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Compartmental immunophenotyping in COVID-19 ARDS: A case series

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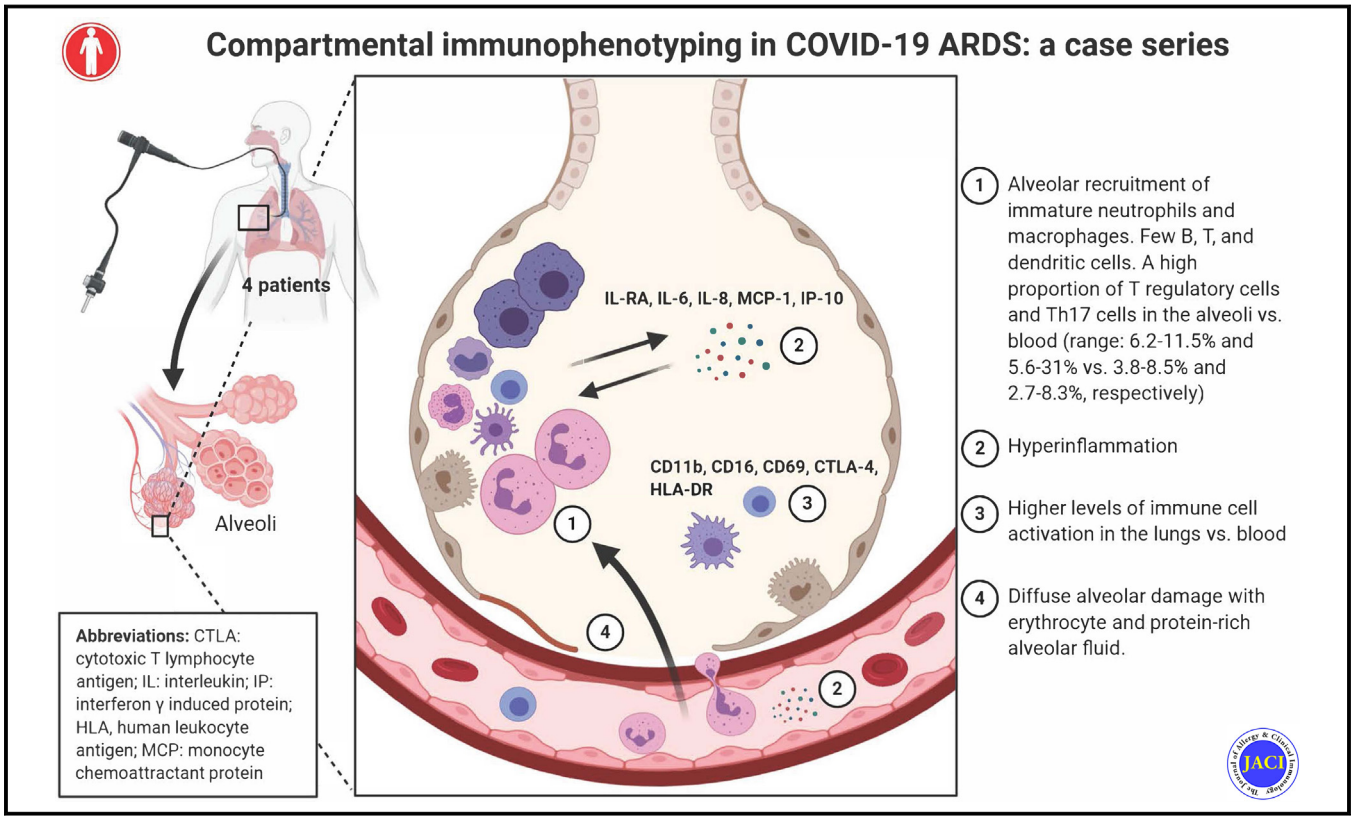
GRAPHICAL ABSTRACT



Background: Severe immunopathology may drive the deleterious manifestations that are observed in the advanced stages of coronavirus disease 2019 (COVID-19) but are poorly understood. Objective: Our aim was to phenotype leukocyte subpopulations and the cytokine milieu in the lungs and blood of critically ill patients with COVID-19 acute respiratory distress syndrome (ARDS).

Methods: We consecutively included patients less than 72 hours after intubation following informed consent from their next of kin. Bronchoalveolar lavage fluid was evaluated by microscopy; bronchoalveolar lavage fluid and blood were assessed by 10-color flow cytometry and a multiplex cytokine panel.

Results: Four mechanically ventilated patients (aged 40-75 years) with moderate-to-severe COVID-19 ARDS were

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This study was supported by the Lundbeck Foundation (grant R349-2020-540).

Disclosure of potential conflict of interest: T. Benfield reports personal fees and nonfinancial support from Bristol-Myers Squibb and Gilead. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication June 17, 2020; revised August 27, 2020; accepted for publication September 3, 2020.

Available online October 23, 2020.

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0091-6749/$36.00

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https://doi.org/10.1016/j.jaci.2020.09.009

included. Immature neutrophils dominated in both blood and lungs, whereas CD4 and CD8 T-cell lymphopenia was observed in the 2 compartments. However, regulatory T cells and T_H17 cells were found in higher fractions in the lung. Lung CD4 and CD8 T cells and macrophages expressed an even higher upregulation of activation markers than in blood. A wide range of cytokines were expressed at high levels both in the blood and in the lungs, most notably, IL-1RA, IL-6, IL-8, IP-10, and monocyte chemoattactant protein-1, consistent with hyperinflammation.

Conclusion: COVID-19 ARDS exhibits a distinct immunologic profile in the lungs, with a depleted and exhausted CD4 and CD8 T-cell population that resides within a heavily hyperinflammatory milieu. (J Allergy Clin Immunol 2021;147:81-91.)

Key words: *Acute respiratory distress syndrome, bronchoalveolar lavage, COVID-19, cytokines, flow cytometry*

In December 2019, a cluster of pneumonia cases with unknown cause was reported in Wuhan in the Hubei Province of China.[1] A novel coronavirus was identified in January as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2),[2] and as of July 29, 2020, nearly 17 million cases of corona virus disease 2019 (COVID-19) with more than 660,000 deaths had been confirmed worldwide.[3] Although recent results support the use of an RNA polymerase inhibitor (remdesivir) and low-dose dexamethasone in hospitalized patients requiring oxygen therapy,[4,5] the mortality of critically ill patients with COVID-19 remains high.[6,7]

A subgroup of patients with COVID-19 progress to severe respiratory failure mimicking acute respiratory distress syndrome (ARDS).[8,9] These patients tend to be older and have more comorbidity than their nonprogressing counterparts,[10] but the underlying pathways leading to increased disease severity are incompletely understood. An important clinical hallmark of invasive disease is lymphopenia, and it has thus been suggested that a severe course of disease is triggered by a dysregulated innate and adaptive immune response and hyperinflammation.[10] Clinical trials of immunomodulatory treatment are currently pending, and to aid this agenda a better understanding of the host immune response to SARS-CoV-2 is warranted.

Although the available evidence favors the presence of severe immunopathology during COVID-19 ARDS, the compartmental transmission of immunoinflammatory processes between blood and lungs remains unexplored, and characterization of distinct leukocyte subpopulations and their cytokine mediators could thus potentially reveal both novel therapeutic targets and guide the timing of treatment. In the present study, we immunophenotyped bronchoalveolar lavage fluid (BALF) and blood of mechanically ventilated patients with moderate-to-severe COVID-19 ARDS. Myeloid cell population as well as T- and B-cell subsets were characterized with focus on cellular differentiation and activation. We further studied a wide spectrum of inflammatory mediators and performed a detailed microbiologic analysis.

Abbreviations used

ARDS:	Acute respiratory distress syndrome
BALF:	Bronchoalveolar lavage fluid
COVID-19:	Coronavirus disease 2019
CTLA-4:	Cytotoxic T lymphocyte–associated protein
ICU:	Intensive care unit
IP-10:	IFN-γ–inducible protein-10
MCP-1:	Monocyte chemoattractant protein-1
PD1:	Programmed cell death protein-1
RTE:	Recent thymic emigrant
SARS-CoV-2:	Severe acute respiratory syndrome coronavirus 2
Treg:	Regulatory T

METHODS

Study population

For inclusion in this cross-sectional study, participants had to be adults (≥18 years) with SARS-CoV-2 confirmed by PCR, with presence of ARDS determined according to the Berlin criteria[11] and less than 72 hours of mechanical ventilation after admittance to the intensive care unit (ICU). All patients were sedated and unable to provide informed consent, which was obtained from their next of kin. The study was approved by the Regional Ethics Committee of Copenhagen (H-20023159) and the Knowledge Center for Data Review of Copenhagen (P-2020-399) and registered at ClinicalTrials.gov (NCT04354584). A Consolidated Standards of Reporting Trials checklist and flow diagram are provided in Table E1 and Fig E1 (both of which are available in the Online Repository at www.jacionline.org), respectively.

Setting

Approximately 2294 patients with COVID-19 have been hospitalized in Denmark (5.8 million inhabitants), with 348 requiring treatment in an ICU as of June 4, 2020.[12] At our hospital (Hvidovre Hospital), a total of 33 patients with severe COVID-19 were treated in the ICU, with all except 1 requiring mechanical ventilation. Of the 17 eligible patients admitted during the study inclusion period, 4 were included in our study. Additional details on study inclusion are provided in the Online Data Supplement in this article's Online Repository (available at www.jacionline.org). We originally intended to include a total of 20 patients, but because of rapidly decreasing admission rates and as this was a descriptive immunopathologic study, we chose to disseminate the findings already at hand.

Bronchoalveolar lavage procedure and initial sample preparation

Arterial blood samples were drawn immediately before the bronchoalveolar lavage procedure, which was performed in a standardized fashion according to current guidelines[13]; the procedure and processing of samples are described in further detail in the Online Data Supplement. In addition, BALF supernatant and EDTA-stabilized plasma were separated and stored at −80 °C for cytokine analyses. A total of 21 healthy controls (9 men and 12 women with a median age of 40 years [range 26-65 years]) were used for comparison of flow cytometry results in blood, and 15 healthy nonsmoking volunteers (all of them men, with a median age 23 years [range 20-27 years]) from a previous study were used for comparison of cytokines in the blood and BALF.[14]

The duration of the bronchoalveolar lavage procedure was between 2 and 4 minutes and there were no procedure-related complications. Bronchoscopy revealed slight erythema and friability of the mucosa and mild-to-moderate edema but no visible secretions. BALF return volumes ranged between 30 and 75 mL, and vol% recovered ranged between 20% and 50%. In all patients, the initial return was colorless and then progressively turned pinker with subsequent aliquots. None of the samples were yellowish or lactescent in color, and no clumps or debris were observed.

Bronchoalveolar lavage cytology

Five cytospins (each containing 1 to 3 drops of BALF) from each of the 4 patients were made. Two of the 5 cytospins were stained with May-Grünwald-Giemsa stain.

TABLE I. Clinical characteristics of patients with COVID-19 ARDS

Characteristic	Patient no. 1	2	3	4
Age (y)/sex	40/M	65/F	72/M	75/M
BMI (kg/m^2)	21	25	33	26
Blood type	A^+RhD^+	A^+RhD^+	A^+RhD^+	A^+RhD^+
Coexisting disorder	None	Asthma	T2D, HT	HT
Duration of symptoms before admission to hospital (d)	4	11	4	3
Symptoms at hospitalization	Dyspnea, fever, cough	Dyspnea, fever, vomiting, diarrhea	Dyspnea, fever, cough, muscle pain	Dyspnea, fever, cough
Time from hospital to ICU admission (d)	3	0	7	12
Time from ICU admission to procedure (d)	3	2	1	3
MRC score before intubation	4	4	4	4
SAPS 3	58	72	69	77
Pao_2/Fio_2 ratio at ICU admission (mm Hg)/before procedure*	98/83	104/108	125/109	190 / 70
Treatment protocols if any	20-0006 ACTT†	20-0006 ACTT†	None	COVACTA WA42380‡
Outcome§	Survived	Died	Died	Survived

ACTT, Adaptive COVID-19 Treatment Trial; *BMI*, body mass index; *COVACTA*, A Study to Evaluate the Safety and Efficacy of Tocilizumab in Patients With Severe COVID-19 Pneumonia; *F*, female; *HT*, hypertension; *M*, male; *MRC*, Medical Research Council; *Pao_2/Fio_2*, ratio of Pao_2 (mm Hg) to fractional inspired oxygen; *SAPS*, simplified acute physiology score; *T2D*, type 2 diabetes.

*Moderate ARDS corresponds to a Pao_2/Fio_2 ratio greater than 100 but less than 200, and severe ARDS corresponds to a Pao_2/Fio_2 ratio less than 100.

†Remdesivir versus placebo.

‡Tociluzumab versus placebo.

§Patients 1 and 2 were referred to another ICU with extracorporeal membrane oxygenation (ECMO) facilities within days after the procedure. Patient 1 was discharged from the hospital after 22 days of ECMO therapy followed by 30 days of hospital admission. Patient 2 was weaned off ECMO but later died due to refractory hypoxemia; patient 3 died within hours after the procedure as a result of cardiogenic shock. Patient 4 was discharged from the ICU but was still hospitalized as of June 7, 2020.

TABLE II. Laboratory data and imaging and adjuvant therapies in patients with COVID-19 ARDS

Variable	Patient no. 1	2	3	4
Laboratory data*				
Leukocyte count ($\times10^9$/L)	3.8	9.8	12.3	10.8
Neutrophil count ($\times10^9$/L)	3.1	8.6	9.9	8.0
Lymphocyte count ($\times10^9$/L)	0.34	0.65	0.92	0.94
Eosinophil count ($\times10^9$/L)	0.2	0.01	0.00	0.65
Platelet count ($\times10^9$/L)	116	225	431	185
Alanine aminotransferase (U/L)	43	115	-	91
Lactate dehydrogenase (U/L)	422	505	494	802
Bilirubin (μmol/L)	39	29	11	16
D-dimer (FEU/L)	16.5	14	-	1.8
Creatinine (μmol/L)	76	59	122	93
Lactate (mmol/L)	3.9	1.5	2.1	1.7
C-reactive protein (mg/L)	340	320	250	30
Procalcitonin (μg/L)	25.6	0.58	20.4	0.73
Plasma albumin (g/L)	34	36	32	25
BALF albumin (mg/L)	2150	726	354	420
Chest radiography findings*	Diffuse bilateral infiltrates	Diffuse bilateral infiltrates	Diffuse bilateral infiltrates	Diffuse bilateral infiltrates
Adjuvant therapies†				
Neuromuscular blockade	Yes	No	No	No
Prone position	Yes	Yes	No	No
Inhaled pulmonary vasodilator	Yes	No	Yes	Yes
Vasopressors	Yes	Yes	Yes	Yes
Renal replacement therapy	No	No	No	No
vv-ECMO	Yes	Yes	No	No
Specific characteristics of mechanical ventilation‡				
Plateau pressure (cm H_2O)	32	28	20	29
Driving pressure (cm H_2O)	17	13	10	19
Compliance (mL/cm H_2O)	19	31	50	25

FEU, Fibrinogen equivalent unit; *vv-ECMO*, venovenous extracorporeal membrane oxygenation.

*Laboratory data and chest radiography before the bronchoscopy procedure.

†Adjuvant therapies applied during the ICU stay.

‡Specific characteristics of mechanical ventilation immediately before the bronchoscopy procedure.

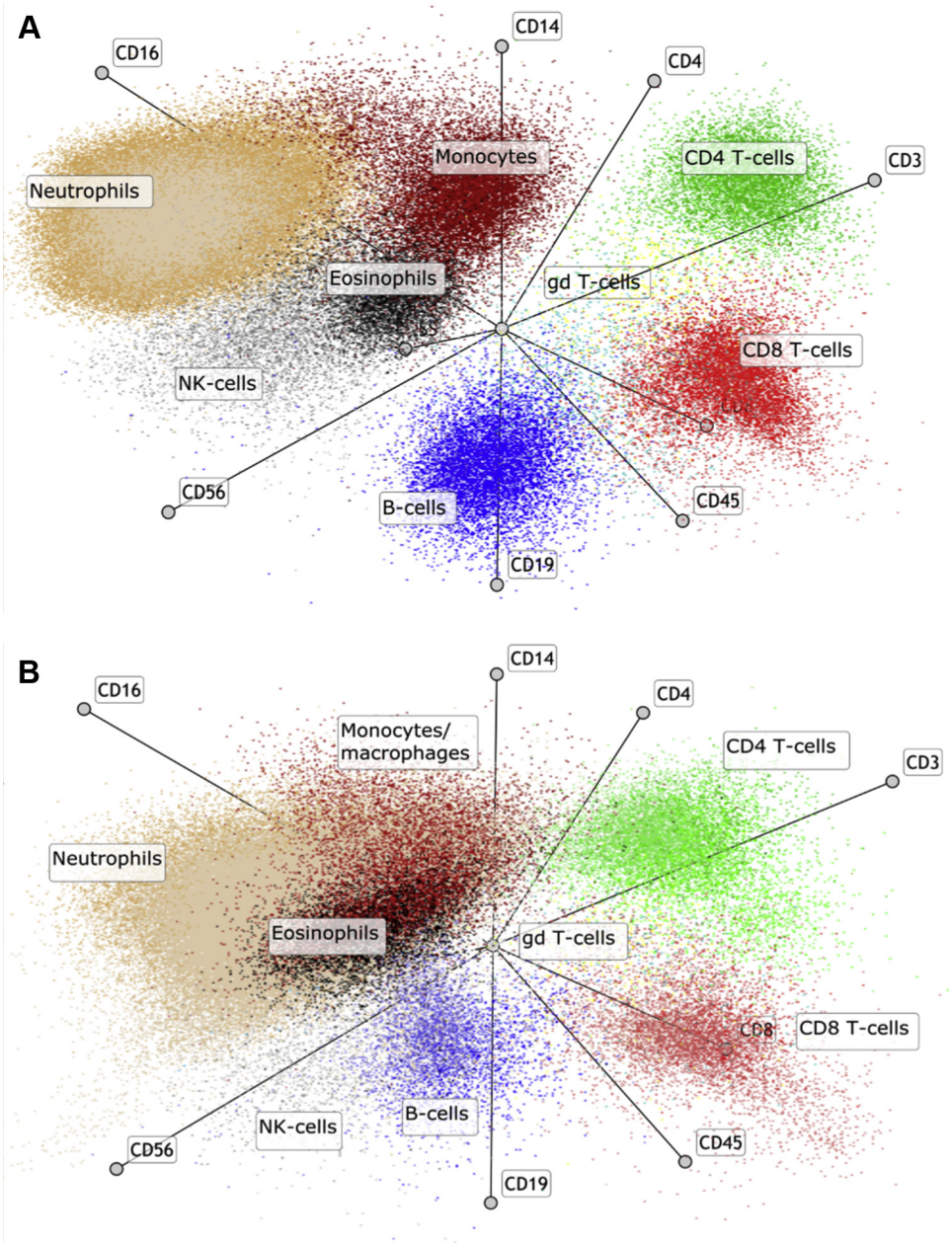

FIG 1. Radar plots of COVID-19 leukocyte subsets in blood (**A**) and BALF (**B**). Data from the same 4 patients are merged for blood (**A**) and BALF (**B**). Axis labels indicate the recognition antibody, and the cluster labels describe the gated cell population. Dim colored dots in clusters indicate activated cells (positive for CD69). BALF is dominated by myeloid cells. In healthy adults,[21] monocytes comprise 65.3% to 95.4%, lymphocytes comprise 3.0% to 32.4%, neutrocytes comprise 0.2% to 4.3%, eosinophils comprise 0.1% to 3.5%, and basophils comprise 0.0% to 0.2% of leukocytes in BALF. $CD4^+$ cells comprise 35% to 79%, $CD8^+$ cells comprise 15% to 57%, B cells comprise 0.0% to 5.3%, and natural killer cells comprise 2% to 8% of the leukocytes. *gd,* γδ.

Flow cytometry

For flow cytometry, we used a 10-color antibody panel consisting of 7 prefabricated customized DuraClone tubes from Beckman Coulter (Beckman Coulter, Brea, Calif) with freeze-dried antibodies. Additional details on flow cytometry, including use of antibodies (see Table E2 in this article's Online Repository at www.jacionline.org) and flow characterization of cell populations (see Table E3 in this article's Online Repository at www.jacionline.org), are provided in the Online Data Supplement. We identified key cell populations to be included in the study *a priori*.

Cytokine and chemokine analyses

The MSD V-Plex Human Biomarker 35 Plex Kit (Meso Scale Discovery platform, [Meso Scale Diagnostics, Rockville, Md]) was used for cytokine and chemokine quantification and run (300 μL of BALF supernatant and

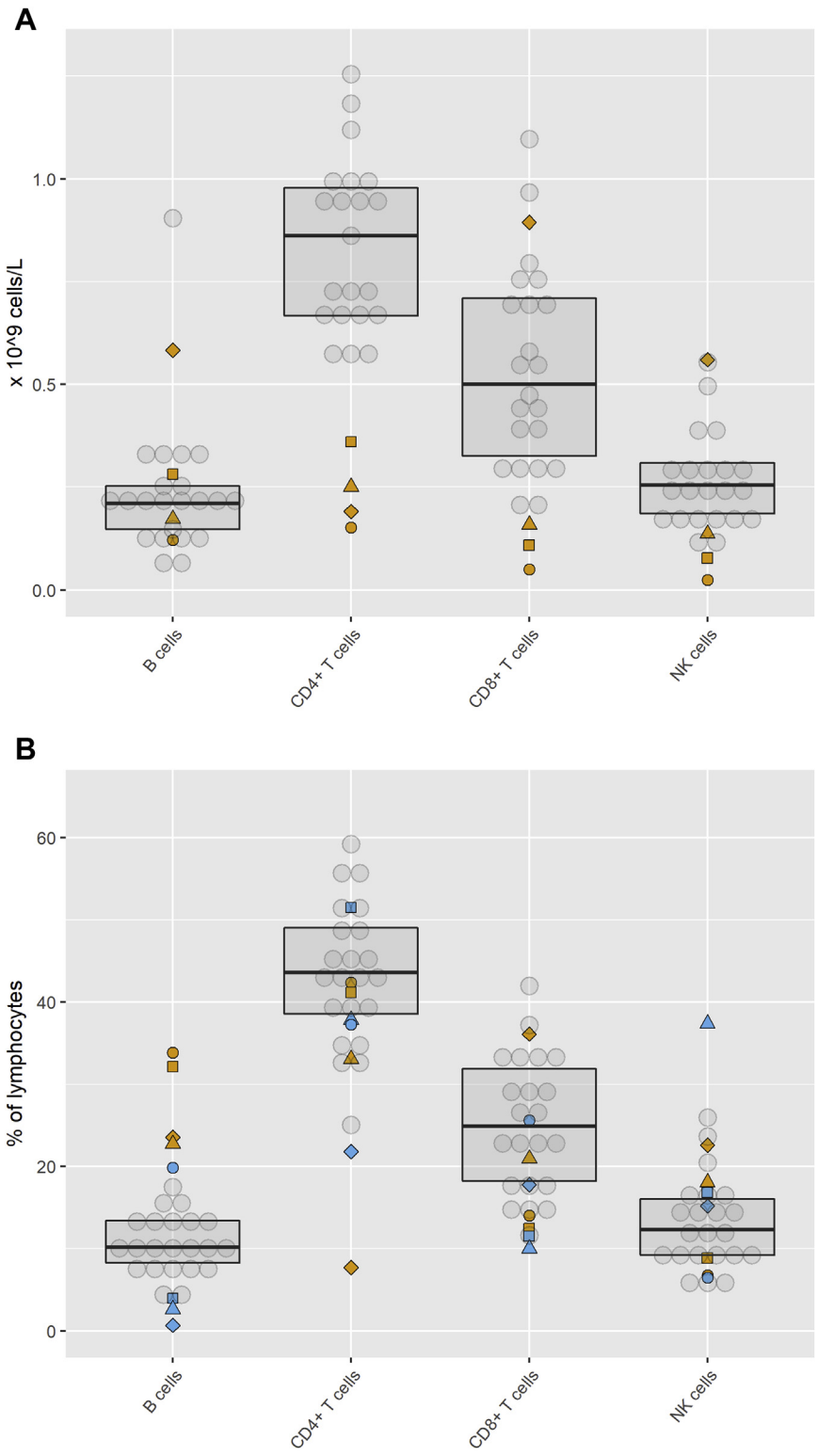

FIG 2. Absolute number and proportion of lymphocyte subpopulations in the blood and BALF. **A,** Absolute number of lymphocyte subpopulations in the blood of patients (*yellow symbols*) and healthy controls (*gray circles*). **B,** Proportion of lymphocyte subpopulations in BALF (*blue symbols*) and blood (*yellow symbols*) of patients and healthy controls (*blood only, gray circles*). Colored symbols (*circle, square, triangle, and diamond*) represent the same patient within the different subpopulations and within the 2 compartments (*blue indicates BALF; yellow indicates blood*). The box plot represents the median and interquartile range for healthy controls (n = 22). *NK*, Natural killer.

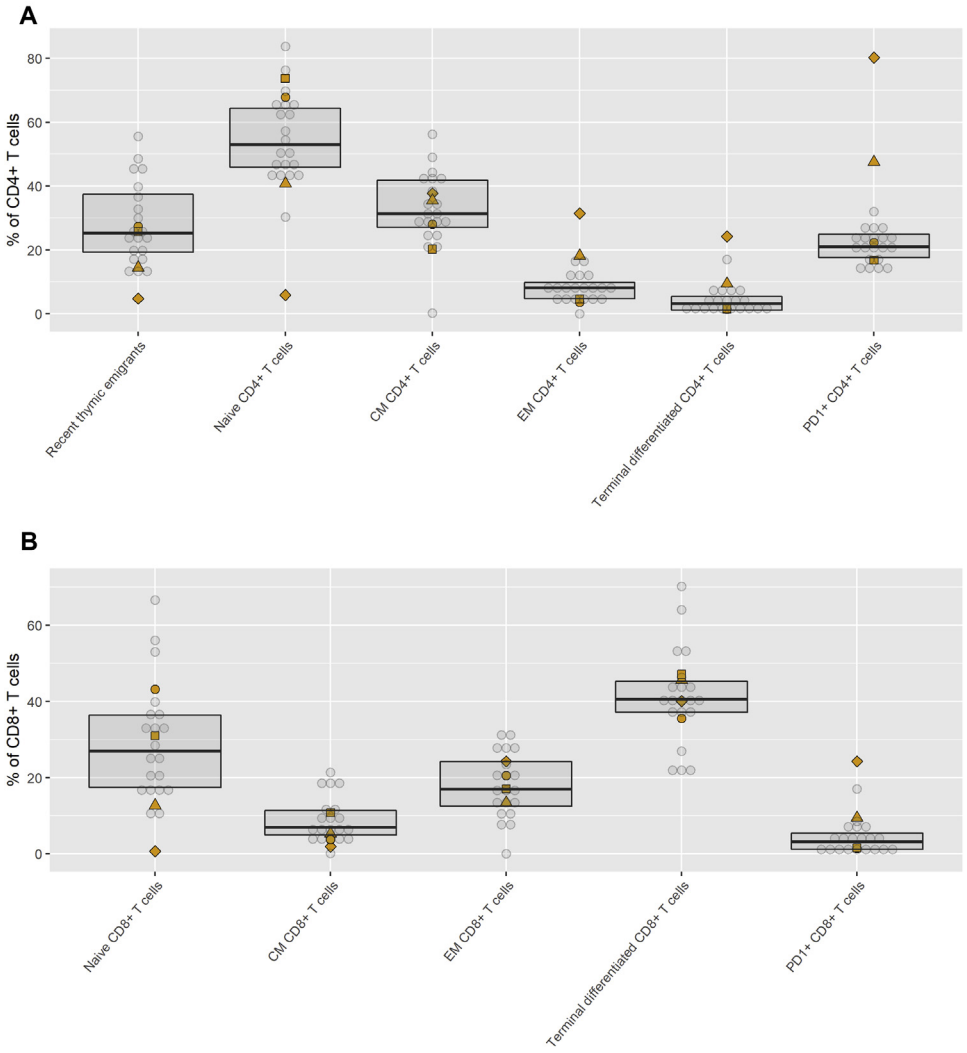

FIG 3. Lymphocyte differentiation in blood. **A,** Proportion of CD4 T-cell subpopulations in blood of patients (*yellow symbols*) and healthy controls (*gray circles*), **B,** Proportion of CD8 T-cell subpopulations in blood of patients (*yellow symbols*) and healthy controls (*gray circles*). Yellow symbols (*circle, square, triangle, and diamond*) represent the same patient within the different subpopulations. The box plot represents the median and interquartile range for healthy controls (n = 22). The antigens used to define subpopulations are depicted in Table E2. *CM*, Central memory; *EM*, effector memory.

300 μL of plasma) according to the manufacturer's protocol, with lower limits of detection ranging between 0.218 and 713 pg/mL. BALF and blood cytokines from patients with COVID-19 and controls were analyzed on the same plates.

Microbiologic assays

SARS-CoV-2 RNA was determined by real-time RT-PCR targeting the ORF1a/b nonstructural region that is unique to SARS-CoV-2. In addition, a BioFire FilmArray Pneumonia Plus Panel (see Table E4 in this article's Online Repository at www.jacionline.org) was used for detection of respiratory pathogens, and 16s and 18s PCR were performed on all BALF samples. A more detailed description of these analyses is provided in the Online Data Supplement.

Statistical analyses

We decided to limit the use of summary statistics and instead present data as case by case. We further decided against generating *P* values because of the small sample size. Figures were generated by using R software, version 3.4.3.

RESULTS

Patient characteristics and bronchoalveolar lavage procedure

Patient characteristics, laboratory data, and specific ICU therapies are depicted in Tables I and II. All patients had moderate-to-severe impairment of oxygenation at ICU admission that deteriorated before the study procedure, and they were treated with broad-spectrum antibiotics (piperacillin/tazobactam or meropenem) at the time of inclusion. None was treated with corticosteroids, antivirals, or immunomodulatory drugs off protocol, but 3 of the 4 were included in a randomized clinical trial before ICU admission (Table I).

Microbiologic evaluation of BALF revealed detectable SARS-CoV-2 RNA at the time of the procedure in all patients. Patient 3 was found to be infected with methicillin-sensitive *Staphylococcus aureus*; the other identified pathogens are listed in Table E5 (in this article's Online Repository at www.jacionline.org).

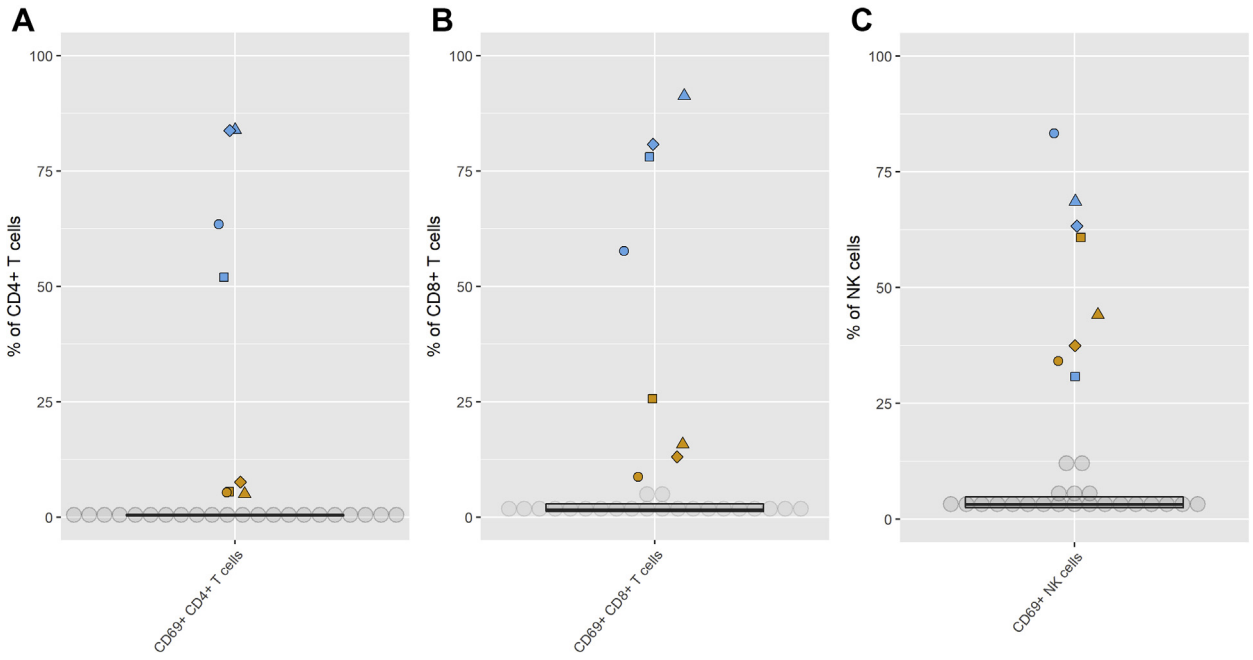

FIG 4. Activation of lymphocyte subpopulations in the blood and BALF. **A,** Proportion of CD4 T-cell CD69 expression in the BALF (*blue symbols*) and blood (*yellow symbols*) of patients and healthy controls (*blood only, gray circles*), B) Proportion of CD8 T-cell CD69 expression in the BALF (*blue symbols*) and blood (*yellow symbols*) of patients and healthy controls (*blood only, gray circles*). **C,** Proportion of natural killer (NK) cell CD69 expression in BALF (*blue symbols*) and blood (*yellow symbols*) of patients and healthy controls (*blood only, gray circles*). Colored symbols (*circle, square, triangle, and diamond*) represent the same patient within the different subpopulations and within the 2 compartments (*blue indicates BALF; yellow indicates blood*). The box plot represents the median and interquartile range for healthy controls (n = 22).

We found no evidence of coinfection with viral or fungal pathogens.

Leukocyte subsets, differentiation, and expression patterns in blood

Radar plots of the leukocyte subsets in the blood (and BALF) are provided in Fig 1. Lymphopenia was observed in all cases (ranging from 0.34 to 0.94 $\times$ 10^9/L), with normal (in 1 of the 4 patients) or increased (in 3 of the 4 patients) neutrophil counts (Table II). Two of the patients had eosinopenia (Table II). B-cell counts were within the normal range (0.09-0.57 $\times$ 10^9/L [Fig 2]), but reduced isotype switching (<3% of the B cells) was observed in 2 patients; 3 patients had increased plasmablast concentrations in their blood, and in 1 of these patients, mainly non–isotype-switched plasmablasts were present (see Fig E2 in this article's Online Repository at www.jacionline.org). T-cell levels were low, with CD4 T-cell counts ranging from 0.19 to 0.36 $\times$ 10^9/L (normal range 0.39-1.7 $\times$ 10^9/L) and CD8 T-cell counts ranging from 0.05 to 0.89 $\times$ 10^9/L (normal range 0.19-1.0 $\times$ 10^9/L) (Fig 2). All patients had low CD4 recent thymic emigrant (RTE) counts (ranging from 0.009 to 0.093 $\times$ 10^9/L), indicating reduced thymic output. The distribution of CD4 T-cell subsets was directed toward central memory, whereas CD8 T-cell subsets were generally directed toward terminal differentiation; 1 patient had severely reduced CD4 RTE counts and a high fraction of both CD4 and CD8 terminal differentiation (Fig 3). Regulatory T (Treg) cell levels were elevated in all cases, whereas T_H17 levels were comparable to those found in healthy controls (see Fig E3 in this article's Online Repository at www.jacionline.org). Neutrophils were primarily $CD10^-$ (ie, immature) (see Fig E4 in this article's Online Repository at www.jacionline.org). In addition, a small population of $CD35^-CD49d^+$ metamyelocytes could be detected in blood. Moreover, the concentration of dendritic cell populations was low (see Fig E5 in this article's Online Repository at www.jacionline.org).

Several activation markers were consistently upregulated in leukocyte subsets in the blood. CD4 and especially CD8 T cells expressed an upregulation of CD69 (Fig 4) and HLA-DR (see Fig E6 in this article's Online Repository at www.jacionline.org). Cytotoxic T lymphocyte–associated protein-4 (CTLA-4) was also weakly upregulated (ranging from 8% to 15%) in CD4 T cells (see Fig E7 in this article's Online Repository at www.jacionline.org). Two of the patients had upregulation of programmed cell death protein (PD1) receptor on both CD4 and CD8 T cells (Fig 3). Histograms depicting fluorescent intensity of CTLA-4, PD1, CD69, and HLA-DR on T cells are shown in Fig E8 (in this article's Online Repository at www.jacionline.org). Monocytes in the blood showed upregulation of CD69, but HLA-DR expression was slightly decreased compared with that in healthy controls (see Fig E9 in this article's Online Repository at www.jacionline.org).

Leukocyte subsets and differentiation and expression patterns in BALF

May-Grünwald-Giemsa–stained BALF cytocentifugates were characterized by a background of erythrocytes and a variable

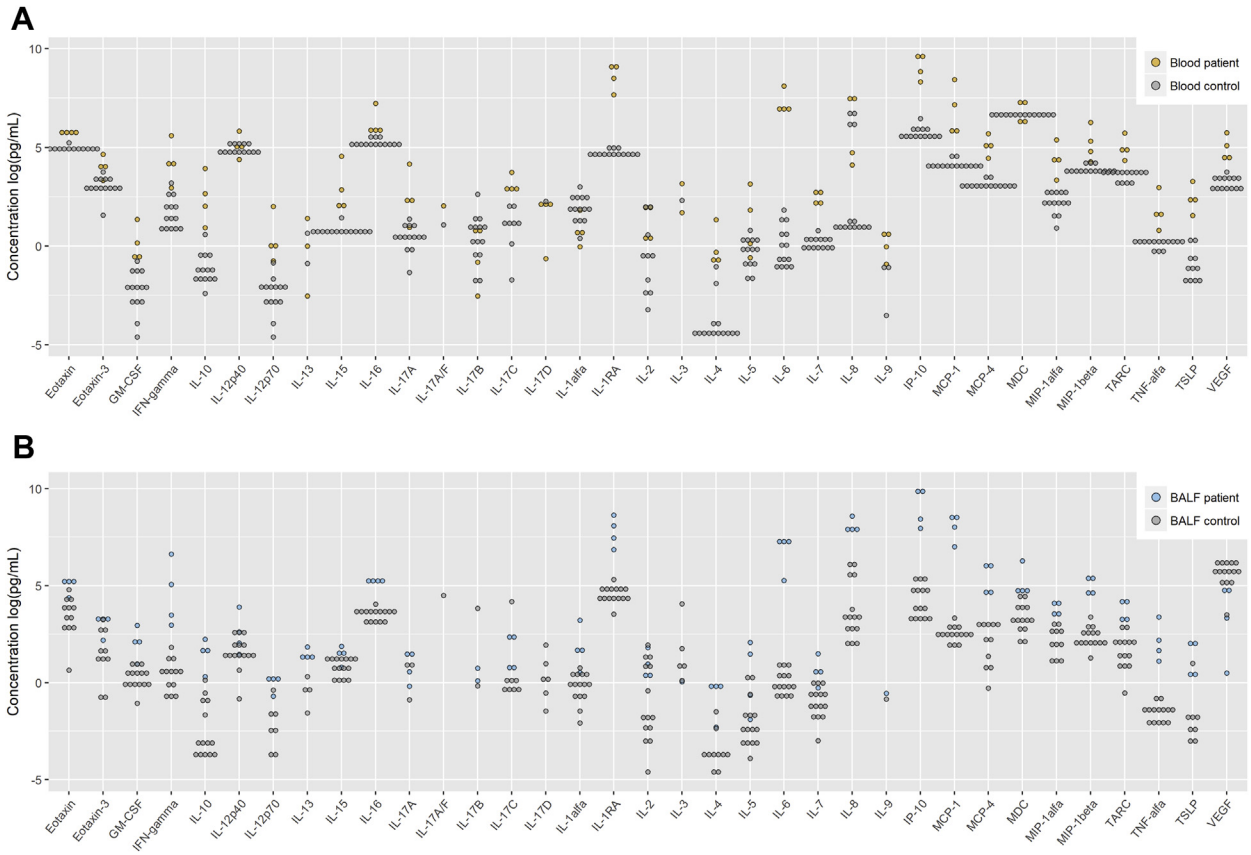

FIG 5. Cytokines and chemokines measured in blood and BALF (log-transformed). The MSD V-Plex Human Biomarker Proinflammatory Panel 1, Cytokine Panels 1 and 2, and Chemokine Panel 1 were used for analyses of cytokines and chemokines. Data are depicted for the blood (**A**) and BALF (**B**) of both patients (*yellow or blue circles*) and healthy controls (*gray circles* [n = 15]). Cytokine data are also depicted on absolute scale in Fig E10. An internal positive control consisting of pooled plasma was used. A few cytokines were not detected in the blood (ie, IL-17F) and BALF (ie, IL-3, IL-9, IL-17D, and IL-17F). *IP*, IFN-γ–induced protein; *MDC*, macrophage-derived chemokine; *TARC*, thymus and activation-regulated chemokine; *TSLP*, thymic stromal lymphopoietin; *VEGF*, vascular endothelial growth factor.

number of neutrophils, macrophages, small lymphocytes, and (to a lesser extent) eosinophils. We did not detect any hemosiderin-laden alveolar macrophages.

In line with this, flow cytometry identified the major components in BALF as neutrophils, monocytes/macrophages, and eosinophils but noticeably few lymphocytes (Fig 1). Further characterization of the lymphocyte subpopulations revealed that the fraction of CD4 and CD8 T cells was specifically reduced (Fig 2) but the proportion of both Treg cells and T_H17 cells was nonetheless higher in BALF than in blood (see Fig E3). Neutrophils were primarily $CD10^-$ (see Fig E4). In BALF, $CD14^+$ monocytes showed marked upregulation of CD69 and HLA-DR expression compared with blood monocytes. In addition, a distinct $CD14^+$ subpopulation with very bright HLA-DR expression and increased autofluorescence identifying the macrophages showed increased expression of CD64 and CD16 and (to a lesser extent) increased expression of CD11b (see Fig E9). Furthermore, as in blood, the concentration of dendritic cell populations was low in BALF (see Fig E5).

CD4 and CD8 T cells and natural killer cells in BALF expressed very high levels of CD69 activation (Fig 4 and see Fig E8), and CTLA-4 was also markedly upregulated on both CD4 (ranging from 25% to 60%) and CD8 (ranging from 25% to 85%) T cells (see Fig E7).

Albumin, cytokines, and chemokines in blood and BALF

Albumin was detected in BALF from all 4 patients (Table II). The levels of several cytokines and chemokines in both blood and BALF were markedly elevated; this was most pronounced for IL-1RA, IL-6, IL-8, IFN-γ–inducible protein-10 (IP-10), and monocyte chemoattractant protein-1 (MCP-1) (Fig 5 and see Fig E10 in this article's Online Repository at www.jacionline.org). Except for the concentration of IL-8, which was higher in BALF than in blood, the concentrations of the inflammatory mediators seemed to be elevated to the same extent in the 2 compartments. A heatmap depicting the correlation between cytokines in the lung and systemic compartment and CRP dynamics before and after study inclusion have been included as supplementary material (see Figs E11 and E12, respectively, in this article's Online Repository at www.jacionline.org).

DISCUSSION

The clinical management of critically ill patients with COVID-19 is hampered by a limited understanding of the underlying pathophysiologic mechanisms, and so far, only a handful of studies have focused on *in vivo* immunopathology, primarily by analyzing the immune system at a transcriptional level in either blood or the lungs. In the present study, we analyzed simultaneously obtained blood and BALF from 4 patients with COVID-19 ARDS, thus allowing for comparison of the 2 distinct immunologic compartments. Our main findings are that although certain aspects of the immune response (notably, the dominance of immature neutrophils and vast increases in a wide array of cytokines) are similar in blood and the lungs, several changes within the T-cell population are markedly compartmentalized. Thus, the depleted T-cell population in the lungs encompassed elevated Treg cells and T_H17 cells and exhibited a prominent upregulation of activation markers.

In all patients we observed macroscopic signs of inflammation but no visible secretions, the latter being in contrast with what was recently found in a larger population of patients with COVID-19 who underwent bronchoscopy.[15] Accordingly, we did not experience macroscopic bleeding and the yield of more erythrocyte-rich BALF with successive aliquots and detectable albumin most likely reflects fragile lung parenchyma and vascular leakage in the more distal airways. No hemosiderin-laden macrophages were found, which suggests diffuse alveolar damage rather than diffuse alveolar hemorrhage, a finding that is supported by autopsy studies in patients with COVID-19.[16,17]

A consistent finding in the present study was the lymphopenia driven by low T cells counts in blood, which appears to be pathognomonic for severe COVID-19.[18,19] Furthermore, 2 of the patients were eosinopenic, which some authors have highlighted as a putative hallmark of COVID-19.[20] Correspondingly, the proportion of lymphocytes in BALF was low, with a concomitant reduction in both CD4 and CD8 T-cell fractions compared with the normal values.[21] A recent single-cell RNA sequencing BALF study in 9 patients with COVID-19 showed a similarly low proportion of T cells in the BALF of patients who were critically ill compared with the proportion in those with moderate disease severity.[22] This has important pathophysiologic implications; assisted by CD4 T cells, both CD8 T and B cells play an important role in viral clearance, and systemic depletion of CD4 and CD8 T cells has previously been shown to be associated with adverse outcomes in SARS-CoV infection.[23] Another consistent finding in the present study was a higher fraction of Treg cells. A high fraction of Treg cells in BALF has previously been shown in non–COVID-19 ARDS, where Treg cells appear to be involved in resolution of lung injury by induction of neutrophil apoptosis, macrophage efferocytosis, and decreasing fibrocyte recruitment.[24]

The $CD161^+$ and $CD196^+$ CD4 T cells seemed to be markedly elevated in BALF compared with blood. T_H17 cells may mediate tissue destruction by recruitment of neutrophils to the lungs. However, these cells have also been found to be elevated in community-acquired pneumonia, and this finding may thus not be specific for COVID-19.[25] However, T_H17 cells were characterized by using the surrogate surface markers CD161 and CD195 without intracellular staining, which may impede correct identification of these cells.

The mechanisms explaining the reduction in circulating CD4 and CD8 T cells in severe COVID-19 remain to be elucidated, but may share similarities with that observed during sepsis-induced immune cell apoptosis.[26,27] Because of the remarkably low CD4 and CD8 fractions in BALF, this is not likely caused by extravasation (ie, compartmentalization to the alveoli). Rather, reduced T-cell production (as indicated by the low CD4 RTE cell count in blood) is likely at play. Several mediators may promote thymic dysfunction/involution, including systemic hyperinflammation *per se*[28,29]; specifically, the presence of high levels of TNF-α and IL-6 in severe COVID-19 could potentially lead to both reduced T-cell production and accelerated T-cell apoptosis.[30] Finally, the increased frequency of Treg cells and T_H17 cells in blood may be explained by a decrease in the number of naive T cells[27] in combination with strong immune activation and differentiation in lung.

We observed a significant upregulation of activation markers on both lung and blood leukocytes, including CD69 and HLA-DR. Notably, the immune checkpoint molecule CTLA-4 was upregulated on both FOXP3-expressing CD4 Treg cells and CD8 T cells in blood, and it was even more pronounced on T cells from BALF. CTLA-4 is constitutively expressed in Treg cells but upregulated on the cell surface only after activation, a phenomenon that is particularly notable in cancers.[31] This finding in COVID-19 ARDS is likely secondary to massive stimulation of the immune system caused by the viral invasion, thus resulting in an inexpedient exhaustion of the immune system. Exhausted T cells are generally defined as PD1- and CD57-expressing CD8 T cells. Thus, the marked PD1 upregulation found not only in patient blood CD8 T cells but also in CD4 T cells likely indicates a more general pronounced exhaustion in patients with COVID-19. Although activation markers were expected to be upregulated in the lungs compared with in the blood, these expression levels far exceeded what has previously been reported in BALF for healthy controls,[32] and they were also higher in the blood of patients with COVID-19 ARDS than in the blood of healthy controls.

Another consistent finding within the BALF cellular compartment was the dominance of neutrophils and (to a lesser extent) monocytes and macrophages ($CD14^+$ with very bright HLA-DR expression and increased autofluorescence), which also displayed pronounced upregulation of surface markers related to activation (CD64, CD16, HLA-DR, CD11b, and CD69). In comparison, blood monocytes showed moderate signs of activation by upregulation of CD64 and CD69 and slight decrease in HLA-DR.

Previous studies in patients with severe SARS-CoV found high levels of proinflammatory cytokines (IL-1, IL-6, and IL-12), T_H1 cytokine INF-γ, neutrophil chemotactic factor (IL-8), MCP-1, and T_H1 chemokine IFN-δ–inducible protein-10 (IP-10).[33-35] A recent study performed genome-wide transcriptome sequencing of RNA obtained from BALF in 3 patients with COVID-19 with unknown disease severity; it reported different expression of 1004 genes, including a high expression of cytokines such as MCP-1, IP-10, MIP-1A, and MIP-1B.[36] In comparison, another transcriptomic analysis of 8 patients with COVID-19 suggested increased expression of proinflammatory genes, including interferon-stimulated gene.[37] In the present study, we found nearly all cytokines to be elevated in the BALF and blood of the 4 patients with COVID-19 compared

with in healthy volunteers. Importantly, the chemokines IL-8, IP-10, and MCP-1 were found in very high concentrations in the lungs and accordingly may elicit and perpetuate local inflammation. Of these, IL-8 exhibited a notable compartmentalized response within the lungs, which when considering the cellular immune response reported here, is consistent with the well-established role of IL-8 in the recruitment of neutrophils to the lungs during acute pulmonary inflammation.[38] The observed cytokine and chemokine levels in both blood and BALF point toward an extensive hyperinflammatory phenotype in severe COVID-19, a phenomenon that some authors have coined a "cytokine storm."[39] However, in addition to proinflammatory cytokines, several anti-inflammatory cytokines (eg, IL-10) were also detected at high levels in BALF. This may be a beneficial response that helps regulate local inflammation, although excess production of anti-inflammatory mediators could potentially also compromise the host's ability to clear SARS-CoV-2.

The current hope is that existing, approved therapies aimed at dampening or inhibiting inflammation, including IL-6, IL-17, and IL-1 receptor blockade, will increase survival. A list of registered off-label trials with respect to the cytokines evaluated in this study is presented in Table E6 (in this article's Online Repository at www.jacionline.org). In addition, recent proteomic studies have revealed that key protein kinases (eg, p38 mitogen–activated protein and casein kinase II) are involved in COVID-19 pathogenesis,[40] which could be targeted by using existing protein kinase inhibitors.

Our study has strengths and limitations. We performed a comprehensive protocoled study with uniform BALF and blood sampling in patients with COVID-19 ARDS. This provided a unique material for in-depth characterization of the leukocyte and cytokine milieu in the lungs and blood, and it allowed for direct intraindividual and interindividual comparison of the 2 compartments. We intended to include additional patients, but the course of the COVID-19 epidemic in Denmark did not allow us to do so. Hence, major limitations of our study are the small sample size and the explorative design. Moreover, we had a control group only for flow cytometry comparison in blood and not for BALF. Our study was observational and cross-sectional, and because of the nature of the design, we are not able to infer causation.

In conclusion, our study provides novel phenotypic insight into the cell composition and inflammatory mediators simultaneously present in the lungs and blood in patients with COVID-19 ARDS. Our findings indicate that although both compartments exhibit a severely hyperinflammatory milieu, in which activated lymphocytes are present in low numbers, the T cells within the lungs are remarkably activated, and dominated by subtypes that function to govern both reparative and destructive processes. Future studies should determine how these findings are related to the clinical course and how protocoled anti-inflammatory therapies may affect the immunophenotype at both the local and systemic level.

We thank all the patients with COVID-19 and their families for their participation. We also thank Anna Louise Sørensen, Ann-Charlotte Lundstedt, Unni Nielsen, and the staff at the Department of Pathology, Microbiology and Clinical Biochemistry for their outstanding laboratory assistance. We also thank the clinical staff and nurses at the Department of Anesthesiology and Intensive Care for their dedicated contribution.

Key messages

- **The identification of a distinct pulmonary immunologic phenotype in COVID-19 ARDS is potentially of use for identification of targets for future anti-inflammatory therapies.**
- **The cellular immune response of COVID-19 ARDS is dominated by immature neutrophils in both the blood and the lungs, with concomitant CD4 and CD8 T-cell lymphopenia and elevated inflammatory chemokine and cytokine levels.**
- **The T-cell profile in the lungs is substantially different from that in blood, with expression of much higher levels of activation and higher frequency of Treg cells and T_H17 cells.**

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