 levels in the plasma of hospitalized COVID-19 patients at admission and during ICU hospitalization. Arunachalam and colleagues [22] found higher levels of bacterial DNA products, as measured by PCR quantification of 16S ribosomal RNA gene product, and LPS levels in severe ICU patients. The increased bacterial products in the peripheral circulation may contribute to the enhanced macrophage activation in infected tissue (i.e. lungs and gastrointestinal tissues) and to the failure in suppressing the hyperinflammation during SARS-CoV-2 infection [9]. In fact, elevated levels of gut leakage markers were previously associated with inflammasome activation which leads to cardiac injury during the course of hospitalization in COVID-19 patients [19]. Corroborating these data, other studies show an important role for endotoxin and sCD14 in heart failure [27,28].

Microbial translocation to the peripheral blood also contributes to an early activation of monocyctic cells. Indeed, we observed higher sCD14 in survivors and non-survivors COVID-19 patients. In line with this, Bowman and coworkers [29] found increased sCD14 levels in COVID-19 patients independent of the severity of disease. Furthermore, the authors revealed that COVID-19 critical ill patients who recovered or deceased had the highest sCD14 and lipopolysaccharide-binding protein (LBP, another marker of endotoxemia) values compared to the other groups [29]. These data highlighted the presence of strong activation of the monocytic lineage on hospital admission due to the SARS-CoV-2 infection in addition to the LPS translocation and inflammatory exacerbation in ICU patients [30]. In accordance with this study, systemic markers of monocyte activation, mainly sCD14 and sCD163, correlated with inflammatory cytokines in a non-intensive care unit COVID-19 cohort patients [31]. Giron and coworkers [32] found strong correlations between markers of tight junction permeability and microbial translocation with systemic inflammatory molecules, which corroborates our data.

It was previously postulated that perturbations in gut microbiota and microbial translocation may exacerbate the severity of hyperinflammation in COVID-19 [33]. Moreover, sCD14 tended to be higher...
at T2 in non-survivors COVID-19 patients. Interestingly, heat map correlations revealed that sCD14 correlated with CCL4/MIP-1β and CCL2/MCP-1, while LPS correlated with CCL5/RANTES and CCL4/MIP-1β.

These results suggest that microbial translocation may contribute to the increases in chemokines, which recruits innate immune cells to the infectious site. In fact, chemokines release is directly involved with the
accompanies clinical deterioration and death of severe COVID-19 patients. The increased LPS and sCD14 markers during the hospitalization were associated with higher systemic levels of IL-6, TNF-α, CCL5/RANTES and CCL2/MCP-1 in non-survivors COVID-19 patients. On the other hand, survivors patients maintained LPS and sCD14 levels at stable values concomitant with increases in IFN-γ and TNF-α levels. Finally, the incubation of THP-1 cells with plasma samples from non-survivors, obtained at the end of hospitalization (T2), increased the expression of TLR4, CCR2, CCR5, and CD69 on the cell surface of monocytic lineage cells, while THP-1 cells incubated with plasma of survivor patients at T2 presented higher CCR5 and CD16 expression.

Here, we hypothesized that plasma factors, such as LPS and inflammatory cytokines and chemokines, may directly impact the phenotype of monocytes. To address this question, we incubated monocytic THP-1 lineage with the plasma samples of hospitalized survivors or non-survivors COVID-19 patients obtained at hospital admission (T1) and in a time compromising 0–72 h before hospital discharge (T2, alive or dead). Interestingly, TLR4, the LPS ligand, increased only in non-survivors COVID-19 patients, which is corroborated by the higher systemic LPS levels in this group. In fact, critically ill COVID-19 patients presented upregulation in the TLR4 expression and higher pro-inflammatory cytokine and chemokine production after monocyte TLR4 stimulation similarly to the observed in bacterial sepsis [40]. Furthermore, the spike protein binds to LPS and lead to TLR4/NF-κb axis activation, modulating the phenotype of monocytes. In this sense, we found increased CD69 expression on THP-1 cells incubated plasma of non-survivors COVID-19 patients collected at T2, indicating an early monocyte activation. However, no difference was observed in the HLA-DR expression on THP-1 cells after incubation with plasma of both groups. Plasma obtained from COVID-19 patients may have no impact on the antigen presentation and long-term activation of monocytes. On the other hand, plasma from COVID-19 survivors collected at T2 moment increased CD16 expression in THP-1 cells. Data from literature described higher percentages of CD14^+CD16^{high} monocytes and very low expression of HLA-DR in all CD14^+ monocyte subsets in severe COVID-19 patients [41–43]. The mobilization of newly bone marrow monocytes associated with continuous higher systemic and organ inflammation along the course of the acute infection, rather than short-term stimulation, may impact on the changes of CD16 and HLA-DR expression in circulating monocytes.

Severe COVID-19 patients presented an inflammatory phenotype of monocytes associated with early activation and downregulation of HLA-DR [42,44]. Monocytes upregulate the CD69 expression, which promotes tissue infiltration and retention [45,46]. Increased activation and CD69 expression in monocytes of severe COVID-19 subjects were associated with diapedesis and tissue infiltration, contributing to the monocytopenia observed in the peripheral circulation of deceased patients [47]. The increased expression of CCR2, CCR5, and CCR7 in THP-1 incubated with plasma at T2 of non-survivors COVID-19 patients may indicate the pro-inflammatory action of soluble factors produced during
Infiltrating monocytes constitute the majority of leukocytes migrating into the infected lungs, contributing to the severe lung inflammation in COVID-19 [44]. In fact, increased transcription of leukocytes migrating into the infected lungs, contributing to the severe SARS-CoV-2 infection. Moreover, THP-1 cells also presented higher increased CCR2 alveolar lavage fluid of severe COVID-19 patients [48]. Furthermore, P.C. Teixeira et al. local response and induces tissue damage [49 which recruit neutrophils and mast cells, exacerbating the inflammatory overexpression of chemokine receptors in severe COVID-19 patients. 

In conclusion, severe COVID-19 is related to increased microbial translocation during hospitalization coexisting with the inflammatory condition of SARS-CoV-2 infection and could lead to higher monocyte activation and worsening clinical outcomes, such as death.

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Declaration of Competing Interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References
[1] G.A. Poland, I.G. Ovsyannikova, R.B. Kennedy, SARS-CoV-2 immunity: review and applications to phase 3 vaccine candidates, Lancet [Internet] 8730 (20) (2020), https://doi.org/10.1016/S0140-6736(20)32151-1.
[2] G. Chen, D. Wu, W. Guo, Y. Cao, D. Huang, H. Wang, et al., Clinical and immunologic features in severe and moderate forms of Coronavirus Disease 2019, (2020) 6–20.

[3] N. Vabret, G.J. Britton, C. Guerber, S. Hegde, J. Kim, M. Kuskis, et al., Immunology of COVID-19: Current State of the Science, Immunity [Internet]. 52 (6) (2020) 910–941, https://doi.org/10.1016/j.immuni.2020.05.003.
[4] C. Lucas, P. Wong, J. Klein, T. Castro, J. Silva, M. Sundaram, et al., Longitudinal immunological analyses reveal inflammatory misfiring in severe COVID-19 patients. 2020.
[5] I. H. Zhang, Z. Kang, H. Dong, X. Xu, J. Wang, Z. Li, et al., The digestive system is a potential route of 2019-nCoV infection: A bioinformatics analysis based on single-cell transcriptomes [Internet]. bioRxiv. bioRxiv; 2020 May 1 (2020) p. 2020.05.05.20231209. Available from: https://doi.org/10.1016/j.cgh.2020.03.075.
[6] I. S., A.R. Intestinal Barrier Function in Health and Disease: Any role of SARS-CoV-2? Microorganisms [Internet]. 2020 Nov 1 (2020) 8(11), 1–2. Available from: https://pubmed.ncbi.nlm.nih.gov/33172188/.
[7] V. Kipkorir, I. Cheruyiui, B. Nguere, M. Miniani, J. Munguti, Prolonged SARS-CoV-2 RNA detection in anal/rectal swabs and stool specimens in COVID-19 patients after negative conversion in nasopharyngeal RT-PCR test [Internet]. vol. 92, Journal of Medical Virology, John Wiley and Sons Inc 2020 (2020) p. 5228–2331. Available from: https://pubmed.ncbi.nlm.nih.gov/32403374/.
[8] Immunological co-ordination between gut and lungs in SARS-CoV-2 infection | Elsevier Enhanced Reader [Internet]. [cited 2021 Aug 9]. Available from: https://reader.elsevier.com/reader/sd/pii/S0167256120308469?token=117C2D1D3681577480FF1E1FA1EC6A949DE56386089838D9C2C60878AC687BD2ED3ADEDA0A606F155C6AxFar&originRegion=us-east-1&originSite=Creation--20210809140924.
[9] T. Zuo, F. Zhang, G.C.T. Liu, Y.K. Yeo, A.Y.L. Li, H. Zhang, et al., Alterations in Gut Microbiota of Patients with COVID-19 During Time of Infection and Post-recovery, Gastroenterology [Internet]. 2020 Sep 1 (2020) 159(3), 944-955.e8. Available from: https://pubmed.ncbi.nlm.nih.gov/32442562/.
[10] P. Srivongraprangson, W. Kulvichit, S. Payangom, T. Piatsikan, A. Chikdamporn, S. Prapangornanonta, et al., Endotoxemia and circulating bacteriome in severe COVID-19 patients [Internet]. medRxiv. medRxiv; 2020 (2020) p. 2020.05.29.20190785. Available from: https://doi.org/10.1101/2020.05.29.20190785.
[11] W.K. Leung, K. To, P.K.S. Chan, H.L.Y. Chan, A.K.L. Wu, N. Lee, et al., Enteric involvement of severe acute respiratory syndrome-associated coronavirus infection, Gastroenterology [Internet]. 125 (4) (2003) 1011–1017. Available from: https://pubmed.ncbi.nlm.nih.gov/15178783/.
[12] B.S. coworkers, J.O. Lee, Role of upregulation of lipopolysaccharide pattern by TLR4 complexes [Internet]. Vol. 45, Experimental and Molecular Medicine. Nature Publishing Group [Internet]. 2013 [2021 May 5] p. 45. Available from: www.nature.com/emm.
[13] S. Fedre, C. Stockmann, P. Freitag, J. Fandrey, Bacterial lipopolysaccharide induces HIP-1 activation in human monocytes via p44/42 MAPK and NF-κB, Biochim. J. (2006).
[14] C. Yue, B. Ma, Y. Zhao, Q. Li, J. Li, Lipopolysaccharide-induced bacterial translocation is intestine site-specific and associates with intestinal mucosal inflammation, Inflammation [Internet]. 35 (6) (2012) 1880–1888. [cited 2021 May 5] Available from: https://pubmed.ncbi.nlm.nih.gov/22821406/.
[15] F. De Oliveira Feitosa De Castro, J.M. Silva, G.P. Dorneles, J.B. De Sousa Barros, C. B.Ribiero, I. Noronha, et al., Distinct inflammatory profiles in HIV-infected individuals under antiretroviral therapy using cannabis, cocaine or cannabis plus cocaine, AIDS. 33 (12) (2019).
[16] Funda DP, L. Tucková, M.A. Fárré, T. Iwase, J. Iwane, M. Hlinková-Hoganov, CD14 is expressed and released as soluble CD14 by human intestinal epithelial cells in vitro: Lipopolysaccharide activation of epithelial cells revisited, Infect. Immun. [Internet]. 69 (6) (2001) 3772–3781 [cited 2021 May 21] Available from: http://iai.asm.org/.
[17] L.B. Giron, H. Dweep, X. Yin, H. Wang, M. Damra, A.R. Goldman, et al., Severe COVID-19 Is Fueled by Disrupted Gut Barrier Integrity, [Internet]. medRxiv. medRxiv; 2020 (2020) p. 2020.11.13.20231209. Available from: https://doi.org/10.1101/2020.11.13.20231209.
[18] H. Höel, H. Heggelund, D.H. Reikvam, B. Stikrud, T. Ueland, A.E. Michelsen, et al., Elevated markers of gut leakage and inflammasome activation in COVID-19 patients with cardiac involvement, J. Intern. Med. [Internet]. 289 (4) (2021) 523–531 [cited 2021 May 5] Available from: https://pubmed.ncbi.nlm.nih.gov/32976665/.
[19] No Title [Internet]. [cited 2021 May 5]. Available from: https://www.who.int/docs/default-source/coronaviruse/clinical-management-of-novel-cov-pdf.pdf.
[20] J.F.P. De Barros, T. Gautier, W. Silva, C. Adrio, H. Choubley, E. Charron, et al., Quantitative lipopolysaccharide analysis using HP/IC/M-MS and its correlation with the limulus amebocyte lysate assay, J. Lipid. Res. [Internet]. 56 (7) (2015) 1363–1369 [cited 2021 May 20]. Available from: https://pubmed.ncbi.nlm.nih.gov/26025073/.
[21] P.S. Arunnachalam, F. Wimmers, C.K. Mok, R.A.P.M. Perera, M. Scott, T. Hagan, et al., Systems biological assessment of immunity to mild versus severe COVID-19 infection in humans, Science (80-) 369 (6580) (2020) 1210–1220.
[22] Xiao, M. Tang, K. Zheng, Y. Liu, X. Li, H. Shao, BRIEF COMUNICATIONS Evidence for Gastrointestinal Infection of SARS-CoV-2, 2020 [cited 2021 May 17]; Available from: https://doi.org/10.1053/j.gastro.2020.02.055.
[23] J.C. Marshall, D. Foster, J.L. Vincent, D.J. Cook, J. Cohen, R.P. Dellinger, et al., Diagnostic and prognostic implications of endotoxemia in critical illness: Results of a randomized controlled trial, J. Clin. Investig. [Internet]. 90 (3) (2002) 527–534 [cited 2021 May 7]. Available from: https://pubmed.ncbi.nlm.nih.gov/15243928/.
