A Granulocytic Signature Identifies COVID-19 and Its Severity

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Background. An unbiased approach to SARS-CoV-2–induced immune dysregulation has not been undertaken so far. We aimed to identify previously unreported immune markers able to discriminate COVID-19 patients from healthy controls and to predict mild and severe disease.

Methods. An observational, prospective, multicentric study was conducted in patients with confirmed mild/moderate (n = 7) and severe (n = 19) COVID-19. Immunophenotyping of whole-blood leukocytes was performed in patients upon hospital ward or intensive care unit admission and in healthy controls (n = 25). Clinically relevant associations were identified through unsupervised analysis.

Results. Granulocytic (neutrophil, eosinophil, and basophil) markers were enriched during COVID-19 and discriminated between patients with mild and severe disease. Increased counts of CD15+CD16+ neutrophils, decreased granulocytic expression of integrin CD11b, and Th2-related CRTH2 downregulation in eosinophils and basophils established a COVID-19 signature. Severity was associated with emergence of PD-L1 checkpoint expression in basophils and eosinophils. This granulocytic signature was accompanied by monocyte and lymphocyte immunoparalysis. Correlation with validated clinical scores supported pathophysiological relevance.

Conclusions. Phenotypic markers of circulating granulocytes are strong discriminators between infected and uninfected individuals as well as between severity stages. COVID-19 alters the frequency and functional phenotypes of granulocyte subsets with emergence of CRTH2 as a disease biomarker.

Keywords. SARS-CoV-2; COVID-19; neutrophil; eosinophil; basophil; CRTH2; immune checkpoint; CD11b; CD16; PD-L1.

The hallmark of COVID-19, the disease caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is the occurrence, in 10%–20% of patients, of a sudden deterioration 7–10 days after the onset of symptoms, increasing the risk of acute respiratory distress syndrome, need for intensive care, and ultimately of death [1]. Studies exploring the immune response suggested that SARS-CoV-2 may induce unique patterns of immune dysregulation [2, 3]. To our knowledge, a systematic approach to SARS-CoV-2–induced immune dysregulation at the phenotype level has not been undertaken so far. Single-cell RNA sequencing of peripheral blood mononuclear cells evidenced phenotypic remodeling affecting innate and adaptive populations [4]. Our aim was to establish a comprehensive, unsupervised map of circulating immune cells in COVID-19 patients using a first-in-class flow cytometry approach for rapid whole-blood assessment. The primary objective was the identification of immunophenotypic patterns most accurately associated with COVID-19 diagnosis and severity. Among the large number of phenotypic markers of circulating immune cells modulated by SARS-CoV-2, those related to granulocyte lineages (neutrophils, basophils, and eosinophils) were strong discriminators between infected and uninfected individuals as well as between different degrees of disease severity. Beside SARS-CoV-2–associated lymphopenia, changes in frequency and activation of granulocyte subsets may be predictive of clinical worsening during COVID-19.

METHODS

Study Design

This open multicenter prospective observational study was conducted in the intensive care unit (ICU) at North Hospital of Marseille and the COVID-19 ward unit at the European Hospital of Marseille.
Patients admitted to ward and ICU with confirmed SARS-CoV-2 infection were included in the study if they fulfilled the criteria: (1) age 18 years or older and (2) a positive SARS-CoV-2 reverse transcriptase-polymerase chain reaction (RT-PCR) in nasopharyngeal swabs or tracheal aspiration. Exclusion criteria were preexisting treatments interfering with immune functions, pregnancy, and missing clinical or laboratory data.

Demographic, clinical, and laboratory data (arterial blood gas analysis, complete blood count, biochemistry, virology) including SARS-CoV-2–related symptoms, date of disease onset, organ support, and medications were collected for each patient upon admission to ICU or conventional ward. The same data were collected on the day of blood sampling. At day 28 after COVID-19 diagnosis, the duration of mechanical ventilation, length of ICU and hospital stays, and ICU and hospital mortality rates were also recorded. The Simplified Acute Physiology Score II (SAPS II) [5], the Sepsis-Related Organ Failure Assessment (SOFA) [6], the National Early Warning Score 2 (NEWS2) [7], and the World Health Organization (WHO) progression scale [8, 9] were calculated at admission and on the day of blood sampling. Patients were classified as mild/moderate (WHO grade 4 and 5, hereafter termed mild) depending on the presence of oxygen supply, while those receiving high-flow oxygen therapy (WHO grade 6) or invasive mechanical ventilation (WHO grade 7–9) were considered as severe.

Samples from healthy blood donors (HBD), serving as controls, were received from Etablissement Français du Sang (EFS), Marseille, France.

Study Approval
The study was conducted in accordance with the Declaration of Helsinki and the French law on research involving humans. It was registered with the French Agence Nationale de Sécurité du Médicament et des Produits de Santé under reference ID-RCB: 2020-A00756-33 and received approval from the national review board Comité de Protection des Personnes Ile de France XI (20027–60604, 25 March 2020). The patients were informed and agreed to participate in this study. Patient enrollment took place from 30 March to 8 April 2020. HBD samples were obtained through an institutional agreement between EFS and UMR-D258 Microbes, Évolution, Phylogénie et Infection.

Flow Cytometry
All antibodies and reagents were obtained from Beckman Coulter. Blood (4 mL) was collected by venipuncture on EDTA-anticoagulated tubes, stored, and delivered at room temperature to the immunology laboratory. Multiparametric flow cytometry was used for immune cell enumeration and immune phenotyping less than 24 hours after blood collection. Each immune phenotyping panel (Table 1) was provided in a premix dry antibody cocktail completed in some cases by the addition of liquid conjugates prior to sample addition. Staining of leukocytes for enumeration was performed by the addition of 100 μL whole blood to the 1M Count tube followed by 15 minutes incubation at room temperature. Lysis of red blood cells was achieved with 2 mL Versalyse (Beckman Coulter) followed by a 15 minutes incubation prior to acquisition. Immune phenotyping followed a similar protocol except for the incubation (20 minutes for leukocyte staining and 10 minutes for red blood cell lysis, in the dark). Lysed cells were washed with 3 mL phosphate-buffered saline (PBS) and the cell pellet resuspended in 0.5 mL PBS ×1, 0.1% formaldehyde. Acquisition was performed with a Navios flow cytometer (Beckman Coulter).

Data Analysis and Statistics
Multiparameter flow cytometry data files were analyzed using the Kaluza software, version 2.1 (Beckman Coulter). Parameters were exported to JMP 14.2.0 software (SAS) for statistical analysis. The response screening platform of JMP, not only yielding a P value but also a false discovery rate (FDR) corrected value, was then used to identify the parameters with the highest discriminative capabilities. Best discriminators were ranked according to the LogWorth of their FDR corrected P values. Multivariate analyses following the principal component analysis (PCA) approach were also conducted with JMP. Nonparametric Wilcoxon rank sum tests, equivalent to Mann-Whitney tests, were also performed by JMP to compare parameter levels across different subgroups of individuals.

The χ² with Yate correction, Fisher exact test, t test, Mann-Whitney test, and Wilcoxon test were used to compare clinical and laboratory variables between the mild and severe groups as appropriate. Statistical significance was defined as P < .05.

RESULTS
Demographic Characteristics of the Study Population
During the study period, 55 confirmed COVID-19 cases were referred to the participating centers. Among them, 19 patients were admitted to the ICU (severe group) and 7 to the conventional ward (mild group). Twenty-five HBDs served as the control group (Supplementary Figure 1). Demographic data are presented in Table 2. Differences were observed between the mild and severe group. Elevated body mass index and hypertension were more frequent in the severe group than in the mild group (P = .005 and .03, respectively). Severity scores, including the WHO progression score and the SOFA score, were significantly higher in the severe group than in the mild group. C-reactive protein was higher in the severe group as compared with the mild group whereas eosinophils and monocytes were significantly lower in severe as compared to mild patients (Table 2). Lymphopenia, defined as a lymphocyte count of less than 1 giga/L, was found in 85% of COVID-19 patients.
comprising 71% of the mild group and 89% of the severe group, a nonsignificant difference (Table 2).

Controls Versus COVID-19 Patients

An unsupervised analysis of circulating leukocyte subsets and immune phenotypic markers yielded more than 100 significant discriminators between COVID-19 patients and controls, with FDR $P$ values less than .05. Further analysis was arbitrarily restricted to the 25 most discriminant markers (Figure 1A). PCA of these 25 markers effectively discriminated COVID-19 patients from controls (Figure 1B). Eleven of 25 were granulocyte-associated markers, followed by lymphocyte, natural killer (NK), and dendritic cell variables (Figure 1A). Enrichment in granulocyte-associated markers affected the 3 granulocytic lineages: neutrophils, eosinophils, and basophils. There was a significant increase in the frequency of CD15+$^+$ granulocytes (mainly comprising neutrophils), an increase in the frequency of CD15+$^+$CD16+ neutrophil subset, and a decrease in the frequency of basophils in COVID-19 patients, as compared with controls (Figure 2A). Two prominent function-associated membrane antigens were modulated in COVID-19 patients as compared to HBD: CRTH2 (CD294), a receptor for prostaglandin D2, whose expression was decreased at the surface of basophils and eosinophils (Figure 2B), and CD11b (aM subunit of integrin CD11bCD18, also known as complement receptor 3, CR3), whose expression was decreased at the surface of neutrophils and basophils (Figure 2C). Hence, SARS-CoV-2 infection was characterized by changes in frequency of granulocyte subsets and alteration of their functional phenotypes with the emergence of CRTH2 as a biomarker for COVID-19.

Effect of Disease Severity

We wondered if the granulocyte signature displayed specific changes associated with disease severity (Figure 3A). Unsupervised analysis followed by PCA of the best markers discriminating between patients with mild and severe COVID-19 (Figure 3B) evidenced the predominance of granulocytic markers (8 of 19 with a FDR $P$ value less than .05). Some of the markers discriminating COVID-19 patients from HBD also discriminated patients with mild disease from those with severe disease. Neutrophil subset frequency was one of these shared markers. The frequency of CD15+$^+$ granulocytes and CD15+$^+$CD16+ neutrophil subset was significantly increased in the severe group ($P$ = .002), while the levels of expression of both CD15 and CD16 were decreased in the severe group as compared with the mild group (Figure 4A). Another shared marker was eosinophil CRTH2 expression, which was profoundly decreased in the severe group (Figure 4B). Hence, COVID-19 severity was associated with a more profound imbalance of granulocyte subsets and functional markers of the disease.

However, severe disease was associated with the emergence of specific markers. Severe disease differed from mild with respect to functional markers of eosinophils and basophils. At the surface of both basophils and eosinophils, the

| Table 1. Dry Antibody Panels for Whole-Blood Flow Cytometry |
|---|---|---|---|---|---|---|---|---|
| Variables | PB | KrO | FITC/AF 488 | PE | ECD | PC 5.5 | PC7 | APC |
| Count | CD45 | CB | CD19 | CD14 | CD4 | CD8 | CD3 |
| Basic | CD45 | CD16 | CD56 | CD19 | CD14 | CD4 | CD8 | CD3 |
| Granulocytes | CD15 | CD45 | CD294 | CD16 | CD33 | CD11b | PD-L1 | Lin |
| T-cell subsets | CD57 | CD45 | CD45RA | CCR7 | CD28 | PD-1 | CD27 | CD4 | CD8 | CD3 |
| Regulatory T-cells | Helios | CD45 | CD45RA | CD25 | CD39 | CD4 | FoxP3 | CD3 |
| B cells | IgM | CD45 | IgD | CD21 | CD19 | CD27 | CD24 | CD38 |
| Innate lymphoid cells | CD45 | CD294 | CD1a/CD3/CD14/CD16/CD19/CD34/CD94/TCRγ/TCRδ/CD123/CD117 | Nkp46 | CD127 | CD161 |
| NK subsets | CD57 | CD45 | CD16 | Nkp46 | Nkp30 | KIR2DL3 | NKG2a | CD56 |
| NK checkpoint 1 | CD16 | CD45 | CD64 | CD137 | PD-1 | CD274 | NKG2A | CD56 |
| NK checkpoint 2 | TIM-3 | CD45 | CD16 | Nkp46 | LAG-3 | HLA-DR | CD69 | NKG2D | CD56 |
| Dendritic cells | HLA-DR | CD45 | CD16 | Lin | CD1c | CD11c | Clec9A | CD123 |

Abbreviations: 7-AAD, 7-aminoactinomycin D (viability marker); AF, AlexaFluor; APC, allophycocyanin; CB, counting beads; ECD, phycoerythrin-Texas red; FITC, fluorescein isothiocyanate; IgD, immunoglobulin D; IgM, immunoglobulin M; KrO, Krome Orange; Lin, lineage; NK, natural killer; PB, Pacific Blue; PC, phycoerythrin cyanin; PD-L1, programmed cell death ligand 1; PE, phycoerythrin.
Table 2. Demographic, Clinical, and Laboratory Data of Enrolled Patients

| Variables | Healthy Donors n = 25 | COVID-19 Patients n = 26 | P | Mild Group n = 7 | Severe Group n = 19 | P |
|-----------|------------------------|--------------------------|---|------------------|---------------------|---|
| **Characteristics** | | | | | | |
| Male | Male | 10 (38) | 20 (73) | **.007** | 5 (71) | 15 (79) | .69 |
| Age, y, median (IQR 25–75) | 45 (31–54) | 66 (57–74) | **<.0001** | 71 (49–75) | 65 (57–74) | .79 |
| BMI, kg/m², median (IQR 25–75) | 28 (25–33) | 24 (23–26) | | 29 (27–36) | | **.005** |
| **Comorbidities** | | | | | | |
| Coronary disease | Coronary disease | 7 (27) | 1 (14) | | 6 (32) | | .6 |
| Hypertension | Hypertension | 17 (65) | 2 (29) | | 15 (79) | | **.03** |
| COPD | COPD | 3 (12) | 0 | | 0 | | .5 |
| Stroke | Stroke | 0 | 0 | | 0 | | |
| Smoker | Smoker | 5 (19) | 0 | | 5 (26) | | .3 |
| Active cancer | Active cancer | 3 (12) | 1 (14) | | 3 (16) | | .5 |
| Immunodepression | Immunodepression | 3 (12) | 1 (14) | | 2 (11) | | 1 |
| Chronic kidney disease | Chronic kidney disease | 0 | 0 | | 0 | | |
| Liver disease | Liver disease | 2 (8) | 1 (14) | | 1 (5) | | .5 |
| Diabetes | Diabetes | 10 (38) | 2 (9) | | 8 (42) | | .7 |
| **At hospital admission** | | | | | | |
| Clinical features | Clinical features | | | | | |
| Temperature, °C, median (IQR 25–75) | 38 (37.4–39) | 37 (36.6–39) | | 38.5 (38–39) | | .08 |
| MAP , mmHg, median (IQR 25–75) | 790 (72–91) | 87 (73–94) | | 78 (69–85) | | .2 |
| Heart rate, bpm, median (IQR 25–75) | 95 (80–107) | 90 (67–95) | | 100 (80–122) | | .2 |
| Respiratory rate, cpm, median (IQR 25–75) | 28 (18–34) | 17 (16–22) | | 32 (27–35) | | **.002** |
| Oxygen low flow | Oxygen low flow | 1 (4) | 1 (14) | | 0 | | .3 |
| Noninvasive ventilation | Noninvasive ventilation | 2 (8) | 0 | | 2 (11) | | 1 |
| Mechanical ventilation | Mechanical ventilation | 17 (65) | 0 | | 17 (89) | | **<.0001** |
| WHO progression scale, median (IQR 25–75) | 135 (108–193) | 275 (271–476) | | 124 (106–172) | | **.007** |
| SpO₂, %, median (IQR 25–75) | 94 (90–95) | 96 (95–97) | | 93 (84–95) | | .003 |
| SpO₂/FiO₂ ratio, median (IQR 25–75) | 186 (154–407) | 457 (452–462) | | 176 (142–194) | | **.0001** |
| PaO₂/FiO₂ ratio, median (IQR 25–75) | 130 (108–193) | 275 (271–476) | | 124 (106–172) | | **.007** |
| SAPS II, median (IQR 25–75) | 54 (43–71) | 54 (38–39) | | 38.5 (38–39) | | .08 |
| NEWS2 score, median (IQR 25–75) | 65 (57–74) | 65 (57–74) | | 65 (57–74) | | .79 |
| SOFA score, median (IQR 25–75) | 32 (27–35) | 32 (27–35) | | 32 (27–35) | | .79 |
| Use of vasopressors | Use of vasopressors | 13 (50) | 0 | | 13 (68) | | **.008** |
| Laboratory data | Laboratory data | | | | | |
| Serum aspartate aminotransferase, UI/L, median (IQR 25–75) | 51 (38–67) | 38 (31–52) | | 54 (33–71) | | .04 |
| Serum alanine aminotransferase, UI/L, median (IQR 25–75) | 33 (22–52) | 24 (14–50) | | 39 (23–52) | | .2 |
| Serum creatinine, µmol/L, median (IQR 25–75) | 72 (62–98) | 65 (58–71) | | 78 (63–100) | | .3 |
| C-reactive protein, mg/L, median (IQR 25–75) | 110 (64–164) | 17 (12–59) | | 150 (88–181) | | **.003** |
| Red blood cells, G/L, median (IQR 25–75) | 4.4 (2.4–6.7) | 4.4 (4.2–4.8) | | 4.4 (4.2–4.8) | | .5 |
| Platelets, G/L, median (IQR 25–75) | 212 (168–263) | 214 (171–307) | | 210 (159–259) | | .8 |
| Neutrophils, G/L, median (IQR 25–75) | 4.3 (3.3–6.4) | 3.8 (2.9–4.5) | | 4.8 (3.4–6.5) | | .15 |
| Basophils, G/L, median (IQR 25–75) | 0.01 (0.01–0.02) | 0.01 (0.01–0.01) | | 0.01 (0.01–0.02) | | .98 |
| Eosinophils, G/L, median (IQR 25–75) | 0 (0.01–0.01) | 0 (0.01–0.04) | | 0 (0.04–0.04) | | .002 |
| Lymphocytes, G/L, median (IQR 25–75) | 0.8 (0.6–1.1) | 1 (0.4–1.6) | | 0.8 (0.6–1) | | .7 |
| Lymphopenia | Lymphopenia | 22 (85) | 5 (71) | | 17 (89) | | .28 |
| Monocytes, G/L, median (IQR 25–75) | 0.4 (0.2–0.5) | 0.5 (0.4–0.7) | | 0.3 (0.2–0.5) | | .02 |
| Neutrophil to lymphocyte ratio, median (IQR 25–75) | 3.9 (3.6–8.2) | 3.9 (1.8–9.3) | | 5.3 (4–7) | | .2 |
| Platelet to lymphocyte ratio, median (IQR 25–75) | 237 (183–346) | 212 (147–346) | | 241 (192–346) | | .7 |
| Laboratory data at blood sample collection | Laboratory data at blood sample collection | | | | | |
| Red blood cells, G/L, median (IQR 25–75) | 3.7 (3.1–4.2) | 3.7 (3.1–4.2) | | 3.3 (3–3.9) | | **.002** |
| Platelets, G/L, median (IQR 25–75) | 318 (243–429) | 236 (150–278) | | 412 (302–458) | | **.003** |
| White blood cells, G/L, median (IQR 25–75) | 9.5 (6.4–12.3) | 5.2 (3.9–5.4) | | 11 (8.3–14) | | **.0002** |
| Neutrophils, G/L, median (IQR 25–75) | 7.7 (4.4–9.1) | 3.6 (2.8–4) | | 8.9 (7–12) | | **.0001** |
expression of checkpoint inhibitors such as programmed cell death ligand 1 (PD-L1) was significantly higher in the severe group than in the mild group (Figure 4C and 4D). Such prominent changes in surface expression of functional granulocytic markers prompted us to ask whether granulocyte alterations correlated with clinical scores. We found that both WHO and SOFA scores correlated positively with innate immune checkpoints such as PD-L1 expression on basophils and eosinophils, and negatively with neutrophil CD11b and eosinophil CRTH2 expression (Figure 5). The level of correlation between immunophenotypic markers and clinical scores was similar to that of clinical scores between them (WHO versus SOFA, $R^2 = 0.567$).

### Partners of the Granulocyte Signature

The granulocytic signature of COVID-19 was not isolated because it was associated with a decreased representation of CD4+ T cells, CD8+ T cells, and plasmacytoid dendritic cells (Supplementary Figure 2). The upregulation of checkpoint inhibitors was not restricted to the granulocyte lineage: PD-L1 expression on monocytes and NK cells, and PD-1 expression on T cells were also increased in the severe group (Supplementary Figure 3).

### DISCUSSION

This study was undertaken as a holistic description of immune cells and markers from COVID-19 whole-blood samples.
A Parameter

| Parameter | Count | P Value | LogWorth | FDR P Value | FDR LogWorth | Change |
|-----------|-------|---------|----------|-------------|---------------|--------|
| % SS^4 CD15^+ Granulocytes | 51    | 1.9E-18 | 17.7116  | 1.24E-16    | 15.9053      |        |
| % SS^4 CD15^+ CD16^+ Granulocytes | 51    | 3.5E-18 | 17.4949  | 1.24E-16    | 15.9053      |        |
| % SS^4 CD16^+ Granulocytes | 50    | 6.5E-13 | 12.1874  | 1.28E-11    | 10.1919      |        |
| % CD3^+ CD56^+ Lymphocytes | 50    | 3.0E-12 | 11.5178  | 5.31E-11    | 10.2747      |        |
| % SS^CRTH2^+ Basophils | 51    | 6.7E-11 | 10.1723  | 9.42E-10    | 9.0261       |        |
| % lineage CD11c^+ HLA-DR^+ CD123^+ pDCs | 51    | 9.2E-11 | 10.0341  | 1.08E-09    | 8.9672       |        |
| MFI^CRTH2^+ SS^CRTH2^+ Eosinophils | 51    | 1.4E-09 | 8.8254   | 1.42E-08    | 7.8471       |        |
| MFI^CD16^+ SS^CD15^+ Granulocytes | 51    | 1.8E-09 | 8.7450   | 1.42E-08    | 7.8471       |        |
| % PD-L1^+ CD56^+ CD3^+ NK Cells | 50    | 1.82E-09 | 8.7380 | 1.42E-08    | 7.8471       |        |
| CD3^+ CD56^+ T cells count (cells/L) | 50    | 2.96E-09 | 8.5282 | 2.07E-09    | 7.6833       |        |
| CD3^+ T cells count (cells/L) | 50    | 9.54E-09 | 8.0205 | 5.96E-09    | 7.2322       |        |
| MFI^CRTH2^+ SS^CRTH2^+ Basophils | 51    | 1.0E-08 | 7.9981   | 5.86E-08    | 7.2322       |        |
| MFI^CD16^+ SS^Granulocytes | 51    | 2.8E-08 | 7.5481   | 1.52E-07    | 6.8169       |        |
| MFI^CD16^+ CD3^+ CD4^+ CCR7^+ PD1^+ T cells | 49    | 3.23E-08 | 7.4913 | 1.61E-07    | 6.7923       |        |
| MFI^CD16^+ CD3^+ CD4^+ CD27^+ CD69^+ T cells | 49    | 7.14E-08 | 7.1463 | 3.33E-07    | 6.4733       |        |
| MFI^CD16^+ lineage CD11c^+ HLA-DR^+ mDCs | 49    | 2.83E-07 | 6.5456 | 1.25E-06    | 5.9046       |        |
| MFI^CD16^+ SS^CD15^+ Granulocytes | 51    | 3.13E-07 | 6.5051 | 1.29E-06    | 5.8905       |        |
| % PD-L1^+ CD56^+ CD3^+ NK Cells | 50    | 1.34E-06 | 6.8274 | 5.22E-06    | 5.2826       |        |
| CD3^+ CD4^+ T cells count (cells/L) | 50    | 3.19E-06 | 6.4957 | 1.18E-05    | 4.9294       |        |
| % CD4^+ SS^CD14^+ Monocytes | 50    | 6.03E-06 | 6.2181 | 2.12E-05    | 4.6740       |        |
| % KG2^+ ^NKp46^+ NK Cells | 50    | 7.51E-06 | 6.1242 | 2.50E-05    | 4.5013       |        |
| % CD3^+ CD4^+ CCR7^+ PD1^+ T cells | 49    | 8.30E-06 | 5.0810 | 2.64E-05    | 4.5284       |        |
| MFI^CD16^+ SS^CRTH2^+ Basophils | 51    | 1.10E-05 | 4.9580 | 3.35E-05    | 4.4746       |        |
| MFI^HLA-DR Monocytes | 51    | 1.26E-05 | 4.9002 | 3.65E-05    | 4.4373       |        |
| MFI^CD16^+ SS^CRTH2^+ Eosinophils | 50    | 1.30E-05 | 4.8846 | 3.65E-05    | 4.4373       |        |

Stronger discriminators between COVID+ subjects and COVID- patients

| Parameter | Count | P Value | LogWorth | FDR P Value | FDR LogWorth | Change |
|-----------|-------|---------|----------|-------------|---------------|--------|
| % CD4^+ SS^CD14^+ Monocytes | 50    | 6.03E-06 | 6.2181 | 2.12E-05    | 4.6740       |        |
| % KG2^+ ^NKp46^+ NK Cells | 50    | 7.51E-06 | 6.1242 | 2.50E-05    | 4.5013       |        |
| % CD3^+ CD4^+ CCR7^+ PD1^+ T cells | 49    | 8.30E-06 | 5.0810 | 2.64E-05    | 4.5284       |        |
| MFI^CD16^+ SS^CRTH2^+ Basophils | 51    | 1.10E-05 | 4.9580 | 3.35E-05    | 4.4746       |        |
| MFI^HLA-DR Monocytes | 51    | 1.26E-05 | 4.9002 | 3.65E-05    | 4.4373       |        |
| MFI^CD16^+ SS^CRTH2^+ Eosinophils | 50    | 1.30E-05 | 4.8846 | 3.65E-05    | 4.4373       |        |

**Figure 1.** Best immunophenotype discriminators between controls and COVID-19 patients. **A**, Ranking of the 25 most discriminant variables between controls and COVID-19 patients resulting from an unsupervised analysis of immunophenotypic markers. Population frequency is expressed in percentage of gated populations and marker expression is expressed as the MFI of those markers on a defined cell subset. **B**, Representation of the principal component analysis results obtained with the 25 most discriminant markers. COVID-19 patients (dots) and controls (triangles) are well separated with no overlap (left). The contribution of each parameter to each cluster is displayed on the right. Abbreviations: Basos, basophils; COVID-19, coronavirus disease 2019; DC, dendritic cell; Eosinos, eosinophils; FDR, false discovery rate; MFI, median fluorescence intensity; Monos, monocytes; NK, natural killer; PD-L1, programmed cell death ligand 1.

Alterations of lymphocyte subsets have been widely reported [4, 10, 11]. Here, a multiparametric flow cytometry approach using whole-blood samples allowed us to assess the features of the cells involved in the innate response, beyond the lymphocyte response. As opposed to monocytes and lymphocytes, granulocyte investigation requires freshly isolated whole-blood samples. A combination of dry antibody panels optimized for whole-blood investigation and the detection of rare events [12] enabled the simultaneous study of more than 100 phenotypic markers. This unbiased approach showed that changes in the frequency of granulocyte subsets and alteration of their functional phenotypes characterize patients during the course of COVID-19.
In previous studies, the neutrophil to lymphocyte ratio was used to predict the degree of disease severity in patients with early-stage COVID-19 [13, 14]. Eosinopenia was reported in patients with severe disease [15, 16] and was also present in our study population. We show here that the increase in neutrophil counts is characterized by the emergence of cells involved in the inhibition of immune responses. At the neutrophil level, the increase in absolute numbers was due to CD15+CD16+ neutrophils, which may have proinflammatory properties [17]. Neutrophils express predominantly the
glycophosphatidylinositol-linked CD16b isoform, also known as low-affinity IgG receptor FcγRIIIb, which acts as a suppressive FcγR receptor [18]. Low fucosylation of anti-SARS-CoV-2 antibodies [19] suggests that increased CD16+ neutrophils in patients with severe disease might contribute to persistent inflammation through synergistic mechanisms.

Neutrophils from COVID-19 patients also expressed lower levels of CD11b as compared to HBD. CD11b is a subunit of the
αMβ2 (CD11bCD18) integrin involved in intercellular adhesion, transmigration, fibrinogen adhesion, and neutrophil-T cell crosstalk during infection [20–22]. CD11b has been found to play a critical role in the resolution of inflammation process [23]. Although neutrophil CD11b is mobilized from intracellular stores to the cell surface upon activation, alterations of circulating neutrophil CD11b expression are reported in autoimmune conditions, for example, low levels of neutrophil CD11b in rheumatoid arthritis [24] and strong associations with systemic lupus erythematosus in genome-wide studies [20]. Thus, altered neutrophil CD11b expression may contribute to the autoimmune and hypercoagulable status reported in COVID-19 and its severe prognosis [25].

At the surface of basophils and eosinophils, we found a high expression of immune checkpoint PD-L1. Immune checkpoints are regulatory molecules involved in tissue repair at the end of an immune response [26], preventing immune-driven diseases, but they can also be subverted by pathogens, including viruses, to reduce the clearance of pathogens during infectious processes [26–28]. Immune checkpoint studies have addressed mainly T lymphocytes, resulting in a relative lack of information on granulocyte immune checkpoint regulation and clinical implications [27, 29]. Upregulation of neutrophil checkpoint molecules including PD-L1 has been associated with poor outcomes in sepsis patients [29, 30]. Our results show that SARS-CoV-2–induced immune dysregulation and immunoparalysis target the first steps of the virus encounter with granulocytic first-line defenses.

Downregulation of basophil and eosinophil CRTH2 (CD294), a high-affinity receptor of prostaglandin D [31], suggests that SARS-CoV-2 infection might be associated with an inhibition of T helper 2 (Th2)-polarized immune responses and decreased chemotaxis of CRTH2⁺ cells. CRTH2, a central activator of eosinophils, basophils, and Th2-type responses in allergy and hypereosinophilic asthma, might be the explanation for decreased ACE2 expression and apparent protection conferred against SARS-CoV-2 infection and severity by such conditions [32–34]. Conversely, CRTH2 deficiency was associated with pulmonary fibrosis in a mouse model [35], suggesting...
a role for CRTH2 and Th2 downregulation in the pathophysiology of post-COVID-19 pulmonary fibrosis. The combination of increases in CD15⁺CD16⁺ neutrophil frequency, checkpoint inhibitor expression, and reduction in basophil and eosinophil CRTH2 suggest that the granulocyte signature may serve as a reliable biomarker for COVID-19 diagnosis and severity assessment.

The strength of our study is the translational dimension. We explored the association between immune status and disease severity assessed with validated scores in 2 distinct, well-characterized patient groups. There were marked clinical differences between the mild and the severe group, notably the recourse to invasive mechanical ventilation required in 89% of the latter. We found a continuum between the decreased counts and surface marker expression of immune cells and the disease severity, suggesting an association between disease severity and impairment of the immune response. Taken together, our data show an early and deep impairment of the immune response, and question the use of drugs that could alleviate the immune response in COVID-19 patients, especially in the most severe forms requiring intensive care unit admission.

Several limitations must be acknowledged. The small size of the study population precludes definitive conclusions. However, our results are homogenous in each subgroup, and consistent with other published COVID-19 cohorts. Second, the timing of blood sampling differed between mild and severe groups; however, it reflected immune status at turning points in disease progression: at diagnosis and upon progression to severity.

In conclusion, we show here that immune exhaustion during SARS-CoV-2 infection markedly affects first-line immune cells (neutrophils, eosinophils, and basophils) as evidenced by increased expression of inhibitory checkpoints, decreased expression of adhesion molecules, and decreased expression of CRTH2. These findings provide further clues for dysregulated induction of adaptive immune responses and the observed risk mitigation in allergic patients. The predominance of inhibitory systems may preclude the efficiency of viral clearance mechanisms. Further pathophysiological investigations of this new COVID-19 granulocytic signature are required in order to better understand and manage this disease.

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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Author contributions. J. L. M., M. L., P. H., D. O., P. M., and J. V. designed the study. A. B., A. B. D., M. M., S. M., and Y. S. conducted experiments and acquired data. M. L., J. A. S., and A. L. enrolled the patients, collected, and analyzed demographic, clinical and laboratory data. J. M. B., T. M., and F. M. provided antibody panels. J. L. M., M. L., J. A. S., A. L., J. V., J. M. B., T. M., F. M., and S. M. analyzed experimental data. M. L., J. L. M., J. V., J. A. S., A. L., and M. M. wrote the manuscript. All authors read and approved the final manuscript.

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Potential conflicts of interest. T. M., J. M. B., and F. M. are employees of Beckman Coulter Life Sciences. J. V. has received speaker and consultancy fees from Meda Pharma, Mylan, Sanofi, Thermo Fisher, and Beckman Coulter, outside this work. M. L. received fees as speaker from MSD and Edwards LifeScience and as consultant from Aiguettant, Amomed, and Gilead. D. O. is cofounder and shareholder of Imcheck Therapeutics, Emergence Therapeutics, and Alderaan.

All other authors report no potential conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Huang C, Wang Y, Li X, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. Lancet 2020; 395:497–506.
2. Giamarellos-Bourboulis EJ, Netea MG, Rovina N, et al. Complex immune dysregulation in COVID-19 patients with severe respiratory failure. Cell Host Microbe 2020; 27:992–1000.e3.
3. Qin C, Zhou L, Hu Z, et al. Dysregulation of immune response in patients with coronavirus 2019 (COVID-19) in Wuhan, China. Clin Infect Dis 2020; 71:762–8.
4. Wilk AJ, Rustagi A, Zhao NQ, et al. A single-cell atlas of the peripheral immune response in patients with severe COVID-19. Nat Med 2020; 26:1070–6.
5. Le Gall JR, Lemeshow S, Saulnier F. A new Simplified Acute Physiology Score (SAPS II) based on a European/North American multicenter study. JAMA 1993; 270:2957–63.
6. Vincent JL, Moreno R, Takala J, et al. The SOFA (Sepsis-Related Organ Failure Assessment) score to describe organ dysfunction/failure. On behalf of the Working Group on Sepsis-Related Problems of the European Society of Intensive Care Medicine. Intensive Care Med 1996; 22:707–10.
7. Smith GB, Redfern MA, Pimentel MA, et al. The National Early Warning Score 2 (NEWS2). Clin Med (Lond) 2019; 19:260.
8. World Health Organization. Infection prevention and control during health care when novel coronavirus (nCoV) infection is suspected, 2020. https://www.who.int/publications/i/item/10665-331495. Accessed 3 October 2020.
9. WHO Working Group on the Clinical Characterisation and Management of COVID-19 Infection. A minimal common outcome measure set for COVID-19 clinical research. Lancet Infect Dis 2020; 20:e192–7.
10. Zhao Q, Meng M, Kumar R, et al. Lymphopenia is associated with severe coronavirus disease 2019 (COVID-19) infections: A systematic review and meta-analysis. Int J Infect Dis 2020; 96:131–5.
11. Tay MZ, Poh CM, Rénia L, MacAry PA, Ng LFP. The trinity of COVID-19: immunity, inflammation and infection. Nat Rev Immunol 2020; 20:363–74.
12. Bourgoïn P, Hayman J, Rimmelé T, Venet F, Malergue F, Monneret G. A novel one-step extracellular staining for flow cytometry: Proof-of-concept on sepsis-related biomarkers. J Immunol Methods 2019; 470:59–63.
13. Ma A, Cheng J, Yang J, Dong M, Liao X, Kang Y. Neutrophil-to-lymphocyte ratio as a predictive biomarker for moderate-severe ARDS in severe COVID-19 patients. Crit Care 2020; 24:288.
14. Yan X, Li F, Wang X, et al. Neutrophil-to-lymphocyte ratio as prognostic and predictive factor in patients with coronavirus disease 2019: A retrospective cross-sectional study [published online ahead of print 26 May 2020]. J Med Virol doi: 10.1002/jmv.26061.
15. Liu F, Xu A, Zhang Y, et al. Patients of COVID-19 may benefit from sustained lopinavir-combined regimen and the increase of eosinophil may predict the outcome of COVID-19 progression. Int J Infect Dis 2020; 95:183–91.
16. Lindsley AW, Schwartz JT, Rothenberg ME. Eosinophil responses during COVID-19 infections and coronavirus vaccination. J Allergy Clin Immunol 2020; 146:1–7.
17. Li Y, Li H, Wang H, et al. The proportion, origin and pro-inflammation roles of low density neutrophils in SFTS disease. BMC Infect Dis 2019; 24:109.
18. Wang TT, Ravetch JV. Functional diversification of IgGs through Fc glycosylation. J Clin Invest 2019; 129:3492–8.
19. Chakraborty S, Edwards K, Buzzanco AS, et al. Symptomatic SARS-CoV-2 infections display specific IgG Fc structures. medRxiv 20103341 [Preprint]. 15 May 2020 [cited 22 September 2020]. Available from: https://doi.org/10.1101/2020.05.15.20103341.
20. Aarts CEM, Hiemstra IH, Béguin EP, et al. Activated neutrophils exert myeloid-derived suppressor cell activity damaging T cells beyond repair. Blood Adv 2019; 3:3562–74.
21. Tak T, Rygiel TP, Karnam G, et al. Neutrophil-mediated suppression of influenza-induced pathology requires CD11b/CD18 (MAC-1). Am J Respir Cell Mol Biol 2018; 58:492–9.
22. Scott NR, Swanson RV, Al-Hammadi N, et al. S100A8/A9 regulates CD11b expression and neutrophil recruitment during chronic tuberculosis. J Clin Invest 2020; 130:3098–112.
23. Pilione MR, Agosto LM, Kennett MJ, Harvill ET. CD11b is required for the resolution of inflammation induced by Bordetella bronchiseptica respiratory infection. Cell Microbiol 2006; 8:758–68.
24. Leite Pereira A, Bitoun S, Paoletti A, et al. Characterization of phenotypes and functional activities of leukocytes from rheumatoid arthritis patients by mass cytometry. Front Immunol 2019; 10:2384.
25. Iba T, Levy JH, Levi M, Thachil J. Coagulopathy in COVID-19 [published online ahead of print 18 June 2020]. J Thromb Haemost doi: 10.1111/jth.14975.
26. Wang Z, Wang S, Goplen NP, et al. PD-1hi CD8+ resident memory T cells balance immunity and fibrotic sequelae. Sci Immunol 2019; 4:eaw1217.
27. Wykes MN, Lewin SR. Immune checkpoint blockade in infectious diseases. Nat Rev Immunol 2018; 18:91–104.
28. Kahan SM, Zajac AJ. Immune exhaustion: past lessons and new insights from lymphocytic choriomeningitis virus. Viruses 2019; 11:156.
29. Patera AC, Drewry AM, Chang K, Beiter ER, Osborne D, Hotchkiss RS. Frontline science: defects in immune function in patients with sepsis are associated with PD-1 or PD-L1 expression and can be restored by antibodies targeting PD-1 or PD-L1. J Leukoc Biol 2016; 100:1239–54.
30. Patil NK, Guo Y, Luan L, Sherwood ER. Targeting immune cell checkpoints during sepsis. Int J Mol Sci 2017; 18:2413.
31. Huang T, Hazen M, Shang Y, et al. Depletion of major pathogenic cells in asthma by targeting CRTh2. JCI Insight 2016; 1:e86689.
32. Jackson DJ, Busse WW, Bacharier LB, et al. Association of respiratory allergy, asthma, and expression of the SARS-CoV-2 receptor ACE2. J Allergy Clin Immunol 2020; 146:203–6.e3.
33. Kimura H, Francisco D, Conway M, et al. Type 2 inflammation modulates ACE2 and TMPRSS2 in airway epithelial cells. J Allergy Clin Immunol 2020; 146:80–88.e8.
34. Carli G, Cecchi L, Stebbing J, Parronchi P, Farsi A. Is asthma protective against COVID-19? [published online ahead of print 1 June 2020]. Allergy doi: 10.1111/all.14426.
35. Ueda S, Fukunaga K, Takihara T, et al. Deficiency of CRTH2, a prostaglandin D2 receptor, aggravates bleomycin-induced pulmonary inflammation and fibrosis. Am J Respir Cell Mol Biol 2019; 60:289–98.