COVID-19–associated Lung Microvascular Endotheliopathy: A "From the Bench" Perspective

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ORIGINAL ARTICLE

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A "From the Bench" Perspective

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Abstract

Rationale: Autopsy and biomarker studies suggest that endotheliopathy contributes to coronavirus disease (COVID-19)-associated acute respiratory distress syndrome. However, the effects of COVID-19 on the lung endothelium are not well defined. We hypothesized that the lung endotheliopathy of COVID-19 is caused by circulating host factors and direct endothelial infection by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).

Objectives: We aimed to determine the effects of SARS-CoV-2 or sera from patients with COVID-19 on the permeability and inflammatory activation of lung microvascular endothelial cells.

Methods: Human lung microvascular endothelial cells were treated with live SARS-CoV-2; inactivated viral particles; or sera from patients with COVID-19, patients without COVID-19, and healthy volunteers. Permeability was determined by measuring transendothelial resistance to electrical current flow, where decreased resistance signifies increased permeability. Inflammatory mediators were quantified in culture supernatants. Endothelial biomarkers were quantified in patient sera.

Measurements and Main Results: Viral PCR confirmed that SARS-CoV-2 enters and replicates in endothelial cells. Live SARS-CoV-2, but not dead virus or spike protein, induces endothelial permeability and secretion of plasminogen activator inhibitor 1 and vascular endothelial growth factor. There was substantial variability in the effects of SARS-CoV-2 on endothelial cells from different donors. Sera from patients with COVID-19 induced endothelial permeability, which correlated with disease severity. Serum levels of endothelial activation and injury biomarkers were increased in patients with COVID-19 and correlated with severity of illness.

Conclusions: SARS-CoV-2 infects and dysregulates endothelial cell functions. Circulating factors in patients with COVID-19 also induce endothelial cell dysfunction. Our data point to roles for both systemic factors acting on lung endothelial cells and viral infection of endothelial cells in COVID-19–associated endotheliopathy.

Keywords: COVID-19; acute respiratory distress syndrome; endothelial permeability; lung endothelial injury

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A complete list of COMET Consortium and Co-ACIT Study Group members may be found before the beginning of the References.

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Endothelial cell dysfunction, or "endotheliopathy," is believed to contribute to the pathogenesis of acute respiratory distress syndrome (ARDS) in severe coronavirus disease (COVID-19), and the endothelium has been proposed as a therapeutic target in severe COVID-19 disease (1, 2). However, despite an unprecedented focus on defining COVID-19 mechanisms (3), published data on the direct endothelial effects of COVID-19 are minimal (4, 5). Endothelial cells regulate vascular barrier function, vasomotor tone, vasculogenesis, coagulation and fibrinolysis, and trafficking of cells and substrates. Infection and injury induce a proinflammatory program in endothelial cells with upregulated production of cytokines, chemokines, procoagulant factors, and proadhesive proteins, as well as glycocalyx damage and vascular hyperpermeability with resultant tissue edema (6, 7). Elevated circulating endothelial cell biomarkers have been reported to associate with increased disease severity and risk of death in COVID-19 (8–12).

The radiographic features of bilateral ground-glass opacities in patients with COVID-19–associated ARDS are compatible with endothelial hyperpermeability (13). Furthermore, autopsies of patients who died of COVID-19 have revealed diffuse alveolar damage with perivascular T cell infiltration, virus within endothelial cells, endothelial injury, capillary microthrombi, and microvascular angiogenesis (14). These findings alongside the high incidence of thrombotic complications (15) implicate endotheliopathy in COVID-19–associated ARDS. The inaccessibility of endothelial cells to direct sampling in real time in humans has impeded progress toward understanding the cause(s) of COVID-19–associated endotheliopathy and its role in driving ARDS.

We hypothesized that COVID-19–associated endotheliopathy results from both viral infection of endothelial cells and secondary endothelial effects of factors in the blood of patients with COVID-19. We therefore tested the effects of live and inactivated virus, as well as sera from patients with and without COVID-19, on the activation and permeability of human lung microvascular endothelial cells (HMVECs), and we quantified serum endothelial biomarkers in patients with and without COVID-19.

Methods

HMVEC Culture, Agonists, and Antagonists

HMVECs (passages 3–6; Lonza) from male and female cadavers deceased of nonseptic causes were cultured (37°C, 5% CO_2) in endothelial culture medium (EGMTM-2 EC Growth Medium). Confluent monolayers were treated with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), spike protein, or sera from patients with and without COVID-19 or healthy volunteers. Some experiments used agonists, antagonists, or inhibitors, including the ACE2 agonist Diminazene aceturate (DMZ, 20 μM; Tocris), angiotensin-converting enzyme (ACE) inhibitor (CAS35115-60-7, 20 μM; Sigma-Aldrich), Toll-like receptor 4 (TLR4) antagonist LPS-RS ultrapure (1 μg/ml; InvivoGen), and TLR3 blocker CU-CPT 4a (10 μM; Tocris).

Viral Reagents

Experiments with live SARS-CoV-2 were performed in a biosafety level 3 laboratory. Live SARS-CoV-2 (strain nCoV/USA-WA1/2020) was provided by Dr. Melanie Ott. To generate viral stocks, Vero E6 cells were exposed to SARS-CoV-2 for 72 hours, and supernatants were stored at −80°C (16). Plaque assays were performed using Vero E6 cells to establish viral stock titers. We purchased inactivated SARS-CoV-2 (strain nCoV/USA-WA1/2019), produced by γ-irradiation or by heating (65°C, 30 min), and recombinant spike glycoprotein (stabilized) from SARS-CoV-2, Wuhan-Hu-1, produced in a baculovirus expression system (American Type Culture Collection).

Patient Enrollment Criteria and Serum Sample Collection

All human studies were approved by the University of California, San Francisco Institutional Review Board (IRB). Sera were collected from acutely ill patients with COVID-19 ($n = 99$) and patients without COVID-19 ($n = 43$) in the emergency department or early after admission to wards or ICUs at the Zuckerberg San Francisco General Hospital and the University of California, San Francisco Medical Center as part of two separate clinical studies: the Co-ACIT study (COVID-19 Associated Coagulopathy, Inflammation, and Thrombosis) (IRB 20-30895; $n = 130$) and the COMET study (COVID-19 Multi-Phenotyping for Effective Therapies; www.comet-study.org/team; IRB 20-30497; $n = 12$). The diagnosis of COVID-19 was based on a positive SARS-CoV-2 PCR test. The Co-ACIT study includes adults (aged >18 yr) undergoing evaluation for possible COVID-19 in the Zuckerberg San Francisco General Hospital emergency department based on fever, respiratory symptoms (cough, shortness of breath, wheezing), gastrointestinal symptoms (diarrhea, nausea/vomiting), and/or changes in taste or smell. The COMET study includes adults admitted to the wards and ICUs with symptomatic COVID-19. Subjects in both studies were followed until discharge from the emergency department or hospital, and a comprehensive collection of patient characteristics, physiological and laboratory parameters, and clinical outcomes was documented. Patients were subclassified into three disease severity categories at Day 1 based on their level of care: mild (ambulatory mild disease: discharged after evaluation in the emergency department), moderate

Author Contributions: J.J. and J.H. conceptualized and designed the study. J.J. acquired and analyzed the data and drafted and revised the manuscript. J.H. provided critical edits to the manuscript. L.R. was responsible for virus propagation and viral titer determination. L.R. and A.S. supervised biosafety level 3 (BSL-3) working conditions. E.L. participated in cell culture, stimulation, and immunoassays. The other authors (Z.A.M., A.T.F., R.J.B., P.K., C.S.C., P.G.W., D.J.E., C.H., M.F.K., C.R.L, M.A.M., and L.Z.K.) were part of patient enrollment in the COMET (COVID-19 Multi-Phenotyping for Effective Therapies) and Co-ACIT (COVID-19 Associated Coagulopathy, Inflammation, and Thrombosis) studies or members of the COMET executive committees and provided critical edits to the manuscript. Studies not requiring BSL-3 laboratory precautions were performed in J.H.'s laboratory, and those requiring BSL-3 laboratory precautions were performed in A.S.'s laboratory.

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This article has a related editorial.

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org.

At a Glance Commentary

Scientific Knowledge on the Subject: The clinical course, autopsy studies, and available data on immune cell functions and circulating concentrations of inflammatory and endothelial biomarkers in patients with coronavirus disease (COVID-19) all point to the endothelium as a target organ in severe COVID-19 disease. Endothelial cell activation and dysregulation ("endotheliopathy") are pathogenically involved in acute respiratory distress syndrome caused by sepsis and tissue injury. Currently, the understanding of the endotheliopathy of COVID-19, including its underlying mechanisms and role in driving acute respiratory distress syndrome, is rudimentary.

What This Study Adds to the Field: This translational-clinical study demonstrates that live severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) can enter primary human lung microvascular endothelial cells (HMVECs) and induce hyperpermeability and a proadhesive and proinflammatory phenotype. Analogous to the heterogeneous course of humans with COVID-19, we noted substantial variability in the responses of HMVECs from different donors. *Ex vivo* treatment with sera from patients with COVID-19 induces HMVEC permeability. Sera from moderately and critically ill patients with COVID-19 had a biomarker signature that was consistent with endothelial activation and dysfunction. Our data, in conjunction with other studies, suggest that COVID-19–associated lung endotheliopathy is caused by the actions of systemic and localized cells and inflammatory factors on endothelial cells, as well as SARS-CoV-2 infection of endothelial cells.

(hospitalized: moderate disease, admitted to the floor), and critical (hospitalized: severe disease, admitted to the ICU) according to guidelines from the World Health Organization Working Group on the Clinical Characterization and Management of COVID-19 (17, 18). Table 1 includes the baseline demographics and characteristics of patients. Sera were also collected from healthy volunteers without fever or respiratory or gastrointestinal symptoms (IRB 10-01981 and 20-30895).

Assessment of Effects of Viral Particles, Spike Protein, and Human Sera on HMVEC Permeability and Biomarker Production

HMVECs were treated with live or inactivated SARS-CoV-2, spike protein, or human sera. Permeability was inferred from transendothelial resistance (TER), which was measured using electric cell substrate impedance sensing (Applied Biophysics), which quantifies the resistance of a cell monolayer to electrical current flow in real time (19, 20). Decreased TER reflects increased permeability. HMVECs were cultured to confluence based on reaching a stable plateau in TER measured at a frequency of 4,000 Hz (20). HMVECs were then incubated with virus or spike protein (four to eight replicates per condition) or 10% sera from patients with COVID-19 and patients without COVID-19 or healthy humans (two replicates per sample). TER was measured repeatedly over 24 hours. Data were quantitatively analyzed by calculating the area between the control curve (normalized resistance, 1) and the experimental condition, called the area under the curve, as an integrative marker of the permeability (21). At 24 hours, IL-6, IL-8, plasminogen activator inhibitor 1 (PAI-1), vascular endothelial growth factor (VEGF), and CCL-2 were quantified in supernatants by ELISA (R&D Systems). Viral PCR was performed at 24 and 72 hours in some experiments. *Escherichia coli* 0111:B4 LPS (1 μg/ml) served as a positive control that reliably induces HMVEC permeability across donors.

Quantification of Biomarkers in Sera from Cohort of 74 Patients

Endothelial biomarkers were quantified in sera from patients with and without COVID-19 collected in the Co-ACIT study during their first medical evaluation in the emergency department before receiving fluids, dexamethasone, remdesivir, or plasma therapy. The specific biomarkers were chosen because they are produced by or directly act on the endothelium, or, as in the case of syndecan-1, reflect endothelial glycocalyx injury. IL-6, IL-8, VEGF (R&D Systems), and syndecan-1 (Abcam) were quantified by ELISA. Other endothelial biomarkers were quantified using multiplex fluorescence-based panels (Eve Technology), including the cardiovascular disease panel (HCVD4-07-0; follistatin, platelet endothelial cell adhesion molecule 1 [PECAM-1], pentraxin-3, sE-selectin, tissue factor, s-thrombomodulin, troponin T) and the endothelial biomarker array (HDHSB10; brain-derived neurotrophic factor [BDNF], cathepsin D, myeloperoxidase, neural cell adhesion molecule [NCAM], PAI-1, platelet-derived growth factor [PDGF]-AA, PDGF-AB/BB, regulated upon activation, normal T cell expressed and secreted [RANTES], soluble intercellular adhesion molecule 1 [sICAM-1], soluble vascular cell adhesion molecule 1 [sVCAM-1]). ACE and ACE2 were quantified by ELISA (R&D Systems).

Flow Cytometry

HMVECs were detached with Accutase, and cells were labeled with fluorescein isothiocyanate CD102 (clone CBR-IC1/2), phycoerythrin-cyanine 7 CD106 (clone 429 MVCAM.A) (BioLegend), eFluor 450 CD31 (clone WM59), allophycocyanin CD62P (clone Psel.KO2.3) (eBioscience), and phycoerythrin CD144 (clone 16B-1) (Invitrogen). A fixation step was used to inactivate live virus before HMVECs underwent staining. Single-cell suspensions underwent flow cytometry the same day (LSRII Fortessa flow cytometer). The data were analyzed with FlowJo software. Endothelial cells were defined as $CD31^{+}CD102^{+}$.

Statistics

Statistics were performed using R software (www.R-project.org). Graphs show means and SDs. Nonparametric Mann-Whitney *U* tests were used for continuous variables; the chi-square test was used for discrete variables; or ANOVA with multiple Tukey's *post hoc* tests were used for comparisons between multiple groups. For analysis of biomarkers in patient sera, to limit multiple testing–related biases, we applied a two-step-up method with a false discovery rate (q) of 5% (22). We also performed a principal component analysis based on 10 endothelial activation and injury biomarkers to assess the main relationships between the pattern of biomarkers, COVID-19 status, and disease severity. All tests were two-sided, and P values less than 0.05 were considered significant.

Table 1. Characteristics at Inclusion of 142-Patient Cohort Whose Sera Were Used in *Ex Vivo* Endothelial Cell Stimulation Assays

Variable	Total Cohort (*N* = *142*)	Non–COVID-19 (*n* = *43*)	COVID-19 (*n* = *99*)	*P* Value
Age, yr, mean ± SD	58 ± 16	59 ± 16	55 ± 19	0.24
Male, *n* (%)	88 (62)	28 (65)	60 (60.6)	0.7
Duration of symptoms before admission, d	6.6 ± 7	5.3 ± 9.6	7.2 ± 5.3	0.14
Ethnicity, *n* (%)				
White	17 (12)	11 (25.6)	6 (6.1)	0.005
Black or African American	17 (12)	10 (23.3)	7 (7.1)	0.01
Hispanic/Latino	71 (50)	12 (28)	59 (59.6)	0.0009
Asian	19 (13.4)	4 (9.3)	15 (15.1)	0.43
Native American	1 (0.7)	1 (2.3)	0 (0)	0.3
Other/not reported	17 (12)	5 (11.6)	12 (12.1)	0.99
Comorbidities, *n* (%)				
Obesity	49 (34.5)	10 (23.3)	39 (39.4)	0.08
Diabetes	41 (28.9)	9 (20.9)	32 (32.3)	0.23
COPD/asthma/CRD	16 (11.3)	9 (20.9)	7 (7.1)	0.02
CKD	14 (9.8)	3 (7)	11 (11.1)	0.55
CAD/CHF	15 (10.6)	6 (14)	9 (9.1)	0.38
Stroke	8 (5.6)	6 (14)	2 (2)	0.009
Liver disease	12 (8.4)	6 (14)	6 (6)	0.18
HIV/AIDS	6 (4.2)	5 (11.6)	1 (1)	0.009
Cancer	13 (9.1)	5 (11.6)	8 (8.1)	0.53
Admission on Day 1, *n* (%)				
ED only/outpatient: mild	42 (29.6)	14 (32.6)	28 (28.3)	0.55
Floor: moderate	60 (43.7)	19 (44.2)	41 (43.4)	0.85
ICU: critical	40 (26.7)	10 (23.3)	30 (28.3)	0.42
Respiratory support on Day 1, *n* (%)				
None	61 (43)	22 (51.2)	39 (39.4)	0.2
NC/NRB	48 (33.8)	15 (34.8)	33 (33.3)	0.84
HFNC	18 (12.7)	3 (7)	15 (15.1)	0.27
CPAP/BiPAP	1 (0.7)	1 (2.3)	0 (0)	0.3
Intubated	14 (9.9)	2 (4.7)	12 (12.1)	0.23
Severity-of-illness scores				
SOFA score, mean ± SD	1.8 ± 3	0.9 ± 1.2	2.4 ± 3.4	0.01
Respiratory SOFA score, mean ± SD	0.63 ± 1.1	0.21 ± 0.47	0.82 ± 1.2	0.02
Diagnosis at admission, *n* (%)				
COVID-19	99 (69.7)	0 (0)	99 (100)	NA
Sepsis (of origin)	18 (12.7)	18 (41.8)	0 (0)	NA
Pneumonia/URI	12 (8.4)	12 (27.9)	0 (0)	NA
UTI	1 (0.7)	1 (2.3)	0 (0)	NA
SSTI	2 (1.4)	2 (4.7)	0 (0)	NA
Other/not reported	3 (2.1)	3 (7)	0 (0)	NA
Asthma/COPD exacerbation	6 (4.2)	6 (14)	0 (0)	NA
Heart failure	8 (5.6)	8 (18.6)	0 (0)	NA
Pulmonary embolism	1 (0.7)	1 (2.3)	0 (0)	NA
Gastrointestinal symptoms	4 (2.8)	4 (9.3)	0 (0)	NA
Other/not reported	6 (4.2)	6 (14)	0 (0)	NA

Definition of abbreviations: BiPAP = bilevel positive airway pressure; CAD = coronary artery disease; CHF = chronic heart failure; CKD = chronic kidney disease; COPD = chronic obstructive pulmonary disease; COVID-19 = coronavirus disease; CPAP = continuous positive airway pressure; CRD = chronic respiratory disease; ED = emergency department; HFNC = high-flow nasal cannula; NA = not applicable; NC = nasal cannula; NRB = non-rebreather mask; SOFA = Sequential Organ Failure Assessment; SSTI = skin and soft tissue infections; URI = upper respiratory infection; UTI = urinary tract infection.

Results

SARS-CoV-2 Enters and Replicates in HMVECs

Using PCR, we detected SARS-CoV-2 in lysates of HMVECs after 24 and 72 hours of exposure to live SARS-CoV-2 at a multiplicity of infection (MOI) of 0.1 and 1 viral particle per cell (Figure 1A). Intracellular viral mRNA concentrations were higher at 72 hours than at 24 hours, indicating viral replication within endothelial cells. Viral mRNA was not detected in lysates of medium-treated HMVECs.

Exposure to Live SARS-CoV-2 Induces HMVEC Permeability

Exposure of HMVECs to SARS-CoV-2 at an MOI of 1, but not at an MOI of 0.01 or 0.1, led to decreased TER, consistent with increased permeability (Figure 1B1–6). Although the magnitude of the permeability induced by SARS-CoV-2 varied between HMVECs from different donors, there were significant differences between the areas under the curve of SARS-CoV-2 at an MOI of 1 versus medium for all six donors (Figure 1B7) ($P < 0.05$).

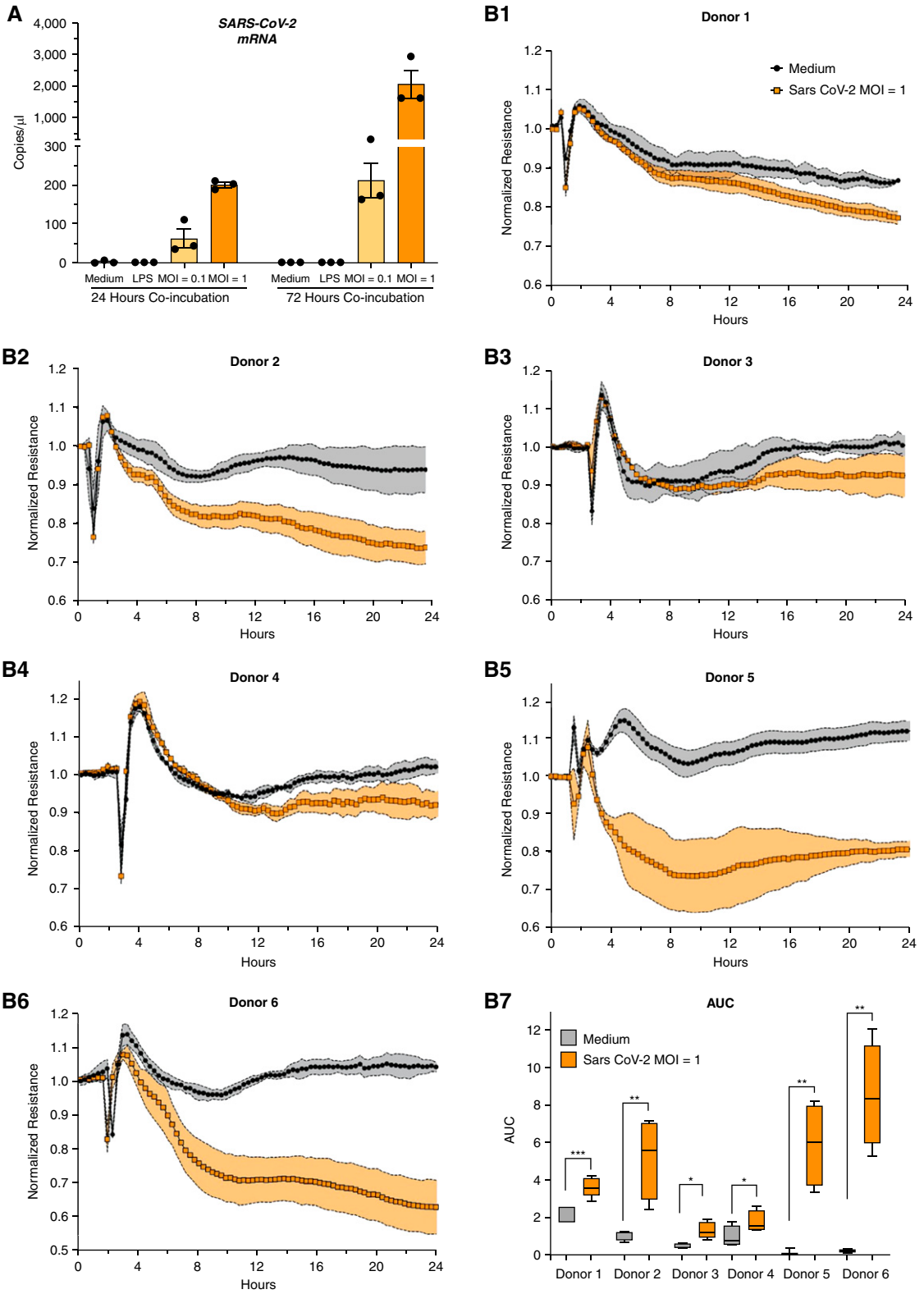

Figure 1. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) human lung microvascular endothelial cell (HMVEC) infection and endothelial permeability. (*A*) SARS-CoV-2 mRNA was detected and quantified in HMVEC lysates after 24 and 72 hours of exposure to live SARS-CoV-2 virus (multiplicity of infection [MOI] of 1; orange, strain nCoV/USA-WA1/2020) using quantitative PCR (TaqMan 2019-nCoV assay). This example shows cells from a single donor; dots are biological replicates. (*B1–B6*) Transendothelial resistance during treatment with medium (black line) or live virus (MOI of 1; orange) in six HMVEC donors. (*B7*) The corresponding area under the curve (AUC) for HMVECs on each of the six donors. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by two-tailed Mann-Whitney *U* test.

Effects of ACE and ACE2 Modulation and Innate Immune Receptor Blockade on SARS-CoV-2–induced HMVEC Permeability

Treatment with an ACE inhibitor did not affect SARS-CoV-2–induced permeability (Figure 2A1–2). Conversely, the ACE2 agonist DMZ reversed the virus-induced permeability of HMVECs from three of six donors (Figure 2A1–2; *see* Figure E1 in the online supplement) ($P < 0.05$). DMZ did not reduce intracellular SARS-CoV-2 concentrations (data not shown), which suggests that it reduces permeability independently of viral entry. Also, DMZ reduced LPS-induced permeability (Figure 2B) ($P < 0.01$), suggesting that its effects are not specific to SARS-CoV-2. Neither TLR4 antagonism using LPS-RS nor TLR3 inhibition using CU-CPT 4a affected virus-induced permeability (data not shown).

SARS-CoV-2 Effects on HMVEC Inflammatory Mediator and Adhesion Molecule Expression

Exposure to live SARS-CoV-2 significantly upregulated IL-6, PAI-1, and VEGF secretion by HMVECs from three of the six donors (donors 2, 5, and 6) and increased IL-8 and CCL-2 secretion by HMVECs from one donor (donor 6) (Figure 3A). On the basis of flow cytometry, there was high variability in the baseline expression of multiple adhesion molecules (VCAM-1 [CD106], ICAM-2 [CD102], P-selectin [CD62P], PECAM-1 [CD31], and VE-cadherin [CD144]) between HMVECs from different donors (Figure 3B). As shown in the heat map in Figure 3B2, exposure to SARS-CoV-2 significantly upregulated surface expression of VCAM-1 by two donors (donors 2 and 4), P-selectin by two donors (donors 2 and 5), and ICAM-2 by one donor (donor 2).

Spike Protein and Inactivated Virus Do Not Induce HMVEC Permeability or Inflammation

There were no significant effects of spike protein (0.1–1,000 ng/ml) or inactivated virus (10^4, 10^5, or 10^6 copies/ml) on permeability (Figure E2A), nor did spike protein or inactivated virus induce IL-6, IL-8, or CCL-2 secretion by HMVECs from any of the three donors tested (Figure E2B). Moreover, on the basis of flow cytometry, neither spike protein nor inactivated virus affected the surface expression of endothelial cell proteins, including CD31 (PECAM-1), CD102 (ICAM-2), CD106 (VCAM-1), CD144 (VE-cadherin), and CD62P (P-selectin) (Figure E2C). These data suggest that spike protein and viral particles do not activate endothelial cell surface innate immune receptors.

Effects of Sera from Patients with and without COVID-19 on HMVEC Permeability and Inflammation

We exposed HMVECs to sera from patients with COVID-19 ($n = 99$) and patients without COVID-19 ($n = 43$) and from healthy volunteers ($n = 18$). Table 1 includes baseline demographics, level of care, and comorbidities of the cohort. Hispanic/Latino individuals were substantially overrepresented, whereas Black or African American and White individuals were substantially underrepresented, among patients with COVID-19 versus patients without COVID-19. Compared with sera from healthy subjects, sera from patients with COVID-19 with mild ($P < 0.05$), moderate ($P < 0.001$), or critical ($P < 0.001$) disease induced reductions in TER, reflecting increased permeability (Figure 4). Sera from critical patients without COVID-19 also induced HMVEC permeability ($P < 0.001$), whereas healthy donor sera did not (Figure 4). There were no differences in permeability induced by sera from patients without and with COVID-19 with comparable illness severities (Figure 4B).

There was considerable variability in the effects of COVID-19 sera on HMVEC secretion of different inflammatory mediators and in the pattern of upregulation of mediators induced by COVID-19 and non–COVID-19 sera (Figure E3). Compared with healthy human sera, sera from critical but not mild or moderate COVID-19 disease increased HMVEC secretion of IL-6 ($P < 0.05$), whereas sera from patients without COVID-19 did not. Exposure to sera from patients with COVID-19 of any disease severity and from mild and moderate patients without COVID-19 significantly upregulated CCL-2 secretion compared with healthy human serum. COVID-19 and non-COVID-19 sera did not affect IL-8 or PAI-1 secretion by HMVECs. Finally, sera from moderate and critical patients with COVID-19, but not patients without COVID-19, significantly increased VEGF secretion (Figure E3).

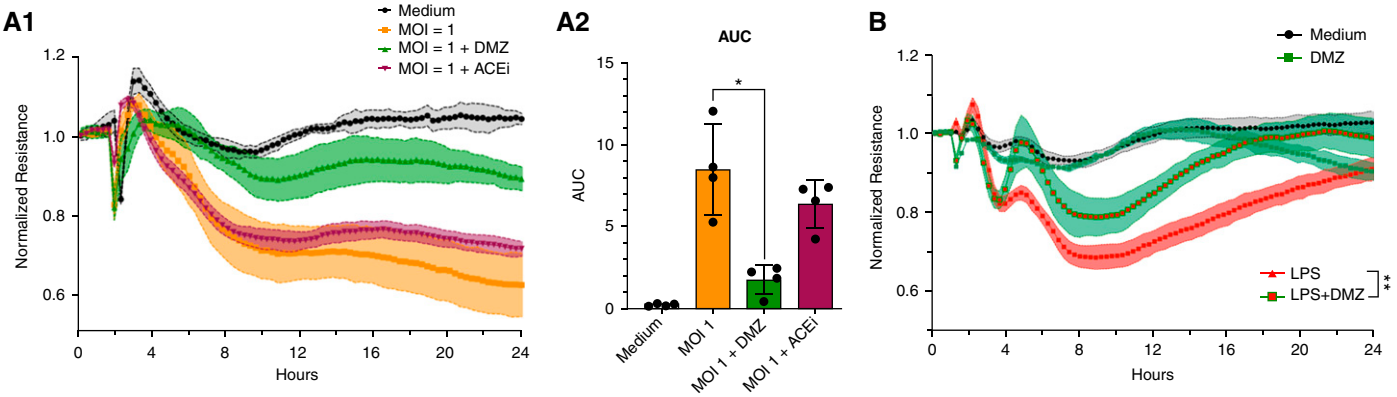

Figure 2. Pharmacological modulation of angiotensin-converting enzyme 2 receptor (ACE2R) pathway and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)-induced human lung microvascular endothelial cell (HMVEC) permeability. (*A1* and *A2*) Representative example of ACE2 agonist (Diminazene aceturate [DMZ] 20 μM; green) and ACE inhibitor (ACEi) effect (20 μM; claret red) on the transendothelial resistance and area under the curve (AUC) of HMVECs treated simultaneously with ACE2 agonists/antagonists and live SARS-CoV-2 (multiplicity of infection [MOI] of 1; orange) infection. (*B*) The stabilizing effects of DMZ on permeability induced by LPS (1 μg/ml). $^*P < 0.05$, $^{**}P < 0.01$ by two-tailed Mann-Whitney *U* test (AUC LPS vs. LPS + DMZ).

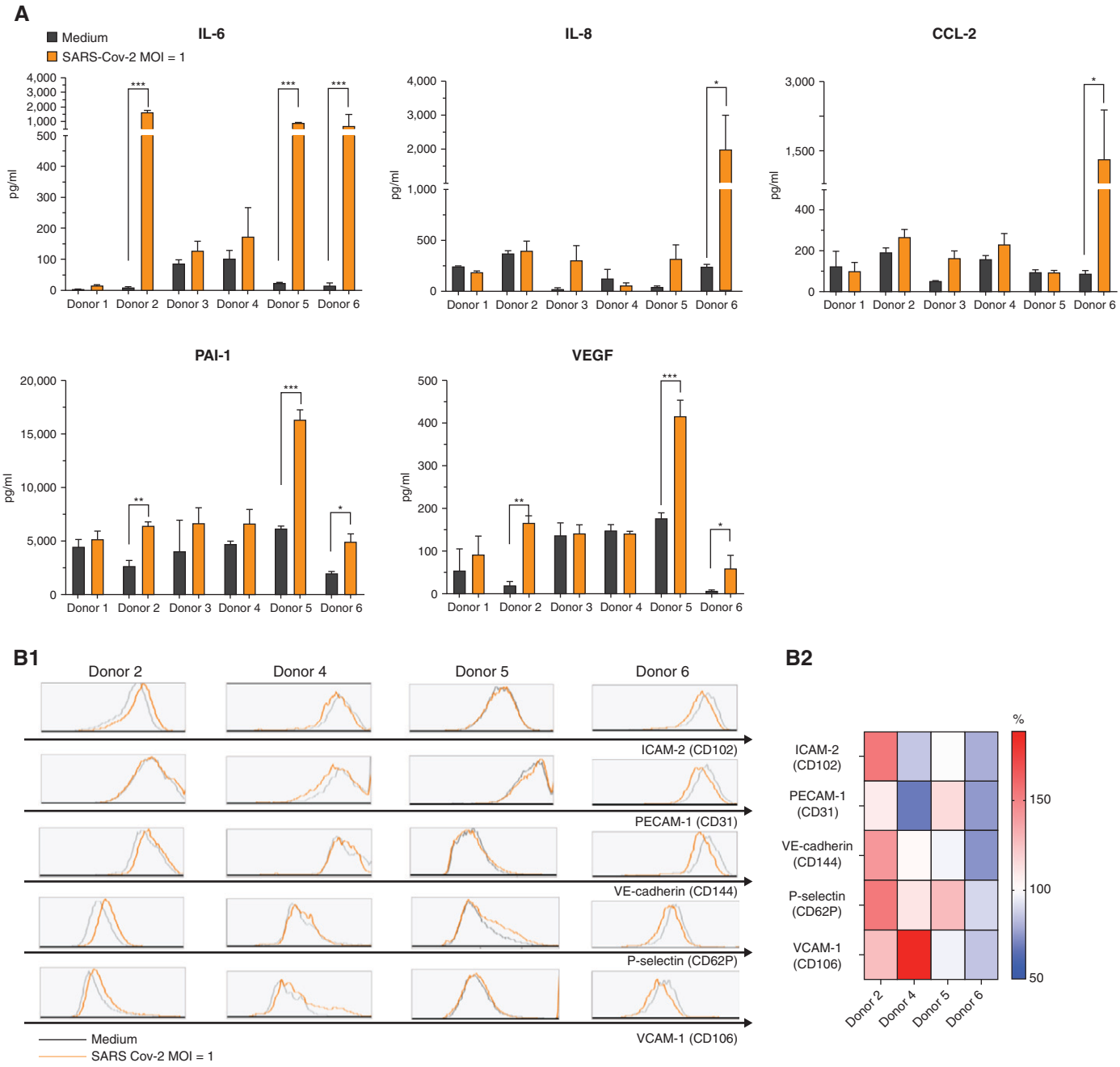

Figure 3. Effect of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection on human lung microvascular endothelial cell (HMVEC) mediator secretion and proadhesive phenotype. (*A*) Concentrations of IL-6, IL-8, CCL-2, plasminogen activator inhibitor 1 (PAI-1), and vascular endothelial growth factor (VEGF) in supernatants of HMVECs from six different donors treated with medium or SARS-CoV-2 (multiplicity of infection [MOI] of 1; orange, strain nCoV/USA-WA1/2020). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ by two-tailed Mann-Whitney *U* test. (*B1*) Histograms for CD102 (ICAM-2), CD31 (platelet endothelial cell adhesion molecule 1 [PECAM-1]), CD106 (vascular cell adhesion molecule 1 [VCAM-1]), CD144 (VE-cadherin), and CD62P (P-selectin) expression on HMVECs from four different donors after 24 hours of treatment with medium (gray) or infection with SARS-CoV-2 (MOI of 1; orange, strain nCoV/USA-WA1/2020). (*B2*) A double-gradient heat map of the relative mean fluorescence intensity on endothelial cells from four different donors after 24 hours of treatment with medium versus virus.

Patients with COVID-19 Have Elevated Serum Endothelial Activation and Injury Biomarkers

Concentrations of endothelial biomarkers in sera from patients with and without COVID-19 are shown in Table E1. IL-6 ($P = 0.001$), IL-8 ($P = 0.003$), VEGF ($P = 0.01$), follistatin ($P = 0.04$), tissue factor ($P = 0.02$), serum thrombomodulin ($P = 0.03$), sVCAM-1 ($P = 0.03$), and syndecan-1 were positively associated with the level of care in COVID-19 and non–COVID-19 groups. Conversely, BDNF ($P = 0.04$) and PDGF-AA ($P = 0.03$) concentrations were negatively associated

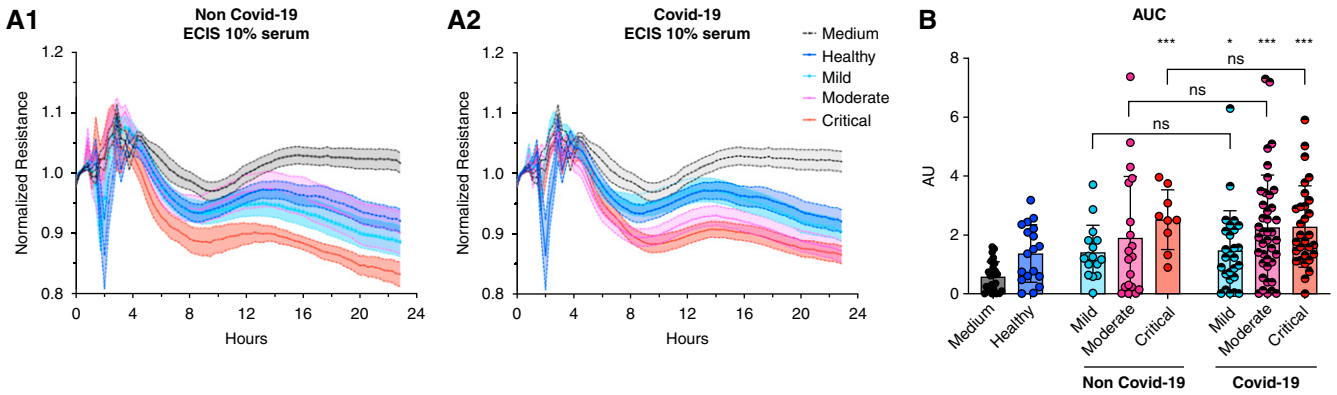

Figure 4. Sera from patients with and without coronavirus disease (COVID-19) induce human lung microvascular endothelial cell (HMVEC) permeability proportionate to their clinical severity. (*A1* and *A2*) Transendothelial resistance tracings of HMVECs during treatment with medium or with sera from healthy volunteers ($n = 18$); patients with COVID-19 with mild, moderate, or critical illness ($n = 99$; mild [$n = 28$], moderate [$n = 41$], critical [$n = 30$]); or patients without COVID-19 with mild, moderate, or critical illness ($n = 43$; mild [$n = 14$], moderate [$n = 19$], critical [$n = 10$]). Each curve represents the mean value of the group ± SD. (*B*) The corresponding electric cell substrate impedance sensing (ECIS) area under the curve (AUC) represents the intensity of sera-induced HMVEC permeability. $^*P < 0.05$, $^{***}P < 0.001$ versus healthy donors by two-tailed Mann-Whitney *U* test.

with level of care (Table E2). Disregarding level of care, patients with COVID-19 had higher sVCAM-1 ($P < 0.0001$), sICAM-1 ($P = 0.006$), NCAM ($P = 0.009$), cathepsin ($P = 0.002$), PAI-1 ($P = 0.0005$), and syndecan-1 ($P = 0.017$) and lower BDNF ($P = 0.03$) than patients without COVID-19 (Figure 5A). ACE and ACE2 serum concentrations were similar between patients without and with COVID-19 (Figure 5A, Table E3). After stratification by level of care, patients with COVID-19 had significantly higher circulating concentrations of sVCAM-1, cathepsin D, sICAM-1, NCAM, PAI-1, and syndecan-1 than patients without COVID-19 (Figure 5B, Table E2). Applying a two-step-up method with a false discovery rate (q) of 5% (22), only sVCAM-1 ($q = 0.0006$), NCAM ($q = 0.015$), sICAM-1 ($q = 0.03$), cathepsin D ($q = 0.003$), syndecan-1 ($q = 0.02$), PAI-1 (total) ($q = 0.003$), and IL-8 ($q = 0.015$) were significantly different between patients with and without COVID-19. An exploratory multivariate analysis by a principal component analysis based on 10 endothelium-related biomarkers and the level of care showed that patients with COVID-19 have increased sVCAM-1, NCAM, sICAM-1, cathepsin D, and PAI-1 but lower BDNF and RANTES in comparison to patients without COVID-19 (Figure 5C).

Discussion

This study highlights the complexity of the endotheliopathy associated with SARS-CoV-2 infection. We found that SARS-CoV-2 infects and induces permeability of HMVECs from all donors, and it variably affects inflammatory mediator secretion by HMVECs from different donors. We observed that *ex vivo* exposure of HMVECs to sera of patients with COVID-19 induces endothelial permeability and variable inflammatory activation. At the time of presentation to the emergency department, enrolled patients with COVID-19 had elevated serum endothelial biomarkers that correlated with their severity of illness. Our data, in conjunction with autopsy findings of SARS-CoV-2 within lung endothelial cells (14) and reports of elevated circulating endothelial biomarkers (8–12), suggest that both viral infection of endothelial cells and secondary effects of circulating factors and intrapulmonary leukocytes mediate COVID-19–associated lung endotheliopathy and, by extension, ARDS. Figure 6 shows our hypothetical model of endothelial cells as targets for SARS-CoV-2 infection and secondary injury.

We found that exposure to sera from patients with mild, moderate, or critical COVID-19 significantly increased HMVEC permeability. This indicates the presence of circulating factors capable of dysregulating endothelial function early during symptomatic COVID-19. We observed differences in the effects of sera from patients with mild, moderate, and severe COVID-19 on HMVEC production of specific proinflammatory mediators. Sera from patients with critical COVID-19 significantly upregulated IL-6 production by HMVECs. We also observed that live SARS-CoV-2 significantly upregulated production of IL-6, as well as PAI-1 and VEGF, by HMVECs from half of the donors. Notably, live SARS-CoV-2 induced the highest degree of permeability in HMVECs from the same donors that produced the most IL-6. Circulating IL-6 levels are predictive of nonsurvival in COVID-19 (9). Our data suggest that enhanced IL-6 secretion by endothelial cells is linked to endothelial dysfunction and is associated with increased endothelial permeability.

We observed large differences in live SARS-CoV-2 effects on permeability and activation of HMVECs from different donors. We hypothesize that intrinsic differences in lung endothelial cell susceptibility to SARS-CoV-2 infection and to the actions of systemic and localized immune factors underlie the variability in the outcomes of COVID-19. Possible sources of the heterogeneity in the responses of different donor HMVECs include genetics, epigenetics, age, concomitant diseases, recent infection or injury, or the cause of the donor's death. Interindividual differences in the ACE/ACE2 system, such as ACE2 polymorphisms or cell surface expression of ACE2 (23–26), could also contribute to variability in the susceptibility to HMVEC infection and downstream inflammation and permeability (27). One study examining SNPs in ACE2 and ACE genes in 297 patients with COVID-19 and 253 patients without COVID-19 reported that the ACE2 rs2285666, the GG genotype, or the G allele was significantly associated with a twofold

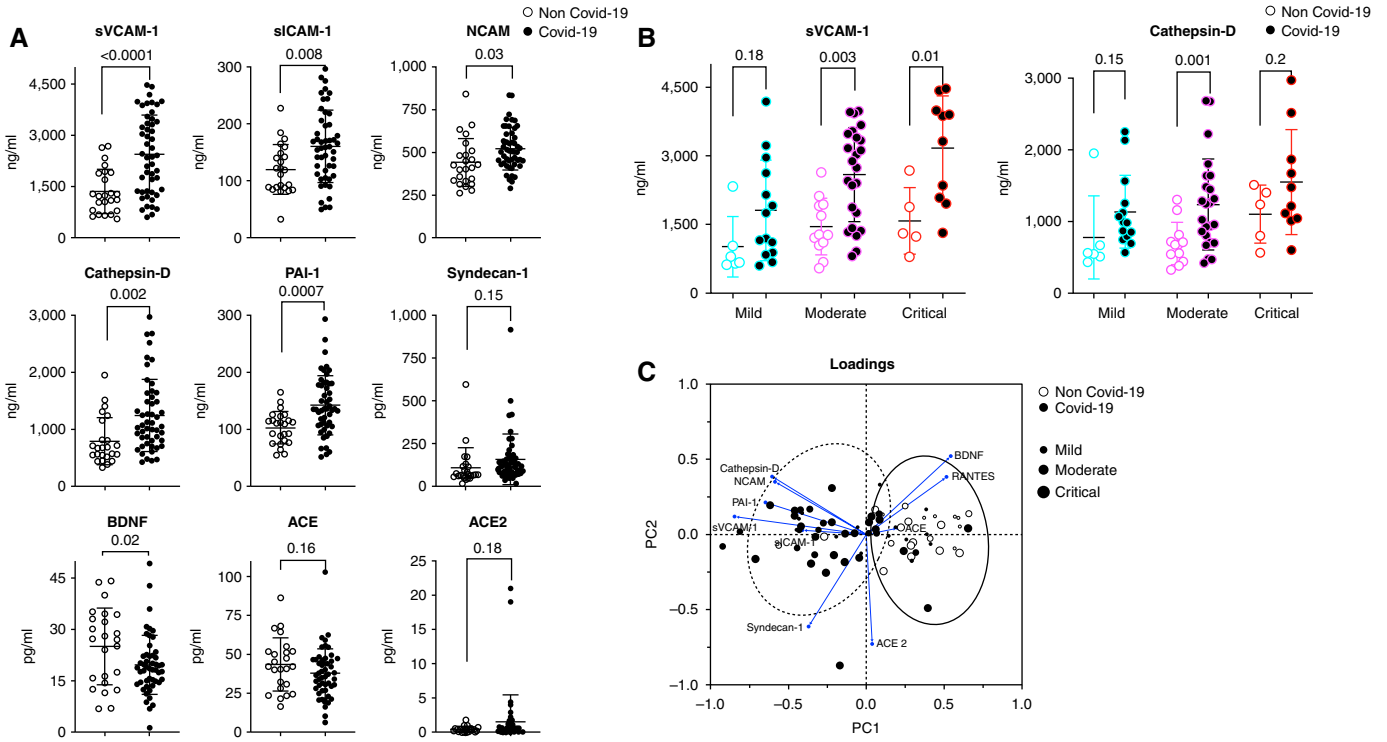

Figure 5. Admission serum endothelial biomarkers in patients with versus without coronavirus disease (COVID-19). (*A*) Comparison of endothelium-related biomarkers in serum from patients with COVID-19 ($n = 50$) and patients without COVID-19 ($n = 24$). *P* values are based on two-sided Mann-Whitney *U* tests. (*B*) Comparison of endothelium-related biomarkers between patients with COVID-19 ($n = 50$) and patients without COVID-19 ($n = 24$), stratified according to the severity of illness. (*C*) Principal component analysis showing representation of COVID-19 and non-COVID-19 populations in the two dimensions (score plot). Scores and loadings are presented in a scatterplot of one principal component (PC) against another. The loadings are represented in a circle of correlations: The closer the arrow of a loading is to the circle (shown in blue), the more the variable is well represented in the space of the two plotted PCs and contributed to the building of these PCs. The first two PCs allowed the discrimination of patients with and without COVID-19, consolidating results of the univariate analysis. Patients with COVID-19 (dotted line ellipse) were characterized by increased cathepsin D, neural cell adhesion molecule (NCAM), soluble vascular cell adhesion molecule 1 (sVCAM-1), soluble intercellular adhesion molecule 1 (sICAM-1), plasminogen activator inhibitor 1 (PAI-1), and syndecan-1. Patients without COVID-19 (solid line ellipse) were characterized by increased brain-derived neurotrophic factor (BDNF), RANTES (regulated upon activation, normal T cell expressed and secreted), and angiotensin-converting enzyme (ACE).

increased risk of SARS-CoV-2 infection and a threefold increased risk of severe or lethal COVID-19 (28). However, it remains unclear whether endothelial cells express ACE2 (29–31). In our study, the ACE2 agonist DMZ reduced SARS-CoV-2–induced permeability, without reducing viral entry into HMVECs. DMZ also reduced LPS-induced permeability. This suggests that activation of ACE2 signaling pathways helps to stabilize the endothelial barrier during acute inflammation caused by multiple processes rather than being specific to COVID-19 (32, 33).

We observed that sera from patients with COVID-19 and patients without COVID-19 with comparable severities of illness induced similar levels of HMVEC permeability. This finding is not surprising, given the known role of endothelial injury and dysfunction in sepsis and ARDS, but it does not answer the question whether the pathogenesis of COVID-19–associated endotheliopathy is analogous to that of sepsis or ARDS. There are a number of reports of elevated blood endothelial biomarkers in COVID-19, and some studies have shown associations between elevated serum endothelial biomarkers, such as PAI-1 and syndecan-1, with organ failure and death (8–12, 34–39). Despite similarities between serum endothelial biomarkers and the degree of HMVEC permeability induced by sera from patients with and without COVID-19, our data suggest that there is a serum endothelial biomarker signature of COVID-19 disease that differs from that of non–COVID-19 disease and includes increased levels of NCAM, sVCAM-1, and sICAM-1. The upregulation of these adhesion molecules may reflect the intensity of lung leukocyte infiltration and the angiocentric alveolar inflammation in COVID-19 ARDS, analogous to sepsis-induced ARDS (40–42). We found that cathepsin D levels were positively associated with the severity of illness and were higher in patients with COVID-19 than in patients without COVID-19. Cathepsin D increases vascular permeability and can activate VEGF (43) and could play a role in the lung edema and neoangiogenesis in COVID-19 (14). Finally, serum BDNF levels were lower in patients with COVID-19 than in patients without COVID-19. BDNF is involved in neuronal survival and differentiation (44) and in vascular stability (45). We speculate that lower BDNF levels may contribute to COVID-19–associated endotheliopathy and neurological dysfunction (46–48).

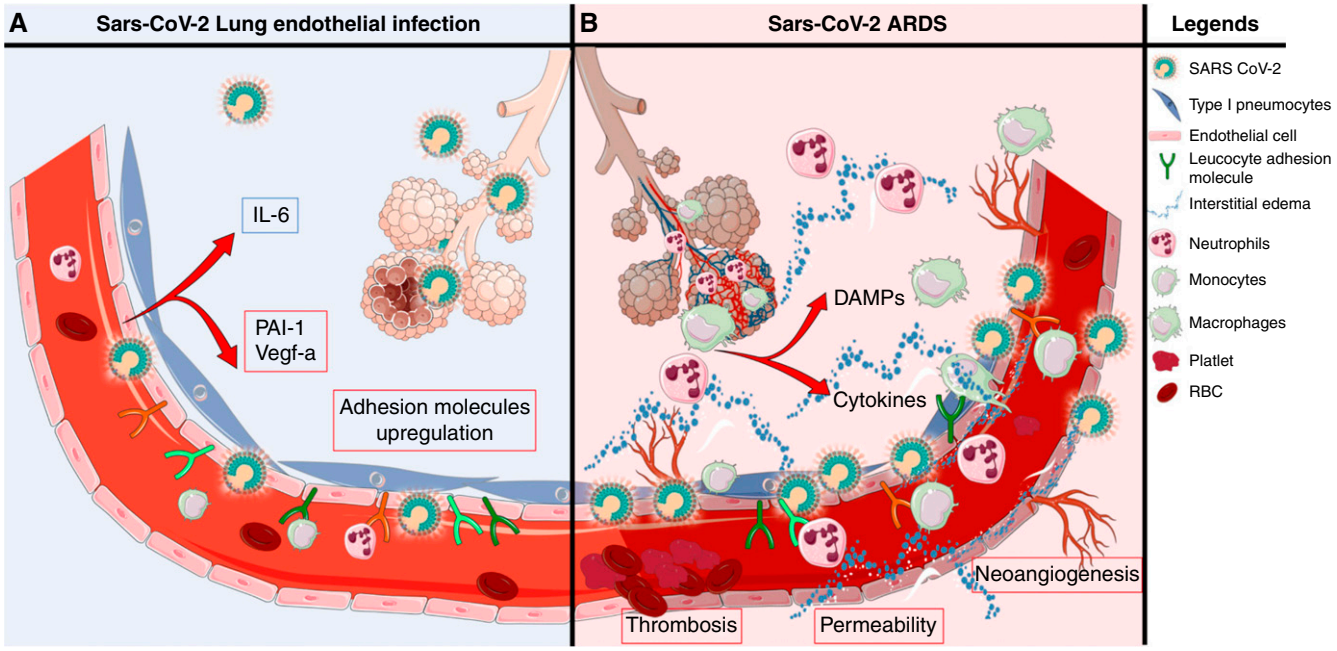

Figure 6. Hypothetical model of coronavirus disease (COVID-19)-associated lung microvascular endotheliopathy. On the basis of our experimental results, we speculate that viral infection and secondary injury of the endothelium by inflammatory mediators and immune cells contribute to the lung endotheliopathy of COVID-19 and, in turn, COVID-19–associated acute respiratory distress syndrome (ARDS). (*A*) Endothelial cell exposure to live severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) could occur early or later in the course of the disease if there is persistence of virus in the host, including in alveolar epithelial cells, which are in proximity to microvascular endothelial cells. In susceptible individuals, immune dysfunction could lead to increased viral load and release by alveolar epithelial cells. In this speculative model, SARS-CoV-2 infection of lung microvascular endothelial cells induces permeability, upregulates surface expression of leukocyte adhesion molecules and secretion of plasminogen activator inhibitor 1 (PAI-1) and vascular endothelial growth factor (VEGF) secretion, and induces low levels of secretion of cytokines and chemokines. In this context, infection of endothelial cells may promote and/or amplify endotheliopathy and ARDS. (*B*) Secondary injury to the lung endothelium is likely to be a principal determinant of COVID-19–induced endotheliopathy. Secondary injury may result from the actions of circulating inflammatory mediators and toxins and of activated pulmonary leukocytes and damaged alveolar epithelial cells. In susceptible individuals, impaired viral clearance and persistent viral replication in epithelial or endothelial cells may promote leukocyte recruitment to the lung and cell death and the release of damage-associated molecular patterns (DAMPs). These DAMPs could, analogously to their roles in sepsis and injury, further exacerbate the endotheliopathy, as well as acute lung injury and ARDS. RBC = red blood cells.

Our study has certain limitations. First, we used patient sera that were collected within the first 24 hours of hospitalization. This limits the ability to define any associated dynamic relationship with outcomes. However, an advantage to using these early serum samples is that none of the patients received fluids, dexamethasone, remdesivir, or plasma before sample collection, which should have minimized biases resulting from dilution or the administration of plasma, immunomodulatory drugs, or antiviral therapy. Second, the study was designed to include symptomatic patients with mild, moderate, and severe COVID-19 to reflect the broader spectrum of the disease, but the low mortality in the COVID-19 group (3 of 99 patients with COVID-19 deceased) does not allow analysis of the predictive value of our measured endpoints on clinical outcome. Third, we observed considerable heterogeneity in the effects of live virus on HMVECs from different donors. This finding limits our ability to draw conclusions about the lung endothelial cells that are generalizable to all individuals. Also, for the serum exposure experiments, we used a single donor's HMVECs because of limitations in the quantity of sera available for testing. This could have created a potential "donor-related bias." This might have been avoided by using an immortalized cell line, but using primary human cells is more clinically relevant. Fourth, we used only one strain of virus for our experiments and cannot rule out the possibility that other variants would have different effects. Last, we tested effects of sera and virus on HMVECs under static conditions. However, COVID-19–induced lung injury undoubtedly involves a complicated and dynamic interplay among multiple cell types, including alveolar epithelial cells, endothelial cells, and leukocytes (12).

In summary, these studies indicate that live SARS-CoV-2 can infect and induce permeability and a proadhesive phenotype of HMVECs and that sera from patients with COVID-19 induce permeability and mild inflammatory activation of HMVECs. Our data, alongside other published studies on circulating biomarkers in COVID-19 and autopsy studies, suggest that both viral infection of endothelial cells and secondary injury and activation of endothelial cells by circulating and localized cells and mediators contribute to COVID-19–associated lung endotheliopathy, as depicted in our hypothetical model in Figure 6. We speculate that live SARS-CoV-2 may have the opportunity to interact with and infect lung endothelial cells longitudinally over the course of the disease, facilitated by persistence of live virus in nearby alveolar epithelial cells (Figure 6A). We further speculate that circulating and local tissue

leukocytes, inflammatory mediators, and damage-associated molecular patterns contribute to COVID-19–associated lung microvascular endotheliopathy (Figure 6B). In some patients, impaired viral clearance and viral replication in epithelial or endothelial cells may exacerbate the endotheliopathy by promoting leukocyte recruitment to the lung and cell death with release of more damage-associated molecular patterns. Further studies on direct endothelial infection by SARS-CoV-2 and on the secondary actions of systemic factors and pulmonary leukocytes in COVID-19–associated endotheliopathy could lead to the identification of endothelial cell–targeted approaches to treat COVID-19 disease. ■

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