

Heterogeneous NLRP3 inflammasome signature in circulating myeloid cells as a biomarker of COVID-19 severity

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Key Points

- Measurement of NLRP3 inflammasome activation in the blood of patients reveals an impaired immature neutrophil response in severe COVID-19.
- Inflammasome signature analysis in circulating myeloid cells allows COVID-19 patients to be stratified and predicts evolution.

Dysregulated immune response is the key factor leading to unfavorable coronavirus disease 2019 (COVID-19) outcome. Depending on the pathogen-associated molecular pattern, the NLRP3 inflammasome can play a crucial role during innate immunity activation. To date, studies describing the NLRP3 response during severe acute respiratory syndrome coronavirus 2 infection in patients are lacking. We prospectively monitored caspase-1 activation levels in peripheral myeloid cells from healthy donors and patients with mild to critical COVID-19. The caspase-1 activation potential in response to NLRP3 inflammasome stimulation was opposed between nonclassical monocytes and CD66b⁺CD16^{dim} granulocytes in severe and critical COVID-19 patients. Unexpectedly, the CD66b⁺CD16^{dim} granulocytes had decreased nigericin-triggered caspase-1 activation potential associated with an increased percentage of NLRP3 inflammasome impaired immature neutrophils and a loss of eosinophils in the blood. In patients who recovered from COVID-19, nigericin-triggered caspase-1 activation potential in CD66b⁺CD16^{dim} cells was restored and the proportion of immature neutrophils was similar to control. Here, we reveal that NLRP3 inflammasome activation potential differs among myeloid cells and could be used as a biomarker of a COVID-19 patient's evolution. This assay could be a useful tool to predict patient outcome. This trial was registered at www.clinicaltrials.gov as #NCT04385017.

Introduction

Severe acute respiratory syndrome (SARS) coronavirus 2 (SARS-CoV-2) is a novel human coronavirus that emerged in December 2019 in Wuhan, China.¹ The virus is responsible for a contagious respiratory illness named coronavirus disease 2019 (COVID-19), which can evolve into life-threatening SARS in some cases.² However, some patients infected by SARS-CoV-2 suffer from mild COVID-19 conditions, reporting only slight cough and low-grade fever, and cases of even asymptomatic carriers have been reported.² As for most viral infections, it is very likely that the outcome of the infection is mainly governed by the interplay between virus and host antiviral immunity.^{3,4} Innate immunity is the first line of defense against pathogen invasion in naive patients. It plays an essential role in restricting viral replication and activating adaptive immunity during the first stages of infection. Innate immune defects have been involved in susceptibility to infection whereas activating mutations can cause autoinflammatory diseases.⁵ Both innate and adaptive immunity work as a continuum that starts by an efficient detection of the pathogen by the innate immune system.⁵ The innate immune detection system of viruses relies on

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pattern recognition receptors (PRRs). PRRs are conserved proteins able to sense pathogen-associated molecular patterns specific to microbes.⁷ Viral nucleic acids as well as viral proteins have been shown to interplay with PRRs.⁸ Among the PRRs, inflammasomes control the maturation of interleukin-1 β (IL-1 β) and IL-18 cytokines.⁹ The NLRP3 inflammasome is the most extensively studied and is activated by either pathogen-associated molecular patterns or damage-associated molecular patterns. Among these triggers, nigericin is a bacterial pore-forming toxin widely used as a specific activator of the NLRP3 inflammasome. The stimulation of NLRP3 by nigericin results in assembly of the inflammasome through recruitment of the adaptor protein apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain (ASC) and the recruitment and activation of caspase-1, which has proteolytic activity and allows the maturation of pro-IL-1 β into active IL-1 β .

Recent studies suggest that the death of COVID-19 patients with no medical history can be attributed to a cytokine storm that is similar to what is observed during sepsis with excessive plasma IL-6 and IL-1 β levels.¹⁰⁻¹² Recent reports have suggested a potential role of NLRP3 inflammasome during the COVID-19 cytokine storm, and clinical evidence of NLRP3 inflammasome involvement during COVID-19 is emerging.¹³⁻²⁰ Furthermore, clinical trials have been designed to dampen either the NLRP3 inflammasome or IL-1 β cytokine-dependent inflammation, but the knowledge concerning NLRP3 inflammasome activation in COVID-19 patients is still limited.

To address this point, we designed an assay to monitor NLRP3-triggered caspase-1 activation in the whole blood of COVID-19 patients. Here, we used this assay to determine the innate immune status of patients by identifying myeloid cell activation profiles and propose these biomarkers as a tool to predict COVID-19 severity.

Methods

Study design and ethics

This prospective study was performed in the Emergency Department, Infectious Diseases Department, and intensive care unit (ICU) of the University Hospital of Nice (Nice, France) as well as in the ICU of Cannes Hospital (Cannes, France) between May and October 2020. A French ethics committee (Comité de Protection des Personnes NORD OUEST-1) approved the study (national registration number 2020-00959-30). The study design is summarized next.

All adult patients managed for COVID-19 in either institution were eligible. COVID-19 diagnosis was confirmed by positive SARS-CoV-2 reverse transcription-polymerase chain reaction on nasopharyngeal swab specimen. The exclusion criteria included pregnancy, breastfeeding, bone marrow aplasia, or HIV infection with a CD4 T-cell count <200/ μ L. Eligible participants provided written informed consent. When required during ICU management, written informed consent was provided by the surrogate decision-maker and confirmed later by patients themselves. The following characteristics of patients were collected: sex, age, and comorbidities; acquired, drug-induced, or congenital immunosuppression; oxygen supply or mechanical ventilation; COVID-19 symptoms; and complete blood cell count for hospitalized patients. COVID-19 disease severity was classified according to World Health Organization (WHO) guidelines.²¹ As daily arterial blood gas is not

performed outside of the ICU, we used the pulse oximetric saturation in oxygen (SpO₂)/fraction of inspired oxygen (FiO₂) ratio (SpO₂/FiO₂) to monitor respiratory dysfunction on the day of inclusion and 48 hours later. When FiO₂ could not be measured, it was calculated as follows: (oxygen flows in liters per minute) \times 0.03 + 0.21.²² Unfavorable outcome was defined as death, ICU transfer, or requirement of mechanical ventilation for patients directly admitted to the ICU. Blood samples from healthy donors were used for comparison and characterization purposes. The recovered COVID-19 patients analyzed were clinically cured and were reanalyzed at a minimum of 29 days after inclusion (mean, 39 days). Blood samples from recovered patients followed the same protocol. Informed consent was provided according to the Declaration of Helsinki following the recommendations of an independent scientific review board. The project has been validated by the Etablissement Français du Sang, the French National Agency for Blood Collection (13-PP-11/CCTIRS no. 14.266).

Statistical analysis

Statistical analysis of flow cytometry data was performed with a Mann-Whitney nonparametric *U* test. Patient characteristics were analyzed using the Fisher's exact test, the χ^2 test, the unpaired Student *t* test, or 1-way analysis of variance where appropriate. Single correlations among SpO₂/FiO₂ ratio and flow cytometry data were evaluated with the Spearman coefficient of correlation. The relationship between SpO₂/FiO₂ ratio and flow cytometry data were checked by visual inspection of scatterplots and outliers were controlled before the analyses.

ELISAs

Plasma from healthy donors or COVID-19 patients was obtained from the sodium citrate collection tubes used for flow cytometry analysis and stored at -80°C so that all samples could be analyzed simultaneously. The cytokine levels in the plasma were determined by enzyme-linked immunosorbent assay (ELISA) using human Quantikine ELISA kits for IL-1 β , IL-1RA, IL-18, and IL-18BPa (R&D Systems) according to the manufacturer's instructions.

Ex vivo stimulation of whole blood and flow cytometry

Whole-blood samples were obtained using sodium citrate collection tubes and analyzed 24 hours later. Peripheral blood was diluted 1/1 with RPMI 1640 medium and treated with 5 μ M nigericin (Invivogen) or vehicle for 30 minutes at 37°C under agitation (500 rpm on Eppendorf ThermoMixer). Caspase-1 activation was detected using the FAM-FLICA Caspase-1 Assay kit (ImmunoChemistry Technologies) according to the manufacturer's instructions. Briefly, cells were incubated with the FAM-FLICA probe for 30 minutes at 37°C before being washed: 1 mL of RPMI 1640 was added to dilute the nonbound probe. Cell-surface markers were stained for 10 minutes in the dark at room temperature using the following recombinant antibodies (1/100; Miltenyi Biotec): CD45-VioGreen (clone REA747), CD14-allophycocyanin (APC)-Vio770 (clone REA599), CD66b-phycoerythrin (PE)-Vio770 (clone REA306), CD16-PE (clone REA423), CD15-APC (clone VIMC6), CD10 APC-Vio770 (clone REA877), and Siglec-8 PE-Vio615 (clone REA1045). Red blood cells were lysed using BD Pharm Lyse buffer (BD Biosciences) according to the manufacturer's instructions. Cells were fixed with 4% paraformaldehyde for 10 minutes. A minimum of 10^5 leukocytes (CD45⁺) were recorded per condition. Cells were analyzed using a MACSQuant 10 flow

cytometer from Miltenyi Biotec. Data were analyzed with FlowJo and GraphPad Prism software. After single cells were gated and debris excluded, peripheral blood mononuclear cells were identified as CD45⁺ cells. Monocytes and granulocytes were then gated as CD14⁺ and CD66b⁺ cells, respectively. The gating strategy used in this study is represented in supplemental Figures 1 and 2.

Results

Patient recruitment

Sixty-six COVID-19 patients and 24 healthy donors were included during the study period; their main clinical characteristics are presented in supplemental Table 1. COVID-19 patients were recruited upon SARS-CoV-2⁺ reverse transcription–polymerase chain reaction; healthy donors were negative for SARS-CoV-2 serological assays. Patients were classified into 4 groups (mild, moderate, severe, and critical) in accordance with WHO guidelines.²¹ None had any acquired, baseline drug-induced, or congenital immunosuppression. Blood from 24 healthy donors with a mean age of 62 years underwent the same assay at the same time, parallel to COVID-19 patients. Eight patients were included during their ICU stay whereas 4 patients included during their management in our Infectious Diseases Ward were subsequently admitted in ICU.

Steady-state caspase-1 activity in circulating myeloid cells

The FAM-FLICA probe (FAM-YVAD-FMK) was previously shown to be a powerful tool for monitoring inflammatory caspase-1 activation in monocytes during bacterial infection.²³ To determine caspase-1 activation levels in multiple blood myeloid cells of COVID-19 patients, we used the FAM-FLICA probe together with specific extracellular immune cell markers (CD45, CD14, CD66b, and CD16) in the blood of patients; analyses were performed by flow cytometry (Figure 1; supplemental Figure 1).

Peripheral blood cells of healthy donors or COVID-19 patients were analyzed for the expression of monocyte and granulocyte surface markers and FAM-FLICA. To monitor caspase-1 activity in monocytes from healthy donors or COVID-19 patients, we first gated the CD45⁺CD14⁺ monocyte population, which was subsequently subdivided into 3 subpopulations: classical monocytes (CD45⁺CD14^{high}CD16⁻), intermediate monocytes (CD45⁺CD14^{high}CD16⁺), and nonclassical monocytes (CD45⁺CD14^{dim}CD16⁺) (Figure 1A; supplemental Figure 1). At steady state, we did not observe a statistical difference in the level of caspase-1 activation in any of these monocyte subsets in COVID-19 patients compared with healthy donors (Figure 1B-D).

We next focused our analysis on 2 subpopulations of granulocytes: CD66b⁺CD16^{high} and CD66b⁺CD16^{dim} (Figure 1E). CD66b⁺CD16^{high} cells of mild to moderate COVID-19 patients as well as severe to critical COVID-19 patients showed decreased caspase-1 activation compared with healthy controls (Figure 1F). We measured lower caspase-1 activation in CD66b⁺CD16^{dim} granulocytes in the severe and critical forms of COVID-19 that was not observed in mild cases (Figure 1G). CD66b⁺CD16^{dim} granulocytes of critical patients showed a twofold decrease in basal caspase-1 activation relative to healthy controls (Figure 1G). Consistent with previous reports and validating our cohort,^{10,24} we measured an increase of inflammasome-related cytokines IL-1 β ,

IL-1RA, IL-18, and IL-18BP α in the serum of COVID-19 patients, which correlated with the severity (supplemental Figure 3). Thus, our assay revealed specific regulations of caspase-1 activation in different myeloid cell populations depending on the clinical severity of COVID-19 patients.

Nigericin-triggered caspase-1 activation in circulating myeloid cells

NLRP3 priming is critical for the activation of the inflammasome.²⁵ To investigate whether myeloid cells could be primed to respond to the SARS-CoV2 infection, we evaluated the activation potential of the NLRP3 inflammasome in COVID-19 patients. To this aim, we incubated 100 μ L of blood samples with the NLRP3 trigger nigericin without lipopolysaccharide, and caspase-1 activation was monitored in myeloid innate immune cells of healthy donors and COVID-19 patients. Monocytes are known to be important innate immune effectors and thought to be key players during COVID-19.¹⁵ We first investigated whether we could monitor the monocyte-priming state by analyzing nigericin-triggered NLRP3 activation in monocytes (Figure 2). We observed increased activation specifically in CD14^{dim}CD16⁺ nonclassical monocytes isolated from severe to critical COVID-19 patients (Figure 2A-B). Interestingly, this effect was inversely correlated with the decreased number of these cells in severe to critical COVID-19 patients (Figure 2C). In contrast, the nigericin-triggered NLRP3 activation in intermediate and classical monocytes was found to be similar to healthy donors and their number remained unchanged (Figure 2D-I). These data reveal differences in the NLRP3 inflammasome–priming state not only between healthy controls vs COVID-19 patients but also depending on the subpopulation and the severity of COVID-19. Next, we investigated nigericin-triggered NLRP3 activation in granulocytes (Figure 3). In contrast to CD66b⁺CD16^{high} granulocytes in which we observed increased caspase-1 activation, we measured impaired response to the NLRP3 inflammasome trigger in CD66b⁺CD16^{dim} cells, which is associated with the severity of symptoms (Figure 3A-E). The proportion of CD66b⁺CD16^{high} granulocytes was found to be increased in correlation with the severity whereas the number of CD66b⁺CD16^{dim} granulocytes stayed similar in COVID-19 patients and in healthy donors. Importantly, CD66b⁺CD16^{dim} cells were found to exhibit a higher response to nigericin treatment in both healthy donors and mild COVID-19 patients (Figure 3B,D,E). Interestingly, CD66b⁺CD16^{dim} granulocytes from healthy donors displayed a fourfold increase in nigericin-triggered caspase-1 activation compared with those who were untreated (Figure 3D). In contrast, we observed that the CD66b⁺CD16^{dim} cell response to nigericin was lost in severe and critical COVID-19 patients (Figure 3B,D,E). We here identified different priming levels or tolerance states of the NLRP3 inflammasome in myeloid cell subpopulations that were specific to severe COVID-19 forms, suggesting that this priming is a consequence of the viral infection. The NLRP3 signature of CD66b⁺CD16^{dim} was noteworthy due to its potential value as a biomarker to stratify patients.

Caspase-1 activation in CD66b⁺CD16^{dim} cells after recovery

Next, we wondered whether the impaired response to nigericin in CD66b⁺CD16^{dim} cells was due to a preexisting susceptibility that could be the cause of the symptom's severity or rather a consequence

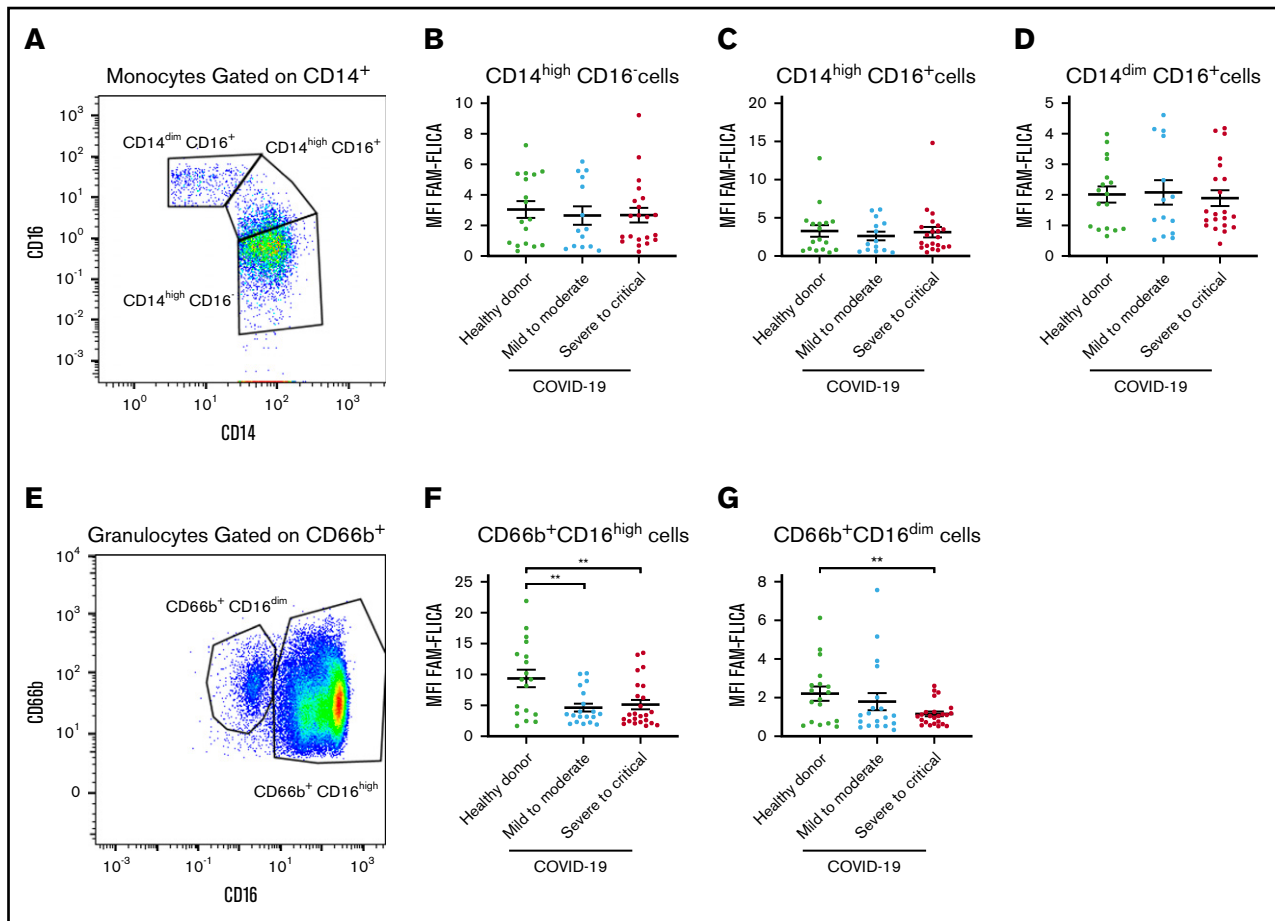


Figure 1. Caspase-1 activation level in myeloid cells in the blood of COVID-19 patients. Whole peripheral blood cells of healthy donors or COVID-19 patients with mild to critical symptoms were stained for active caspase-1 (detected using the FAM-FLICA probe) and for CD45, CD14, CD16, and CD66b markers. Cells were immunophenotyped by flow cytometry. Leukocytes were defined as CD45⁺ and were analyzed for monocyte and granulocyte surface markers. (A-D) Monocytes were defined as CD14⁺ and subpopulations were gated as indicated in panel A using CD14 and CD16 markers. The indicated monocyte subsets were analyzed for the mean fluorescence intensity (MFI) of FAM-FLICA corresponding to the activation of caspase-1 (B-D). (E-G) Granulocytes were defined as CD66b⁺ and the different subsets were gated as indicated using CD66b and CD16 markers (E). (F-G) The indicated granulocyte subsets were analyzed for the FAM-FLICA MFI. ***P* ≤ .01.

of the infection. To address this question, after recovery, we reanalyzed the blood of patients after a mean time of 39 days following inclusion using the same settings (Figure 4). Our data revealed that the CD66b⁺CD16^{dim} cells of recovered patients had a restored nigericin-triggered caspase-1 activation potential (Figure 4A-B). Although 1 patient (number 12) still presented low nigericin-triggered caspase-1 activation (Figure 4B), both severe and critical patients tested had recovered the capacity to respond to nigericin treatment (Figure 4A-B). In addition, nonclassical CD14^{dim}CD16⁺ monocytes isolated from recovered severe to critical COVID-19 patients showed a capacity to respond to nigericin treatment that was similar to controls (Figure 4C). The reversibility of nigericin-triggered caspase-1 activation in CD66b⁺CD16^{dim} granulocytes and CD14^{dim}CD16⁺ monocytes of recovered patients reinforced our interest of this value as a biomarker of COVID-19 severity.

Identification of immature neutrophils as a severity marker of COVID-19 patients

We further attempted to characterize the CD66b⁺CD16^{dim} cells impaired in the NLRP3 inflammasome response in the most severe

forms of COVID-19. We observed that these cells showed differential CD45-expression levels, suggesting the presence of 2 different populations with a respective proportion depending on the severity of COVID-19 (Figure 5A). Indeed, we found that CD45 is highly expressed in the CD66b⁺CD16^{dim} cells of healthy donors and patients with mild cases of COVID-19 whereas, in patients with severe and critical COVID-19, we observed low CD45 expression (Figure 5A). CD66b⁺CD16^{dim} cells could be either eosinophils or immature neutrophils depending on their CD45-expression pattern. To discriminate between these populations, we introduced the CD15, Siglec-8, and CD10 markers in our immunophenotyping panel (supplemental Figures 1 and 2). Siglec-8 was used to identify eosinophils, and CD15 and CD10 were used as markers of mature neutrophils.²⁶ In accordance with the CD66b⁺CD16^{dim}CD45^{high} cell profile, we observed that the majority of Siglec-8-expressing cells in healthy donors and their proportion decreased in severe forms of COVID-19 (Figure 5B). In the same severe patients, we observed an increased number of CD66b⁺CD16^{dim}CD15⁺CD10⁻ immature neutrophils in accordance with the CD66b⁺CD16^{dim}CD45^{dim} profile that we found increased in the severe forms (Figure 5C).

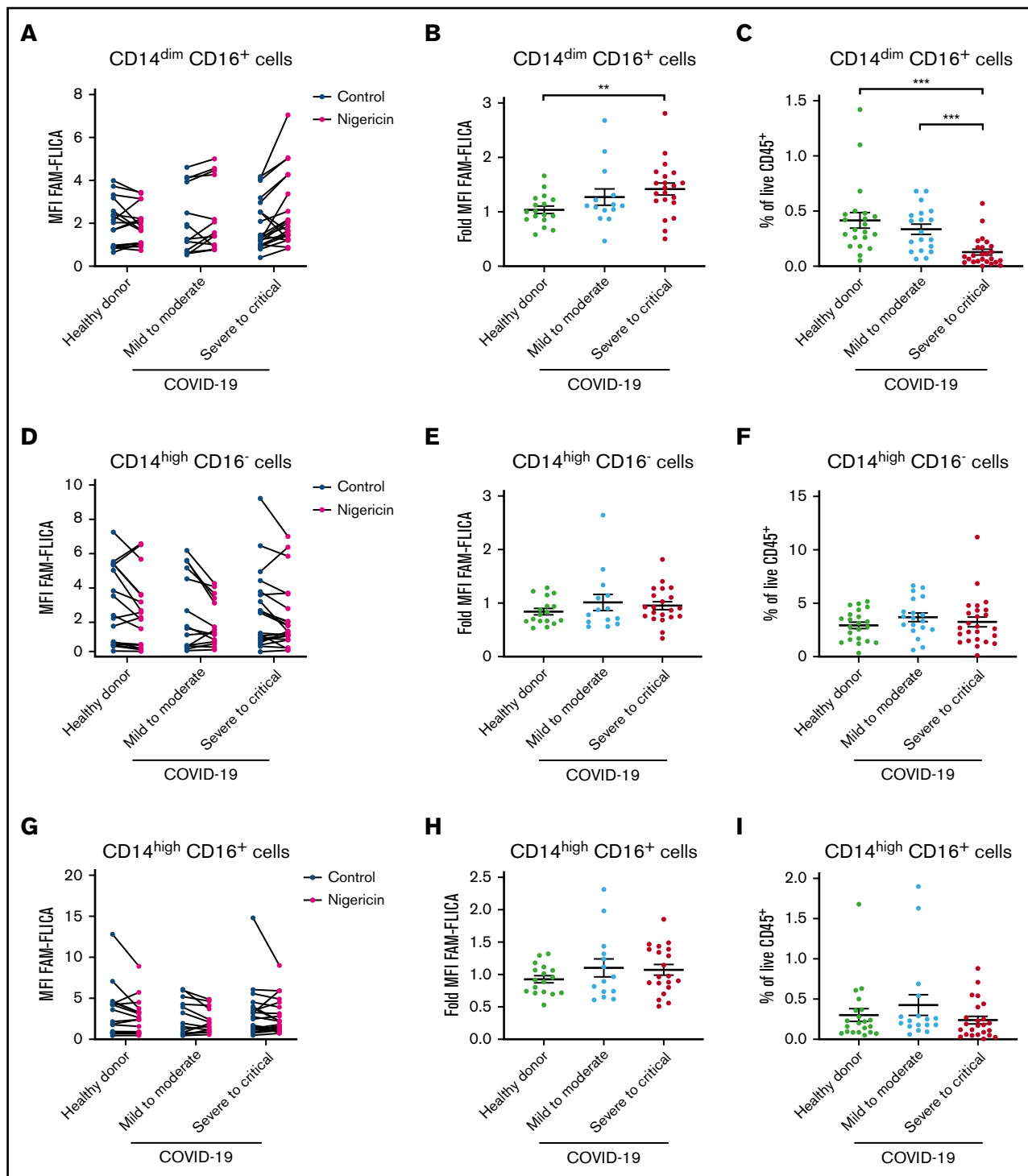


Figure 2. Nonclassical monocyte disappearance and increased nigericin-triggered caspase-1 activation in nonclassical monocytes are associated with COVID-19 severity. Whole peripheral blood cells of healthy donors or COVID-19 patients were analyzed by flow cytometry using CD45, CD14, and CD16 markers. Whole peripheral blood was treated with vehicle (control) or nigericin (5 μ M) for 30 minutes and monocyte subsets were analyzed for FAM-FLICA MFI (caspase-1 activation) (A,D,G) and nigericin-induced fold of FAM-FLICA MFI compared with control (B,E,H). Leukocytes were defined as CD45⁺ (C,F,I) and the frequency of monocyte subsets among leukocytes was analyzed: CD14^{dim}CD16⁺ nonclassical monocytes (C) CD14^{high}CD16⁻ classical monocytes (F) and CD14^{high}CD16⁺ intermediate monocytes (I). ** $P \leq .01$; *** $P \leq .001$.

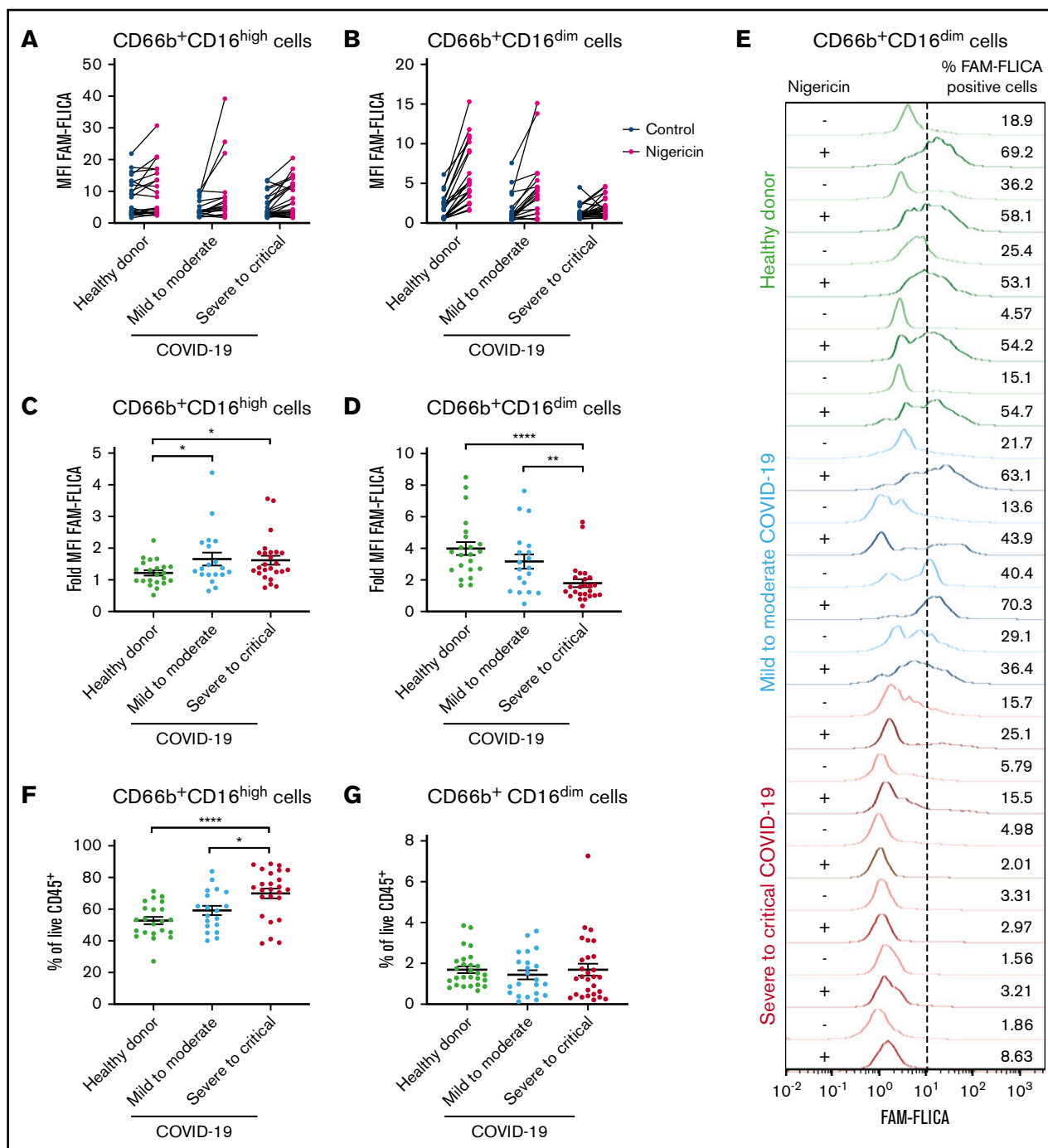


Figure 3. CD66b⁺ CD16^{high} granulocytes display increased nigericin-triggered caspase-1 activation in severe COVID-19 whereas CD66b⁺ CD16^{dim} granulocytes of severe COVID-19 lost their capacity to respond to the NLRP3 stimulation. Whole peripheral blood cells of healthy donors or COVID-19 patients were analyzed by flow cytometry using CD45, CD66b, and CD16 markers. (A-E) Whole peripheral blood was treated with vehicle (control) or nigericin (5 μ M) for 30 minutes and granulocyte subsets were analyzed for FAM-FLICA MFI (caspase-1 activation) (A-B) and nigericin-induced fold of FAM-FLICA MFI compared with control (C-D). (E) Histogram of FAM-FLICA signal in CD66b⁺CD16^{dim} cells. Light colors, FAM-FLICA in the control condition; dark colors, FAM-FLICA in the nigericin-treated condition. The dotted line represents the gate used to determine the percentage of FAM-FLICA⁺ cells. (F-G) Leukocytes were defined as CD45⁺ and the frequency of granulocyte subsets among leukocytes was analyzed. * $P \leq .05$; ** $P \leq .01$; **** $P < .0001$.

Importantly, both eosinophils and immature neutrophils were found to have impaired inflammasome activation in severe and critical forms (Figure 5D-G). Critical patients who recovered

from COVID-19 showed a restored CD45 profile with a marked disappearance of CD66b⁺CD16^{dim}CD45^{dim} cells (Figure 5H). Interestingly, patient 12, found to have a low recovery rate in the

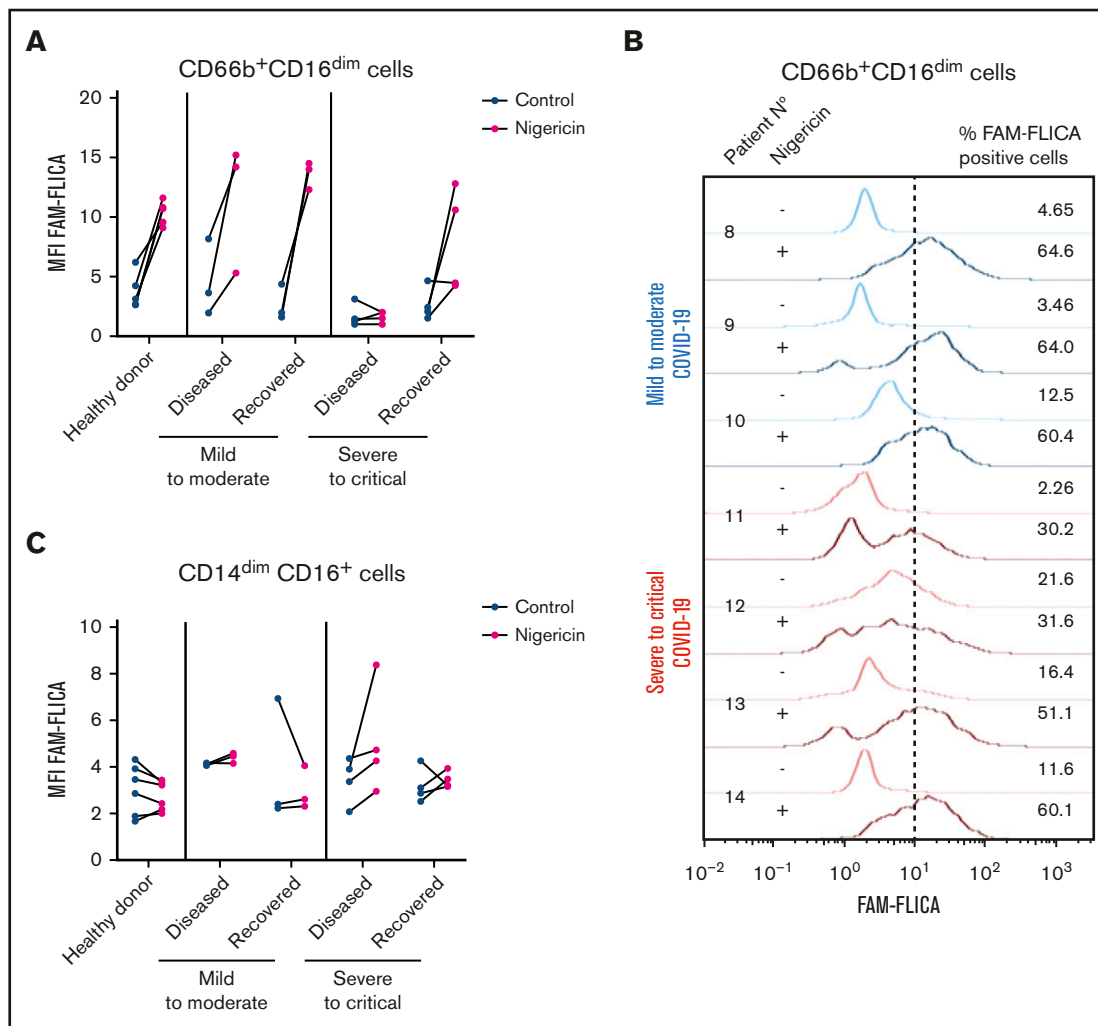


Figure 4. Myeloid cell response to NLRP3 inflammasome stimulation in recovered COVID-19 patients. Peripheral blood cells of recovered COVID-19 patients were collected 30 to 50 days after the first analysis. Whole peripheral blood cells of recovered COVID-19 patients were analyzed by flow cytometry using CD45, CD16, and CD66b markers. (A) Whole peripheral blood was treated with vehicle (control) or nigericin (5 μ M) for 30 minutes and CD66b⁺CD16^{dim} granulocytes were analyzed for FAM-FLICA MFI (caspase-1 activation). (B) Histogram of FAM-FLICA signal in CD66b⁺CD16^{dim} cells. Light colors, FAM-FLICA in the control condition; dark colors, FAM-FLICA in the nigericin-treated condition. The dotted line represents the gate used to determine the percentage of FAM-FLICA⁺ cells. (C) Whole peripheral blood was treated with vehicle (control) or nigericin (5 μ M) for 30 minutes and CD14^{dim}CD16⁺ were analyzed for FAM-FLICA MFI (caspase-1 activation).

nigericin-triggered caspase-1 response, still had a profile with numerous CD66b⁺CD16^{dim}CD45^{dim} cells (Figure 5H).

Inflammasome myeloid cell response as a biomarker of COVID-19 evolution

We here identified biological parameters significantly associated with the level of disease severity at inclusion. The severity biomarkers we identified as statistically robust were the decreased number of nonclassical monocytes and the decreased nigericin-triggered caspase-1 activation in CD66b⁺CD16^{dim} granulocytes (Figures 2C and 3B). We next evaluated whether these parameters can be used as biomarkers to predict both the evolution of the patient during the 2 days following inclusion and the final outcome. The immediate evolution of the disease was evaluated by the patient's oxygen requirement, calculated by the SpO₂/FiO₂ ratio on the day of inclusion (day 1) (Figure 6A-C) and 48 hours later (day 3)

(Figure 6D-F). For this purpose, only hospitalized patients were included in this analysis. Both the decreased number of non-classical monocytes (Figure 6A,D) and the nigericin-triggered caspase-1 activation in CD66b⁺CD16^{dim} granulocytes (Figure 6B,E) correlate with the SpO₂/FiO₂ ratio at day 1 and day 3 after inclusion. However, the correlation with the SpO₂/FiO₂ ratio was stronger with our caspase-1-based score (C1B score) defined as: (the percentage of CD14^{dim}CD16⁺ monocytes) \times (fold FAM-FLICA in CD66b⁺CD16^{dim} granulocytes) (Figure 6C,F).

We thus investigated whether the biological values obtained at day 1 of inclusion could predict the final outcome. Unfavorable outcome was defined as patient death, ICU transfer, or requirement of mechanical ventilation for patients directly admitted to the ICU. Taken alone, the decreased number of nonclassical monocytes was significantly associated with the final outcome of the patients (Figure 6G) and this association was statistically reinforced when

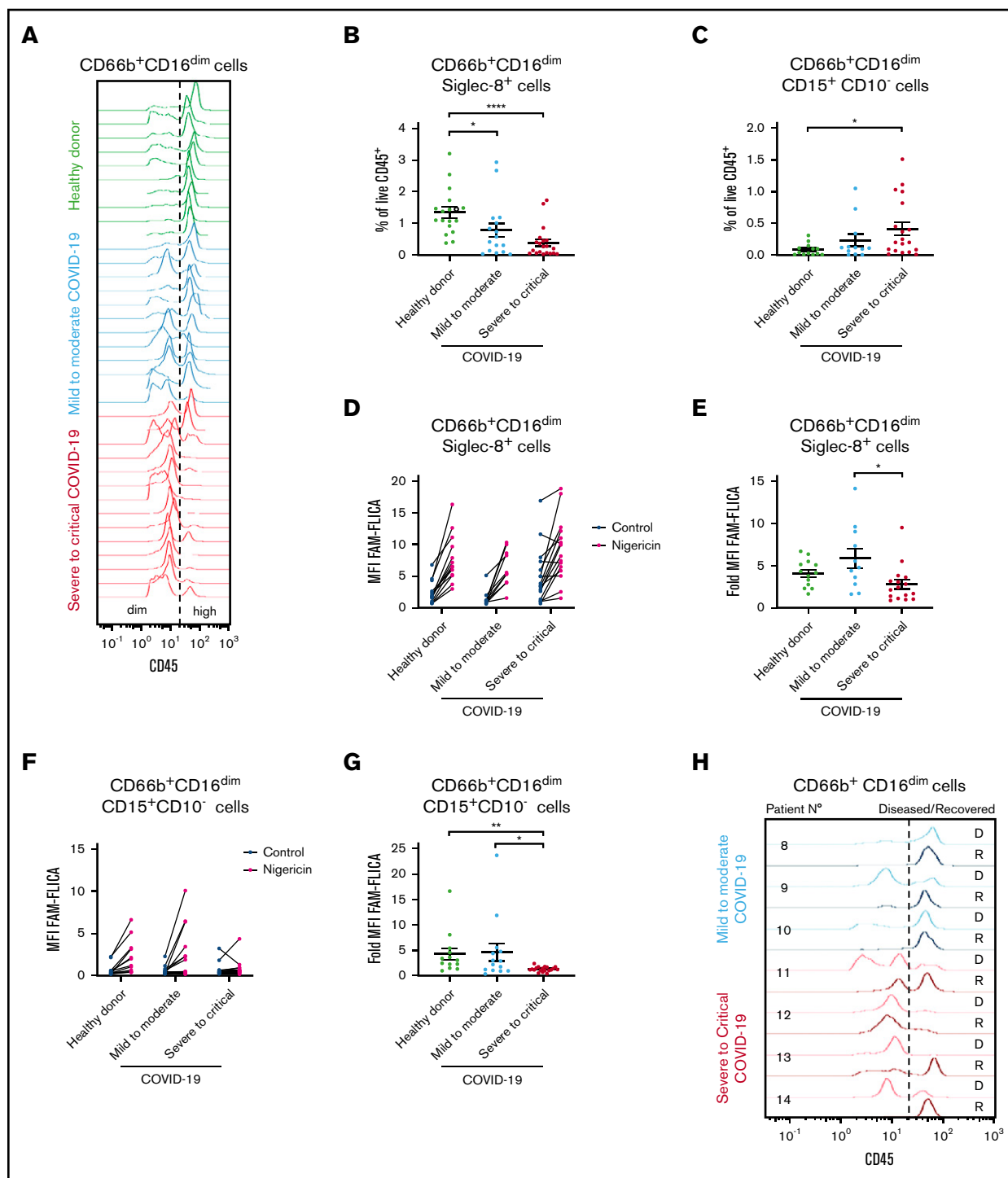


Figure 5. Severe COVID-19 is associated with eosinophil disappearance and accumulation of immature neutrophils with impaired nigericin-triggered caspase-1 activation. Whole peripheral blood cells of healthy donors or COVID-19 patients were analyzed by flow cytometry using CD45, CD66b, CD16, and Siglec-8 or CD45, CD66b, CD16, CD10, and CD15 markers. (A) CD66b⁺CD16^{dim} cells were analyzed for CD45 expression. (B) CD66b⁺CD16^{dim} cells were analyzed for CD66b and Siglec-8 (eosinophil marker) and the frequency of CD66b⁺CD16^{dim}Siglec8⁺ eosinophils among leukocytes was determined. (C) CD66b⁺CD16^{dim} cells were analyzed for CD10 (marker of mature neutrophil) and CD15 (neutrophil marker) and the frequency of CD66b⁺CD16^{dim}CD15⁺CD10⁻ immature neutrophils among leukocytes was determined. (D-G) Whole peripheral blood was treated with vehicle (control) or nigericin (5 μ M) for 30 minutes and eosinophils or immature neutrophils were analyzed for FAM-FLICA MFI (caspase-1 activation) (D,F) and nigericin-induced fold of FAM-FLICA MFI compared with control (E,G). (H) Peripheral blood cells of recovered COVID-19 patients were collected 30 to 50 days after the first analysis. Whole peripheral blood cells of recovered COVID-19 patients were analyzed by flow cytometry using CD45, CD16, and CD66b markers. CD66b⁺CD16^{dim} cells were analyzed for CD45 expression. * $P \leq .05$; ** $P \leq .01$; **** $P < .001$. D, diseased; R, recovered.

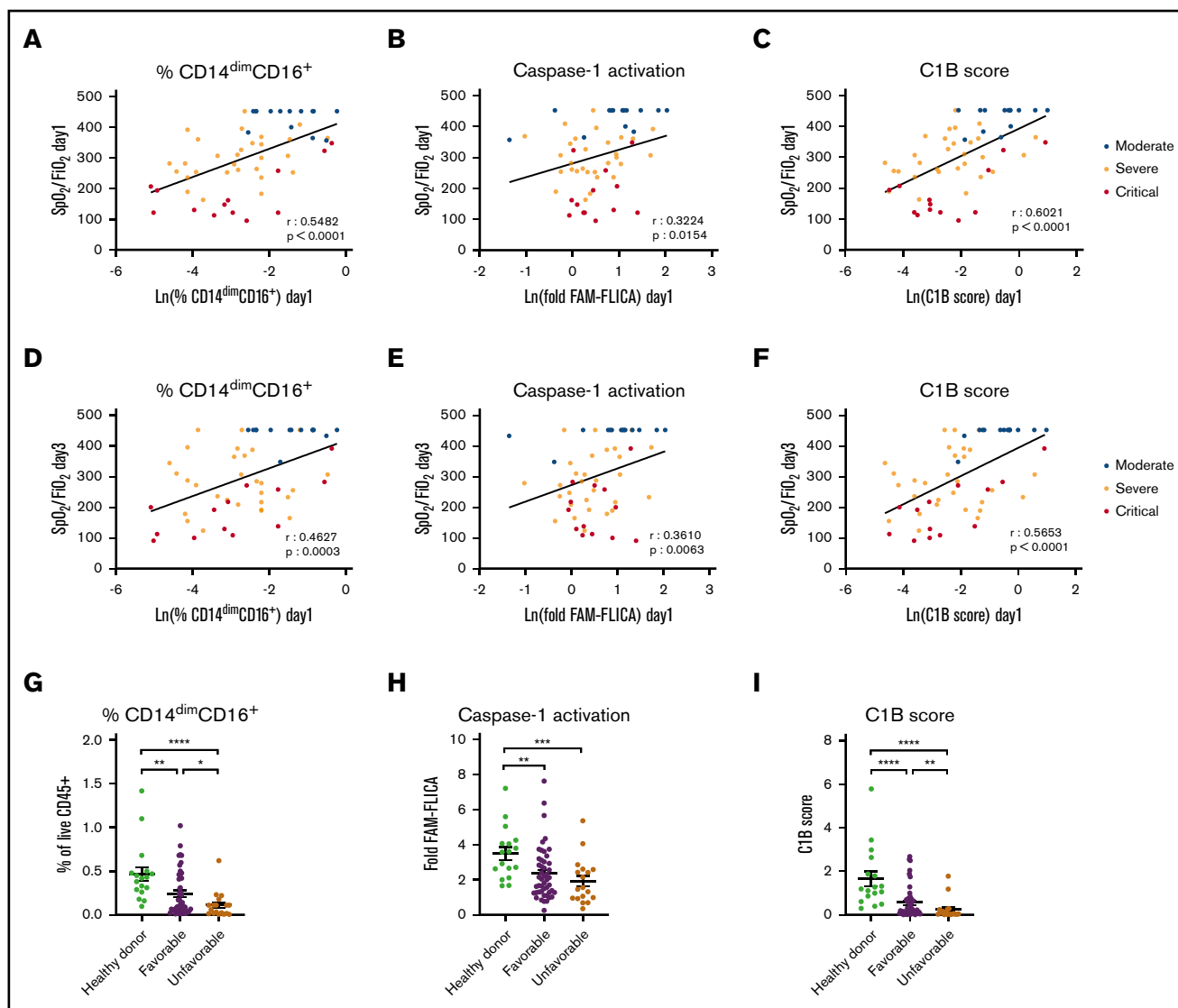


Figure 6. C1B score is associated with the final outcome of the patient and predicts patients' evolution. (A-F) Correlation between (A,D) the percentage of CD14^{dim}CD16⁺ nonclassical monocytes of live CD45⁺ cells, (B,E) the nigericin-triggered fold of FAM-FLICA in CD66b⁺C16^{dim} cells compared with control, (C,F) the C1B score (defined as the percentage of CD14^{dim}CD16⁺ × fold FAM-FLICA in CD66b⁺CD16^{dim} cells) and the SpO₂/FiO₂ ratio at (A-C) day 1 and (D-F) day 3 of inclusion. The line represents the linear regression. Each dot represents a COVID-19 patient and the color its condition at the day of inclusion (blue, moderate; orange, severe; and red, critical). (G-I) Nigericin-triggered fold of FAM-FLICA in CD66b⁺C16^{dim} cells compared with control (H), percentage of CD14^{dim}CD16⁺ nonclassical monocytes of live CD45⁺ cells (G) and C1B score (I) as previously defined, observed in healthy donors or COVID-19 patients with favorable or unfavorable outcome. **P* < .05; ***P* < .01; ****P* < .001; *****P* < .0001. *r*, Spearman coefficient.

the nigericin-triggered caspase-1 activation in CD66b⁺ CD16^{dim} granulocytes was used in the C1B score (Figure 6H-I).

In conclusion, we here described an assay that allows either the monitoring of basal caspase-1 activation or the activation of the NLRP3 inflammasome triggered by nigericin in blood myeloid cells obtained from healthy donors and COVID-19 patients. This assay allowed us to determine nonclassical monocytes as major NLRP3-responsive myeloid cells specifically in severe forms of COVID-19. Our results showed that the CD66b⁺CD16^{dim} cells of COVID-19 patients were decreased both in the basal level of caspase-1 activation as well as in nigericin-triggered caspase-1 activation in severe to critical patients. We show that patients who recovered

from COVID-19 had restored nigericin-triggered caspase-1 activation potential in CD66b⁺CD16^{dim} cells. Finally, we specifically identify the emergence of immature neutrophils that display a strong defect of NLRP3 inflammasome activation in response to nigericin in these patients. Importantly, we provide proof of concept that caspase-1 and NLRP3 inflammasome monitoring in circulating myeloid cells could be used to stratify COVID-19 patients and predict their evolution.

Discussion

The involvement of inflammasomes controlling IL-1 β maturation during the COVID-19 cytokine storm is under extensive investigation, and

drugs inhibiting inflammasomes are expected to dampen this detrimental inflammation. Strategies directly targeting the inflammasome components or the IL-1 β -signaling pathways are currently being evaluated in clinical trials.³ Among them, the use of IL-1R antagonist anakinra in COVID-19 patients has been reported to reduce both mortality and ICU admission, providing first evidence of the importance of this pathway during the COVID-19 cytokine storm.²⁷ Here, by using a probe that labels active caspase-1, we investigated whether myeloid cells in the blood of COVID-19 patients had modulated caspase-1 activation, a hallmark of inflammation, and whether this response is related to the severity of COVID-19 symptoms.

Recent studies have shown increased NLRP3 inflammasome activation in patients with severe COVID-19 by measuring either the increased caspase-1 activity or pyroptotic cell death in peripheral blood mononuclear cells.^{19,20} Here, we extended this finding and precisely defined nonclassical monocytes of COVID-19 patients as the NLRP3 inflammasome most responsive to myeloid cells. Indeed, our study revealed that both basal and triggered inflammasome activation differ among myeloid cell populations. We identified nonclassical monocytes as a myeloid population with a COVID-19 severity signature. Indeed, the nigericin-triggered NLRP3 inflammasome activation of nonclassical monocytes was increased in severe forms. Our data indicated nonclassical monocytes as the major NLRP3 inflammasome-responsive/primed cells in COVID-19 patients and suggest that their decreased proportion in severe forms may be a consequence of pyroptotic cell death occurring downstream of caspase-1 activation. In contrast, we measured lower basal caspase-1 activation in granulocytes of COVID-19 patients. As a major result of our study, we observed that the nigericin-triggered caspase-1 activation of CD66b⁺CD16^{dim} granulocytes inversely correlated with the severity of the symptoms of COVID-19 patients. Here, our data indicated that, in severe and critical patients, CD66b⁺CD16^{dim} granulocytes are not able to respond to the NLRP3 inflammasome stimulation. This result suggests that CD66b⁺CD16^{dim} granulocyte cells could be either exhausted or paralyzed. Interestingly, paralysis of the NLRP3 inflammasome was previously observed in patients during sepsis.²³ Another possibility to explain this absence of responsiveness is that severe or critical COVID-19 patients exhibited a more immature subset of neutrophils associated with an altered response. Such a situation was previously observed during sepsis: CD66b⁺CD16^{dim} neutrophils are released from the bone marrow and display less immune functionality.²⁸ Interestingly, in severe and critical COVID-19 patients, we identified the emergence of CD66b⁺CD16^{dim}CD15⁺CD10⁻ immature neutrophils favoring this hypothesis, reinforcing the parallel between the cytokine storm observed in the severe forms of COVID-19 and that seen during sepsis. Recent reports have indicated the increased number of immature neutrophils in severe forms of COVID-19.²⁹⁻³¹ Complementing these studies, we here provide evidence for impaired function of these cells in correlation with the severity of COVID-19.

Strikingly, in patients who recovered from COVID-19, we found that CD66b⁺CD16^{dim} granulocytes had restored a normal response to nigericin treatment. These data show that the tested

COVID-19 patients did not constitutively exhibit an NLRP3 inflammasome impairment; rather, this reduced response is a consequence of SARS-CoV-2 infection. This observation is in agreement with the occurrence of the NLRP3 inflammasome transient paralysis observed during sepsis.²³ Additionally, these data suggest that bone marrow stem cells, responsible for neutrophil generation, are not altered in recovered COVID-19 patients.

An interesting hypothesis that might explain the heterogeneous cell response of the NLRP3 inflammasome is differential expression of NLRP3 in COVID-19 patients. Supporting this hypothesis, single-cell RNA-sequencing data comparing healthy donors to COVID-19 patients showed increased NLRP3 expression in monocytes.^{32,33} Further studies will be necessary to precisely investigate the transcriptional regulation of inflammasome components with regard to the different myeloid cell population and the severity of the disease.

We here identified the C1B score by combining the parameters our test determined as the most correlated with COVID-19 severity. The analysis of these parameters allowed us to identify the C1B score. For patients included in our cohort, we found that the C1B score predicted the worsening of a patient's clinical status in the next 2 days as well as their final outcome, using a rapid flow cytometry-based test requiring only 100 μ L of blood. We believe that the test should be validated in various conditions and geographic areas for the strength of its prognosis value in various contexts.

By monitoring caspase-1 activation directly in the myeloid cells of COVID-19 patients, we provide first evidence of the involvement of myeloid cells, caspase-1, and the NLRP3 inflammasome complex during COVID-19 disease. We believe that our results will serve as a springboard for future development of a clinical test to be used for personalized medicine or to analyze biomarkers to predict COVID-19 severity. Such a test would have an important impact on the management of COVID-19 patient flow at hospitals during the pandemic period and would be helpful in therapeutic decisions involving immunomodulatory drugs.

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Authorship

Contribution: J. Courjon, D.C., J.D., J. Contenti, and A.R. recruited patients and collected patient samples; J. Courjon, O.D., D.C., C.T., A.D., C.L., S.V., R.L., C.P.-E., G.G., S.I., and A.J. performed and analyzed experiments; J. Courjon, O.D., A.R., L. Bailly, L.Y.-C., P.M., O.V., J.D., V.G., M.C., P.A., S.I., A.J., and L. Boyer interpreted the results; J. Courjon, O.D., and L. Boyer wrote the manuscript; L.Y.-C., S.I., P.A., and A.J. reviewed the manuscript; all authors edited the manuscript; and A.J. and L. Boyer supervised the project.

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