Platelet-monocyte interaction amplifies thromboinflammation through tissue factor signaling in COVID-19

Running Title: TF signaling amplifies inflammation in COVID-19

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Supplemental Methods

Patient care

Severe COVID-19 was defined as those critically ill patients, presenting viral pneumonia on computed tomography scan and requiring oxygen supplementation through either a nonrebreather mask or mechanical ventilation. Patients with acute respiratory distress syndrome (ARDS) were managed with neuromuscular blockade and a protective ventilation strategy with low tidal volume (6 mL/kg of predicted body weight) and limited driving pressure (less than 16 cmH_2O). Optimal PEEP was calculated based on the best lung compliance and PaO_2/FiO_2 ratio. In those with severe ARDS and PaO_2/FiO_2 ratio below 150 despite optimal ventilatory strategy, prone position was initiated. Our management protocol included antithrombotic prophylaxis with enoxaparin 40 to 60 mg per day. Patients did not receive antivirals or other anti-inflammatory or anti-platelet drugs. The SARS-CoV-2-negative control participants were not under anti-inflammatory or anti-platelet drugs for at least two weeks before blood sampling.

Platelet and monocyte isolation

Blood samples were drawn into acid-citrate-dextrose (ACD) and centrifuged (150 x g, 20 min, room temperature) to obtain the platelet-rich plasma (PRP). PRP was supplemented with 100 nM of Prostaglandin E_1 (PGE_1, Cayman 13010) and recentrifuged (500 x g, 20 min, room temperature) to pellet the platelets. Platelets were resuspended in 2.5 mL of phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) containing 2 mM EDTA, 0.5% human serum albumin and 100 nM PGE_1, and incubated with anti-CD45 antibodies (1:200) for 10 min and magnetic beads-conjugates (1:100) for additional 15 min, followed by magnetic removal of leukocytes for 10 min (Human CD45+ depletion kit, StemCell). Purified platelets were washed in 25 mL of PSG (5 mM $C_8H_{18}N_2O_6S_2$, 145 mM NaCl, 4 mM KCl, 50 µM Na_2HPO_4, 1 mM $MgCl_2 \cdot 6H_2O$, 5.5 mM glucose; pH 6.8) containing 100 nM PGE_1, centrifuged again (500 x g, 20 min, room

temperature) and resuspended in medium 199 (M199, Lonza 12-117F) to the concentration of 10^9 platelets/mL.

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood after PRP was removed (bottom cell layer after the first centrifugation abovementioned) using Ficoll-Paque (GE Healthcare) gradient centrifugation. The PBMC were resuspended in PBS containing 1 mM EDTA and 2 % fetal bovine serum to the concentration of 10^8 cells/mL and incubated with anti-CD14 antibodies (1:10) for 10 min and magnetic beads-conjugates (1:20) for additional 10 min, followed by magnetic recovery of monocytes for 5 min. Recovered monocytes were resuspended in PBS containing 1 mM EDTA and 2 % fetal bovine serum and subjected to two more rounds of selection in the magnet according to the manufacturer's instructions (Human CD14+ selection kit, Easy Sep; StemCell). The purity of monocyte preparations (>98% CD14+ cells) was confirmed through flow cytometry.

Flow cytometry

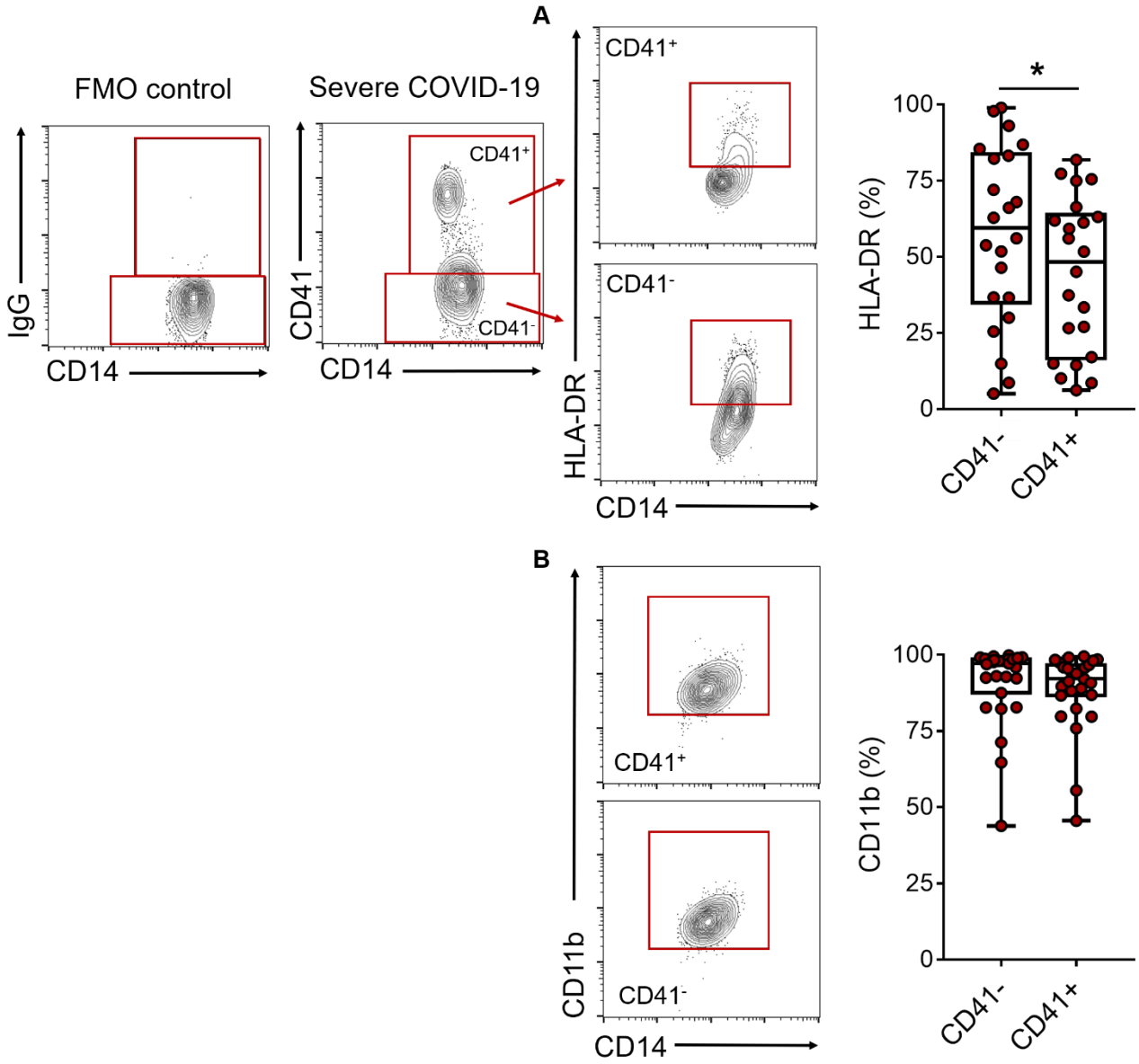
Platelet-monocyte aggregates were evaluated as previously described[36]. Whole blood samples were incubated for 10 min with FACS lysing buffer (BD Biosciences) and then centrifuged at 500 x g for 15 min. The supernatant was discarded and cells were resuspended in HEPES-Tyrode (HT) buffer (10 mM HEPES, 137 mM NaCl, 2.8 mM KCl, 1 mM $MgCl_2.6H_2O$, 12 mM $NaHCO_3$, 0.4 mM Na_2HPO_4, 5.5 mM glucose, 0.35% BSA [pH 7.4]). Monocytes were labeled with FITC-conjugated CD16, PE-conjugated anti-TF, peridinin-chlorophyll-conjugated CD14 and APC-conjugated anti-CD41 (BD Pharmingen), or with FITC-conjugated HLA-DR, PE-conjugated anti-CD11b, peridinin-chlorophyll-conjugated CD14 and APC-conjugated anti-CD41 (BD Pharmingen) for 30 min at room temperature and fixed with 4% paraformaldehyde. Platelets or monocytes labeled with each antibody separately were used for appropriate color compensation and isotype-matched IgG conjugated with the same fluorochromes were used as the negative controls. Flow cytometer (BD FACSCalibur) was used to acquire 5,000 CD14+ gated events. Acquired data were further analyzed using FlowJo software.

Single-cell RNAseq

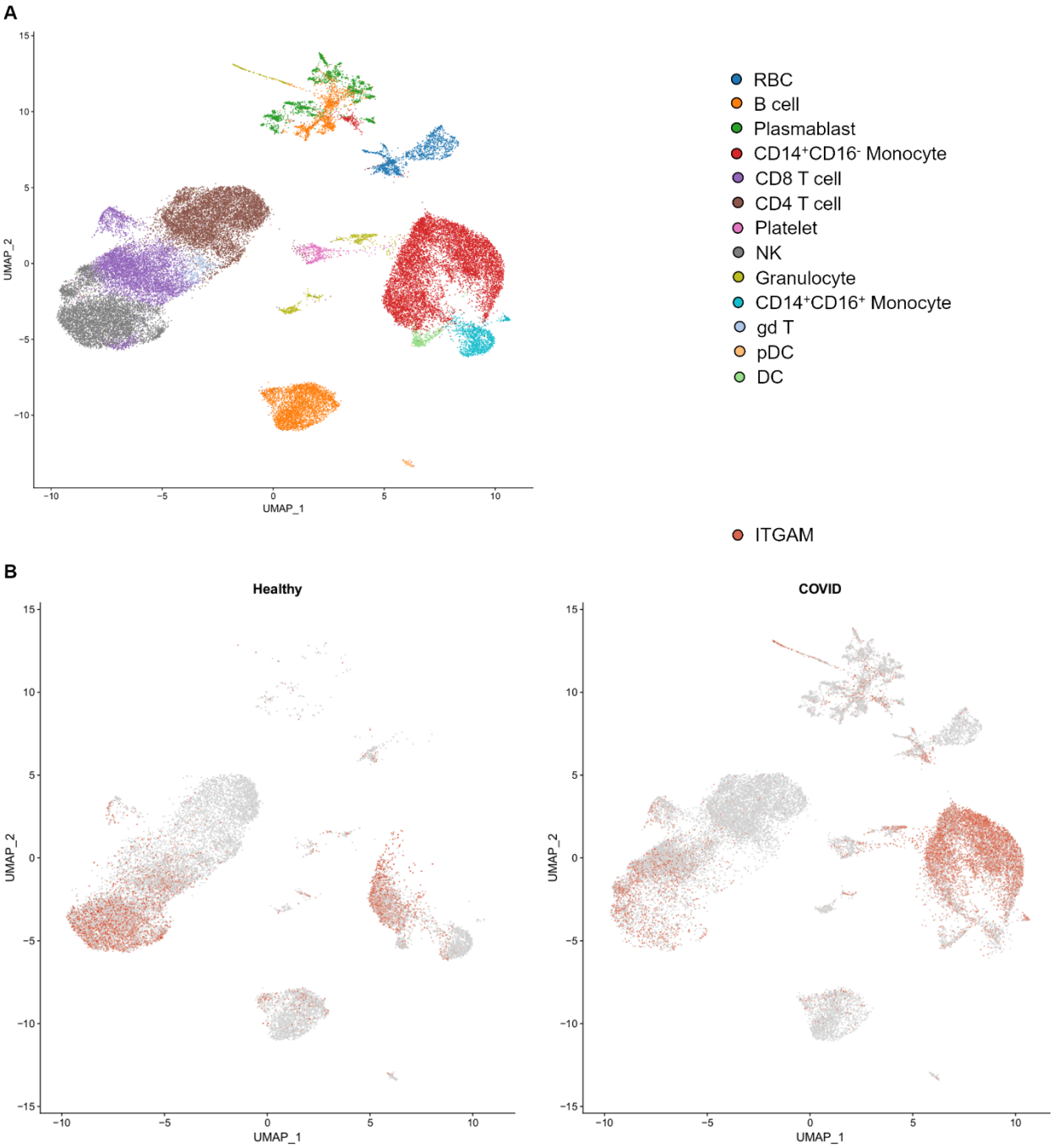
We analyzed single-cell transcriptomic public available data from peripheral blood mononuclear cells (PBMCs) from seven patients hospitalized for COVID-19, four of whom had acute respiratory distress syndrome, and six healthy controls[38]. The dataset generated by the original authors is publicly available at https://www.covid19cellatlas.org. Briefly, the dataset was downloaded and the RDS seurat object was imported into R environment version v4.0.5 and Seurat v4.0.1[39]. CD14+ and CD16+ monocyte cells were selected for downstream analysis based on the cell type annotation provided by the authors. Differential expression analysis was conducted using the FindMarkers function of the seurat package using Wilcoxon test to compare CD14+ Monocyte cells of COVID-19 patients to healthy control cells and also CD16+ monocyte cells of COVID-19 patients to healthy control cells. Differentially expressed genes were identified considering genes expressed in at least 10 % of cells, FDR < 0.05 and |avg_logFC| > 0.25.

Quantification of inflammatory mediators

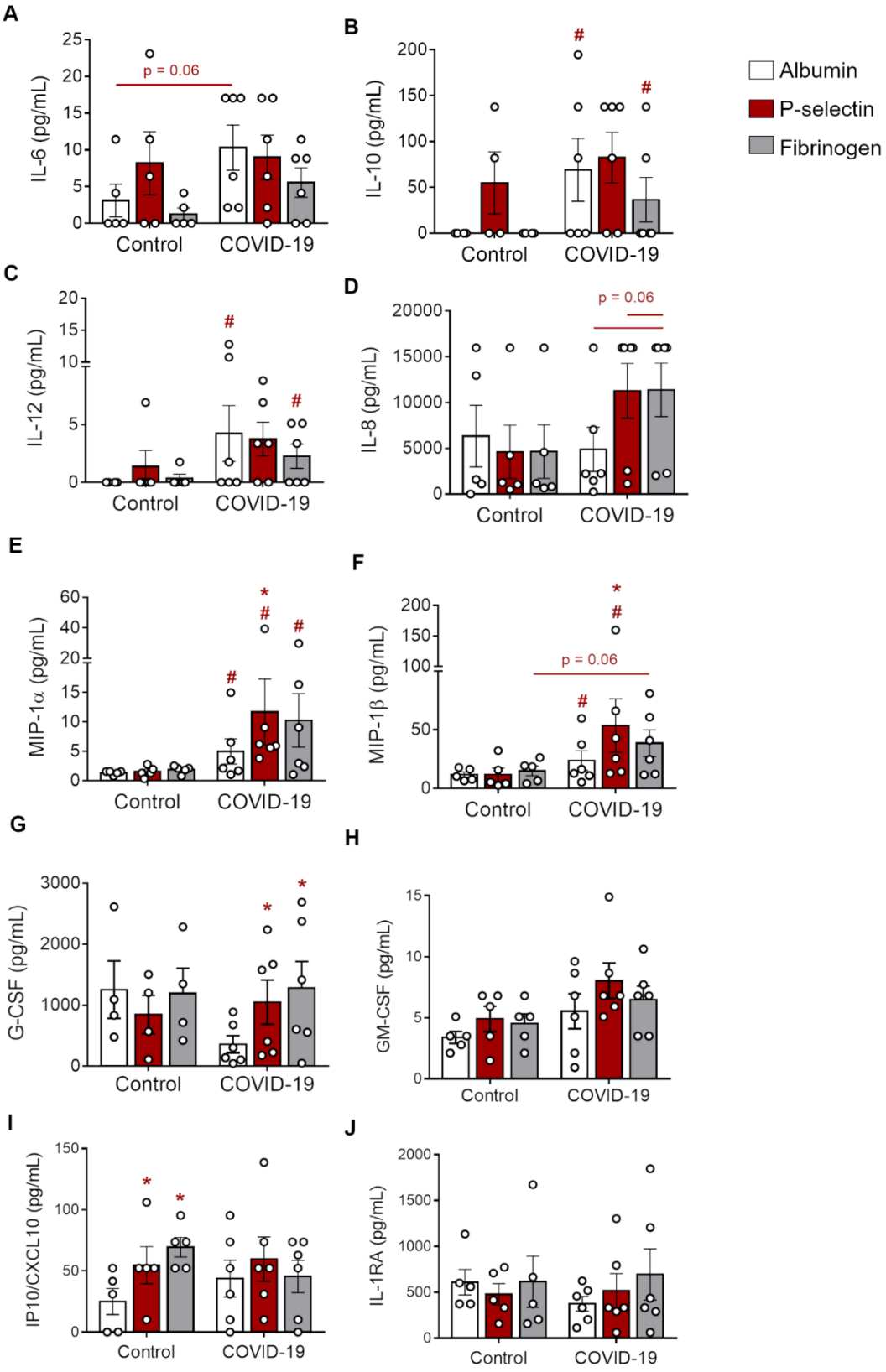
The concentrations of cytokines, chemokines and eicosanoids were quantified in the supernatants from platelets, monocytes and platelet-monocyte interactions using standard commercially available Multiplex, ELISA and EIA kits according to the manufacturer's instructions. (Bio-Rad, R&D systems and Cayman chemicals, respectively).



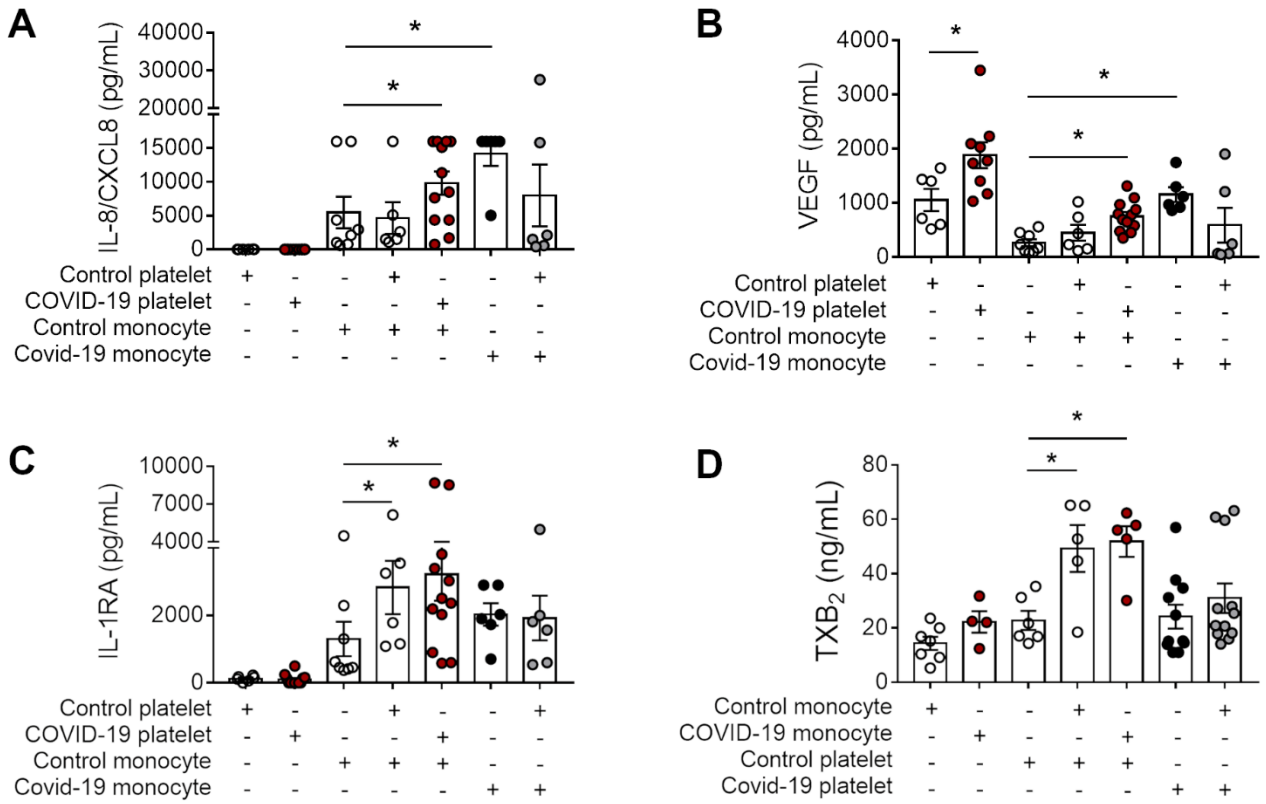
Supplemental Figure S1: The percentage of HLA-DR (**A**) and CD11b (**B**) surface expression on monocytes that were complexed with platelets (CD14+CD41+) or circulating freely (CD14+CD41-) in severe COVID-19 patients. The horizontal lines in the box plots represent the median, the box edges represent the interquartile ranges and the whiskers indicate the minimal and maximal value in each group. * indicates $p < 0.05$ between selected groups.



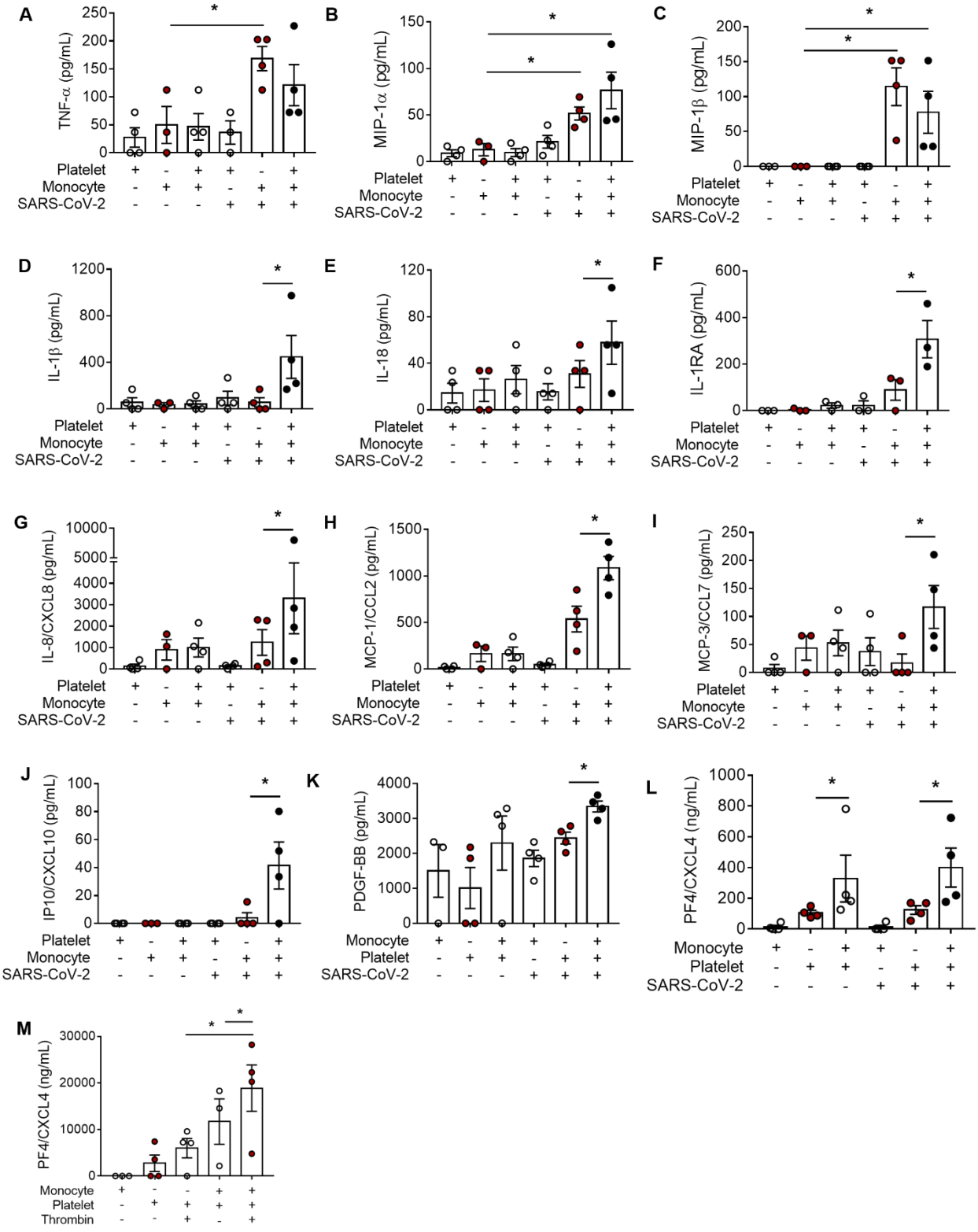
Supplemental Figure S2: Single-cell RNA-seq data showing expression pattern of ITGAM in monocytes from severe COVID-19 patients compared to healthy individuals. (**A-B**) UMAP dimensionality reduction embedding of peripheral blood mononuclear cells (PBMCs) colored by the expression of leukocyte specific markers (**A**) and ITGAM (**B**).



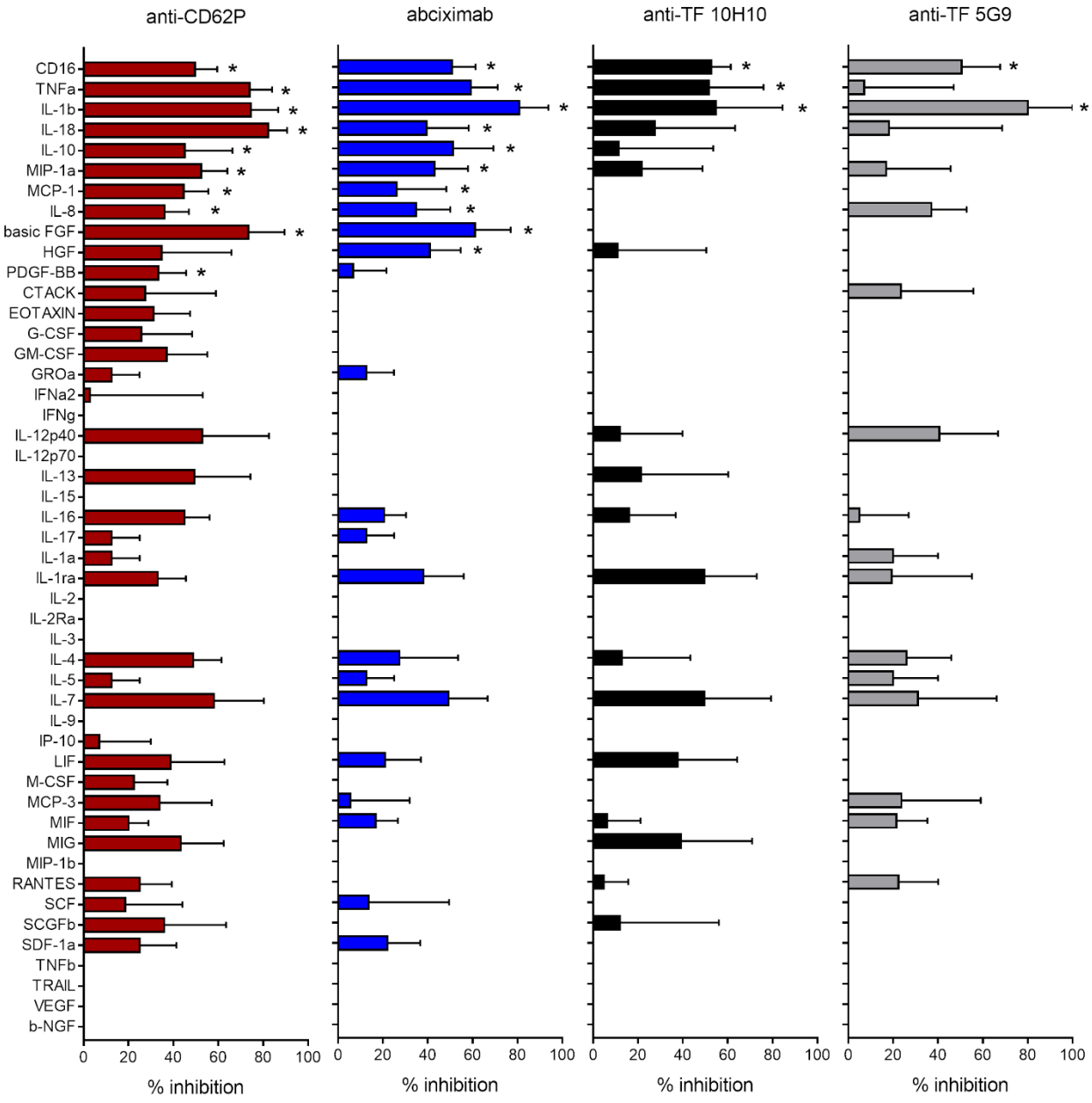
Supplemental Figure S3: Monocytes from severe COVID-19 patients or control participants were adhered on recombinant human albumin, P-selectin or fibrinogen coated surfaces. (**A-J**) The concentration of (**A**) IL-6, (**B**) IL-10, (**C**) IL-12, (**D**) IL-8/CXCL8, (**E**) MIP-1α, (**F**) MIP-1β, (**G**) G-CSF, (**H**) GM-CSF, (**I**) IP-10/CXCL10 and (**J**) IL-1RA in each condition is shown. Bars represent mean ± standard error of the mean of monocytes from 5 independent control participants and 6 independent severe COVID-19 patients. # indicates $p < 0.05$ compared to monocytes from control participants in the same condition; * indicates $p < 0.05$ compared albumin.



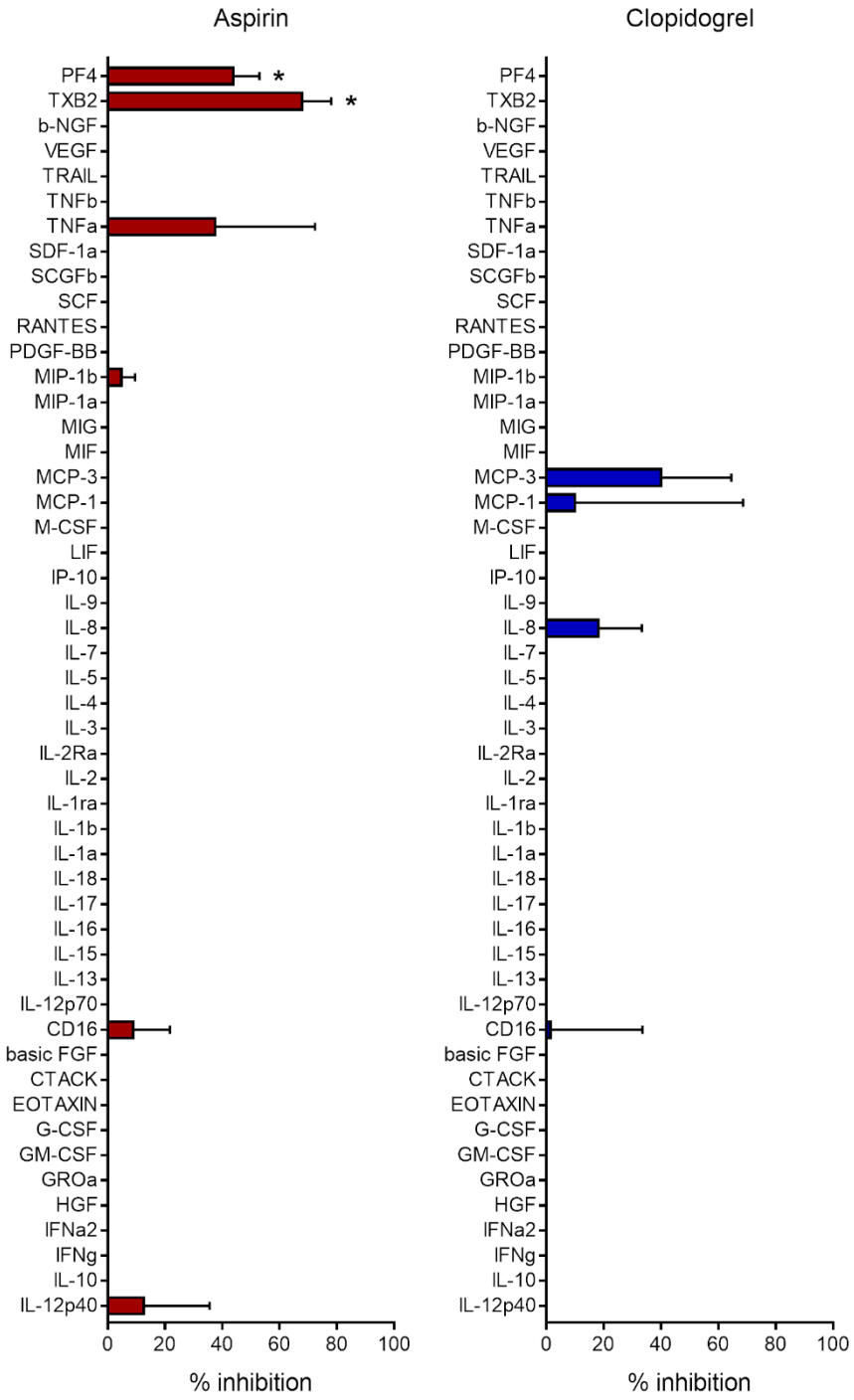
Supplemental Figure S4: Monocytes from healthy volunteers (control monocyte) were incubated with platelets from severe COVID-19 patients (COVID-19 platelets) or from heterologous healthy volunteers (control platelets) for 18 hours and the indicated inflammatory mediators were quantified in the supernatants. Monocytes from COVID-19 patients (COVID-19 monocyte) were also incubated with platelets from healthy volunteers (control platelets). The concentration of (**A**) IL-8/CXCL8, (**B**) VEGF, (**C**) IL-1RA and (**D**) TXB_2 are shown. Bars represent mean ± standard error of the mean of 6-12 platelet and monocyte combinations from COVID-19 patients or control participants. All experiments were repeated with cells from 2 independent control participants exposed to platelets or monocytes from the same COVID-19 patients with similar results, and a representative data from one of the donors is shown. * indicates $p < 0.05$ between selected groups.



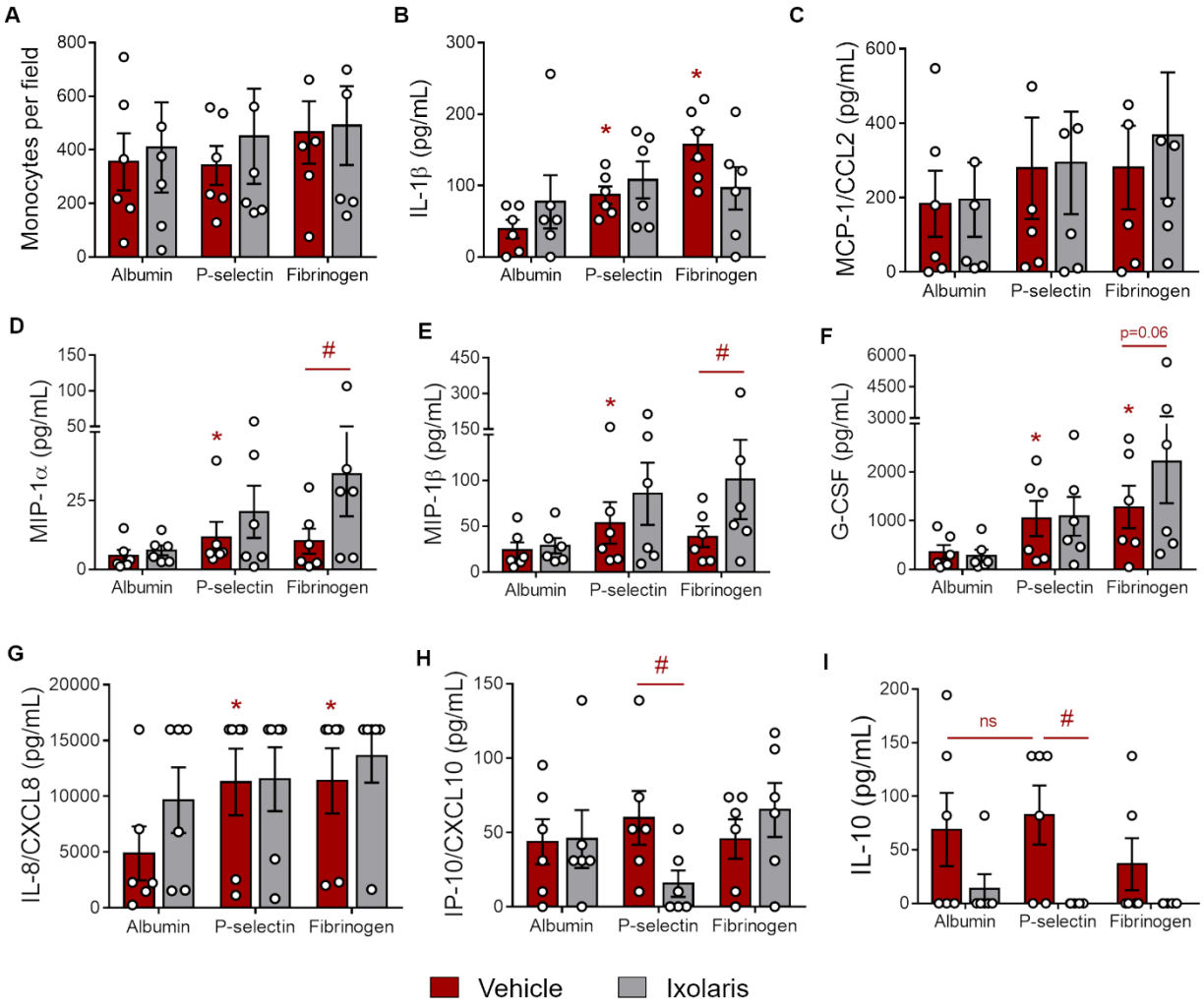
Supplemental Figure S5: Platelets, monocytes and platelet-monocyte co-cultures were kept uninfected or infected with SARS-CoV-2 overnight. The concentrations of (**A**) TNF- α, (**B**) MIP-1α, (**C**) MIP-1β, (**D**) IL-1 β, (**E**) IL-18, (**F**) IL-1RA, (**G**) IL-8/CXCL8, (**H**) MCP-1/CCL2, (**I**) MCP-3/CCL7, (**J**) IP10/CXCL10, (**K**) PDGF and (**L**) PF4/CXCL4 in each condition is shown. (**M**) Platelets were kept unstimulated or stimulated with thrombin (0.5 U/mL) in the presence or absence of autologous monocytes. The concentration of PF4/CXCL4 in each condition is shown. of Bars represent mean ± standard error of the mean of platelets and/or monocytes from 4 independent donors. * indicates $p < 0.05$ compared to uninfected platelets or between selected groups.



Supplemental Figure S6: Monocytes from healthy volunteers were incubated with platelets from severe COVID-19 patients in the presence of anti-P-selectin (anti-CD62P) neutralizing antibody, the anti-α_{IIb}/β_3 antibody abciximab, anti-TF clone 10H10, anti-TF clone 5G9 or isotype matched IgG. The percent inhibition on monocyte CD16 expression and inflammatory mediator release is shown for each condition. Bars represent mean ± standard error of the mean of monocytes of monocytes exposed to platelets from 3-6 independent COVID-19 patients. * indicates $p < 0.05$ compared to IgG.



Supplemental Figure S7: Monocytes from healthy volunteers were incubated with platelets from severe COVID-19 patients in the presence of the anti-platelet drugs aspirin (AAS), clopidogrel or DMSO (vehicle). The percent inhibition on inflammatory mediator release is shown for each condition. Bars represent mean ± standard error of the mean of monocytes exposed to platelets from 3-6 independent COVID-19 patients. * indicates $p < 0.05$ compared to vehicle.



Supplemental Figure S8: Monocytes from severe COVID-19 patients were plated on recombinant human albumin, P-selectin or fibrinogen-coated surfaces in the presence or absence of Ixolaris. (A) The number of monocytes adhered on each condition is shown. (**B-I**) The concentration of (**B**) IL-1β, (**C**) MCP-1/CCL2, (**D**) MIP-1α, (**E**) MIP-1β, (**F**) G-CSF, (**G**) IL-8/CXCL8, (**H**) IP-10/CXCL10 and (**I**) IL-10 secreted at each condition is shown. Bars represent mean ± standard error of the mean of monocytes from 5 independent control participants and 6 independent severe COVID-19 patients. * indicates $p < 0.05$ compared to albumin; # indicates $p < 0.05$ between vehicle and Ixolaris.

Table S1: Characteristics of COVID-19 patients admitted to the ICU based on the requirement of invasive mechanical ventilation or noninvasive oxygen supplementation.

Characteristics[1]	Noninvasive O_2 Supplementation (N=16)	Mechanical ventilation (N=30)	p value[2]
Age, years	38 (26 – 54)	50 (34 – 59)	0.1319
Sex, male	8 (50 %)	15 (50 %)	1
SAPS II	35 (30 – 46)	64 (58 – 73)	**< 0.0001**
PaO_2/FiO_2 ratio	457 (243 – 516)	146 (109 – 189)	**0.0072**
Vasopressors[3]	0 (0 %)	16 (53 %)	**0.0002**
Time from symptom onset to blood sample, days	10 (6 – 13)	12 (8 – 17)	0.2927
28-day mortality	0 (0 %)	18 (60 %)	**< 0.0001**
Comorbidities			
Obesity	6 (38 %)	4 (13 %)	0.0740
Hypertension	8 (50 %)	17 (57 %)	0.7604
Diabetes	4 (25 %)	12 (40 %)	0.3516
Cancer	0 (0 %)	4 (13 %)	0.2820
Chronic heart disease	1 (6.3 %)[4]	3 (10 %)[5]	1
Laboratory findings on admission			
Leukocytes, x 1000/µL	9.3 (7.4 – 12.8)	13.5 (10.6 – 20.8)	**0.0366**
Lymphocytes, cells/µL	1016 (786 – 1613)	1092 (487 – 1514)	0.4728
Monocytes, cells/µL	582 (259 – 768)	692 (534 – 921)	**0.0235**
Platelets, x 1000/µL	245 (179 – 342)	190 (142 – 236)	0.0717

[1]Numerical variables are represented as the median and the interquartile range, and qualitative variables as the number and the percentage.

[2]The qualitative variables were compared using the two tailed Fisher exact test, and the numerical variables using the t test for parametric and the Mann Whitney U test for nonparametric distributions.

[3]Dopamine, epinephrine/norepinephrine, vasopressin or phenylephrine.

[4]Coronary artery disease.

[5]Congestive heart failure.

Table S2: Characteristics of COVID-19 patients admitted to the ICU according to the 28-day mortality outcome as survivors or nonsurvivors.

Characteristics[1]	Survivors (N=28)	Nonsurvivors (N=18)	p value[2]
Age, years	57 (41 – 64)	60 (52 – 76)	0.1795
Sex, male	12 (43 %)	11 (61 %)	0.3651
Respiratory support			
Mechanical ventilation	12 (43 %)	18 (100 %)	**< 0.0001**
SAPS II	56 (37 – 64)	68 (59 – 79)	**0.0007**
PaO_2/FiO_2 ratio	172 (148 – 401)	139 (107 – 178)	**0.0374**
Vasopressor[3]	5 (18 %)	11 (61 %)	**0.0042**
Time from symptom onset to blood sample, days	9 (4 – 13)	10 (3 – 14)	0.6083
Comorbidities			
Obesity	8 (29 %)	2 (11 %)	0.2736
Hypertension	15 (54 %)	10 (56 %)	1
Diabetes	9 (32 %)	7 (39 %)	0.7543
Cancer	2 (7 %)	2 (11 %)	0.6386
Chronic heart disease	2 (7 %)	2 (11 %)	0.6386
Laboratory findings on admission			
Leukocytes, x 1000/µL	12,4 (9,1 – 14)	16,1 (9,7 – 16,2)	**0.0434**
Lymphocytes, cells/µL	1067 (888 – 1533)	934 (328 – 1647)	0.5692
Monocytes, cells/µL	582 (448 – 801)	738 (570 – 957)	**0.0351**
Platelets, x 1000/µL	198 (162 – 329)	187 (135 – 227)	0.1021

[1]Numerical variables are represented as the median and the interquartile range, and qualitative variables as the number and the percentage.

[2]The qualitative variables were compared using the two tailed Fisher exact test, and the numerical variables using the t test for parametric and the Mann Whitney U test for nonparametric distributions.

[3]Dopamine, epinephrine/norepinephrine, vasopressin or phenylephrine.