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HIGHLIGHTED ARTICLE

Frontline Science: COVID-19 infection induces readily detectable morphologic and inflammation-related phenotypic changes in peripheral blood monocytes

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Abstract

Excessive monocyte/macrophage activation with the development of a cytokine storm and subsequent acute lung injury, leading to acute respiratory distress syndrome (ARDS), is a feared consequence of infection with COVID-19. The ability to recognize and potentially intervene early in those patients at greatest risk of developing this complication could be of great clinical utility. In this study, we performed flow cytometric analysis of peripheral blood samples from 34 COVID-19 patients in early 2020 in an attempt to identify factors that could help predict the severity of disease and patient outcome. Although we did not detect significant differences in the number of monocytes between patients with COVID-19 and normal healthy individuals, we did identify significant morphologic and functional differences, which are more pronounced in patients requiring prolonged hospitalization and intensive care unit (ICU) admission. Patients with COVID-19 have larger than normal monocytes, easily identified on forward scatter (FSC), side scatter analysis by routine flow cytometry, with the presence of a distinct population of monocytes with high FSC (FSC-high). On more detailed analysis, these $CD14^{+}CD16^{+}$, FSC-high monocytes show features of mixed M1/M2 macrophage polarization with higher expression of $CD80^{+}$

ABBREVIATIONS: ACE2, angiotensin-converting enzyme 2; ALT, alanine aminotransferase; ARDS, acute respiratory distress syndrome; COVID-19, coronavirus disease 2019; CT, computed tomography; FABP4, fatty acid binding protein-4; FBS, fetal bovine serum; FMO, fluorescence minus one; FSC, forward scatter; FSC-high, higher forward scatter; GFP, green fluorescent protein; Hb, hemoglobin; ICU, intensive care unit; IFN, Interferon; IL-1, interleukin-1; IL-6, interleukin-6; JAK, Janus kinase; LDH, lactate dehydrogenase; PBMCs, peripheral blood mononuclear cells; RT-PCR, reverse transcriptase-polymerase chain reaction; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SSC, side scatter; VL, Visceral Leishmaniasis; WHO, World Health Organization.

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and CD206$^+$ compared with the residual FSC-low monocytes and secretion of higher levels of IL-6, IL-10, and TNF-α, when compared with the normal controls. In conclusion, the detection and serial monitoring of this subset of inflammatory monocytes using flow cytometry could be of great help in guiding the prognostication and treatment of patients with COVID-19 and merits further evaluation.

KEYWORDS
COVID-19, flow cytometry, forward scatter, monocyte, morphology

1 | INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) caused an outbreak of coronavirus disease 2019 (COVID-19).[1,2] First recognized in December 2019, it was declared a pandemic by the World Health Organization (WHO) on 11 March 2020 as it has rapidly spread across the world. Over 6 months since it was first recognized, the pandemic shows no sign of abating, resulting in severe pressure on healthcare services worldwide, a significant death toll, and global economic disruption.

COVID-19 patients experience different manifestation of disease ranging from asymptomatic infection to varying severity of pneumonia, all the way to acute respiratory distress syndrome (ARDS) and sepsis with multiorgan failure and death. Initial clinical features at disease onset are fever (77%–98%), dry cough (46%–82%), myalgia or fatigue (11%–52%), and dyspnea (3%–31%).[3] The majority of COVID-19 patients develop pneumonia, which can proceed in up to 20%–30% of cases to respiratory failure requiring intubation and ventilatory support. In those COVID-19 patients who go on to develop pneumonia, dyspnea develops a median of 8 days after onset of illness. Radiographic abnormalities (CT or chest X-ray) consisting of ground-glass opacities and focal consolidation are seen in patients with pneumonia. Major causes of death include respiratory failure and myocardial damage due to myocarditis. Acute kidney injury, secondary infection, and coagulopathy are each seen in approximately 50% of nonsurvivors. Mortality increases with age and in patients with underlying comorbidities, such as hypertension, diabetes mellitus, coronary heart disease, chronic lung disease, and cancer. According to a recent retrospective report from Wuhan, clinical predictors of increased mortality on multivariate analysis included advanced age, progressive organ failure, and elevated D-dimer levels on admission.[4] Other factors significantly associated with poor outcome by univariate analysis included elevated levels of serum ferritin, IL-6, alanine aminotransferase (ALT), lactate dehydrogenase, and highly sensitive cardiac troponin I, as well as reduced levels of lymphocytes, hemoglobin, platelets, and serum albumin.

Primary control of viral infection requires a complex and multifaceted immune response. The interaction of SARS-CoV-2 with the immune system and the subsequent contribution of dysfunctional immune responses have been reported to be essential for disease progression.[5,6] Among these aspects of immune dysregulation, the most commonly recognized is lymphopenia (significantly reduced CD4$^+$ and CD8$^+$ T cells), which is seen in up to 85% of severe COVID-19 patients.[3,6–8] As CD8$^+$ T cells are important for killing virus-infected cells, whereas CD4$^+$ T cells are crucial to prime CD8$^+$ T cells by producing cytokines, excessive elimination of these cells could result in an uncontrolled inflammatory response.[6] On the other side, an excessive inflammatory response to SARS-CoV-2 is thought to be a major cause of disease severity and death in COVID-19 patients.[9] There is growing evidence implicating excessive alveolar monocyte/macrophage activation and associated cytokine storm with the pathophysiology of severe SARS-CoV-2 disease-related complications.[10,11] Despite this, there are few reports to date relating to abnormalities of peripheral blood monocytes in patients with COVID-19. Herein we describe novel observations in relation to changes in monocyte morphology and activation status, which correlate with the prognosis and severity of COVID-19 infection and which can be readily quantified by flow cytometry with the concurrent measurement of forward scatter (FSC) and side scatter (SSC), which measure cell size and complexity, respectively. Specifically, we have identified the presence of a population of monocytes with higher FSC (FSC-high), which are not typically seen in healthy donors and other types of viral infections. Further analysis reveals that these FSC-high monocytes show morphologic and phenotypic characteristics of macrophages, and secrete high levels of IL-6, IL-10, and TNF-α.

2 | MATERIALS AND METHODS

2.1 | Patients

In this study, 34 cases of COVID-19 patients (see Supplemental Table 1 for supporting information) in the period from 18 February 2020 to 24 April 2020 were studied from Xi'an No.8 Hospital (Shaanxi Provincial Infectious Disease Hospital) and the First Affiliated Hospital of Xi'an Jiaotong University, which are the designated hospital for COVID-19 by the local government (Shaanxi province, China). All COVID-19 patients were diagnosed according to the WHO interim guidance (https://www.who.int/publications-detail/infection-prevention-and-control-during-health-care-when-novel-coronavirus-(ncov)-infection-is-suspected-20200125) and the Guide of Diagnosis and Treatment of COVID-19 (6th edition, in Chinese) published by the National Health Commission of China (http://www.nhc.gov.cn/yzygj/s7652m/202002/54e1ad5c2aac45c19eb541799bf637e9.shtml), and confirmed by SARS-CoV-2 nucleic acid testing of nasal and pharyngeal throat swab specimens using real-time RT-PCR assay and by chest computed

tomography (CT). This study was approved by the Ethics Commissions of Xi'an No.8 Hospital and the First Affiliated Hospital of Xi'an Jiaotong University (2020-07), with a waiver of informed consent due to a public health outbreak investigation. The study was registered at http://114.255.48.20, which is the Medical Research Registration Information System of the National Health Commission of the People's Republic of China.

2.2 | Blood samples

Peripheral blood samples from the first 2 days of COVID-19 patients on admission were collected in K_3-EDTA-containing tubes for laboratory assessments according to the doctor's instruction. The blood samples were processed within 6 h after collection.

2.3 | Flow cytometry analysis

For membrane staining, 50 μL K3-EDTA anticoagulant whole blood cells were incubated with a panel of fluorochrome-labeled antibodies or unstained/FMO (fluorescence minus 1) controls for 15 min at room temperature. Erythrocytes were further lysed by mixing with 450 μL BD FCAS lysing solution (BD Biosciences, San Jose, CA, USA) for 5–10 min. After washing cells by adding 2–3 mL PBS and centrifuging for 5 min at 400 *g*, the cells were resuspended in 400 μL PBS and examined by a flow cytometer (BD FACScanto^{TM} II; BD Biosciences) using the FACSDiva v. 6.1 software. The data were analyzed by FlowJo software (Version 7.6.1; Tree Star, Inc., Ashland, OR, USA).

For intracellular staining, 100 μL K_3-EDTA anticoagulant whole blood was first aliquoted per assay tube, then surface marker staining was performed, as mentioned above. After lysis of RBCs and rinsing with PBS, the samples were then fixed with 1% paraformaldehyde in PBS for 15 min, permeabilized with 0.1% saponin for 30 min at room temperature. After rinsing the samples with PBS, the cells were incubated with antibodies for staining intracellular molecules for 30 min. Next, the cells were washed by centrifugation in 3 mL PBS twice. Resuspended cells in 400 μL PBS were analyzed on flow cytometer.

Indirect staining was performed as follows to investigate the expression of the SARS-CoV-2 angiotensin-converting enzyme 2 (ACE2) on monocyte/macrophage cell lines. Briefly, human monocytic cell lines THP-1 and U937, as well as murine macrophage cell line RAW264.7, were cultured in RPMI1640 medium supplemented with 10% FBS and antibiotics, or high glucose DMEM medium supplemented with 10% FBS but without antibiotics (for RAW264.7), at 37°C in a 5% CO_2 air incubator. Half million of log-phase cells were then collected to incubate with Human Fc Block^{TM} (BD Biosciences) at 4°C for 15 min. After blocking, the cells were stained with the primary ACE2 antibody at 4°C for 30 min. After washing with PBS, the cells were further stained with FITC-labeled secondary antibody at 4°C for 30 min. The washed and resuspended cells were finally analyzed on the flow cytometer. The final staining volume for all samples was 50 μL. For flow cytometric analysis of the ACE2 expression on human peripheral monocytes, 50 μL anticoagulant whole blood cells were blocked with Human Fc Block^{TM} first, then stained with the primary ACE2 antibody and the secondary antibody as described above. Samples stained with the secondary antibody alone were used as a negative control.

A full list of antibodies used in this study is included as Supplemental Table 2.

2.4 | SARS-CoV-2 pseudovirus entry assay

Pseudovirus (FNV-SARS-CoV-2-S) expressing SARS-CoV-2-Spike protein and GFP was bought from Biodragon (Beijing, China). Following host cell entry mediated by the interaction between spike protein on pseudovirus capsid and ACE2 on host cells, it will express bright green fluorescence in the host cell nucleus. To evaluate the entry of the FNV-SARS-CoV-2-S pseudovirus into monocyte cell lines THP-1 and U937, macrophage cell line RAW264.7, as well as monocytes in human PBMCs isolated by a density gradient centrifugation method using Ficoll-Paque (Sigma–Aldrich Inc., St. Louis, MO, USA), 5 μL pseudovirus (10^7 TU/mL) was added to 2×10^4 cells in 100 μL serum-free medium in 96-well plate. After centrifugation at 1000 *g* for 60 min, the cells were cultured for 8–18 h, then detected by flow cytometry. The monocytes in PBMC were gated firstly on mononuclear cells and secondly on $CD14^+$.

2.5 | Wright's staining of the blood smear

The peripheral blood smears were made and stained with Wright's stain using a SP1000i (Sysmex, Kobe, Japan) automated smear-maker-stainer system. Examination of stained blood smears was semiautomated with a digital cell morphology system CellaVision DM96 (CellaVision AB, Lund, Sweden), following the manufacturer's instructions.

2.6 | Survival analysis for discharging of COVID-19 patients from hospitals

To investigate the potential effects of FSC-low monocytes and FSC-low/FSC-high monocytes on the discharge of COVID-19 patients from hospital, we performed Kaplan–Meier survival analysis. Thirty COVID-19 patients were categorized into high FSC-low monocytes % and low FSC-low monocytes % groups based on the median of FSC-low monocytes % values. Survival curves were obtained for both groups, and log-rank test was implemented to examine the statistical significance of the difference in survival curves for the 2 groups. Similar analysis was also conducted for FSC-low/FSC-high monocytes. In addition, Cox models were also fitted to adjust the potential effects of age and gender.

2.7 | Statistical analysis

Statistics values are presented as means ± SD. Data were analyzed by using GraphPad Prism version 6.04 (GraphPad Software, San Diego, CA, USA). An unpaired *t*-test was used to compare the mean between 2 independent groups. A one-way ANOVA was used to

TABLE 1 Morphologic changes of monocytes in peripheral blood of HD and COVID-19

	Percentage of total monocytes in peripheral blood leukocytes	The ratio of typical/atypical monocytes in 200 counted leukocytes
Healthy donor	4.2%	9/0
Patient 1	7.9%	3/12
Patient 2	4.6%	2/5
Patient 3	2.1%	1/3
Patient 4	4.0%	0/8

compare the means of more than 2 groups. $P < 0.05$ was considered statistically significant.

3 | RESULTS

3.1 | A specific FSC-high population can be identified in the peripheral blood of COVID-19 patients

To better understand the impact on the immune response of SARS-CoV-2/COVID-19, we originally sought to investigate changes of immune cells in the peripheral blood in COVID-19 patients using flow cytometry. Unexpectedly, in the tested COVID-19 patients, we found the presence of a specific population (FSC-high) right next to the population of monocytes, when using the FSC and SSC parameters (Fig. 1A). In contrast, this population is virtually absent in healthy donors (Fig. 1B and C). To validate the nature of the FSC-high population, we further performed Wright-Giemsa staining on blood smears. As shown in Fig. 1D, we confirmed the presence of an increased number of larger, atypical, vacuolated monocytes, not normally seen in the peripheral blood of healthy individuals. Table 1 shows that while the percentages of monocytes in COVID-19 patients are still in the normal range from 2% to 8% in total WBC count, there was an increased proportion of larger and atypical monocytes.

3.2 | The FSC-high population in COVID-19 expresses higher levels of macrophage markers

Next, we analyzed the expression of the phenotypic markers on both FSC-low and FSC-high populations using flow cytometry. As shown in Figs. 2A and 2B, both FSC-low and FSC-high cells are strongly positive for CD14 and CD16, suggesting that they belong to the monocyte lineage. More importantly, we observed an increase of CD16 in the FSC-high population. Similarly, the expression of CD80 in the FSC-high cells was higher than in the FSC-low cells. Moreover, the expression of CD206 in FSC-high cells was higher than that in FSC-low cells and healthy donors (Fig. 2C). As CD80 is considered a marker typical of M1 polarization and CD206 is considered to be typical of M2 polarization, it suggests that the FSC-high monocytes had features of both M1 and M2 polarization. To further confirm this suspicion, we performed intracellular staining to validate the expression of M1/M2-specific cytokines in peripheral blood monocytes. As shown in Fig. 2D and E, the expression levels of M1-specific cytokines IL-6 and TNF-α in monocytes from COVID-19 patients were significantly higher than that of healthy donors. Similarly, M2-specific cytokine IL-10 was found to be highly expressed by the monocytes from COVID-19 patients, but no statistically significant difference was found. Collectively, these findings may suggest that the peripheral monocytes in COVID-19 patients are inflammatory, and start to differentiate into macrophages.

3.3 | The monocytes in COVID-19 contain a decreased number of classical subset with an increase in intermediate and nonclassical subsets

As immune functions are tightly associated with monocyte subpopulations, we further compared the distribution of the classical, intermediate, and nonclassical subsets between COVID-19 patients and healthy donors by counting the percentage of $CD14^{++}CD16^{-}$, $CD14^{++}CD16^{+}$, and $CD14^{+}CD16^{++}$ cells in FSC-low and FSC-high populations. As shown in Fig. 3, in contrast to healthy donors, the number of classical monocytes in COVID-19 patients decreased, but the number of intermediate and nonclassical monocytes increased.

3.4 | SARS-CoV and SARS-CoV-2 receptor ACE2 is strongly expressed in monocytes

ACE2 is the entry receptor for both the SARS-CoV and SARS-CoV-2.[12–15] To speculate whether directly viral infection could trigger the observed changes in monocytes, we further investigated whether monocytes express ACE2 and, therefore, could be infected by the SARS-CoV-2. By staining ACE2 on human monocytic cell lines THP-1 and U937, as well as murine macrophage cell line RAW264.7, we demonstrated that all these monocyte/macrophage cell lines are ACE2 positive (Fig. 4A). Moreover, we also found that the monocytes in the peripheral blood of healthy donors and COVID-19 patients are ACE2 positive (Fig. 4B–D). However, the levels of ACE2 on the peripheral monocytes from COVID-19 patients are significantly lower than healthy donors (Fig. 4E). Importantly, by using a pseudovirus system expressing viral spike protein, we further demonstrated that SARS-CoV-2 could direct infect these ACE2-positive monocyte cell lines and primary peripheral monocytes (Fig. 5).

3.5 | Survival analysis showed that the amount of FSC-low and the ratio of FSC-low/FSC-high monocytes were associated with time to discharge from hospital for patients with COVID-19

As shown in Fig. 6, significant differences in time to discharge from hospital were identified between COVID-19 patients with low and high levels of FSC-low monocytes% ($\chi^2 = 9.87$, $P = 0.0017$; Fig. 6A) and FSC-low/FSC-high monocytes% ($\chi^2 = 12.08$, $P = 0.0005$; Fig. 6B). The differences were still significant after being adjusted for age and

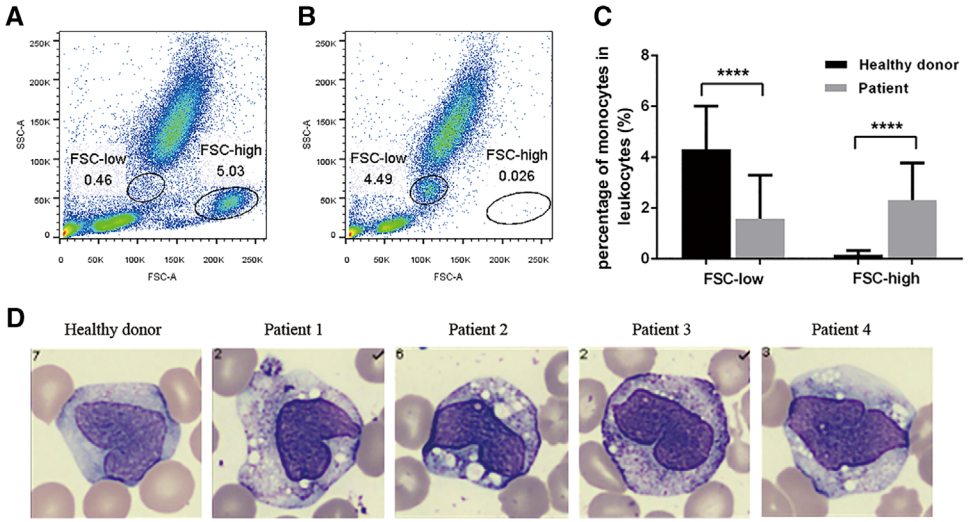

FIGURE 1 **Flow cytometry analysis of the peripheral blood identified a FSC-high population in COVID-19 patients.** (**A** and **B**) Representative pictures of flow cytometry FSC/SSC parameters showing a specific population in the peripheral blood of COVID-19 patients (**A**) and healthy donors (**B**). (**C**) Statistical analysis of the percentage of FSC-low and FSC-high population in healthy controls and COVID-19 patients (HD $n = 16$, patient $n = 34$, $^{****}P < 0.0001$). (**D**) Representative pictures of peripheral blood films showing the FSC-high monocytes in COVID-19 patients (×1,000 magnification)

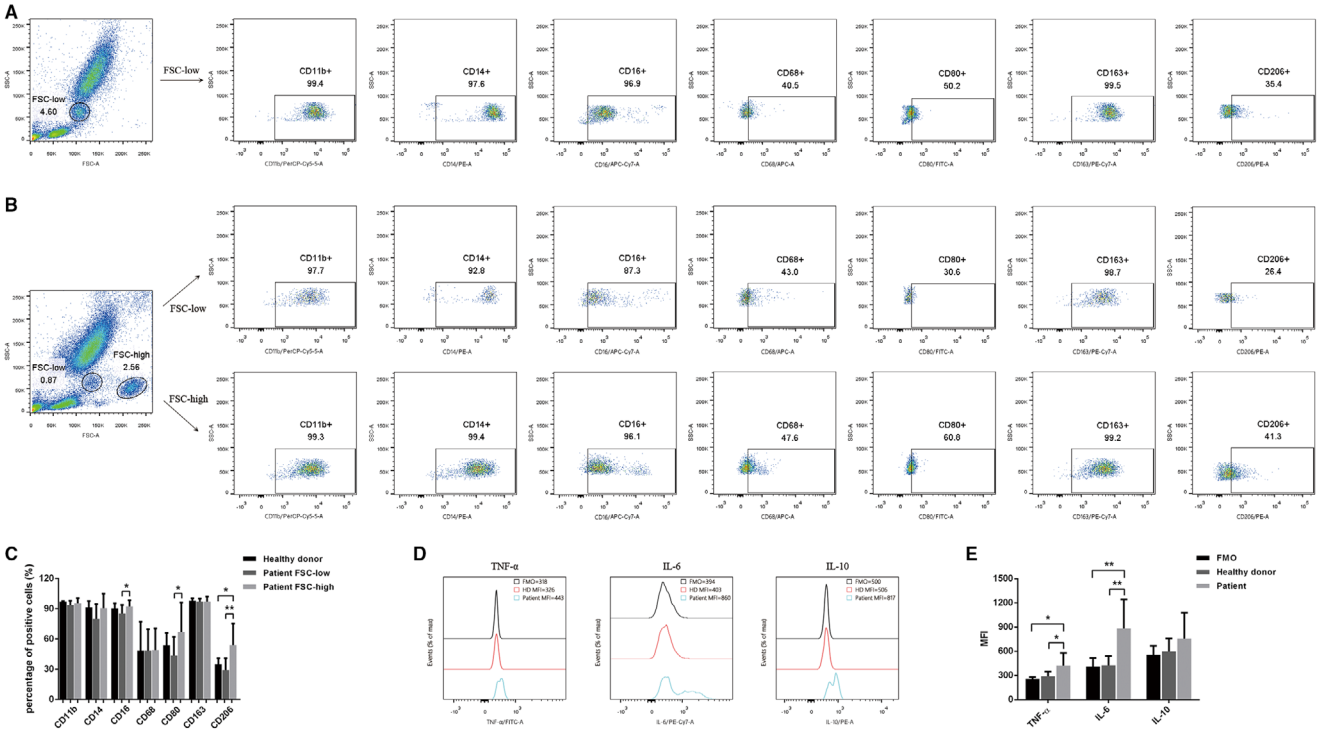

FIGURE 2 Flow cytometry analysis of the expression of the macrophage markers on the monocytes in peripheral blood of COVID-19 patients. (**A**) Representative flow cytometry results show the expression of monocyte/macrophage-related markers in the FSC-low population in healthy donors. (**B**) Representative flow cytometry results show the expression of monocyte/macrophage related markers in FSC-low and FSC-high population in COVID-19 patients. (**C**) Statistical analysis of the expression of the macrophage markers on the monocytes in healthy donors and COVID-19 patients (HD $n = 8$, patient $n = 14$, $^{*}P < 0.05$, $^{**}P < 0.01$). (**D**) Representative intracellular staining flow cytometry results show the expression of M1/M2-associated cytokines. (**E**) Statistical analysis of the expression levels of M1/M2-associated cytokines in peripheral blood monocytes from healthy donors and COVID-19 patients (HD $n = 6$, patient $n = 15$, $^{*}P < 0.05$, $^{**}P < 0.01$)

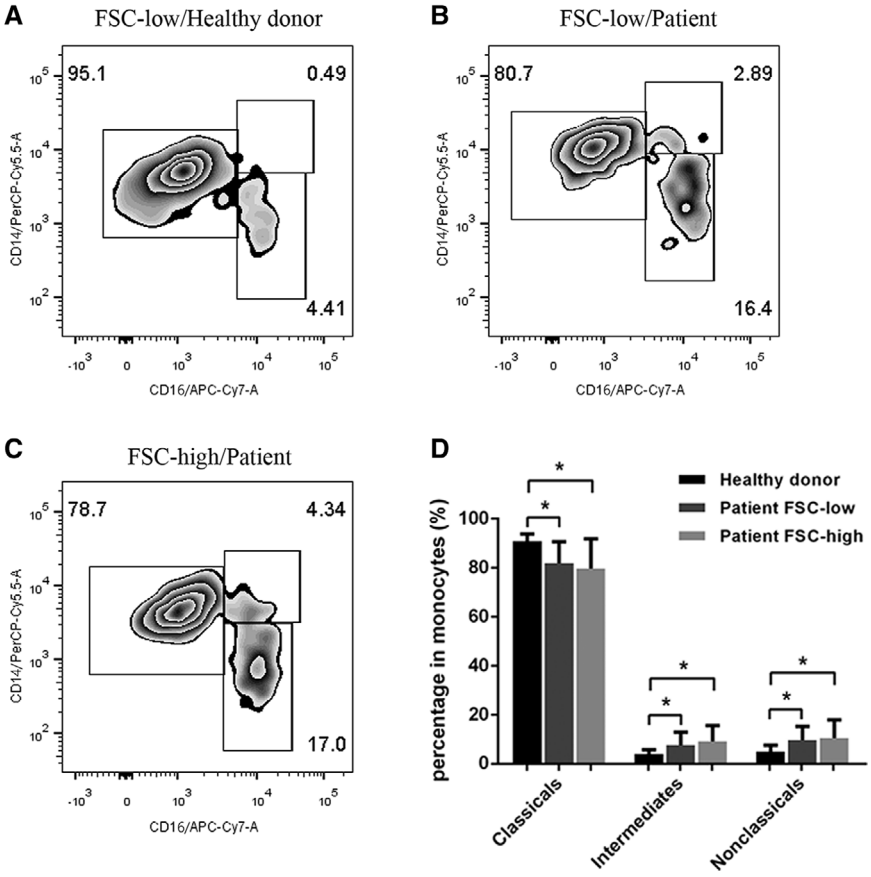

FIGURE 3 The percentage of the 3 kinds of monocyte subsets (classical/intermediate/nonclassical) in FSC-low and FSC-high populations. (**A**–**C**) Representative flow cytometry results show the percentage of the classical/intermediate/nonclassical monocytes in FSC-low of healthy controls (**A**), FSC-low of COVID-19 patients (**B**), and FSC-high of COVID-19 patients (**C**). (**D**) Statistic analysis shows that COVID-19 patients had a reduction in classical monocytes with a higher proportion of intermediate and nonclassical monocytes, compared with the healthy controls (HD $n = 9$, patient $n = 21$, $^*P < 0.05$)

gender by the Cox models for FSC-low monocytes% ($Z = -2.96$, $P = 0.0031$) and FSC-low/FSC-high monocytes% ($Z = -3.34$, $P = 0.0008$). Patients with higher level of FSC-low or FSC-low/FSC-high monocytes spent less time in hospital averagely.

3.6 | COVID-19 patients admitted to the ICU showed a significantly higher ratio of FSC-high population

Of the 34 COVID-19 patients included in this study, 5 were admitted to the intensive care unit (ICU). Focusing on the total number of monocytes and the percentage of FSC-low and FSC-high, we observed a noticeable difference from the non-ICU patients. As shown in Fig. 6C, 3 representative ICU patients have lower number of total monocytes; of these, the FSC-high subsets are the majority. Moreover, the average length of hospital stay of the 5 ICU patients is 40.60 ± 7.89, significantly longer than the average 15.68 ± 2.67 days of the non-ICU patients ($P < 0.0001$).

4 | DISCUSSION

Here, we report our findings in 34 patients with confirmed COVID-19 infection with varying degrees of severity. We identified a distinct change in the morphology and function of monocytes that was predictive of severity of disease, likelihood of ICU admission, length of hospital stay, and full recovery. These monocytes were characterized by a shift in FSC on flow cytometry as well as a relative increase in intermediate and nonclassical monocytes. These FSC-high monocytes express higher level of macrophage markers CD80 and CD206, which represent an inflammatory monocyte subset not typically seen in healthy controls.

In a review of the causes and consequences of cytokine storm and immunopathology in patients with human coronavirus infections that predated the identification of COVID-19, the followings were identified as the major causes of an exuberant inflammatory response: rapid virus replication, hCoV infection of airway and/or alveolar epithelial cells, delayed type I IFN responses, and monocyte/macrophage and neutrophil accumulation.[16] It was highlighted that both SARS-CoV and MERS-CoV encode multiple proteins that antagonize IFN responses and that an early antagonism of the IFN response might delay or evade the innate immune response.[16,17] Furthermore, the delayed IFN signaling could further orchestrate inflammatory monocyte/macrophage responses and sensitize T cells to apoptosis resulting in a further dysregulated inflammatory response.[17] These inflammatory macrophages accumulate in the lungs and are the likely source of proinflammatory cytokines and chemokines associated with fatal disease induced by human coronavirus infections, such as SARS and COVID-19. Autopsy findings from patients with COVID-19 further mirror these findings.[18] Interestingly, as we confirmed, monocytes in both healthy donors and the COVID-19-infected patients are ACE2 positive. In an animal model, SARS-CoV infection of monocytes was shown to lead to abortive replication and a delayed-type I IFN response in these cells.[17] Indeed,

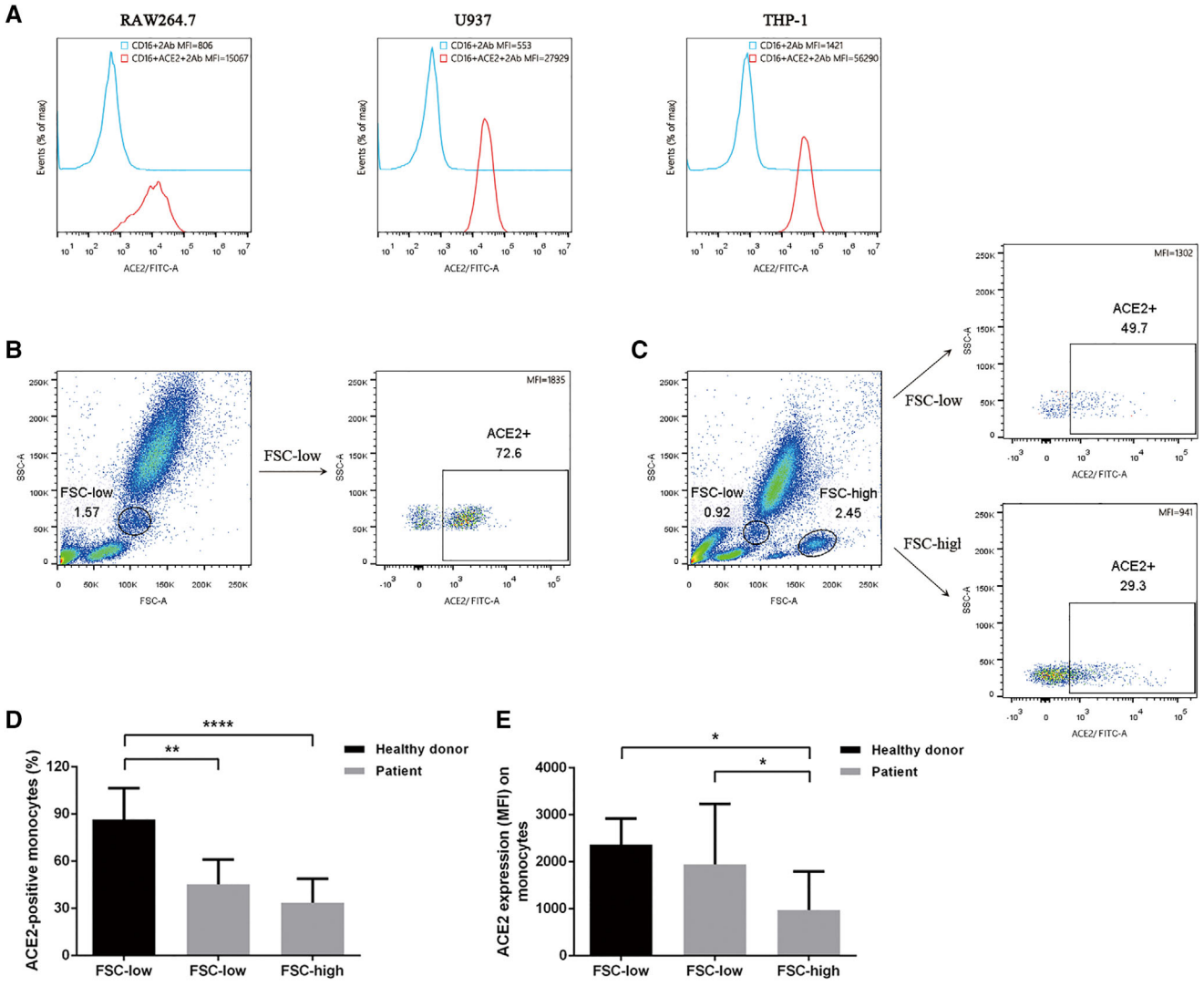

FIGURE 4 **SARS-CoV-2 receptor ACE2 is positively expressed on the surface of the monocytes.** (**A**) Representative flow cytometry results show the expression of ACE2 on monocyte/macrophage cell lines ($n = 3$). (**B** and **C**) Representative flow cytometry results show the expression of ACE2 on monocytes in peripheral blood of the healthy controls (B) and COVID-19 patients (**C**). (**D** and **E**) Statistical analysis shows that patients had lower expression of ACE2 on their monocytes, compared with the healthy donors (HD $n = 3$, patient $n = 13$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$)

the SARS-CoV virus has been found to infect monocytes, though replication was poor.[19] As ACE2 is the receptor used by COVID-19 to gain entry to cells, and most of the monocytes are in the peripheral blood; this may suggest the possibility that SARS-CoV2 may directly infect monocytes. In this regard, a key prerequisite is the presence of SARS-CoV virus in the blood. Using the recommended RT-PCR protocol for throat swab specimens, we failed to detect the presence of the virus in the blood of COVID-19 patients, consistent with the previously report by a German group.[20] After optimizing the viral RNA extraction protocol by using a TaKaRa MiniBEST Extraction Kit, we successfully amplified the virus from the serum of COVID-19 patients (3 out of 12), and the Ct value of positive samples range from 38.71 to 36.29, suggesting a relatively low viral load in blood compared with that in swab samples. By optimizing the extraction protocol, another Chinese group also succeeded in detecting the virus in the serum,[21] suggesting that with the use of more sensitive assays, viremia may be more common than previously thought. Notably, the detectable serum SARS-CoV-2 viral load was found to correlate with drastically increased IL-6 levels in COVID-19 patients tightly.[22] These findings highly suggest that SARS-CoV-2 could directly invade peripheral monocytes. Moreover, the activation of monocytes by SARS-CoV was associated with inflammatory activation and alterations in immune function-related gene expression.[23,24] Inflammatory cytokines such as IFN-α, GM-CSF, IL-6, or TNF-α can also stimulate the differentiation of monocytes to macrophages. We have found that treatment high doses of IL-6 or TNF-α are capable of inducing macrophage differentiation in normal peripheral blood samples within 2 days, whereas GM-CSF plus IFN-α requires 5–7 days (data not shown). Similar to our findings, Zhou et al.[25] recently reported on the presence of a significantly higher percentage of $CD14^{+}CD16^{+}$ inflammatory monocytes in the peripheral blood of COVID-19 patients (both non-ICU and ICU) compared with normal healthy controls. Gómez-Rial

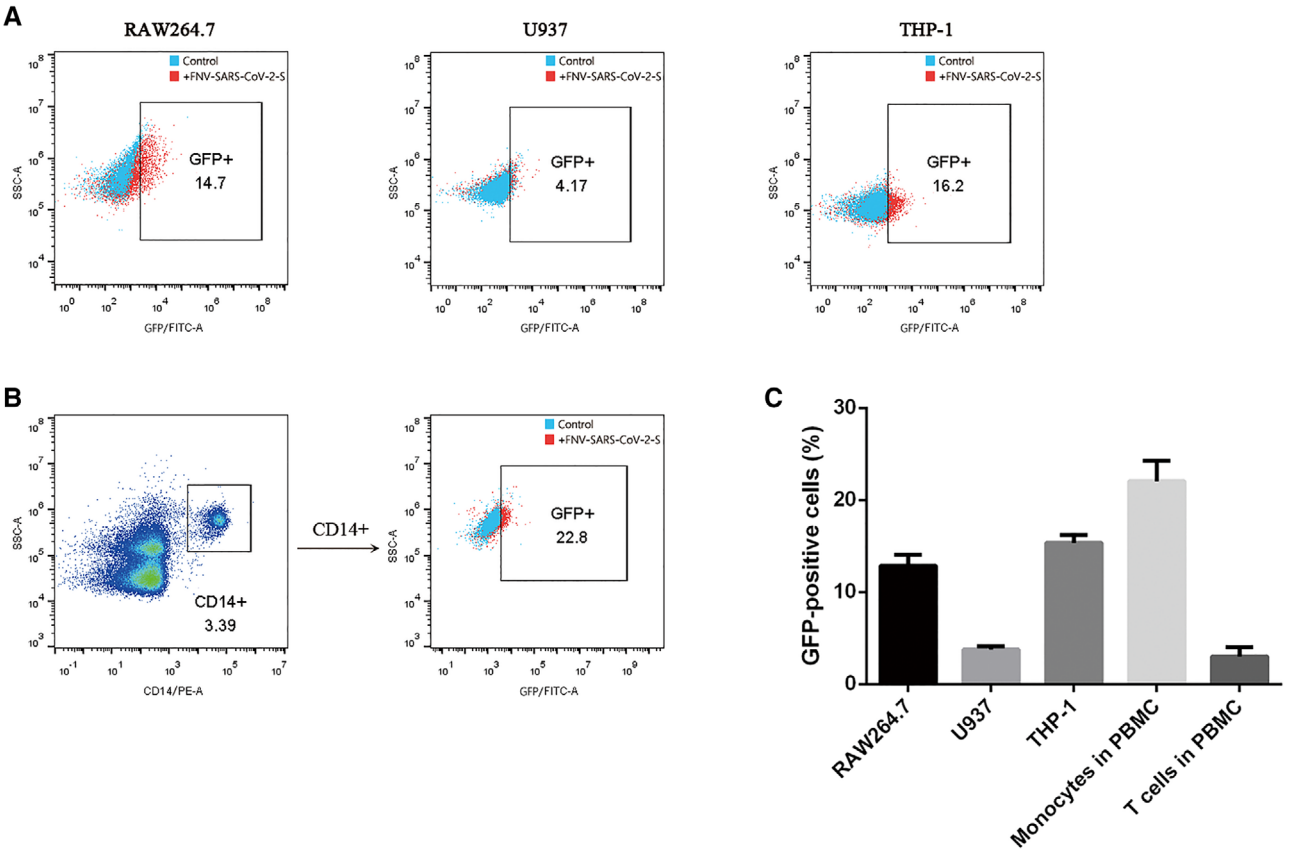

FIGURE 5 **FNV-SARS-CoV-2-S pseudovirus infects monocytes.** (**A**) Representative flow cytometry results show the infection percentage of the different monocyte and macrophage cell lines. The infected GFP-positive cells were detected at the timepoints of 8 (for U937 and THP-1 cells) or 16 (for RAW264.7) hours after the pseudovirus infection ($n = 3$). (**B**) Representative flow cytometry results show the infection of pseudovirus to cultured primary peripheral monocytes. Sixteen hours after the infection, the PBMCs were stained with anti-CD14 to gate on monocyte ($n = 3$). (**C**) Statistical analysis of the transduction efficiency of FNV-SARS-CoV-2-S pseudovirus in different monocytes

et al.[26] found that sCD14 and sCD163, 2 serum markers of monocyte/macrophage activation are significantly increased in COVID-19 patients, and positively correlated with the level of inflammatory cytokine IL-6. This is also supported by recent data relating to single cell RNA sequencing of immune cells from bronchoalveolar lavage and PBMC samples of patients with COVID-19.[27,28] In lung, Ficolin 1 expressing monocyte-derived macrophages, supplant fatty acid binding protein-4 (FABP4) expressing alveolar macrophages as the predominant macrophage subset in the lungs of patients with ARDS.[27] In peripheral blood, there is a unique monocyte subpopulation, which are highly inflammatory and enormous chemokine producers implicated in cytokine storm.[28] These data strongly suggest that targeting migration and differentiation of monocyte/macrophage, or selective cytokine blockade might improve the treatment outcomes of COVID-19.[29,30] Indeed, Tocilizumab, an IL-6 receptor antibody approved for treatment of rheumatoid arthritis as well as CAR-T-associated cytokine release syndrome, has been conducted in the treatment of severe COVID-19 patients with promising results.[31–33] Other treatments for cytokine storm, such as the IL-1 receptor antagonist Anakinra and the JAK inhibitors, may also prove to be useful for treating COVID-19.[34,35]

Given the central role that monocytes appear to play in COVID-19 infection, it is important to recognize the limited information provided by most routine automated blood analyzers. Indeed, the contribution of monocytes to the patient's pathology may be overlooked as patients with severe disease can be monocytopenic.[6] This may well reflect the migration of the inflammatory monocytes/macrophages into the lungs and other affected organs. Although morphologic examination of peripheral blood films revealed somewhat larger, atypical, vacuolated monocytes, these findings are not very specific. We have shown that simple assessment of FSC by flow cytometry in the context of COVID-19 infection can rapidly identify those patients with an increasing proportion of large, activated, IL-6 and TNF-α secreting monocytes, who have severe disease and are at greatest risk of ICU admission. In contrast, patients with a high proportion of normal monocytes have better prognosis with earlier recovery and discharge from hospital. These findings appear to be relatively specific for COVID-19 as we have not seen a similar pattern in healthy donors and the patients with other viral illnesses, such as H1N1 influenza, HIV, or Hantavirus. However, in the peripheral blood of patients with visceral leishmaniasis (VL), we observed a population of FSC-high monocytes (see Supplemental Fig. 1). As the pathogenesis of VL is tightly related to the

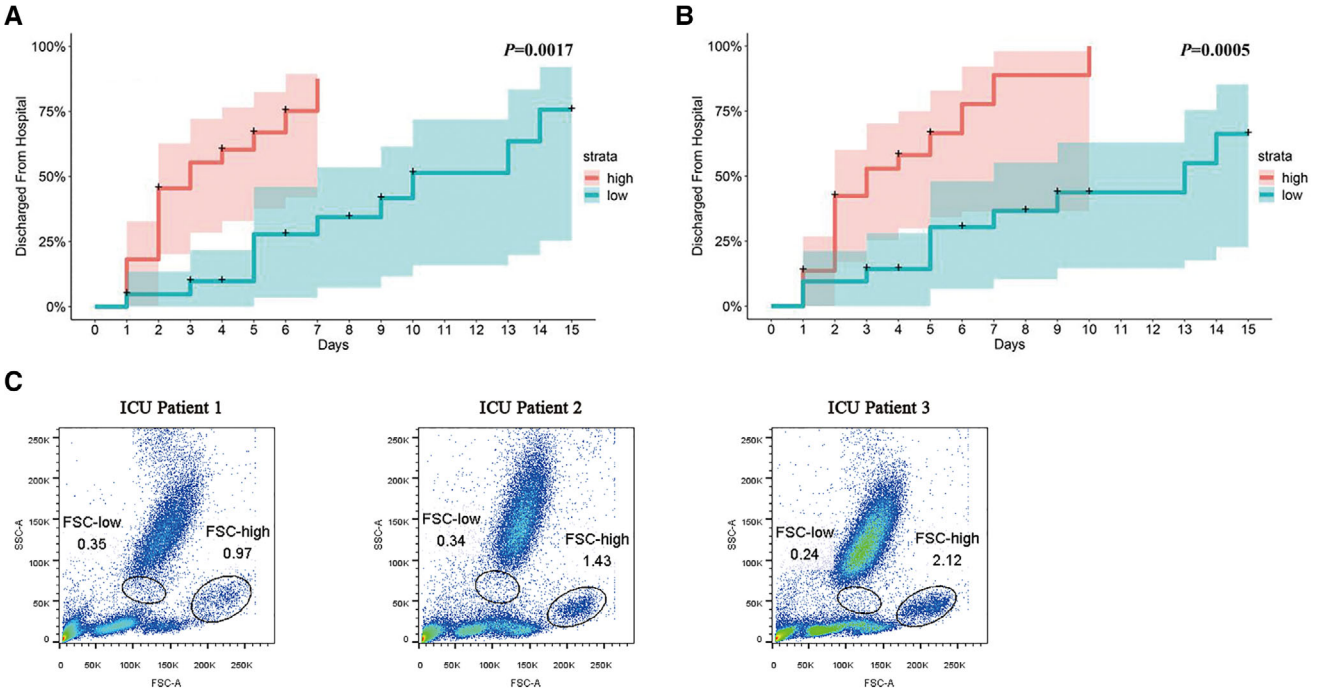

FIGURE 6 **Kaplan–Meier (KM) survival curves obtained from time to discharge from the hospital of the COVID-19 patients stratified by the amounts of FSC-low monocytes and ratio of FSC-low/FSC-high monocytes.** (A) KM survival curves stratified by the amounts of FSC-low monocytes. (B) KM survival curves stratified by the ratio of FSC-low/FSC-high monocytes. (C) The typical FSC/SSC images of the 3 ICU COVID-19 patients

infection of monocytes by Leishmania parasites, it is reasonable that the appearance of FSC-high may be caused by the entry of Leishmania into monocytes.

In conclusion, in patients with severe COVID-19 infection, monocyte activation and the associated inflammatory response is associated with characteristic changes that can be rapidly identified by a simple blood-based flow cytometry-based assay, and serially monitored. Although we acknowledge the limitations of our study, given the small sample size, we feel nevertheless that our findings could be of great help in guiding prognostication and treatment of patients with COVID-19 and merit further evaluation and confirmation in future studies.

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AUTHORSHIP

J.H., M.O'D., and A.H. designed the experiments, supervised the project, and wrote the manuscript. J.H., D.Z., R.G., L.L., H.L., Y.W., and T.D. performed the experiments. H.Q., Y.L., and T.C. collected the samples and the information. D.Z., L.L., Y.W., T.Z., J.W., and Z.L. analyzed the data. All authors accept full responsibility and accountability for the contents of this article.

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DISCLOSURES

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional information may be found online in the Supporting Information section at the end of the article.

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