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COVID-19 rapidly increases MDSCs and prolongs innate immune dysfunctions

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Short title: MDSCs and innate immune response in COVID-19

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Abbreviations: ARDS: acute respiratory distress syndrome, MDSCs: myeloid derived suppressor cells; PMN-MDSCs: polymorphonuclear-MDSCs; M-MDSCs: monocytic-myeloid-derived suppressor cells.

Inflammatory and danger signals stimulate hematopoiesis and the generation of myeloid-derived suppressor cells (MDSCs) that suppress innate and adaptive immune responses [1]. High levels of blood MDSCs are associated with nosocomial infections, morbidity and mortality in critically ill patients with sepsis [2]. Severe COVID-19 is characterized by exuberant inflammation, leading to a cascade of immune-related manifestations. Lymphopenia and impaired immune effector cell functions contribute to COVID-19 pathogenesis and increase the risk of secondary infections and death [3]. While increased expression of MDSCs has been reported in COVID-19 patients [4-7], scare studies performed long-term, longitudinal analyses in recovered patients.

To get insights into the dynamic of MDSCs, we analyzed polymononuclear-MDSCs and monocytic-MDSCs (PMN-MDSCs and M-MDSCs), which are the two main subgroups of MDSCs [1], in 56 COVID-19 patients analyzed at hospitalization and in 21 patients analyzed 3 months later. Patients with moderate COVID-19 (n=45) and severe COVID-19 (n=11, 2 died) were similar for age, gender, underlying diseases, and history of immunosuppressive therapy. Patients with severe COVID-19 had higher leukocyte counts ($p=0.024$) and longer hospital stay than patients with moderate COVID-19 ($p<0.001$) (**Table S1**). Ten age- and sex-matched healthy individuals were used as controls.

Blood samples were analysed by flow cytometry followed and unsupervised clustering to quantify leukocyte subpopulations with a specific emphasis on PMN-MDSCs and M-MDSCs (**Fig. S1** and [8]). At study inclusion, patients expressed significantly less lineage positive (Lin^+: CD3, CD7, CD19 or CD56 positive) cells, DCs and classical, intermediate and non-classical monocytes than healthy controls, but 4-fold more PMN-MDSCs ($p=0.03$) and 2-fold more M-MDSCs ($p=0.01$) (**Fig. 1A**). These data are in line with previous observations [4-7]. Interestingly, counts of PMN-MDSCs, M-MDSCs and leukocytes were normal in patients (14 moderate and 7 severe COVID-19) analyzed 3 months after diagnosis.

At study inclusion, PMN-MDSCs and M-MDSCs counts were 10- and 4-fold higher in severe than in moderate COVID-19 patients ($p=0.0013$ and 0.0014) (**Fig. 1A**). Other cell-

populations were similar in severe and moderate COVID-19 patients. PMN-MDSCs and M-MDSCs levels correlated with each other (ρ=0.43; p=0.03). PMN-MDSCs inversely correlated with lymphocyte counts (ρ=-0.37; p=0.025) (**Fig. 1B**). A similar, but not statistically significant, inverse correlation was detected between MDSCs and $CD4^+$ and $CD8^+$ T cells and T regulatory cells (**Fig. S2**). Since the levels of M-MDSCs in blood, but not in the airways, correlated with COVID-19 severity [5], the quantification of MDSCs in peripheral blood may represent an interesting biomarker of COVID-19.

Thirty-three cytokines/chemokines/growth factors (measured using a 49-multiplex bead assay) were detected in the serum of COVID-19 patients (**Fig 1C**), in line with the notion that massive release of cytokines is associated with COVID-19 pathophysiology [3]. PMN-MDSCs and M-MDSCs correlated positively with most mediators (53/66 of positive associations). Eight associations were statistically significant after correction for multiple testing. PMN-MDSCs and M-MDSCs correlated with epidermal growth factor (EGF; ρ=0.47/0.44; p=0.01/0.02) and hepatocyte growth factor (HGF; ρ=0.42/0.46; p=0.02/0.01). M-MDSCs correlated with IL-1β, IL-7, platelet-derived growth factor-BB (PDGF-BB) and vascular endothelial growth factor (VEGF) (ρ=0.42, 0.38, 0.56, 0.40; p=0.03, 0.05, <0.0001, 0.03) (**Fig. 1C**). Interestingly, EGF, HGF, PDGF-BB and VGEF have been shown to expand and chemo-attract MDSCs, and IL-1β and IL-7 to stimulate myelopoiesis and sustain the expansion and T cell-suppressing activity of MDSCs [1, 2]. Thus, the inflammatory milieu in COVID-19 patients contains mediators that promote the generation and the activity of MDSCs. Based on data from the oncology field, tyrosine kinase inhibitors targeting EGF and HGF pathways represent therapies for controlling MDSCs in COVID-19.

To assess whether the changes in MDSCs might be related to immunological effects, blood was stimulated with LPS and R848. Intracellular cytokine staining followed by flow cytometry analysis was used to quantify the proportion of monocytes and DCs producing TNF and IL-6 (**Fig. 2**). In healthy controls, 0.02% and 4.3% of monocytes produced TNF and IL-6 at baseline, 24% and 17% in response to LPS, and 79% and 46% in response to R848,

77 respectively. The percentage of blood monocytes producing TNF and IL-6 in response to LPS
78 and R848 was 1.3-4.9-fold lower in COVID-19 patients (LPS: $p<0.001$; R848: $p<0.05$). The
79 reduction was more striking in severe than in moderate COVID-19 patients. The impaired
80 response of monocytes persisted 3 months (**Fig. 2A-B**). In healthy controls, 0.6% of DCs
81 produced TNF and IL-6 at baseline, 38% and 36% in response to LPS, and 68% and 58% in
82 response to R848. TNF and IL-6 positive DCs were 2.1-5.1-fold lower in COVID-19 patients
83 ($p<0.001$), more impaired in severe than in moderate COVID-19 patients. Impaired cytokine
84 response persisted 3 months (**Fig. 2C-D**).

85 Finally, we assessed whether the defects observed in monocytes and DCs reflected
86 impaired production of cytokines by whole blood. Upon stimulation with LPS and R848, 17/24
87 and 13/24 of cytokines were detected at lower concentrations in blood from patients than in
88 blood from healthy controls (**Fig. 2E**). Interestingly, 6/24 and 7/24 of the cytokines were
89 detected at lower concentrations in patients analyzed after 3 months, implying prolonged
90 immunological defects. Patients with moderate and severe COVID-19 were similarly affected.

91 Overall, MDSCs represented 10-15% of blood leukocytes, peaked in severe COVID-19
92 patients, and were associated with cytokine levels, lymphocytopenia, worse outcome, and
93 impaired cytokine production by monocytes and DCs. These observations support the
94 assumption that an exuberant immune response to SARS-CoV-2 infection exacerbates the
95 development of immunosuppression limiting anti-microbial defenses. Three months after
96 inclusion, leukocyte counts were back to normal but whole blood, monocytes and DCs still
97 displayed reduced cytokine production, revealing long-term immune disturbances. In a similar
98 way, it has been reported that MDSCs were normalized while cellular abnormalities were
99 uncovered several weeks after SARS-CoV-2 infection [9]. Whether MDSCs play a role in
100 persistent immune dysfunctions is unknown, but would involve long-lasting imprinting
101 independent from MDSCs elevated counts. For example, the suppressive activity of MDSCs
102 might vary over time as reported during sepsis in mice and humans [2]. Overall, failure to
103 restore immune homeostasis in COVID-19 patients may be a driver of long-COVID and post-

acute COVID-19 syndrome, increasing the risk of infections. Long COVID is reminiscent of the post-sepsis syndrome characterized by immunosuppression associated with persistent low-grade inflammation [10].

Our work has several limitations. The number of patients was rather small, which may have limited the detection of differences or correlations. While there is no perfect phenotyping protocol of MDSCs, additional markers might have been used to trace MDSCs. However, we elected to minimize analytical variations by labeling whole blood quickly after drawing and analyzing flow cytometry data by unsupervised clustering. Finally, we have not assessed the immunosuppressive capacity of MDSCs. Yet, this has been reported in many studies, and MDSCs of COVID-19 patients were shown to inhibit the proliferation and cytokine production by T cells [4-6].

To conclude, our data suggest that MDSCs in peripheral blood represent biomarkers to stratify COVID-19 patients. Targeting MDSCs and/or immune dysfunctions might proof useful to counterbalance immunosuppression, reduce nosocomial and long-term infections, and decrease late mortality in severe COVID-19 patients.

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Conflict of Interest Statement: The authors do not have conflicts of interest regarding this manuscript.

Detailed about materials and methods, ethics, author contributions and data availability are found in the supporting information.

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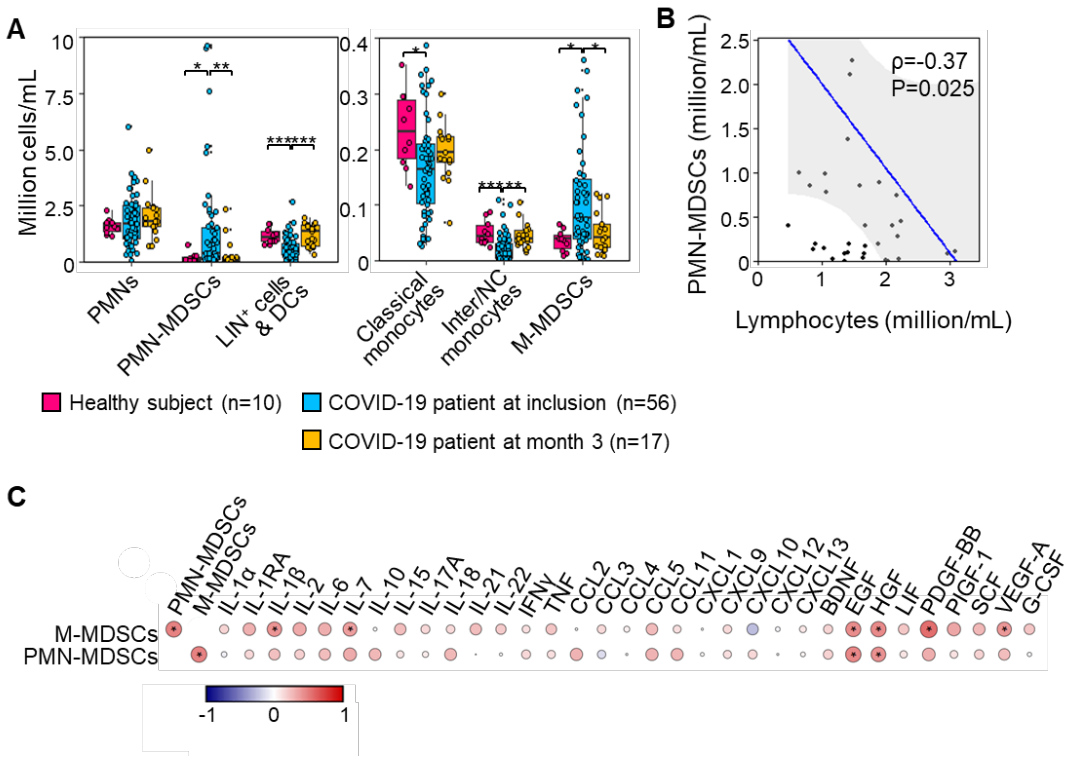

Figure 1. MDSCs in COVID-19 patients. (**A**) Cell populations in healthy controls and COVID-19 patients at study inclusion and after 3 months. Inter/NC monos: Intermediate/non-classical monocytes, Lin: lineage, DCs: dendritic cells. Boxplots show median, upper and lower quartiles. Whiskers show 5-95 percentiles. Each dot represents an individual sample. *p<0.05, **p<0.01, ***p<0.001. (**B**) Scatterplot showing an inverse correlation between PMN-MDSCs and lymphocytes. (**C**) Correlation plots of PMN-MDSCs, M-MDSCs and 33 serum mediators calculated using Spearman's Rank-Order Correlation controlled for FDR. *p<0.05.

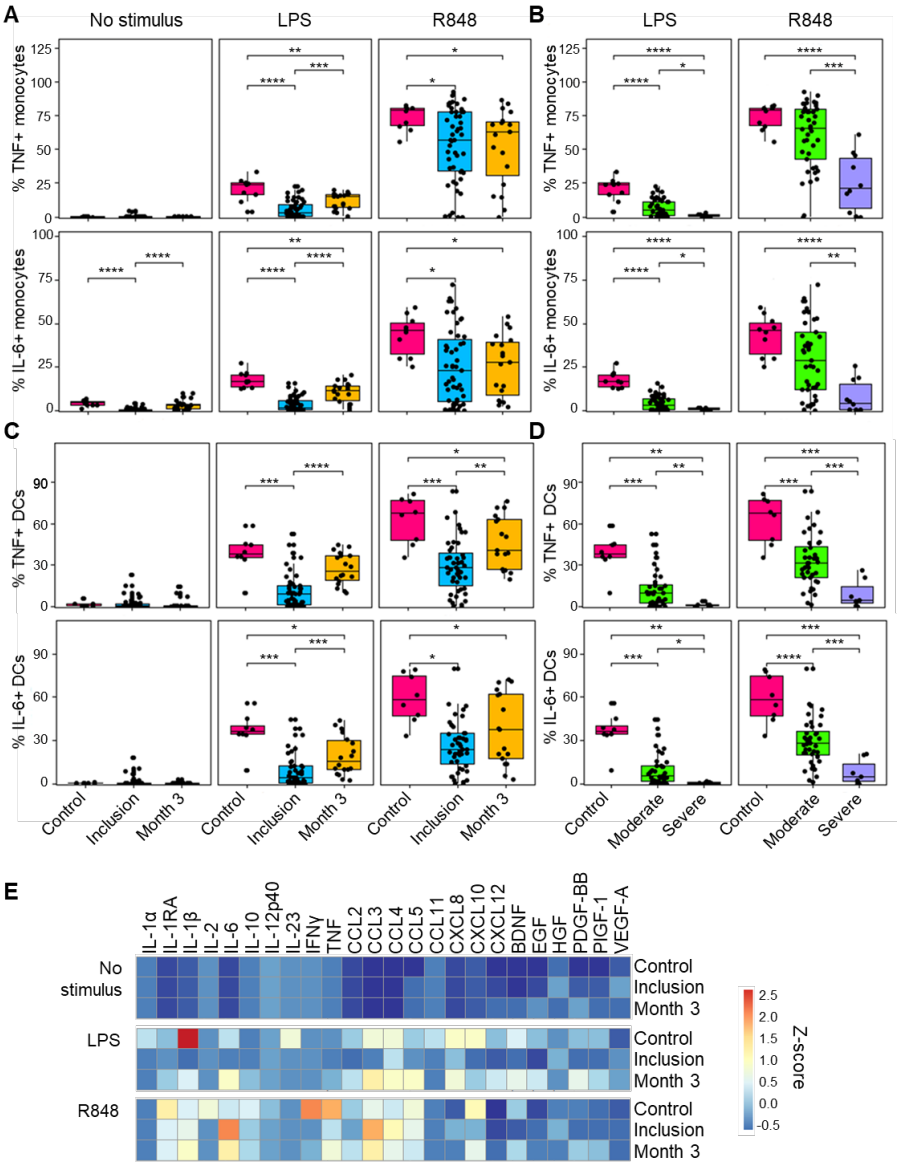

Figure 2. Cytokine response by monocytes, DCs, and whole blood in COVID-19 patients. Blood was obtained from healthy controls and COVID-19 patients at study inclusion and after 3 months. Blood was exposed for 4 hours (**A-D**) or 24 hours (**E**) to LPS (100 ng/mL) and R848 (5 µg/mL). (**A-D**) Cells were stained for intracellular cytokines and markers to identify monocytes and DCs, and analyzed by flow cytometry. Results are percentages of TNF^+ and $IL\text{-}6^+$ cells within monocytes (**A-B**) and DCs (**C-D**). Boxplots show median, upper and lower quartiles, whiskers 5-95 percentiles. Each dot is one sample. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. (**E**) Blood supernatants were used to quantify mediators by multiplex bead assay. Results are expressed as a heat map scaled expression plot in healthy controls (n=5) and COVID-19 patients at inclusion (n=13) and after 3 months (n=12).

Supporting Information

Rapid increase of myeloid-derived suppressor cells and prolonged innate immune dysfunctions in patients with COVID-19

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Materials and methods

Subjects and ethic statement

Fifty-six hospitalized PCR-confirmed SARS-CoV-2 infected adult patients were enrolled in the Lausanne University Hospital (LUH) COVID-19 cohort study (Lausanne, Switzerland) during the time period of 01-04-2020 and 30-10-2020. The exclusion criterion for study enrolment was pregnancy. We did not exclude patients based on comorbidities including malignancies. Moderate COVID-19 was defined as hospital admission without the need for intubation, while severe COVID-19 was defined as hospital admission with mechanical ventilation for respiratory failure and/or death. Blood samples were collected at study inclusion. A second sample was collected 3 months later in 21 patients (14 moderate and 7 severe COVID-19). A control group comprised 10 age- and sex-matched healthy individuals. Exclusion criteria for healthy controls were prior diagnosis of SARS-CoV-2 infection, acute or chronic viral hepatitis, autoimmune disease, immunodeficiency and use of immunomodulatory drugs. The study was approved by the Commission cantonale d'éthique de la recherche sur l'être humain, Canton de Vaud,

Switzerland (CER-VD, Lausanne, Switzerland). Study participants provided written informed consent. Blood samples were treated fresh, in general within less than 1 hour.

Detection of MDSCs in whole blood by flow cytometry

One hundred microliter of EDTA-anticoagulated blood were incubated for 20 minutes at room temperature in the dark with a cocktail of antibodies directed against CD3, CD7, CD11b, CD14, CD15, CD16, CD19, CD33, CD45, CD56, CD135 and HLA-DR. Samples were diluted with 2 mL 1 x 1-step Fix/Lyse solution (eBioscience™, Thermo Fisher Scientific, Waltham, MA, USA), washed once with cell stain medium (CSM: PBS containing 0.5% BSA and 0.02% sodium azide) and acquired using an Attune NxT Flow Cytometer (Thermo Fisher Scientific). Debris, and doublets were excluded using manual gating (**Fig. S1A**), followed by FlowSOM unsupervised clustering using the biexponential transformed expression levels of CD11b, CD14, CD15, CD16, CD33, CD45, HLA-DR and lineage markers (CD3, CD7, CD19, CD56). Metaclustering was set on 30 populations manually merged into populations based on biological knowledge as represented in tSNE plots (**Fig. S1B**). PMN-MDSCs were identified based on their relatively low expression levels of CD16 and CD11b when compared to mature neutrophilic granulocytes and corresponded to $CD11b^{+}$ $CD14^{-}$ $CD15^{+}$ $CD16^{+}$ $CD33^{-}$ $HLA\text{-}DR^{-}$ cells [1]. M-MDSCs were identified based on low expression levels of HLA-DR [1], and corresponded to $CD11b^{+}$ $CD14^{+}$ $CD15^{-/low}$ $CD16^{-}$ $CD33^{+}$ $HLA\text{-}DR^{-/low}$ cells (**Fig. S1C**). Reagents used for flow cytometry analyses are described in **Table S2**.

Blood cytokines, chemokines, growth factors and T cell populations

Serum concentrations of cytokines (IL-1α, IL-1RA, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17A, IL-18, IL-21, IL-22, IL-23, IL-27, IL-31, IFN-α, IFN-β, LIF, LT-α, TNF), chemokines (MCP-1/CCL2, MIP-1α/CCL3, MIP-1β/CCL4, RANTES/CCL5, Eotaxin-1/CCL11, GRO-α/CXCL1, IL-8/CXCL8, MIG/CXCL9, IP-10/CXCL10, SDF-1/CXCL12, BCA-1/CXCL13) and growth factors (NGF-β, BDNF, EGF, FGF-2, HGF, PDGF-BB, PIGF-1, SCF, VEGF-A, VEGF-D, BAFF, GM-CSF, G-CSF) were determined by multiplex bead assay using the Luminex xMAP Technology (Luminex Corporation, Austin, TX) and a BioPlex 200 array

reader (Bio-Rad Laboratories, Hercules, CA) as previously described [2]. Blood T cell populations were profiled by mass cytometry as thoroughly detailed in [2].

Whole blood stimulation assay

Three hundred µL EDTA-anticoagulated blood were incubated for 4 hours at 37°C with or without 100 ng/mL *Escherichia coli* O55:B5 ultrapure ultrapure lipopolysaccharide (LPS), or 5 µg/mL R848, 100 ng/mL. Brefeldin A (5 µg/mL, Invitrogen, Carlsbad, CA) was added at the beginning of incubation [3]. To analyze monocytic cells, 100 µL of reaction mixtures were incubated with LIVE/DEAD™ reagent and antibodies directed against CD14, CD16, CD19, CD33, CD56, HLA-DR and PD-L1/CD274. To analyze DCs, 200 µL of reaction mixtures were incubated with LIVE/DEAD™, Anti-Human Lineage Cocktail 2 (Lin-2, containing anti-CD3, CD14, CD19, CD20 and CD56 antibodies), and anti-CD1c, CD11c, CD16, CD123, HLA-DR and PD-L1/CD274 antibodies. After 20 minutes of incubation at room temperature in the dark, samples were diluted with 2 mL 1x 1-step Fix/Lyse Solution, washed with CSM, incubated for 10 minutes with CSM containing 0.3% saponin (Sigma-Aldrich, Saint Louis, MI), incubated for 20 minutes with CSM containing antibodies directed against TNF, IL-6 and IL-10, washed and acquired using an Attune NxT Flow Cytometer. Reagents are described in **Table S2**. Debris, doublets and dead cells (LIFE/DEAD^{TM}) were excluded by manual gating (**Fig. S1D**). SSC-A intermediate, CD33^{+}, CD3^{-} and CD20^{-} cells were selected by manual gating before applying FlowSOM unsupervised clustering (metacluster set at 20) based on the expression of HLA-DR, CD14, CD16, CD33, CD56 and SSC-A. A second round of clustering was applied to distinguish classical monocytes (HLA-DR^{+} CD14^{+} CD16^{-}), non-classical and intermediate monocytes (HLA-DR^{+} CD14$^{+/-}$ CD16^{+}), and M-MDSCs (HLA-DR$^{+/-}$ CD14^{+} CD16^{-}). To analyze DCs, HLA-DR^{+} and Lin2^{-} cells were selected by manual gating before applying FlowSOM (metacluster set at 12). Patients with < 30 DCs/mL were excluded. FlowSOM with metacluster set at 12 was applied to monocytes and DCs to analyze intracellular cytokine (data not shown). To quantify cytokine release by whole blood, 30 µL of EDTA-anticoagulated blood was incubated for 24 hours at 37°C with or without LPS, and R848 as described above.

Supernatants were used to quantify mediators by multiplex bead assay using the Luminex xMAP Technology.

Statistical analyses and softwares

Manual gating was performed with FlowJo™ Software version 10.6.2 (Ashland, OR). Statistical analyses and figure design were performed using R v.3.6.0 (R Foundation for Statistical Computing, Vienna, Austria). Baseline characteristic comparisons were made using Mann-Whitney U, Chi square or Kruskall-Wallis tests for skewed variables and student's t-test or Chi square for normal distributed variables. Cytokine and flow cytometry data were compared using the Kruskall-Wallis test, Mann-Whitney U, or Spearman's rank correlation controlling for False Discovery Rate (FDR) using the Benjamini and Hochberg step-up procedure. A 2-tailed $p<0.05$ was considered statistically significant.

Ethics

The study was approved by the Commission cantonale d'éthique de la recherche sur l'être humain, Canton de Vaud, Switzerland. We collected blood samples after written informed consent provided by study participants.

Author contributions

ITS, CT, and TR designed the study. PAB, JDC, MP, GP, TC and TR designed the LUH-COVID19 cohort. JR provided clinical characteristics of patients. ITS, CT, NA, DLR, and MP processed the samples. ITS analyzed raw data. All the authors interpreted the data. ITS and TR wrote the manuscript. All the authors revised the manuscript.

Data availability statement

The data that support the findings of this study are available from the corresponding author on reasonable request. Restrictions apply to due to privacy or ethical restrictions.

Supplementary Table 1. Patient's characteristics

Characteristic	Control	Moderate COVID-19	Severe COVID-19	3 months after study inclusion
Number of subjects	10	45	11	21
Gender, male	7 (70%)	31 (70%)	7 (64%)	15 (71%)
Age [years]	58 [55-65]	62 [53-74]	60 [48-63]	61 [54-75]
Charlson comorbidity index	-	3 [1.8-6]	2 [1-4.5]	2 [1-6]
Immunosuppressive drugs	-	7 [16%]	1 [5.3%]	1 [4.8%]
Days of symptoms before inclusion	-	7 [5-10]	7 [5-11]	9 [6.5-11]
Length of hospital stay[#]	-	3.5 [1.8-6.5]	24 [21-27]***	5 [2.5-17]
Death	-	-	2 (18%)	-
Leukocytes [x 10^9 cells/L]	3.4 [3.2-3.7]	3.0 [2.4-4.7]	7.0 [3.3-10.9]*	3.9 [3.2-4.8]
PMN-MDSCs [x 10^9 cells /L]	0.1 [0.07-0.2]	0.2 [0.08-1.0]	2.3 [0.6-8.6]**	0.1 [0.06-0.2]
M-MDSCs [x 10^9 cells /L]	0.04 [0.02-0.05]	0.05 [0.03-0.1]	0.22 [0.11-0.31]**	0.04 [0.02-0.07]

Data are n (%) or median [IQR]. [#]Excluding non-survivors, from moment of inclusion to hospital discharge. Statistics between moderate and severe COVID-19 patients: *p<0.05, **p<0.01, ***p<0.001.

Supplementary Table 2. Reagents

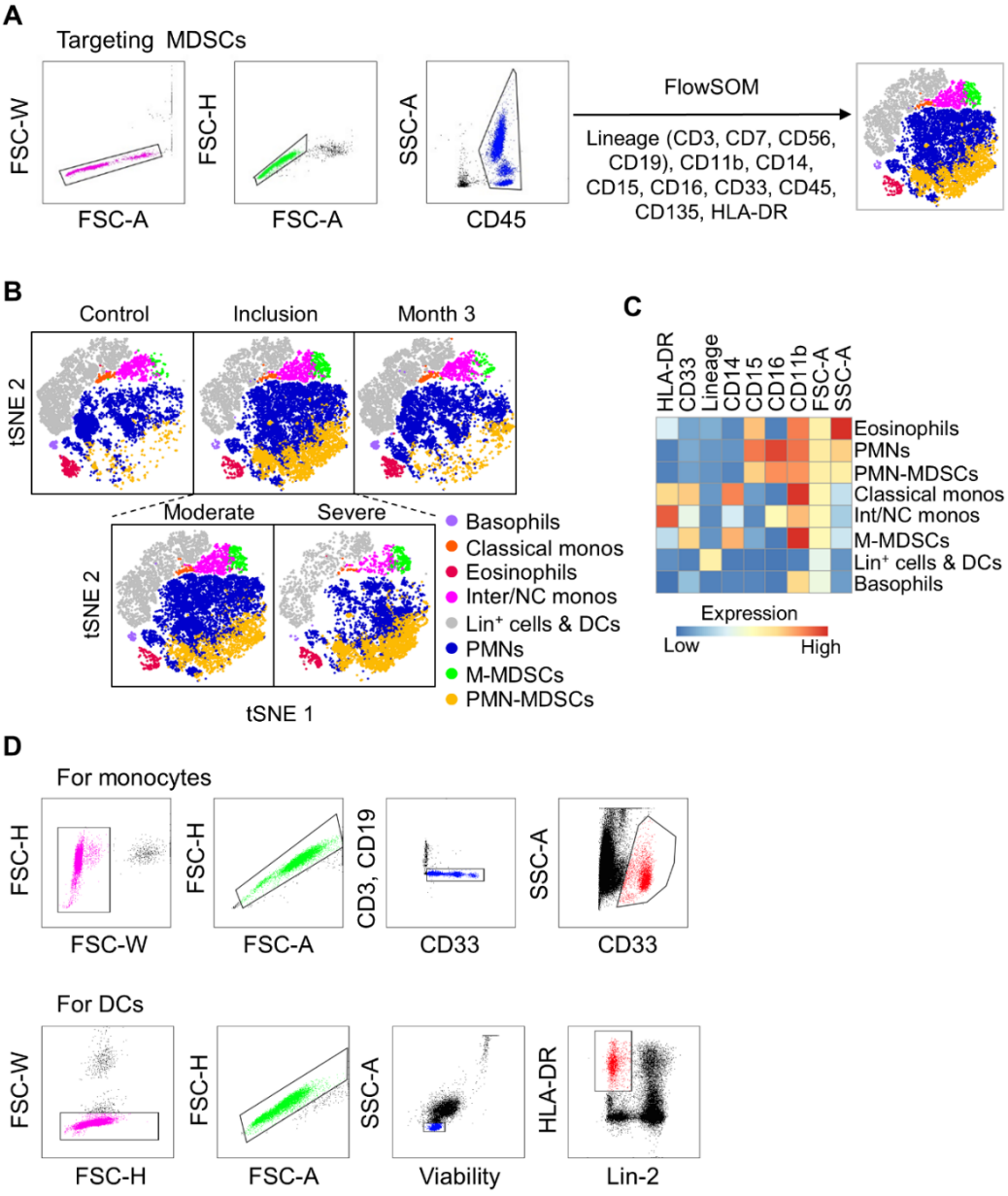
Antibodies and live/dead viable reagent used in flow cytometry

Target	Clone	Fluorochrome	Company	Reference
CD1c	L161	AF700	Biolegend	331530
CD11b	Bear1	PC-7	Beckman Coulter	A54822
CD11c	B-ly6	PE-TXR	BD Pharmingen	562393
CD123	6H6	BV711	Biolegend	306030
CD135	BV10A4H2	PE	Biolegend	313305
CD14	18D11	FITC	ImmunoTools	21620143
CD14	RMO52	APC-AF750	Beckman Coulter	B92421
CD15	80H5	Pacific Blue	Beckman Coulter	B49218
CD16	3G8	PB	BD Pharmingen	558122
CD16	3G8	ECD	Beckman Coulter	B49216
CD19	SJ25C1	APC-C7	BD Pharmingen	557791
CD19	J3.119	AlexaFluor 700	Beckman Coulter	B76284
CD274	MIH1	PE-Cy™7	BD Pharmingen	558017
CD3	SP34	APC-C7	BD Pharmingen	557757
CD33	WM33	BV711	BD Pharmingen	563171
CD33	D3HL60.251	APC	Beckman Coulter	IM2471
CD45	J33	Krome orange	Beckman Coulter	B36294
CD56	HCD56	AF700	Biolegend	318316
CD56	HCD56	AlexaFluor 700	Biolegend	318316
CD7	M-T701	AlexaFluor 700	BD	561603
HLA-DR	REA332	APC-Vio770	Miltenyi Biotec	130-104-871
HLA-DR	Immu-357	PE-TXR	Beckman Coulter	B94238
HLA-DR	Immu-357	FITC	Beckman Coulter	IM1638U
IL-10	JES3-9D7	PE	BD Pharmingen	559337
IL-6	MQ2-13A5	PerCP/Cy5.5	Biolegend	501117
Lin-2	Multiple	FITC	BD	643397
LIVE/DEAD™		Fixable Aqua	Invitrogen	L34957
TNF-α	MAb11	APC	Biolegend	307626

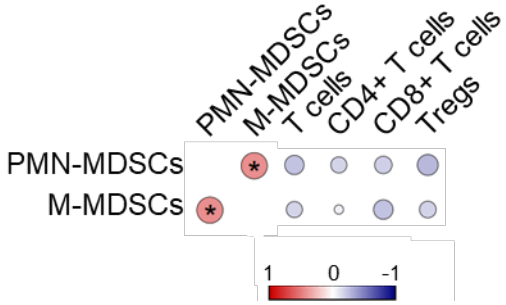
Other reagents

Name	Company	Reference
1-step Fix/Lyse Solution (10X)	eBioscience	00-5333-57
Bovine serum albumin	Sigma-Aldrich	A7906
Brefeldin A	Invitrogen	B7450
Escherichia coli O55:B5 ultrapure lipopolysaccharide	Invivogen	tlrl-pb5lps
R848	Invivogen	tlrl-r848-5
Saponin	Sigma-Aldrich	SAE0073-10G
Sodium azide	Sigma-Aldrich	71289

Supplementary Figures



Supplementary Figure 1. Gating strategy and clustering analyses. Blood was obtained from 10 healthy subjects and 56 COVID-19 patients (45 with moderate COVID-19, 11 with severe COVID-19) at study inclusion and after 3 months (n=17) and analyzed by flow cytometry and, for MDSCs, unsupervised clustering using FlowSOM. (**A, D**) Gating strategy to exclude debris, doublets and non-hematopoietic cells to analyze blood MDSCs, monocytes and DCs by flow cytometry. (**B**) t-SNE plots of leukocyte populations. (**C**) Expression levels of cell surface markers and FSC-A/SSC-A of leukocyte populations.



Supplementary Figure 2. Correlation plots of PMN-MDSCs, M-MDSCs, and lymphocyte populations (n=48). Correlations were calculated using Spearman's Rank-Order Correlation controlled for FDR. *p<0.05.

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