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MAJOR ARTICLE

Dysregulated Innate and Adaptive Immune Responses Discriminate Disease Severity in COVID-19

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The clinical spectrum of COVID-19 varies and the differences in host response characterizing this variation have not been fully elucidated. COVID-19 disease severity correlates with an excessive proinflammatory immune response and profound lymphopenia. Inflammatory responses according to disease severity were explored by plasma cytokine measurements and proteomics analysis in 147 COVID-19 patients. Furthermore, peripheral blood mononuclear cell cytokine production assays and whole blood flow cytometry were performed. Results confirm a hyperinflammatory innate immune state, while highlighting hepatocyte growth factor and stem cell factor as potential biomarkers for disease severity. Clustering analysis revealed no specific inflammatory endotypes in COVID-19 patients. Functional assays revealed abrogated adaptive cytokine production (interferon-γ, interleukin-17, and interleukin-22) and prominent T-cell exhaustion in critically ill patients, whereas innate immune responses were intact or hyperresponsive. Collectively, this extensive analysis provides a comprehensive insight into the pathobiology of severe to critical COVID-19 and highlights potential biomarkers of disease severity.

Keywords. COVID-19; disease severity; biomarkers; cytokines; proteomics; innate immunity; adaptive immunity; flow cytometry; exhaustion markers.

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) has spread rapidly across the world and was officially declared a pandemic in March 2020 [1]. The clinical spectrum of coronavirus disease 2019 (COVID-19), caused by SARS-CoV-2, varies from asymptomatic disease or mild respiratory symptoms to severe pneumonia, respiratory failure, and death [2]. So far, more than 80 million people have been infected, leading to more than 1 800 000 deaths worldwide [3]. Although much has been learned about the pathogenesis of COVID-19 in a very short time, the complex dysregulation of the immune system involved in progression of this disease still remains incompletely understood.

The most severe complication of COVID-19 is respiratory failure due to acute respiratory distress syndrome (ARDS), requiring ventilatory support in the intensive care unit (ICU). Inflammation plays a central role in the pathogenesis of ARDS [4]. Evidence suggests that an exuberant innate immune response induced by SARS-CoV-2 characterizes more severe disease, as illustrated by higher concentrations of circulating proinflammatory cytokines in critically ill (ICU) COVID-19 patients as compared to non-ICU patients [2, 5–7]. Furthermore, severely ill patients display a compromised adaptive immune system, characterized by T-cell exhaustion and profound lymphopenia [5, 8–10]. These findings point towards dysregulation of both innate and adaptive immunity and the degree of perturbance might be associated with disease severity, potentially leading to the development of clinically useful biomarkers. Therefore, in this study, we integrated plasma cytokine

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measurements and proteomics to explore the inflammatory response in hospitalized patients with severe (non-ICU) and critical (ICU) COVID-19. Furthermore, ex vivo functional evaluation of innate and adaptive immune responses in COVID-19 patients and healthy controls was performed to provide a comprehensive understanding of the host response in COVID-19.

METHODS

Study Design and Patients

All patients aged ≥ 18 years with polymerase chain reaction (PCR)-confirmed or clinically presumed COVID-19 admitted to medical wards and ICU in the Radboud University Medical Center between 5 March 2020 and 21 April 2020 were eligible for enrolment. Presumed infection was defined based on clinical signs and symptoms, specific computed tomography (CT) findings, and clinical expert consensus [11]. Disease severity was defined according to the patient's need for intensive care at the time of plasma sampling (critical in ICU vs severe in non-ICU patients). Because 37/38 (97.4%) of the ICU patients received invasive mechanical ventilation (1 patient received ventilatory support by Optiflow), ward of hospitalization was considered a good and pragmatic representation of disease severity in our study performed during the high-intensity health care situation of the first wave of the pandemic in the Netherlands. For ex vivo peripheral blood mononuclear cell (PBMC) stimulation experiments and flow cytometry, sex-matched healthy controls were recruited for comparison. Demographic characteristics of healthy controls are provided in Supplementary Table 1. A graphical overview of the study design is provided in Supplementary Figure 1.

Ethical Approval

All applicable study protocols were approved by the local ethics board before initiation of the study. All patients admitted to hospital (or their representatives) either provided verbal informed consent for (non-ICU wards) or did not object to (ICU) participation before enrollment. This study was performed in accordance with the latest version of the declaration of Helsinki, the International Conference on Harmonisation Good Clinical Practice guidelines and local regulations.

Sample Processing and Data Collection

Plasma was obtained from ethylenediaminetetraacetic acid (EDTA) blood by centrifugation and stored at either −20°C for later enzyme-linked immunosorbent assay (ELISA) or at −80°C for later proteomics analysis.

Clinical data were obtained from patients' medical files and processed in encoded form in electronic case report forms (Castor electronic data capture).

Cytokine and Chemokine ELISAs

Interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) plasma concentrations were measured using commercially available ELISA (Quantikine ELISA kits, R&D Systems) according to the manufacturer's instructions. Cytokine concentrations in supernatants of ex vivo PBMC experiments were assessed by commercially available ELISA (DuoSet ELISA kits, R&D Systems for TNF-α, IL-1β, IL-6, IL-1 receptor antagonist (IL-1Ra), IL-17, and IL-22 and Sanquin Reagents for interferon-γ [IFN-γ]). For all cytokines, measured values below the lower limit of detection (provided in the Supplementary Methods) are represented by this lowest detection value.

Proteomics Analysis

Circulating plasma protein expression was assessed using the commercially available multiplex proximity extension assay from Olink Proteomics AB [12]. Proteins from 3 different panels were measured (Inflammation, Cardiometabolic, and Cardiovascular II), resulting in data on 269 different proteins in total.

To improve the chance of true positive discoveries, we validated the findings by assessing immune biomarkers in 2 cohorts: a discovery cohort for identification of proteins differentially expressed in ICU patients as compared to non-ICU patients, and a validation cohort to validate the findings from the discovery cohort.

Measurements were performed on 2 batches on separate occasions. The first batch included plasma samples donated between 18 and 25 March 2020, the second batch included plasma samples donated between 23 March 2020 and 23 April 2020. Because the second batch encompassed samples from a larger number of patients, patients whose samples were measured at this time were retrospectively assigned to the discovery cohort, whereas those whose samples were measured in the (smaller) first batch were retrospectively assigned to the validation cohort.

Proteins are expressed on a $\log_2$-scale as normalized protein expression values, and were normalized using bridging samples to correct for batch variation. A more detailed description of the proteomics analysis is provided in the Supplementary Methods.

PBMC Isolation and Ex Vivo Stimulation

A detailed description of PBMC isolation and ex vivo stimulation experiments is provided in the Supplementary Methods. In short, PBMCs were isolated from EDTA blood by Ficoll-Paque PLUS differential density gradient centrifugation using SepMate (Stemcell Technologies) isolation tubes. Cells were washed with phosphate-buffered saline (PBS), resuspended in supplemented Roswell Park Memorial Institute (RPMI) 1640 Dutch modified culture medium (Gibco; Thermo Fisher Scientific), and counted using a Sysmex XN-450 automated differential hematology analyzer (Sysmex Corporation). Isolated PBMCs were added to 96-well round-bottom plate wells (Greiner Bio-One International) and incubated with RPMI, lipopolysaccharide (LPS; serotype 055:B5, Sigma-Aldrich), or heat-killed *Candida albicans* yeast cells (strain UC820) for 24 hours at 37°C and 5% CO_2 to assess TNF-α,

IL-1β, IL-6, and IL-1Ra production. For 7 days' stimulation experiments (to assess IFN-γ, IL-17, and IL-22 production), wells were supplemented with 10% pooled human serum. After incubation, supernatants were collected and stored at −20°C before ELISA measurements were performed.

Flow Cytometry

Whole-blood cell counts were obtained using a Coulter Ac-T Diff cell counter (Beckman Coulter), which was calibrated daily. Whole blood (1 to approximately 1.5 mL) was incubated in lysis buffer to lyse erythrocytes. Remaining leukocytes were washed twice with PBS and resuspended in PBS plus 0.2% bovine serum albumin (Sigma-Aldrich) to achieve a final concentration of 5×10^6/mL. Cell suspension (200 µL) was transferred for cell surface staining for cluster of differentiation (CD) 45, CD8, CD4, and CD279. More detailed information on the fluorochrome conjugate monoclonal antibodies used is provided in the Supplementary Methods. All reagents were titrated and tested before they were used in the current study. Stained cells were measured on a 10-color Navios flow cytometer (Beckman Coulter) equipped with 3 solid-state lasers (488 nm, 638 nm, and 405 nm). HLA-DR expression on monocytes was detected using the Anti-HLA-DR/Anti-Monocyte Quantibrite assay (BD Biosciences). This assay is described in more detail in the Supplementary Methods. Flow cytometry data were analyzed using Kaluza Analysis Software version 2.1 (Beckman Coulter).

Statistical Analysis

For continuous variables, groups were compared using Mann-Whitney *U* test or Kruskal-Wallis test with Dunn's multiple comparison test, assuming non-Gaussian distribution of variables. Nominal variables were compared using the χ^2 or Fisher exact test, as appropriate. A P value < .05 was considered statistically significant. Receiver-operating characteristic (ROC) analyses for the performance of biomarkers in distinguishing disease severity were performed by designating values from non-ICU patients as control values and those from ICU patients as patient values. Differential expression analysis of Olink proteins between ICU and non-ICU groups was performed using the R package *limma*, applying a linear model with age and sex as covariates. *Limma* uses an empirical Bayes method to moderate the standard errors of the estimated log fold changes [13]. The Benjamini-Hochberg procedure was applied to correct for multiple testing, and a false discovery rate < 0.05 was considered statistically significant. Statistical analyses were performed using GraphPad Prism 5 or 8 for Windows (GraphPad Software) or R/Bioconductor (https://www.R-project.org/).

RESULTS

Patient Cohort and Characteristics

In total, 147 hospitalized COVID-19 patients donated plasma, 38 of whom were admitted to ICU and 109 to non-ICU clinical wards. Patient characteristics at hospital admission are provided in Table 1. Demographic characteristics did not differ between the ICU and non-ICU groups at the time of hospitalization. However, in ICU patients, time to plasma sampling was significantly longer, comorbid pulmonary and autoimmune diseases were significantly less frequent, and C-reactive protein (CRP) levels and CT severity scores at admission were significantly higher.

Inflammatory Markers in Plasma

At the time of plasma sampling, ICU patients displayed a significantly higher median concentration of CRP, D-dimer, and ferritin versus non-ICU patients (CRP ICU, 248 mg/L vs non-ICU, 82 mg/L; D-dimer ICU, 2665 ng/mL vs non-ICU, 1250 ng/mL; ferritin ICU, 1608 µg/L vs non-ICU, 915 µg/L; Figure 1A and 1B and Supplementary Figure 2). Furthermore, plasma levels of IL-6 were significantly higher in ICU versus non-ICU patients (median concentration 182.0 pg/mL and 40.0 pg/mL, respectively; Figure 1C). Although TNF-α plasma levels were relatively low in both groups, they were also significantly higher in ICU patients (ICU median, 18.5 pg/mL vs non-ICU, 16.0 pg/mL; Figure 1D), although differences were small.

In-Depth Proteomics Analysis

For proteomics analysis, the discovery cohort consisted of 101 (83 non-ICU and 18 ICU) and the validation cohort of 46 patients (26 non-ICU and 20 ICU; Supplementary Figure 1). Patient characteristics in the 2 cohorts were generally well matched (Supplementary Table 2). However, the discovery cohort contained relatively fewer ICU patients than the validation cohort (17.8% vs 43.5%, respectively, P = .002).

After quality control, normalization of all assessed proteins for all samples, and correction for age and sex as covariates, 40 proteins were found to be significantly higher in ICU patients versus non-ICU patients, whereas 24 were significantly lower (false discovery rate < 0.05; Figure 2A). In the validation cohort, this was the case for 19 and 30 proteins, respectively. Overlap analysis and correcting P values for multiple testing revealed 27 proteins overlapping between the 2 cohorts that were significantly differentially expressed in ICU versus non-ICU patients (Figure 2B). Among these, hepatocyte growth factor (HGF; $\log_2$ fold change [logFC], 1.39; adjusted P value = 1.19×10^{-6}), chemokine (C-C motif) ligand 20 (CCL20; logFC, 1.41; adjusted P value = 5.48×10^{-5}), and IL-6 (logFC, 1.44; adjusted P value = 1.61×10^{-3}) were upregulated most strongly and most significantly in ICU patients as compared to non-ICU patients. In contrast, stem cell factor (SCF; logFC, −1.43; adjusted P value = 3.14×10^{-7}), delta and notch-like epidermal growth factor-related receptor (DNER; logFC, −0.49; adjusted P value 3.38×10^{-6}), vascular endothelial growth factor D (VEGFD; logFC, −0.80; adjusted P value 4.0610×10^{-6}), and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL; logFC, −0.63; adjusted P value 1.61×10^{-4}) were most significantly downregulated in critically ill versus less severely ill COVID-19 patients.

Table 1. Patient Characteristics at Hospital Admission

Characteristic	Total (n = 147)	Non-ICU[a] (n = 109)	ICU[a] (n = 38)	*P* Value (Non-ICU vs ICU)
Age, y	66 (54–73)	66 (52–73)	67 (57–73)	.945
Male sex, n (%)	99 (67)	71 (65)	28 (74)	.333
BMI, kg/m^2	26.9 (23.8–29.3)	26.5 (23.7–29.3)	27.6 (25.0–29.9)	.342
BMI > 30 kg/m^2, n (%)	30 (21.1)	22 (21.2)	8 (21.1)	.9896
Time from first COVID-19 symptoms to hospital admission, d	7 (5–10)	7 (5–10)	5 (6–10)	.770
Time from hospital admission to plasma sampling, d	3 (2–4)	2 (2–3)	4 (3–6)	<.001
PCR-proven COVID-19, n (%)	138 (94)	103 (94)	35 (92)	.6956
Comorbidities				
Hematological malignancy	13 (8.8)	10 (9.2)	3 (7.9)	1.000
Solid organ malignancy	31 (21.1)	23 (21.1)	8 (21.1)	.995
SCT	6 (4.1)	5 (4.6)	1 (2.6)	1.000
SOT	7 (4.8)	7 (6.4)	0 (0)	.191
Pulmonary disease, including COPD	35 (23.8)	31 (28.4)	4 (10.5)	.028
Cardiovascular disease, including hypertension	84 (57.1)	64 (58.7)	20 (52.6)	.514
Hypertension	57 (38.8)	41 (37.6)	16 (42.1)	.625
Diabetes mellitus	31 (21.1)	21 (19.3)	10 (26.3)	.359
CKD, requiring RRT	1 (0.7)	1 (0.9)	0 (0)	1.000
CKD, no RRT	9 (6.1)	9 (8.3)	0 (0)	.112
Autoimmune disease, including IBD	23 (15.6)	22 (20.2)	1 (2.6)	.009
HIV/AIDS	1 (0.7)	1 (0.9)	0 (0)	1.000
Liver disease	6 (4.1)	6 (5.5)	0 (0)	.339
Other	130 (88.4)	101 (92.7)	29 (76.3)	.007
WBC, × 10^9/L	7.1 (5.2–9.3)	6.9 (4.5–9.2)	7.5 (6.0–10.3)	.180
Neutrophils, × 10^9/L	5.6 (3.5–7.7)	5.6 (3.3–8.0)	5.8 (4.4–7.4)	.741
Lymphocytes, × 10^9/L	0.8 (0.5–1.1)	0.7 (0.5–1.1)	0.8 (0.4–1.2)	.815
Monocytes, × 10^9/L	0.4 (0.3–0.7)	0.4 (0.3–0.8)	0.4 (0.2–0.5)	.242
CRP, mg/L	82 (44–151)	72 (40–115)	139 (87–225)	<.001
Ferritin, µg/L	795 (377–1468)	785 (378–1385)	1025 (227–2157)	.414
D-dimer, ng/mL	870 (533–1733)	890 (525–1875)	650 (370–1995)	.649
CO-RADS	5 (5–6)	5 (5–6)	5 (5–6)	.144
CT severity score	12 (9–15)	12 (9–15)	17 (13–19)	.002

Data are presented as median with interquartile range (IQR) or n (%).

Abbreviations: BMI, body mass index; CKD, chronic kidney disease; COPD, chronic obstructive pulmonary disease; CO-RADS, Dutch COVID-19 reporting and data system; COVID-19, coronavirus disease 2019; CRP, C-reactive protein; CT, computed tomography; HIV, human immunodeficiency virus; IBD, inflammatory bowel disease; ICU, intensive care unit; PCR, polymerase chain reaction; RRT, renal replacement therapy; SCT, stem cell transplantation; SOT, solid organ transplantation; WBC, white blood cell count.

[a]Classification based on the location of the patient at the time of plasma sampling.

Receiver-Operating Characteristic Analyses

ROC analyses for discriminating ICU from non-ICU patients were performed on differential expression of HGF and SCF and compared to CRP, D-dimer, ferritin, and plasma concentrations of IL-6, as determined by ELISA. Comparison of the area under the curve (AUC) for CRP (0.8724), D-dimer (0.8206), ferritin (0.6684), IL-6 (0.8797), and differential expression of HGF (0.8696) and SCF (0.8385) revealed that the latter 3 demonstrated strong potential in discriminating disease severity, with AUC values similar to those for CRP and D-dimer and higher than that of ferritin (Supplementary Figure 3).

Clustering Analysis of Differential Protein Expression

To identify any potential inflammatory endotypes in COVID-19, unsupervised hierarchical clustering analysis was performed on those proteins that were significantly differentially expressed between ICU and non-ICU patients in the discovery cohort. This revealed no significantly different protein signatures between patients: no specific inflammatory endotypes were identified (Figure 3).

Cytokine Production Capacity and Flow Cytometry

Immune cells' cytokine production and markers of activation and exhaustion were assessed in patients with COVID-19 and compared to healthy controls (HCs). In general, immune cells of COVID-19 patients did not demonstrate significant differences in the production capacity of TNF-α, IL-1β, IL-6, and IL-1Ra as compared to HCs upon stimulation with LPS or *C. albicans* (Figure 4A–4D). However, ICU patients demonstrated a significantly lower TNF-α response upon stimulation with *C. albicans* as compared to HCs (HC median, 2198.0 pg/mL [interquartile range, IQR, 1427.0–3002.0 pg/mL] vs ICU median, 430.9 pg/mL [IQR, 174.3–1140.0 pg/mL] vs non-ICU median, 441.2 pg/mL [IQR, 319.9–1955 pg/mL]; HC vs ICU $P = .0231$; Figure 4A). No significant differences were observed between ICU and non-ICU patients.

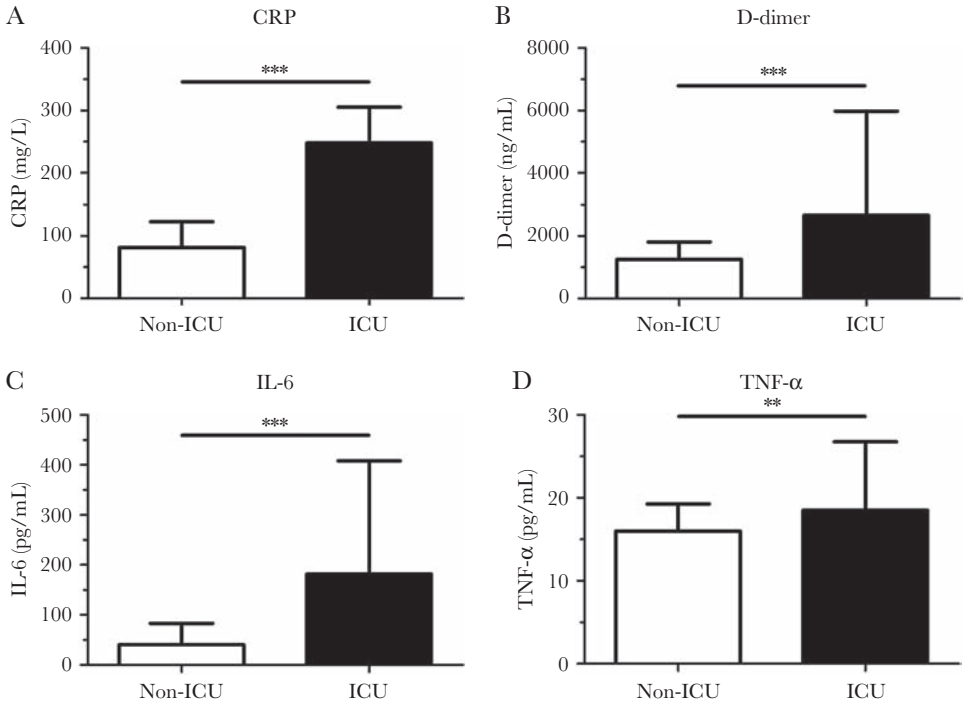

Figure 1. Critically ill COVID-19 patients demonstrated higher levels of inflammatory markers than severely ill patients. *A*, CRP concentrations were significantly higher in COVID-19 patients admitted to the ICU (median, 248 mg/L; IQR, 149–306 mg/L) compared to those admitted to the ward (median, 82 mg/L; IQR, 47–123 mg/L; $P < .0001$) at the time of plasma sampling (non-ICU n = 108; ICU n = 37). This pattern was also observed for (*B*) D-dimer concentrations (ICU median, 2665 ng/mL; IQR 1780–5978 ng/mL vs non-ICU median 1250 ng/mL; IQR, 785–1810 ng/mL, $P < .0001$; non-ICU n = 93; ICU n = 36), all of which were determined in the course of routine clinical care. *C*, Measurements of circulating IL-6 in patient plasma by ELISA revealed significantly higher concentrations in COVID-19 patients admitted to the ICU than in patients admitted to the ward (median, 182.0 pg/mL; IQR, 90.25–408.0 pg/mL vs 40.0 pg/mL; IQR, 23.0–83.3 pg/mL, respectively; $P < .0001$; non-ICU n = 102; ICU n = 33). *D*, Measurements of circulating TNF-α levels in plasma demonstrate significantly higher concentrations in ICU patients as compared to non-ICU patients (ICU median, 18.5 pg/mL; IQR, 16.0–26.8 pg/mL vs non-ICU median, 16.0 pg/mL; IQR, 16.0–19.3 pg/mL; $P = .0082$; non-ICU n = 98; ICU n = 30). Data are median with IQR. Non-ICU and ICU groups were compared using Mann-Whitney *U* test. $^{**}P < .01$. $^{***}P < .0001$. Abbreviations: COVID-19, coronavirus disease 2019; CRP, C-reactive protein; ELISA, enzyme-linked immunosorbent assay; ICU, intensive care unit; IL-6, interleukin-6; IQR, interquartile range; TNF-α, tumor necrosis factor-α.

In contrast, after 7 days of incubation with *C. albicans*, patients' PBMCs displayed a severely defective production of the T helper lymphocyte-derived cytokines IFN-γ (median concentrations in HC, non-ICU, and ICU were 133.6 pg/mL, 37.2 pg/mL, and 19.5 pg/mL, respectively), IL-17 (median concentrations in HC, non-ICU, and ICU were 693.4 pg/mL, 532.1 pg/mL, and 80.2 pg/mL, respectively) and IL-22 (median concentrations in HC, non-ICU, and ICU were 2604.0 pg/mL, 627.9 pg/mL, and 156.0 pg/mL, respectively; Figure 5A–5C), with trends towards lower cytokine production in the more severely ill (ICU) patients. Of note, the percentage of lymphocytes within the PBMC fraction differed significantly among groups (median, 79.5% in HC, 51.2% in non-ICU, and 35.6% in ICU) and the percentage of neutrophils in this fraction was significantly higher in the ICU group versus HC group (median, 27.2% and 0.8%, respectively; Figure 5D). Furthermore, the percentage of monocytes was significantly different between HC and non-ICU patients, but not between HC and ICU patients (median, 18.4% in HC, 39.8% in non-ICU, and 30.7% in ICU).

Flow cytometry of whole-blood samples from a subset of patients and HCs demonstrated significantly lower HLA-DR expression on $CD14^+$ monocytes in ICU patients as compared to non-ICU patients and HCs (median mean fluorescent intensity area values, 15 794, 30 825, and 33 039, respectively; Figure 6A). Although most patients demonstrated values in the lower normal range or just below, none displayed values comparable to those seen in patients with bacterial sepsis [14]. CD279 (also known as programmed cell death protein 1, PD-1) expression on $CD4^+$ T cells, ranging from naive to effector memory cells reexpressing CD45RA (TEMRA), however, was significantly upregulated in both non-ICU and ICU patients as compared to HCs (Figure 6B–6C), indicative of lymphocyte exhaustion (median percentage of TEMRA $CD4^+$ cells positive for CD279 in HC, 1.9%; non-ICU, 14.7%; and ICU, 19.1%).

DISCUSSION

Our study provides several important findings. Our comprehensive approach demonstrates that critically ill COVID-19 patients are characterized by higher plasma concentrations of CRP, D-dimer, ferritin, IL-6, and TNF-α compared to less severely ill patients. In parallel, in-depth analysis of differential protein expression highlights several potential biomarkers of

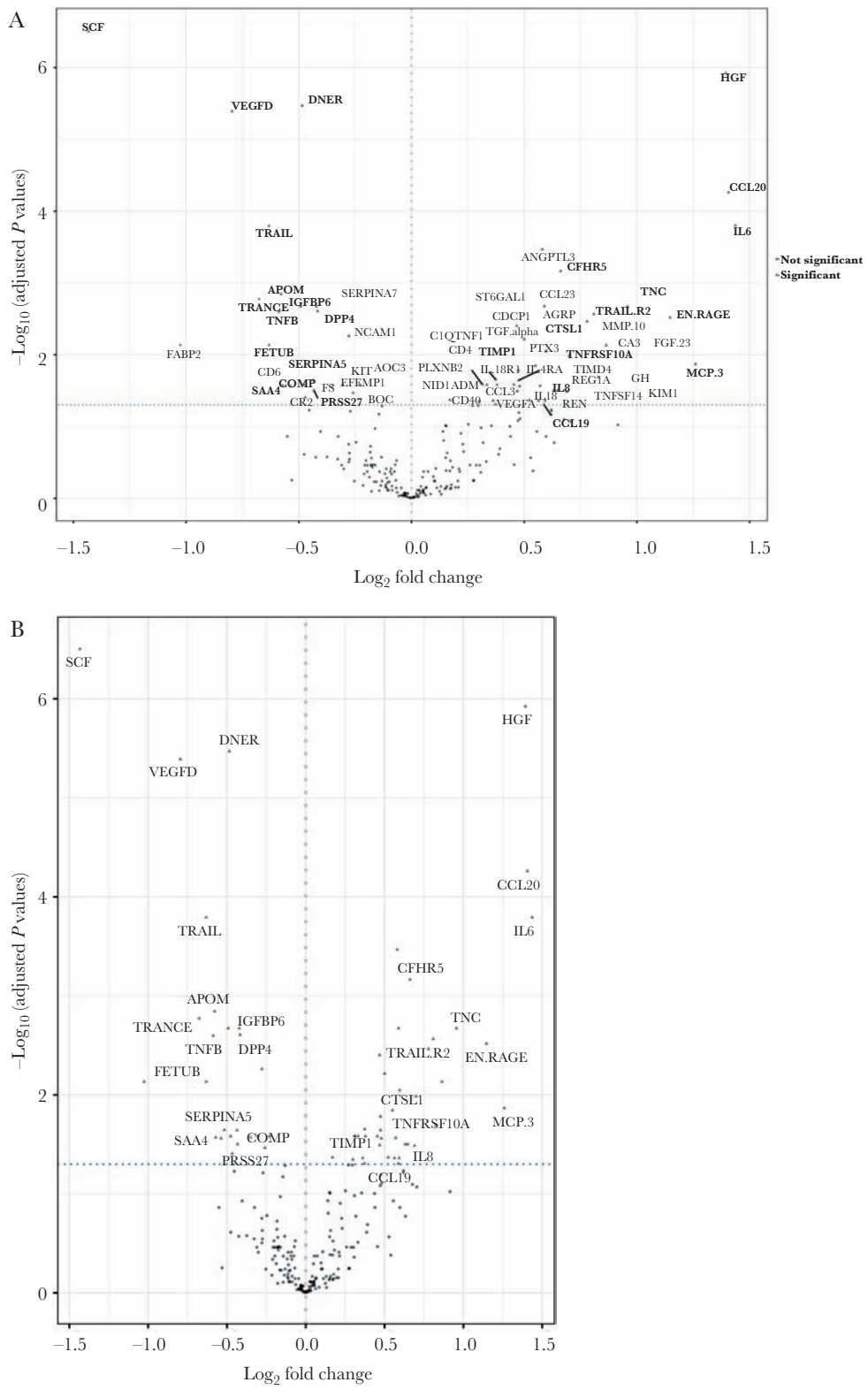

Figure 2. Proximity extension assay demonstrates differential protein expression in plasma according to COVID-19 disease severity. *A*, Volcano plot of differential expression of proteins in the discovery cohort (n = 101), with age and sex included as covariates. Results from all 3 employed protein panels are displayed (Inflammation, Cardiometabolic, and Cardiovascular II panel) as $\log_2$ fold change of expression in ICU patients compared to non-ICU patients, plotted against adjusted *P* values. Proteins significantly differentially expressed in both the discovery and validation cohort are displayed in bold. *B*, After overlap analysis of differential protein expression in both the discovery and validation cohorts, 27 proteins were significantly up- or downregulated in ICU patients, as compared to non-ICU patients with adjusted *P* values < .05 after correction for multiple testing. Most significantly upregulated proteins were HGF, CCL20, and IL-6; most significantly downregulated proteins were SCF, DNER, VEGFD, and TRAIL. Abbreviations: CCL20, chemokine (C-C motif) ligand 20; COVID-19, coronavirus disease 2019; DNER, delta and notch-like epidermal growth factor-related receptor; HGF, hepatocyte growth factor; ICU, intensive care unit; IL-6, interleukin-6; SCF, stem cell factor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; VEGFD, vascular endothelial growth factor D.

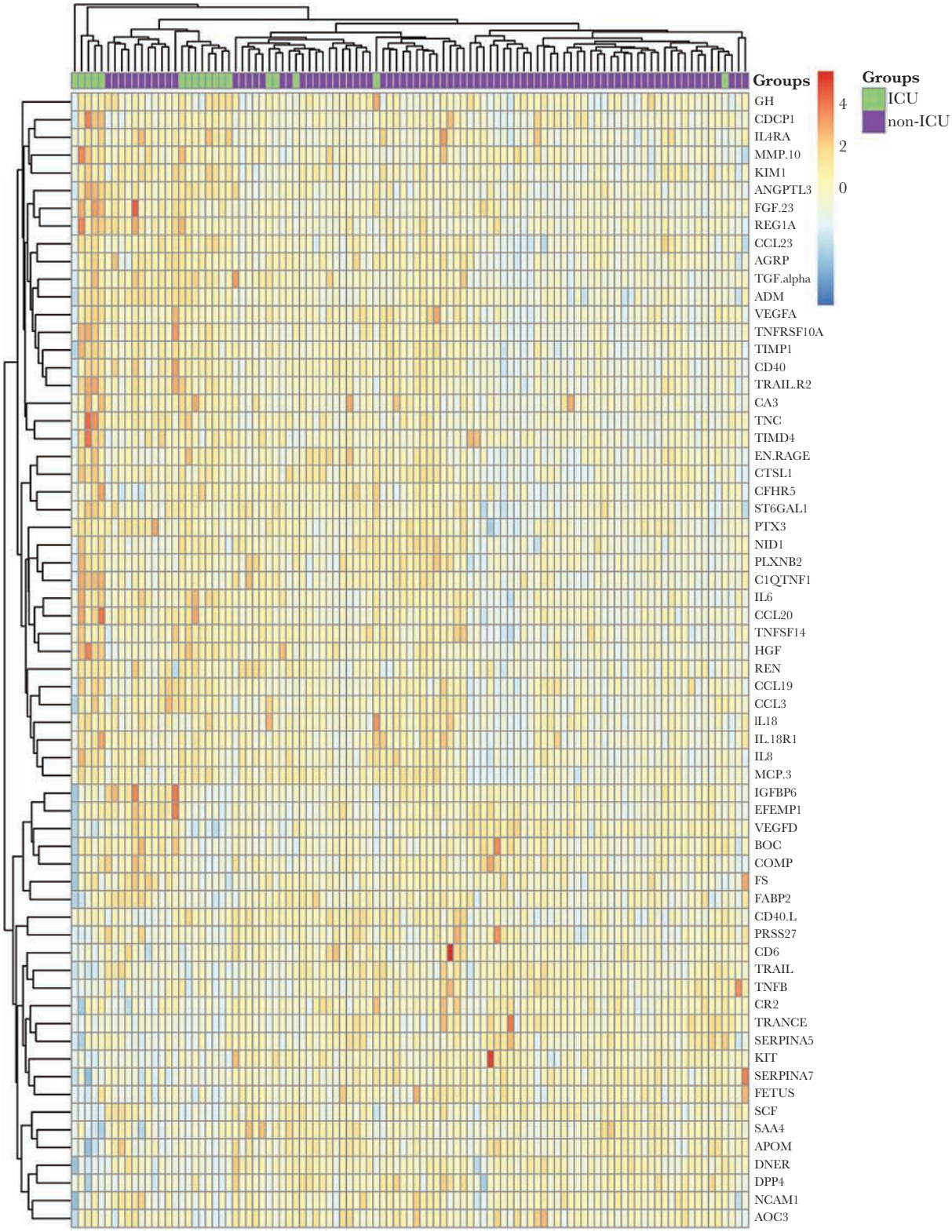

Figure 3. Heatmap of significantly differentially expressed proteins demonstrates no specific inflammatory endotypes. Unsupervised hierarchical clustering of significantly differentially expressed proteins after multiple testing in the discovery cohort does not identify specific patient endotypes. Abbreviation: ICU, intensive care unit.

disease severity. Of these, HGF and SCF can differentiate between critical and severe illness with approximately equal discriminatory performance as CRP, D-dimer, and circulating IL-6, and better performance than ferritin. Next, clustering analysis of differential protein expression demonstrates that patients do not form clusters based on specific inflammatory

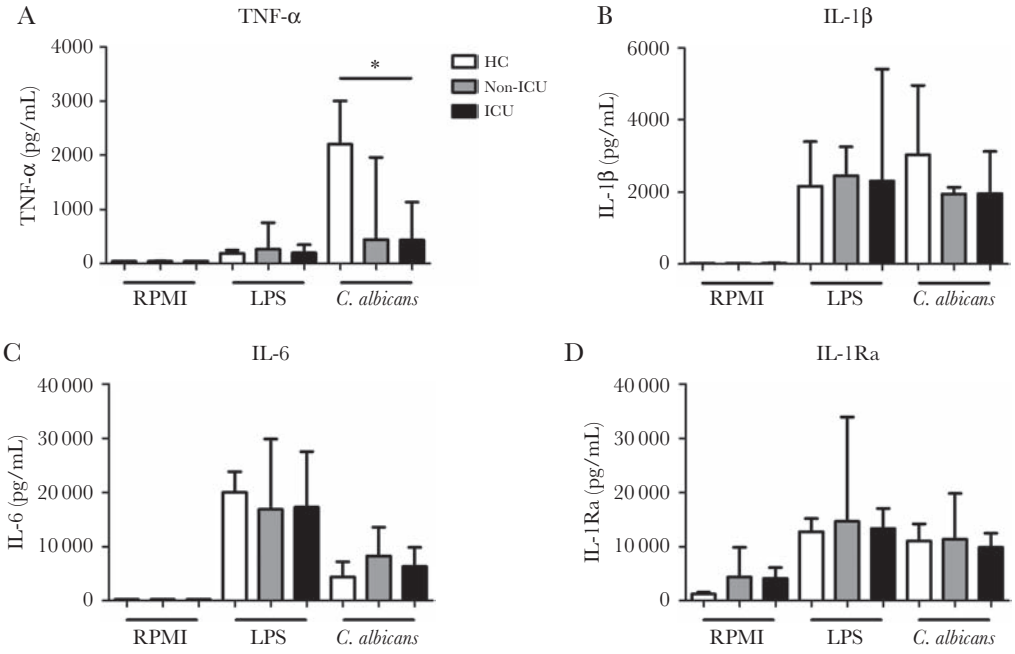

Figure 4. Innate cytokine production in COVID-19. Production of the innate cytokines TNF-α (*A*), IL-1β (*B*), IL-6 (*C*), and IL-1Ra (*D*) was not significantly different between healthy controls and COVID-19 patients admitted to non-ICU wards or ICU after stimulation with LPS or heat-killed *Candida albicans* yeast cells, with the exception of TNF-α production in response to *C. albicans* stimulation (*A*). Cytokine concentration values are provided in Supplementary Table 3. HC n = 8; non-ICU n = 8; ICU n = 9; for IL-1Ra RPMI, non-ICU n = 5; ICU n = 6. Data are median with IQR. All groups were compared using Kruskal-Wallis test with Dunn's post test comparing all pairs of columns. **P* < .05. Abbreviations: COVID-19, coronavirus disease 2019; HC, healthy controls; ICU, intensive care unit; IL, interleukin; IQR, interquartile range; LPS, lipopolysaccharide; RPMI, RPMI 1640 Dutch modified culture medium; TNF-α, tumor necrosis factor-α.

endotypes. Furthermore, patients' innate immune cells show equal or even higher proinflammatory cytokine production after ex vivo stimulation, whereas adaptive cytokine production is significantly decreased in a seemingly severity-dependent manner. Moreover, patients' $CD4^+$ T cells display increased expression of PD-1, a marker of apoptosis and T-cell exhaustion. On the other hand, HLA-DR expression on monocytes is significantly lower than in healthy controls. Collectively, all these findings point towards a general concept of a homogeneous inflammatory state in patients with COVID-19, combined with compromised T-cell immune responses.

The observed relationship between the degree of elevation of proinflammatory markers and disease severity is in line with previous studies and has been recognized early in the COVID-19 pandemic [2, 5–7]. One may argue that differences in severity might be attributed to longer disease duration, as time from hospital admission to blood sampling differed between these 2 groups (4 days in ICU vs 2 days in non-ICU patients). However, while this may have been a potential confounder theoretically, our population shows remarkable consistency in disease severity over time: only 3/109 non-ICU patients made a transition to the ICU during study follow-up, indicating a deterioration in disease severity. In-depth proteomics analysis revealed a multitude of potential markers of disease severity, confirming that IL-6 is strongly upregulated in critically ill patients. Among these, prominently upregulated proteins include HGF and CCL20. Other studies have also shown increased HGF plasma concentrations in COVID-19 related to disease severity [15, 16] and have demonstrated upregulated expression of HGF induced by proinflammatory cytokines [17] and in viral infections such as hepatitis B [18]. CCL20 is a chemokine with a strong chemotactic effect on lymphocytes [19] and is also upregulated by proinflammatory cytokines. Therefore, its upregulation in critical COVID-19 might serve as a compensatory mechanism for lymphopenia, because multiple studies demonstrate a correlation between disease severity and degree of lymphopenia [2, 8]. In contrast, CCL20 upregulation might cause lymphocyte abundance in the tissues [20], leading to lymphocyte depletion in the peripheral blood.

In our proteomics analysis, SCF was most strongly downregulated. This ligand of the c-Kit receptor is a crucial factor in maintaining hematopoietic stem cells and lymphoid progenitor cells [21, 22]. Furthermore, a positive correlation between SCF and specific COVID-19 neutralizing antibody titers has recently been demonstrated [23]. One could speculate that reduced SCF expression might contribute to the observed lymphopenia in COVID-19 and lower antibody titers.

Additional ex vivo functional analysis confirmed an enhanced innate cytokine response in COVID-19 patients (with

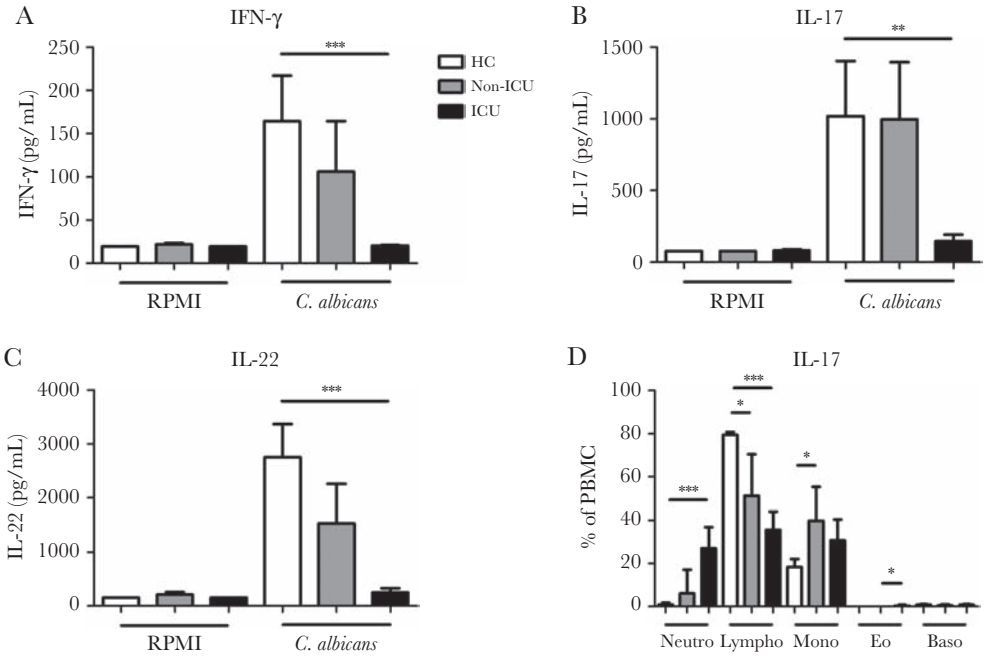

Figure 5. Adaptive cytokine production in COVID-19. Production of the lymphocyte-derived cytokines IFN-γ (*A*), IL-17 (*B*), and IL-22 (*C*) in response to *Candida albicans* was severely abrogated in COVID-19 patients as compared to healthy controls. Furthermore, a trend of less cytokine production with increasing disease severity was observed for IFN- γ (*A*) and IL-22 (*C*). Cytokine concentration values are provided in Supplementary Table 4. HC n = 8; non-ICU n = 8; ICU n = 10. *D*, Cellular composition of the PBMC fraction after differential density gradient centrifugation differed between healthy controls, non-ICU, and ICU COVID-19 patients with regard to the percentage of neutrophils, lymphocytes, monocytes, and eosinophils. Cellular fraction values are provided in Supplementary Table 5. HC n = 8; non-ICU n = 8; ICU n = 10. Data are median with IQR. All groups were compared using Kruskal-Wallis test with Dunn's post test comparing all pairs of columns. $^{*}P < .05$. $^{**}P < .01$. $^{***}P < .001$. Abbreviations: Baso, basophilic granulocytes; COVID-19, coronavirus disease 2019; Eo, eosinophilic granulocytes; HC, healthy controls; ICU, intensive care unit; IFN-γ, interferon-γ; IL, interleukin; IQR, interquartile range; Lympho, lymphocytes; Mono, monocytes; Neutro, neutrophilic granulocytes; PBMC, peripheral blood mononuclear cell; RPMI, RPMI 1640 Dutch modified culture medium.

the exception of the TNF-α response to *C. albicans*), and monocytic HLA-DR expression was only mildly decreased as opposed to the severe immune suppression seen in bacterial sepsis [14]. However, adaptive cytokine production was severely abrogated with apparent correlations with disease severity. This is in line with a recent study demonstrating impaired IFN-γ production in lymphocytes after stimulation with anti-CD3/anti-CD28 [24]. Adaptive immune system dysfunction was further supported by findings of increased expression of PD-1 on $CD4^{+}$ T lymphocytes, consistent with previous data [10, 25]. In conclusion, our findings point towards a disease state characterized by a hyperinflammatory innate immune system and a defective adaptive immune system due to profound lymphopenia, exhausted T cells, and decreased functionality. These findings are supported by a very recent study on the systems biology of severe versus mild COVID-19 patients, which has also shown a combination of increased systemic inflammation, low HLA-DR expression on monocytes, and a defective interferon pathway [26].

Our study has several limitations. First, due to the explorative nature of our study, direct conclusions on causality between immunological profiles and disease severity cannot be inferred. However, we identified several potential biomarkers of severity, warranting further investigation, especially regarding their possible pathophysiological role in disease course and severity. Second, plasma sampling was not performed at predefined time points as a consequence of this study's pragmatic design. Differences in hospitalization duration at the time of sampling might have influenced our results. Additionally, no correction for other potential confounders, such as comorbidity and medication use, was performed due to this approach. Third, we classified disease severity according to admission to ICU versus non-ICU wards, which might differ from classifications employed by other studies. Fourth, in the proteomics analysis, patient assignment to the discovery or validation cohort was not completely random, but instead ultimately based on the date of plasma donation. Although introduction of bias due to this approach cannot be excluded, the fact that no significant changes in diagnostic work-up or therapeutic management of COVID-19 occurred between the sample collection periods of either cohort appears to minimize this risk. Last, in our ex vivo stimulation experiments, cellular composition of the PBMC fraction differed between the groups. Contamination with low-density granulocytes after Ficoll density centrifugation has been described before in sepsis [27]. To what degree cytokine production was influenced by these differences is unclear. As

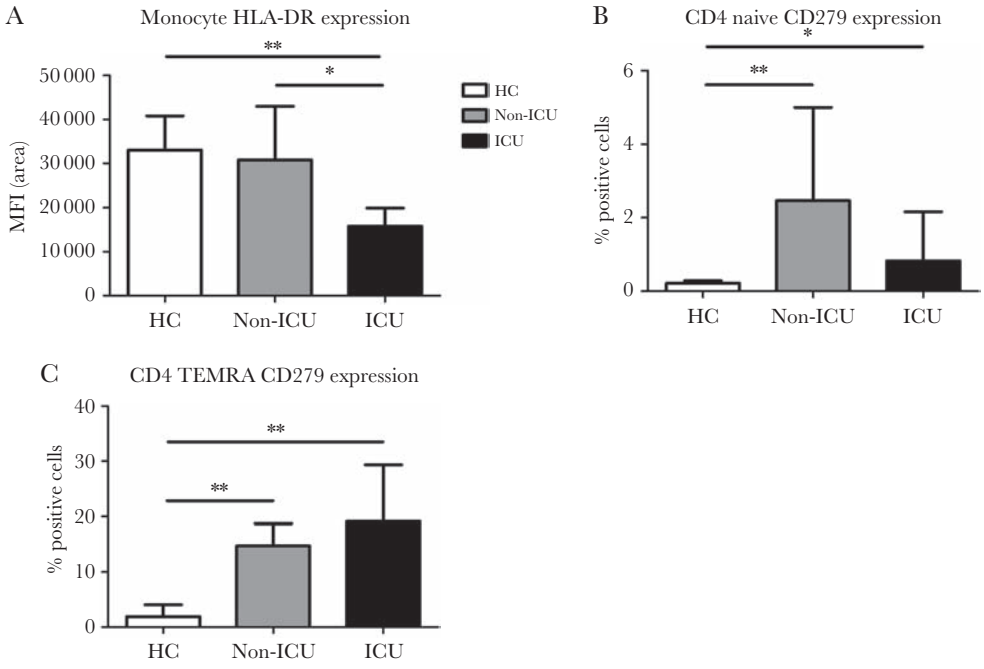

Figure 6. Monocyte HLA-DR expression and CD4$^+$ lymphocyte CD279 expression in COVID-19. *A*, CD14$^+$ Monocytes obtained from ICU COVID-19 patients displayed significantly lower expression of HLA-DR by flow cytometry than healthy controls and non-ICU COVID-19 patients. HC n = 5; non-ICU n = 11; ICU n = 7. *B* and *C*, CD279 (PD-1) was expressed on a significantly higher percentage of CD4$^+$ lymphocytes from COVID-19 patients admitted to non-ICU wards or ICU than in healthy controls, as shown for naive (*B*) and terminally differentiated (*C*) CD4$^+$ lymphocytes. HLA-DR and CD279 expression data are provided in Supplementary Table 6. HC versus non-ICU $P = .0059$ and HC versus ICU $P = .0021$. HC n = 10; non-ICU n = 11; ICU n = 7. Data are median with IQR. All groups were compared using Kruskal-Wallis test with Dunn's post test comparing all pairs of columns. $^*P < .05$. $^{**}P < .01$. Abbreviations: CD, cluster of differentiation; COVID-19, coronavirus disease 2019; HC, healthy controls; ICU, intensive care unit; IQR, interquartile range; MFI, mean fluorescent intensity; PD-1, programmed cell death protein 1; TEMRA, T effector memory cell reexpressing CD45RA.

the experiments were performed with PBMCs isolated from EDTA blood, and depletion of intracellular calcium has been suggested to impact cytokine production capacity, an impact on the overall strength of cytokine production capacity cannot be fully excluded, although potent cytokine release was observed in this study.

In conclusion, our integrated and extensive approach demonstrates essential differences in innate and adaptive immune responses between severely and critically ill COVID-19 patients, presenting potential biomarkers of disease severity and elucidating its pathobiology. It further highlights a severely dysfunctional adaptive immune response, in the presence of a hyperinflammatory innate immune system. Further investigations of the crosstalk between innate and adaptive immunity in COVID-19 and their relationship with disease severity are highly warranted.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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