Single-cell immune profiling reveals long-term changes in myeloid cells and identifies a novel subset of CD9⁺ monocytes associated with COVID-19 hospitalization

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Abstract
Coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection can result in severe immune dysfunction, hospitalization, and death. Many patients also develop long-COVID-19, experiencing symptoms months after infection. Although significant progress has been made in understanding the immune response to acute SARS-CoV-2 infection, gaps remain in our knowledge of how innate immunity influences disease kinetics and severity. We hypothesized that cytometry by time-of-flight analysis of PBMCs from healthy and infected subjects would identify novel cell surface markers and innate immune cell subsets associated with COVID-19 severity. In this pursuit, we identified monocyte and dendritic cell subsets that changed in frequency during acute SARS-CoV-2 infection and correlated with clinical parameters of disease severity. Subsets of nonclassical monocytes decreased in frequency in hospitalized subjects, yet increased in the most severe patients and positively correlated with clinical values associated with worse disease severity. CD9, CD163, PDL1, and PDL2 expression significantly increased in hospitalized subjects, and CD9 and 6-Sulfo LacNac emerged as the markers that best distinguished monocyte subsets amongst all subjects. CD9⁺ monocytes remained elevated, whereas nonclassical monocytes remained decreased, in the blood of hospitalized subjects at 3–4 months postinfection. Finally, we found that CD9⁺ monocytes functionally released more IL-8 and MCP-1 after LPS stimulation. This study identifies new monocyte subsets present in the blood of COVID-19 patients that correlate with disease severity, and links CD9⁺ monocytes to COVID-19 progression.

KEYWORDS
CyTOF, long-COVID-19, nonclassical monocytes, SARS-CoV-2, Slan, T cells

Abbreviations: cDC, classical dendritic cells; cMo, classical monocytes; COPD, chronic obstructive pulmonary disease; COVID-19, coronavirus disease 2019; CyTOF, cytometry by time-of-flight; DC, dendritic cell; DN T cells, double negative T cells; DP T cells, double positive T cells; GLMM, Generalized Linear Mixed Model; immature Mo, immature monocyte; iMo, intermediate monocyte; INR, international normalized ratio; ITU, intensive treatment unit; LDH, lactate dehydrogenase; LIMMA, Linear Models for Microarray Data; nMo, nonclassical monocyte; pDCs, plasmacytoid dendritic cells; PSGL-1, Selectin P ligand-1; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; Slan, 6-Sulfo LacNac; UMAP, Uniform Manifold Approximation and Projection plot.
1 | INTRODUCTION

Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome (SARS)-coronavirus-2 (SARS-CoV-2), has claimed over 6 million lives globally. COVID-19 patients present along a spectrum of severity, ranging from those with asymptomatic or mild flu-like disease to those with critical disease and acute respiratory distress syndrome, which requires hospitalization and mechanically assisted ventilation.

An inflammatory immune response marked by lymphopenia, delayed T cell and antibody responses, increased numbers of monocytes and neutrophils with defective IFN antiviral responses, decreased plasmacytoid dendritic cells (pDCs), and increases of proinflammatory cytokines like TNF-α and IL-6 have all been correlated with increased COVID-19 severity and worse patient outcomes. Also, expansion of CD14+ HLA-DR+ and immunosuppressive immature monocytes has been observed in BMCs from severely ill COVID-19 patients. A general loss of intermediate and/or nonclassical monocytes has also been observed in patients with acute and severe cases of COVID-19, with an increased proportion of nonclassical monocytes and DCs migrating to the lungs. We have recently identified 8 monocyte subsets in healthy humans, and we and others have observed that monocyte subsets can have unique contributions to the progression of disease. Detailed analyses of monocyte subsets in COVID-19 are still just emerging.

Herein, we utilized cytometry by time-of-flight (CyTOF) to identify monocyte (Mo) and DC subsets from healthy subjects and COVID-19 patients that could regulate the immune response to SARS-CoV-2 infection. We also tracked monocyte frequencies at approximately 3 months postinfection in some of the hospitalized subjects. Together, these data generate a more detailed picture of how immune cells change during SARS-CoV-2 infection and identify new subsets of monocytes that may influence the clinical progression of COVID-19.

2 | METHODS

2.1 | Patient samples

This study was approved by the Berkshire Research Ethics 20/SC/0155 (UK) and the Human Institutional Review board of the LJI. Written, informed consent was obtained for all subjects. Healthy subjects (n = 8) donated blood at the San Diego Blood Bank or the LJI Clinical Core. Hospitalized subjects (n = 20) were treated in a teaching hospital in England, either in a general ward or the intensive treatment unit (ITU). SARS-CoV-2 infection was confirmed by reverse transcriptase polymerase reaction or detection of anti-spike protein antibodies. Single-cell transcriptome analysis of virus-reactive CD4+ and CD8+ memory T cells was performed by our colleagues using many of the same COVID-19 patient samples in this study.

2.2 | Sample processing

PBMCs from recovered COVID-19 individuals or healthy subjects were barcoded with CD45 and stained with an established monocyte/T cell panel. Samples were assayed in 5 CyTOF runs. A technical control of healthy PBMCs was CD45 barcoded and spiked into each sample. Data were bead-based normalized using the Matlab-based NormalizerR2013a_Win64. Only live cells were used for analysis.

2.3 | CyTOF analysis

Each CyTOF sample was debarcoded using a deconvolution algorithm implemented in the CATALYST Bioconductor package. Data were normalized using an arcsinh transformation (cofactor = 5). Batch correction was done using a quantile normalization method for the pooled distribution of each batch (a pair of sample and spike-in control) in the function normalizeBatch from the CYDAR Bioconductor package (Figure S1). We used the FlowSOM clustering method with default parameters to identify major cell types from CD45+ live cells. Cell types were identified using lineage markers. Consensus clustering was used to justify the optimal number of clusters from k = 2–30 to based on the relative decrease in area under the cumulative distribution function curve. Clusters were merged based on the similarity of their representative protein markers. Heatmaps of median protein expression and Uniform Manifold Approximation and Projection plot (UMAP) deduction were generated using CATALYST.

2.4 | Monocyte isolation

Healthy donor blood was obtained from the LJI Clinical Core. Whole blood was centrifuged at 500xg for 10 min. Plasma was discarded and remaining blood was mixed 1:1 with PBS (Corning Inc, Corning, NY) with 2% FBS (Omega Scientific, Tarzana, CA), layered on lymphoprep (Stemcell Technologies, Vancouver, BC) and centrifuged at 1200xg for 10 min. Theuffy coat was isolated and enriched for monocytes using the MACS Pan Human Monocyte Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, North Rhine-Westphalia). 5 x 10⁶ monocytes were sorted by flow cytometry using CD14-FITC (Biolegend, San Diego, CA clone M5E2), CD16-BV421 (Biolegend clone B73.1), CD9-PE (Biolegend clone H19a), Dump-PerCP-Cy5.5 (CD19 (Biolegend clone HIB19), CD3 (BD Pharmingen clone UCHT1), CD56 (Biolegend clone MEM-188) and CD66b (Biolegend clone G10FS)), and Live/Dead-Yellow (Invitrogen).

2.5 | Cytokine assays

0.5 x 10⁶ CD9+ and CD9− sorted monocytes were plated onto a 24-well plate in DMEM (Gibco, Waltham, MA) with 10% FBS. Cells
were stimulated for 16 h with 100 ng/ml of LPS (Enzo Biochem, Farmingdale, NY) or equal volume of vehicle control (DMSO). Supernatants were collected and measured for cytokine production using the LEGENDplex human essential immune response kit (Biolegend) and analyzed using LEGENDplex software.

### 2.6 Statistical analysis

Linear modeling, using the Linear Models for Microarray Data (LIMMA) package, was performed to detect statistically significant differentially expressed markers, and a Generalized Linear Mixed Model (GLMM), used through a lme4 package, was used to determine statistically significant differential cell population abundances. Correlations of T cell and monocyte/DC subset frequencies with clinical parameters were performed using Spearman rank correlation tests. Cytokine data were analyzed using GraphPad-PRISM software.

### 3 RESULTS AND DISCUSSION

#### 3.1 Immunoprofiling of PBMCs from COVID-19 patients

In order to examine the immune compartment after SARS-CoV-2 infection, PBMCs and clinical data were obtained from 7 nonhospitalized and 20 hospitalized subjects with COVID-19 and 8 healthy subjects who were age- and sex-matched with the hospitalized subjects (Figures 1(A) and 1(B)). Comorbidities in hospitalized COVID-19
FIGURE 2  CyTOF-mediated identification of changes in immune cell cluster frequencies and surface marker expression in convalescent COVID-19 subjects. CD45<sup>+</sup> Dump<sup>–</sup> leukocytes from healthy and COVID-19 subjects clustered and projected onto a UMAP (A). Expression of cell surface markers projected onto the UMAP of (A) as feature plots (B). Heatmap displaying each cluster’s scaled median expression for 34 markers (C). Box and whisker plots showing median expression of CD9 and CD45RA within the CD45<sup>+</sup> Dump<sup>–</sup> cells for healthy, nonhospitalized, and hospitalized subjects (D). Box and whisker plots of individual cell clusters as a proportion of CD45<sup>+</sup> Dump<sup>–</sup> cells between healthy, nonhospitalized, and hospitalized subjects (E). Statistically significant ($p \leq 0.05$) changes were calculated using adjusted $p$ values generated after a multiple-comparisons correction. Changes in cluster frequencies were calculated using GLMM while changes in marker expression were calculated with LIMMA.

Patients were primarily cardiovascular in scope, including prior histories of myocardial infarction and hypertension (Table S1). PBMCs were analyzed using a 38-marker CyTOF panel focused on identifying monocyte and DC subsets (Figure 1(C)).
FIGURE 3  Changes in monocyte and dendritic cell surface marker expression, subset frequencies and monocyte subset cytokine release in COVID-19 subjects. Monocyte and dendritic cell clusters from Figure 2(A) were subclustered and displayed in their own UMAP (A). Heatmap displaying each subcluster’s scaled median expression of 34 markers (B). Box and whisker plots showing median expression of cell surface markers within all monocytes and dendritic cells used in this subclustering analysis for healthy, nonhospitalized, and hospitalized subjects (C). Box and whisker plots of individual cell clusters as a proportion of all cells in this subclustering analysis between healthy, nonhospitalized, and hospitalized subjects (D). CD9+ and CD9− human monocytes were sorted from healthy human blood and 0.5 × 106 monocytes were incubated with 100 ng/ml of LPS or vehicle control for 16 h. Cell supernatants were collected and used in Luminex analysis for cytokine release quantification (E). Stacked violin plots displaying median expression of CD9, Slan, PDL1, and PDL2 in healthy, nonhospitalized, and hospitalized subjects in each subcluster of monocytes and dendritic cells. The dots represent each individual’s median expression levels and the black horizontal bars represent the median expression levels of each condition (F). Statistically significant (p ≤ 0.05) changes were calculated using adjusted p values generated after a multiple-comparisons correction. Changes in cluster frequencies were calculated using GLMM, changes in marker expression were calculated with LIMMA and changes in cytokine release were calculated with one-way ANOVA with a post hoc Tukey’s test.
3.2 Changes in protein expression and immune cell frequencies during COVID-19

Using the CyTOF data after batch correction (Figure S1), we first assessed changes in global cell surface marker expression and immune cell frequencies in individuals stratified by their hospitalization and infection status (healthy, nonhospitalized, hospitalized). Clusters were identified by referencing their median expression of 35 cell surface markers (Figures 2(A)–(2C)).

Of the 35 surface markers, 16 were primarily markers of cell identity and lineage, so we chose to examine changes in expression of the remaining 19 markers (Table S2). Within these 19 markers, the expression of CD9 and CD45RA significantly increased in the hospitalized subjects (Figure 2(D)). Out of the 13 major immune cell clusters identified within the CD45+ cells for all subjects, the hospitalized subjects displayed a significant increase in the frequency of cMo, CD3+ cMo, iMo, CD56+ T cell, and CD14+ immature monocyte clusters compared with nonhospitalized subjects, and a decrease in the frequency of the nMo cluster compared with the healthy subjects (Figure 2(E)). Notably, none of the changes in cell cluster frequencies in this or subsequent analyses could be attributed to sex or age amongst the donors (Figure S2).

3.3 Subclustering of monocytes and DCs and characterization of CD9+ monocytes

All monocytes and DCs identified in Figure 2(A) were subclustered to obtain a more detailed characterization of the heterogeneity within these populations (Figure 3(A)). Identical subclustering and analysis was also performed for the T cell clusters (Figure S3). When comparing the median expression of the Table S2 markers, we found that expression of CD64, CD163, CD36, CD9, PDL1, and PDL2 all significantly increased, whereas CD95 and 6-Sulfo LacNAc (Slan) expression significantly decreased and HLA-DR trended lower in hospitalized subjects (Figures 3(B) and 3(C)). The increases in PDL1 and PDL2 expression
suggest that these monocytes and DCs may be more immunosuppressive and capable of inhibiting T cell activity than their counterparts in healthy individuals.\(^26,27\) Our finding that expression of HLA-DR trended lower while CD163 increased has been seen by others\(^6,7\) and has been suggested to cause monocytes to be less effective at presenting antigen. CD9 is a tetraspanin that serves as a scaffolding protein for a wide variety of cell surface receptors.\(^28\) For example, CD9 is an important mediator of cell adhesion to the endothelium, MHCII presentation, T cell activation, and LPS-induced TLR4 signaling in myeloid cells.\(^29\)–\(^32\) CD9 mRNA expression increases within classical monocytes of subjects with COVID-19 compared with healthy controls,\(^18\) but this is the first time that CD9 has also been observed to be affected at a protein level.

Our prior work on human cardiovascular disease demonstrates that monocytes can be classified into easily defined subsets with unique phenotypes.\(^14\) After subclustering the monocytes and DCs in our COVID-19 cohort, cMos grouped into 5 separate clusters. While examining markers defining the cMo subsets, we found that CD9 divided the majority of cMos into CD9\(^{hi}\) and CD9\(^{lo}\) or CD9\(^{–}\) clusters (Figures 3(B) and 3(D)). CD9 has not previously been shown to differentiate...
Changes in monocyte and dendritic cell subsets and surface marker expression in moderate and severe hospitalized COVID-19 subjects. Box and whisker plots of individual cell clusters as a proportion of all cells in the Figure 3 subclustering analysis for moderate and severe hospitalized COVID-19 subjects (A). Box and whisker plots showing median expression of cell surface markers within all monocytes and dendritic cells used in the Figure 3 subclustering analysis for moderate and severe hospitalized COVID-19 subjects (B). Statistically significant ($p \leq 0.05$) changes were calculated using adjusted p-values generated after a multiple-comparisons correction. Changes in marker expression were calculated with LIMMA.

Correlations between cell cluster frequencies and clinical parameters of clotting and inflammatory cell death in hospitalized COVID-19 subject blood

After identifying clusters that significantly changed in frequency between subjects, we asked which of these clusters may influence disease progression by correlating their frequencies with known clinical parameters related to COVID-19 severity in the hospitalized subjects (Figures 4A and 4B). While classical monocytes did not significantly correlate with any clinical measurement, nonclassical monocyte frequencies significantly correlated with higher D-Dimer, INR, and LDH levels within COVID-19 hospitalized patients (Figure 4A). The Slan$^{hi}$ nMo and CD33$^{int}$ HLA-DR$^{lo}$ nMo subclusters that decreased in frequency within the hospitalized condition (Figure 3(D)) were positively correlated with INR, LDH, and D-Dimer levels in hospitalized COVID-19 patients (Figure 4B). In opposition to the nonclassical monocytes, the CXCR3$^+$ PDL1$^+$ cMo subcluster that increased in the hospitalized subjects (Figure 3(D)) was negatively correlated with INR, LDH, and D-Dimer values (Figure 4B). Considering their heightened expression of PDL1 and CXCR3, it is possible that these cMos could both respond to IFN-inducible chemokines and travel to sites of infection while also suppressing T cell activity. Interestingly, each of the nonclassical monocyte clusters that positively correlated with clinical values of more severe disease were also clusters that significantly decreased in the hospitalized subjects compared with the healthy or nonhospitalized subjects (Figures 3(C) and 3(D)). Likewise, the classical monocyte clusters that negatively correlated with the clinical values was significantly increased in the hospitalized subjects (Figures 3(C) and 3(D)).

We next looked more closely at hospitalized subjects by separating them into moderate disease or severe disease groups (Table S1).
FIGURE 6 Comparison of immune cell cluster frequencies in hospitalized COVID-19 patient blood through time and characterization of CD9+ monocyte cytokine release. PBMCs from healthy (n = 8) and SARS-CoV-2-infected hospitalized (n = 11) individuals collected at the initial blood draw plotted in Figure 1(A) (Day 0) or approximately 3 months after initial blood collection (~Day 90). CyTOF files were gated in Flowjo as shown in Figures S4 and S5. Relative changes in immune cell frequencies between healthy, hospitalized at Day 0 and hospitalized at ~Day 90 PBMCs were calculated using GLMM and displayed in box and whisker plots (A–H). Statistically significant (p ≤ 0.05) changes calculated using one-way ANOVA with Tukey's post hoc test for the comparisons between healthy, Day 0 or ~Day 90 hospitalized subjects or Welch's T-test for the comparison between only Day 0 or ~Day 90 hospitalized subjects.

When we compared changes in overall marker expression and monocyte and DC subcluster frequencies between the moderate and severe disease subjects, each nMo cluster except for the CD3+ nMos, significantly increased in the severe disease subjects (Figure 5(A)). These increases in cluster frequencies could explain the positive correlations between nMo subset frequencies and the clinical values associated with more severe COVID-19 and thus strengthen the argument that these cells may be pathogenic during COVID-19. Similarly, the increase of CD163 and decrease of CD95 expression in the severe patients matches data in Figure 3(C), giving more credence to changes in these markers as strong indicators of severe disease (Figure 5(B)). However, the decrease in CD64 expression contradicts the increase in CD64 expression in Figure 3(C), suggesting that CD64 is not an ideal marker for severe COVID-19. The decrease in CXCR3 expression in severe
hospitalized subjects, coupled with decreased CXCR3 expression in the T cells of hospitalized subjects (Figure S3(B)), matches well with other data showing decreased CXCR3 expression in lung-infiltrating T cells in severe COVID-19.34 CXCR3 is a receptor for CXCL9, CXCL10, and CXCL11, which are all IFN-inducible genes and contribute to T cell migration and homing.35,36 and the IFN response is one of the most vital antiviral responses and critical for control of COVID-19.19,37 Taken together, these findings help identify immune cell clusters that may influence COVID-19 disease severity and suggest that nonclassical monocytes likely contribute to inflammatory cell death and clotting during COVID-19.

3.5 | Longitudinal changes of immune cell frequencies in COVID-19

While it is important to understand the acute pathogenesis of COVID-19 and its effects on immune cell populations, there can also be long-lasting and debilitating symptoms of infection in what has been termed long-COVID-19.38–40 Given the timeframe needed to study long-COVID-19, there are still relatively few studies that have examined long-term effects of COVID-19 on immune cell populations. Fortunately, we were able to study longitudinal changes in the immune compartment after obtaining blood samples from hospitalized subjects approximately 90 days (Day 90) after their initial blood draw (Day 0). We applied a gating scheme to identify major cell types in our CyTOF data at both timepoints. These data showed that the frequency of pDCs in the hospitalized individuals increased at 90 days postinfection (Figure 6(A)). However, there were no significant changes in T cell, total monocyte, cMo, or iMo frequencies between timepoints in the hospitalized individuals (Figures 6(B)–6(E)). nMos, which decreased in frequency after acute infection, remained decreased during the 3-month timeframe (Figure 6(F)). Further, the CD9+ monocytes remained elevated over time in hospitalized COVID-19 subjects compared with healthy individuals (Figure 6(G)). While the Slan+ nMos did not significantly increase at Day 90 compared with Day 0, they were no longer significantly decreased in frequency compared to healthy controls (Figure 6(H)). These results suggest that SARS-CoV-2 infection results in long-term changes in the immune cell compartment, and it is important to determine whether or not these changes impact symptoms experienced by patients with long-COVID-19.

Herein, we have generated a detailed picture of how monocytes and DCs are altered after SARS-CoV-2 infection of varying severity. Our study could be improved by adding more subjects. However, even with the current number of subjects we had enough statistical power to find significant novel changes in immune cell frequencies and make correlations between disease severity and immune cell subsets. In summary, we identified new cell clusters and markers that we predict contribute to immune response to SARS-CoV-2 and could be predictive of disease severity. We also classified CD9 as a marker that can both differentiate between subsets of monocytes and perhaps influence the response to viral infection for the first time. While decreases in nonclassical monocytes have previously been correlated with more severe disease and worse patient outcome, our data identify subsets of nonclassical monocytes that correlate with inflammatory cell death and clotting in hospitalized subjects and are enriched in the hospitalized subjects with the most severe disease. These data reinforce the need for further investigation into the functions of the monocyte subsets detailed here during SARS-CoV-2 infection. Finally, these data suggest that changes in nonclassical monocyte and CD9+ monocyte frequencies after SARS-CoV-2 infection can be long-lasting and potentially contribute to long-COVID-19 symptoms. We believe these findings can direct future research in the SARS-CoV-2 field and lead to better diagnostics and treatment options for both acute and long-COVID-19.

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AUTHORSHIP
W. J. P. designed and performed experiments, wrote the manuscript, analyzed all data, and generated the figures. L. E. P. designed and performed the experiments and contributed to writing the manuscript. A. A. analyzed all CyTOF data and performed correlational analyses. N. A. G. helped analyze data and generate figures. D. J. A. participated in study design, contributed to writing and editing of the manuscript, and participated in helpful discussions. C. J. H. helped perform experiments and analyze data. C. E. O. assisted with experimental design. H. Q. D. helped with study design and analysis. R. W. assisted with antibody conjugations and CyTOF analysis. P. V. participated in helpful discussions. S. J. C. recruited and cared for patients and obtained blood samples. C. H. O. led the clinical study in the UK, edited the manuscript, and participated in helpful discussions. C. C. H. directed the study and edited the manuscript. W. J. P. and L. E. P. contributed equally to this work.

DISCLOSURE
The authors declare no conflict of interest.

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SUPPORTING INFORMATION
Additional supporting information can be found online in the Supporting Information section at the end of this article.

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