# CXCL10-dependent epithelial-vascular cross-talk for endothelial activation following Sars-CoV-2 infection

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**ABSTRACT**

The vasculature is heavily impacted by SARS-CoV-2 infection. Conflicting results exist concerning the mechanisms by which the SARS-CoV-2 virus acts on the vasculature. The presence of the virus within endothelial cells has been reported in patient samples. However, the ACE2 receptor was not detected in endothelial cells when analyzed by RNAseq analysis. Thus, how SARS-CoV-2 contributes to vascular inflammation and whether cross-talk between epithelial cells and endothelial cells is involved are unclear. Therefore, we investigated the interaction between SARS-CoV-2 and the vasculature using 2D and 3D *in vitro* models, as well as our previously developed 3D vesseloid model. We first determined the suitability of the 3D vesseloid model for our study and then assessed whether SARS-CoV-2 is able to directly infect endothelial cells. In the absence of ACE2 in endothelial cells, no infection was detected. When ACE2 was overexpressed in endothelial cells, low uptake of viral particles by endothelial cells was observed without efficient viral production. We then explored the possibility that an indirect effect of SARS-CoV-2 infection involves epithelial-endothelial cell cross-talk. After infection of the epithelial cells, a significant inflammatory response was detected in the endothelial cells. Furthermore, we investigated the cytokines possibly implicated and identified CXCL10 as the most highly expressed proinflammatory cytokine and explored its function in this context. Finally, the clinical relevance of our findings was confirmed by evaluating CXCL10 and alternative cytokine dosages in blood samples from SARS-CoV-2-infected patients, which were validated *in silico* in an independent patient cohort.

## INTRODUCTION

The coronavirus disease 2019 (COVID-19) pandemic erupted in December 2019 and rapidly evolved into one of the most complex and disruptive public health crises of the 21st century. The virus responsible for severe acute respiratory syndrome spread globally, triggering a pandemic in 2020. This situation has exposed vulnerabilities in the global healthcare system, disrupted economies, and endangered the lives of millions of people. [1]

Postmortem biopsy studies first focused on lung tissue and revealed endothelial injuries with cytoplasmic vacuolization, cell detachment in pulmonary arteries and swelling [2] [3] and an accumulation of infiltrating immune cells, such as macrophages and lymphocytes [4].

Analysis of Covid-19 patient serum samples revealed increased production of proinflammatory cytokines, a phenomenon called “cytokine storm”, associated with high circulatory levels of shed adhesion molecules, such as VCAM-1 or ICAM-1, in the plasma of patients with COVID-19 [5]. The cause of this cytokine storm may be attributed to a deregulated immune response leading to acute respiratory distress syndrome [6]. This syndrome is characterized by a severe inflammatory response, resulting in blood vessel inflammation, impairment of the alveolar-capillary barrier, increased vascular permeability, and disruption of gas exchange, leading to edema and death [7]. Furthermore, vascular thrombotic events are also well documented during SARS-CoV-2 infection, reflecting endothelial cell activation and leading to vascular injury. However, the specific molecular mechanisms involved are not fully understood.

SARS-CoV-2 entry into cells requires the ACE2 receptor. The ACE2 receptor was initially identified for its role in regulating blood pressure and cardiovascular homeostasis but has

become a major research focus due to its essential role as a virus entry point. Studies have shown that ACE2 is widely distributed in various tissues and is found mainly in the heart, lung, kidneys, and gastrointestinal tract, which could explain the diversity of symptoms encountered in COVID-19 patients [8] [9]. However, studies have reported contradictory findings regarding the presence of ACE2 receptors in endothelial cells [10][11]. In addition, some studies have described the presence of Covid-9 RNA in coronary artery plaque or lung endothelial cell macrophages [12] [13] [14], while other studies have reported endothelial cell injury without the presence of SARS-CoV-2 particles in postmortem samples from COVID-19 patients [3].

Therefore, it is imperative to understand the mechanisms governing the effects of this virus on the vascular system. This understanding is crucial for the development of long-term treatment strategies for COVID-19 patients and for anticipating the evolution of new variants.

As part of our study, we used various experimental settings, including 2D and 3D endothelial cell models as well as cocultures with a lung epithelial cell line. We also compared our findings to the clinical situation by the dosage of patients' blood samples and via *in silico* analysis of an external cohort. Taken together, our data point to an indirect mechanism of action for SARS-CoV-2 on the vascular endothelium involving epithelial-endothelial cross-talk.

## MATERIALS AND METHODS

### Viruses and cell lines

Vero-E6 cells and Calu-3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal calf serum and gentamicin (50 µg/mL) at 37°C in a humidified $CO_2$ incubator. HUVECs, HUAECs, and HCAECs were maintained in Endothelial Cell Growth Medium 2 (EGM2; C-22111, PromoCell) in a cell culture dish coated with 0.5% gelatin solution. Human monocytic THP-1 cells were cultured in RPMI-1640 medium (Life Technologies) supplemented with 10% FBS, 2 mM L-glutamine and 1% penicillin and streptomycin. Differentiation of THP-1 cells into M2 macrophages was performed with 10 mM phorbol 12-myristate 13-acetate (PMA) for 24 hours.

The SARS-CoV-2 strain BetaCoV/France/IDF0372/2020 was supplied by the National Reference Centre for Respiratory Viruses hosted by the Pasteur Institute through the European Virus Archive (EVAg platform). Agreement to work with infectious SARS-CoV-2 was obtained, and all work with infectious SARS-CoV-2 was performed in a Class II Biosafety Cabinet under BSL-3 conditions at the UB'L3 facility (TBMcore, Bordeaux).

### Vesseloid production

Vesseloids are engineered blood vessels. They were produced using a microfluidic technology adapted from [15],[16]. In our seminal work [17], we used a coculture of HUVECs and smooth muscle cells to achieve vesseloid contractility. Here, HUVECs were used only for vesseloid production, which was carried out according to the same procedure. Briefly, by using a 3D printer coextrusin device, we produced hollow alginate fibers whose inner walls were coated with ECM (Matrigel) and filled with HUVECs. Cells spontaneously self-organize to create a lumen within less than a day.

**Virus Production**

The SARS-CoV-2 strain was produced by infecting Vero E6 cells at a multiplicity of infection of $10^{-2}$ PFU and then incubating the cells at 37°C in a humidified $CO_2$ incubator until a cytopathic effect was observed (approximately 72 h). The culture supernatant was clarified by centrifugation (5 min at 1,500 rpm), and aliquots were stored at -80°C. All the viral stocks were sequenced to confirm that no mutations were detected during culture, and Sanger sequencing was used to determine the full-length spike sequence [21]; moreover, whole-genome sequencing was performed with Oxford Nanopore technology. Stock titers were determined by adding serial dilutions to $2 \times 10^4$ Vero E6 cells in supplemented DMEM in a 96-well plate. Eight replicates were performed. The plates were incubated at 37°C, after which the cytopathic effects were examined. Cytopathic effects were quantified using the CellTox™ Green cytotoxicity assay (Promega) according to the manufacturer's instructions and a Victor Nivo reader (Perkin-Elmer). The $TCID_{50}$ per ml was calculated according to the method of Reed and Muench [19]. The viral titer expressed in PFU per ml was mathematically converted from $_{\text{the TCID50}}$ per ml determination using the Poisson equation as follows: PFU/ml = -ln 0.5 * $TCID_{50}$/ml.

**Infection of vesseloid and Calu-3 epithelial cells**

Calu-3 cells were seeded in a 24-well plate to obtain 100 000 cells per well on the day of infection. Infection was performed with SARS-CoV-2 at a multiplicity of infection of $10^{-1}$ PFU. Vesseloids were placed on cell culture inserts and inserted into wells containing SARS-CoV-2-infected Calu-3 cells until infection was stopped.

**Vesseloid and supernatant processing**

RNA extraction from vesseloids was performed using TRI Reagent (TR118, Euromedex). Vesseloids were first washed with 500 µL of DMEM and then incubated with 1 mL of ReLeSR (100-0484, StemCell) for 5 min to dissolve the alginate. The cells were pelleted by centrifugation (5 min at 1,500 rpm) and treated with 600 µL of TRI Reagent for 10 min. For microscopic observation, the vessels were fixed with 4% paraformaldehyde (PFA) for 20 min and washed with DMEM.

Five hundred microliters of culture supernatant was either inactivated with 0.5% Triton for cytokine measurement or with lysis buffer for RNA extraction according to the manufacturer's recommendations (High Pure Viral RNA Kit, Roche).

**Quantification of SARS-CoV-2 RNA by RT–qPCR**

For RT–qPCR quantification of viral RNA, total RNA was isolated using the High Pure Viral RNA Kit (Roche) according to the manufacturer's instructions. Viral RNA was quantified using a GoTaq 1-Step RT–qPCR Kit (Promega). SARS-CoV-2 N gene RNA was amplified using forward primers (N-gene F: CGCAACAGTTCAAGAAATTC; N-gene R: CCAGACATTTTGCTCTCAAGC). Serial dilutions of SARS-CoV-2 RNA were used to construct a standard curve. The amplification program began with an RT step for 15 min at 50°C, followed by a denaturation step for 10 min at 95°C, 10 s at 95°C, 10 s at 60°C, and 10 s at 72°C (40 cycles). The melting curve was obtained by a temperature increase of 0.5°C/s from 60°C to 95°C.

**Quantification of gene expression by RT–qPCR**

Total RNA was isolated from cells using TRI Reagent (TR118, Euromedex) according to the manufacturer's instructions. Approximately 1 µg of total RNA was reverse transcribed into complimentary DNA (cDNA) using a high-capacity cDNA reverse transcription kit (4368814, Applied Biosystems™). cDNAs were amplified using specific primers for the genes of interest. Tubulin (TUB R: TAGAGCTCCAGCAGGATT; TUB F: GAGTGCATCTCCATCCACGC) was used as an internal control. We applied the real-time PCR method using the Eurobiogreen qPCR Mix (GAEMMX02H 0T, Eurobio) for CXCL10: (TUB R : GGCCTTCGATTCTGGATTCA ; TUB F : GCCAATTTTGTCCACGTCGTTG); VCAM-1 (VACM-1 R : ACATTCATATACTCCCGCATCCTTC ; VACM-1 F : CGTCTTGGTCAGCCCTTCCT) ; ICAM-1 (ICAM-1 R : CCCATTATGACTGCGGCTGCTA ; ICAM-1 F : AGGCCACCCCAGAGGACAAC) ; IL6 (IL6 R : GCAAGTCTCCTCATTGAATCC ; IL6 F : GGCACTGGCAGAAAACAACC) ; IL8 (IL8 R : AACCCTCTGCACCCAGTTTTC ; IL8 F: TGCAGCTCTGTGTGAAGGTG) ; Ngene (N-gene R CCAGACATTTTGCTCTCAAGC ; N-gene F CGCAACAGTTCAAGAAATTC). Real-time PCR was performed on an AriaMx Real-time PCR System using Agilent Aria 1.71 software (Agilent).

**Cytokine array assay**

Cytokine profiling was performed using a Proteome Profiler Array Human (XL) Cytokine Array Kit (ARY022B, R&D Systems), which detects 105 human cytokines simultaneously. We used cell culture supernatants collected from infected vesseloids and cocultures (SarS-CoV-2-infected Calu-3 cells and vesseloids) according to the manufacturer's instructions. Arbitrary values of cytokine pixels were calculated for each dot plot and were normalized to the reference spots and measured using ImageJ software.

**Luminex dosages**

Blood biomarkers were measured using Luminex technology and ELISA according to the manufacturers' instructions: Angiopoietin-2, VEGFR1, and CD31 were measured using a custom 3Plex (Biotechne); CXCL10, IL8, IL6, and VEGF were measured using a custom 4plex (Biotechne); and CD142 (ab108903, Abcam) and VE-Cadherin (DCADV0, Biotechne) were measured using commercial ELISA. All the measurements were performed on the CHU hospital research platform (PARS) on samples stored at -80°C in the centralized biobank (Bordeaux Biothèques Santé Centre de Ressources Biologiques, Bordeaux University Hospital) blinded to any other data.

**Western blot**

The cells were washed twice with PBS and dissolved in lysis buffer (10 mM Tris-HCl, pH 7.4; 150 mM NaCl; 0.5% NP-40; 1% Triton X-100; and 1 mM EDTA supplemented with protease and phosphatase inhibitor cocktails (Roche)). The protein concentration was quantified with a Pierce™ BCA Protein Assay Kit (Thermo Scientific). The cell lysates were resuspended in Laemmli buffer 4X (62.5 mM Tris (pH 6.8), 10% glycerol, 2.5% SDS, and 2.5% β-mercaptoethanol). All the samples were boiled for 5 minutes, subjected to electrophoresis and transferred. The proteins were electroblotted on nitrocellulose membranes. The membranes were incubated with intercept buffer (927-70001, LICOR) for 1 h and probed overnight at 4 °C with primary antibodies. Anti-ACE2 (38241S, Cell Signaling) 1/1000; anti-SRC (Tyr416) (6943S, Cell Signaling) 1/1000; anti-SRC (2109S, Cell Signaling) 1/1000; anti-p-p44/42 MAPK (Erk1/2) (4370 L, Cell Signaling); anti-p44/42 MAPK (Erk1/2) (9102S, Cell Signaling) 1/1000; anti-p-AKT (Ser473) (9271S, Cell Signaling); anti-AKT (9272S, Cell

Signaling); and anti-vinculin (VLN01) (11394493, Invitrogen). The goat anti-rabbit IR Dye 680CW (926-68021, LI-COR Biosciences), goat anti-rabbit IR Dye 800CW (926-32211, LI-COR Biosciences) and goat anti-mouse IR Dye 800CW (926-32210, LI-COR Biosciences) were used for imaging at 1/10 000 and on an Odyssey infrared imaging system (LI-COR Biosciences, Nebraska, US). Analysis was performed using Image Studio Lite 5.2 software.

**Immunofluorescence**

Cells were seeded on glass coverslips and fixed for 15 min with 4% paraformaldehyde at room temperature. The cells were permeabilized for 5 min with 0.5% Triton X-100, washed with PBS and incubated for 1 hour with blocking buffer (PBS containing 0.5% Triton X-100 and 5% BSA) at room temperature. The cells were incubated with primary antibodies against VE-CAD (D87F2) (2500S; Cell Signaling) diluted 1:500 in blocking buffer for 1 hour at room temperature, washed with PBS, and incubated with secondary antibodies conjugated to Alexa Fluor® 488-AffiniPure Donkey Anti-Rabbit IgG (H+L) (711-545-152; Jackson ImmunoResearch) and phalloidin (diluted in blocking buffer). DAPI (1:1000) was used to label the nuclei (10374168, Fisher Scientific). Coverslips were mounted using Prolong Gold antifade reagent (11559306, Fisher Scientific).

Vesseloids were fixed in 4% PFA overnight at 4°C. Then, vesseloids were cut into sections approximately 1 cm long. The cells were permeabilized at room temperature in Dulbecco's modified Eagle's medium (DMEM; P04-05545; PAN Technology) supplemented with 0.1% Triton X-100 and incubated with 1% BSA and 2% SVF in DMEM for 1 hour at 4°C under agitation. Vesseloids were incubated with the primary antibody against cleaved caspase 3 (Asp175) (9661; Cell Signaling) 1/200 at 4°C overnight with agitation. Vesseloids were then washed with DMEM 3 times for 15 minutes and incubated with the secondary antibodies

Alexa Fluor® 488-AffiniPure Donkey Anti-Rabbit IgG (H+L) (711-545-152, Jackson ImmunoResearch) 1/200 and DAPI 1/500 diluted in blocking buffer for 2 hours at 4°C with agitation.

Vesseloid fragments were fixed in a cellview cell culture dish (627860, Greiner bio-One) with 1% low agarose (BP160500, Fisher Scientific) and covered with DMEM.

**Imaging**

Confocal images of vesseloids were acquired either with an inverted Nikon Eclipse Ti microscope and acquisition software (NIS-Element, NIS) or with a Leica DFC7000 and acquisition software (Leica Las X). Image analysis was performed with ImageJ.

**Permeability assay**

HUVECs were cultured on 6-well transwell insert plates until 100% confluence. The cells were treated for 1 hour with 1 µg/ml recombinant human CXCL10 protein (300-12; Human CXCL10, Gibco). FITC-conjugated dextran was administered to the upper compartments of the inserts at 25 µg/ml. Every 20 minutes after dextran addition, the fluorescence signal in the lower compartments was measured using a fluorescence microplate reader (Infinite® 200 PRO, Tecan) at an excitation wavelength of 485 nm and an emission wavelength of 538 nm.

**Migration assay**

For chemotactic migration assays, 8.0 µm polycarbonate membrane Transwell inserts were used in 6-well plates (Corning). One thousand activated primary THP1 cells were plated in 500 µL of serum-free RPMI medium in the top chambers. Then, the bottom chambers were filled with 2 ml of serum-free RPMI supplemented with 1 µg/ml human CXCL10 recombinant

protein (300-12; human CXCL10; Gibco) or VEGF-A (BT-VEGF; R&D Systems). The cells were cultured overnight at 37°C and 5% CO2, fixed with 4% PFA for 15 minutes and stained with DAPI. Then, the wells were washed with PBS, and 10 images per well were acquired at 10x magnification. Migrated cells per field were counted using ImageJ software. For inhibitor assays, cells were seeded in the top chamber, and 6 nM SCH 546738 (HY-10017, MedChemExpress) or 10 nM PP2 (#73901, Cell Signaling) was added.

**Bioinformatic analysis**

To study the expression of the CXCL10 gene in SARS-CoV-2 patients, we analyzed publicly available RNA-seq datasets retrieved from the Gene Expression Omnibus (GEO) repository (GSE152075). The dataset contains gene expression profiles from 484 individuals (430 patients with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and 54 controls). Differential expression analysis was performed to compare the expression between the COVID-19 and control groups using the R package DEseq2 v1.42.0 [20]. For further analysis, patients were divided into three groups according to the expression of CXCL10: low, medium, and high. Correlations between gene expression patterns were estimated with the Pearson correlation method. The coefficients representing the M1 and M2 macrophage signatures were obtained by calculating the mean of the min–max normalized expression values of the genes used for the analysis among all individuals in a group.

**Statistical analysis**

For continuous variables, the data are presented as the mean ± SD. Differences were considered significant if *P* was less than 0.05. Between-group comparisons were performed with the Mann–Whitney *U* test or *t* test, depending on the sample size for continuous

variables. In cases where more than 2 groups were compared, 1-way ANOVA was performed, followed by Tukey's multiple comparison test. All analyses were performed using Prism 6.0 software (GraphPad).

## RESULTS

### Absence of direct effects of SARS-CoV-2 on the vascular endothelium

To determine the impact of the virus on the vascular endothelium, we cultured various human endothelial primary cell lines, including human umbilical vein endothelial cells (HUVECs), human umbilical artery endothelial cells (HUAECs), and human coronary artery endothelial cells (HCAECs). No ACE2 protein expression was detectable in these cell types when analyzed by Western blotting. In contrast, the expression levels of these genes in the epithelial human lung cancer cell line Calu-3 (Figure 1A) were very high. To investigate whether ACE2 could serve as a functional receptor in endothelial cells for SARS-CoV-2 entry, we overexpressed ACE2 in a human umbilical vein endothelial cell line (ACE2-HUVECs) (Figure 1A). ACE2-HUVECs were then used as the cellular component in the vesseloid model and compared to non-ACE2-overexpressing HUVECs (WT-HUVECs). The vesseloid model was described by our group in a previous publication and consists of a 3D tubular structure harboring an endothelial cell lining that mimics the configuration of a physiological vessel [17]. SarS-CoV-2 infection was performed, and the mRNA levels of the N protein, the nucleocapsid of the virus, in the supernatant were analyzed. In ACE2-HUVECs, viral infection was very low, and minimal viral particle production was observed after 3 or 4 days in the supernatant (Figure 1B). In Calu-3 cells, the N protein concentration was approximately two log greater (Figure 1B). A time-dependent analysis of the infection efficiency was further performed (with an MOI of 1 or an MOI of 0.1) on Calu-3 cells, and the peak N gene expression was detected at 3 days post infection (Sup Figure 1A). The behavior of control HUVECs, HCAECs and HUAECs was similar to that of ACE2-HUVECs (Figure 1B; Sup Figure 1B).

To corroborate these findings, we analyzed the mRNA levels of the N protein, the nucleocapsid of the virus, in WT-HUVEC vesseloids and in the epithelial cell line Calu-3 at 3 days after SARS-CoV-2 infection. Significantly lower levels were detected in vesseloids than in Calu-3 cells (mean control vesseloid = 1 +/- 0 and SARS-CoV-2 vesseloid = 72.41 +/- 16.30 vs mean control Calu-3 = 1 +/- 0 and mean Sars-2 Calu-3 = 9114 +/- 9754) (Figure 1C).

These findings indicate that endothelial cells were not efficiently infected when the ACE2 receptor was present.

Subsequently, we examined whether the virus has an impact on endothelial cell viability in vesseloids after 3 days of SARS-CoV-2 infection (MOI=1) and performed immunostaining for cleaved caspase 3 protein detection. However, cleaved caspase-3 expression remained unchanged, regardless of whether the cells were exposed to the virus. These results indicated that the virus did not directly induce endothelial cell death (mean control vesseloid = 0.2162 +/- 0 vs mean SARS-CoV-2 vesseloid = 0.1015 +/- 0.02141) (Figure 1D). This finding further supports the contention that endothelial cells are not directly infected by the SARS-CoV-2 virus.

**Epithelium-dependent effects of SARS-CoV-2 infection on endothelial cells**

To determine the mechanisms of endothelial activation/inflammation, we cocultured vesseloids with Calu-3 cells. Calu-3 cells were plated in the bottom chamber and infected with SARS-CoV-2 for 4 hours (MOI=1). After several washes, the vesseloids were placed in the upper part of the transwell insert, and cocultures were maintained for more than 3 days (Figure 2A). Analysis of the RNA expression of the vascular adhesion marker VCAM-1 in the vesseloids revealed significantly greater inflammation in the cocultured vesseloids than in the untreated vesseloids (mean number of SARS-CoV-2 vesseloids = 1.050 +/- 0.1212 vs

mean number of SARS-CoV-2 vesseloid cocultures = 11.36 +/- 3.536) (Figure 2B). An increase in intercellular adhesion molecule (ICAM-1) was also observed in cocultured vesseloids (Sup Figure 2A). This observation demonstrated cross-talk between infected epithelial Calu-3 cells and endothelial cells even when the cells were included in the 3D vesseloids. This finding may mirror the *in vivo* situation of endothelial cell activation during SARS-CoV-2 infection.

To determine the molecular mechanism involved, we compared inflammatory cytokine profiles in the supernatants of vesseloids alone or in coculture with Calu-3 cells infected with SARS-CoV-2 via a cytokine array (R&D Systems). The CXCL10 chemokine was strongly increased in the coculture condition (Figure 2C). We discovered that CXCL10 mRNA expression was also highly increased in vesseloids cocultured with infected Calu-3 cells compared to vesseloids cultured alone. Furthermore, the expression of this cytokine was also increased in infected Calu-3 cells cultured alone (mean SARS-CoV-2 vesseloid = 1.050 +/- 0.1212 vs mean SARS-CoV-2 Calu-3 = 212.9 +/- 290.2 vs mean SARS-CoV-2 vesseloid coculture = 116.2 +/- 89.41) (Figure 2D). To validate these findings, we analyzed the amount of CXCL10 in the culture media of vesseloids alone, of Calu-3 cells or during coculture. We observed a significant increase in CXCL10 in the culture medium when vesseloids were cocultured with infected Calu-3 cells (mean number of SARS-CoV-2 vesseloid cocultures = 56869 +/- 47787) (Figure 2E). For Calu-3 cells infected alone, we detected a slight increase in CXCL10 in the culture medium (mean SARS-CoV-2 Calu-3 = 1323 +/- 1079) (Figure 2E). When vesseloids were directly infected with SARS-CoV-2, CXCL10 levels remained unchanged (mean SARS-CoV-2 vesseloid = 3.810 +/- 0.4424) (Figure 2E). These data are in agreement with the CXCL10 mRNA expression observed in both endothelial cells in the vesseloids and infected Calu-3 cells (Figure 2D). As controls, IL6 and IL8 protein concentrations in the culture supernatant were also determined. In Calu-3 cells, IL6 expression was increased but

was not further increased when the cells were cocultured with vesseloids. For CXCL8, no significant further increase was observed above the background level in Calu-3 cells cultured alone or in combination with vesseloids (Sup Figure 2B). These findings highlight the essential role of CXCL10 in the crosstalk between endothelial cells and the infected epithelium.

**Mechanistical insights into the activity of chemokines involved in COVID-19 pathophysiology**

To understand the process of endothelial alterations following SARS-CoV-2 infection, we stimulated HUVECs with recombinant CXCL10, the most highly expressed cytokine in our study.

First, endothelial cell permeability was tested via an *in vitro* permeability assay in which a confluent monolayer of HUVECs was cultured in the upper part of the insert and treated with CXCL10. Carrier or VEGF was used as a negative or positive control, respectively. FITC-dextran was then added to the upper part of the transwell plate, after which the FITC-dextran was collected in the bottom chamber, after which the optical density was measured. We observed a significant increase in endothelial permeability in response to CXCL10 and VEGF compared to that in the control carrier condition (mean control = 15.17 +/- 7.522 vs mean CXCL10 = 53.13 +/- 12.22 vs VEGFA-A mean = 30.13 +/- 4.029; at 60 min) (Figure 3A).

To decipher the biological mechanisms underlying the increase in permeability, we analyzed endothelial cell–cell junctions by immunostaining, focusing on the endothelial-specific junction marker VE-cadherin. Stimulation with CXCL10, similar to VEGF, disrupted VE-cadherin continuous localization at endothelial intercellular contacts in confluent HUVEC monolayers, reflecting destabilization of cell–cell junctions (control mean = 0.1056 +/-

0.02289 vs CXCL10 mean = 0.3584 +/- 0.06868 vs VEGF-A mean = 0.3131 +/- 0.06017) (Figure 3B and 3C). Destabilization of cellular junctions appears to be a major factor contributing to vascular permeability and is directly controlled by CXCL10.

**CXCL10-dependent myeloid recruitment following SARS-CoV-2 infection**

Given the impact of CXCL10 on innate immune cell biology and dynamics and on immuno-vascular modulation under pathological conditions and the deleterious outcomes of this cell population following SARS-CoV-2 infection, we investigated its effect on the recruitment of peripheral myeloid cells (Figure 4). To this end, we investigated the effect of CXCL10 on the migration of activated human THP1 monocytes via a transwell assay to determine the ability of THP1 cells to follow the CXCL10 gradient. Our transwell assay revealed significant monocyte polarization toward the CXLC10 gradient compared to that in the control group (control mean = 16.21 +/- 3.951 vs CXCL10 mean = 61.33 +/- 8.425) (Figure 4A and 4B).

To determine the downstream signaling pathway responsible for this migration, we treated THP1 cells with CXCL10 for various durations and analyzed the downstream substrates by western blot analysis. We focused our analysis on the Src pathway, which was previously reported to be a potent myeloid cell migration downstream cue [18]. Src phosphorylation was significantly increased after CXCL10 stimulation in THP1 cells after 10 minutes (control meal = 1 +/- 0 vs 10 min mean = 2.5443 +/- 1.572) (Figure 4C and 4D). As a control, the ERK pathway, which is responsible for cell proliferation and survival in myeloid cells in response to CXCL10 [22], was also studied and found to be activated, with a peak phosphorylation occurring at 15 minutes. To activate cell signaling, CXCL10 binds to CXCR3 at the cell membrane. CXCR3 was expressed mainly in HUVECs and resting or activated THP-1 cells but was expressed at much lower levels in Calu-3 cells (Sup Figure 3). We therefore tested

whether CXCR3 blockade is able to inhibit THP-1 cell migration. Indeed, the CXCR3 antagonist SCH 546738 significantly inhibited THP-1 cell migration. The same result was observed when PP2, an inhibitor of Src family kinases, was used (control mean = 26.33 +/- 2.215 vs CXCL10 mean = 93.53 +/- 9.101 vs CXCL10 + SCH mean = 43.10 +/- 5.734 vs CXCL10 + PP2 mean = 37.73 +/- 8.768) (Figure 4E). These results showed that CXCL10 plays a crucial role in the recruitment of myeloid cells via activation of the SRC/MAP kinase pathway.

**Clinical correlations**

To corroborate our results and test whether our *in vitro* results reflected clinical conditions, we analyzed the serum of two patient subgroups from the COLCOV cohort, each of which presented moderate or severe symptoms following COVID-19 infection, and quantified a number of circulating cytokines, including not only CXCL10 but also IL6 and IL8, for comparison (Figure 5). Circulating CXCL10 levels were significantly increased in patients with SARS-CoV-2 infection. This was already the case in patients with mild infection. Furthermore, an increase in IL6 was also observed, but only in patients with severe infection. IL8 expression was not altered (CXCL10 control mean = 9.484 +/- 4.197; CXCL10 moderate mean = 208.9 +/- 156.5; and CXCL10 severe mean = 199.9 +/- 183.6) (Figure 5A).

To confirm the results from our biological and clinical analyses, we investigated CXCL10 expression in an independent cohort of COVID-19 patients *in silico*. To this end, we analyzed the differences in CXCL10 expression between COVID-19 patients and non-COVID-19 individuals in an RNA-seq cohort (GSE152075) available online, and statistically significant differences were found. CXCL10 expression was highly upregulated in COVID-19 patients compared to controls. We also assessed the association between the expression of CXCL10 and genes well known to be involved in endothelial activation (ICAM and F3) and

inflammation (CXCL8). The expression of these genes was significantly correlated with CXCL10 expression in COVID-19 patients (Figure 5B).

In parallel, we analyzed the expression of genes related to M1 and M2 macrophage signatures in patients with low, medium, or high CXCL10 expression. We observed an increase in the mean Max-Min-normalized expression of the M1 and M2 genes, which was similar to the CXCL10 expression pattern (Figure 5C). We also calculated Pearson correlation coefficients to determine the possible tendency toward a positive correlation between CXCL10 expression and M1/M2 macrophages, representing genes in SARS-CoV-2 patients, compared to those in the control group. These findings are consistent with our *in vitro* analysis and support the general concept of CXCL10 cross-talk between epithelial and endothelial cells during COVID-19.

**DISCUSSION**

In this article, we explored the interaction between SARS-CoV-2 and the vascular endothelium. Our findings revealed that the virus is unable to infect endothelial cells directly in the presence or absence of ACE2. Instead, we observed indirect endothelial cell activation, which is mediated by CXCL10. Furthermore, CXCL10 also serves as an endothelial cell-derived chemoattractant for myeloid cells. Serum analysis of COVID-19 patients supported this contention since CXCL10 levels were elevated in both moderate and patients with severe COVID-19. This was further supported by the *in silico* analysis of an independent patient cohort.

Multiple studies have reported conflicting results regarding the role of the vascular endothelium in the pathogenesis of COVID-19 [23] [24] [14] [3].

Analysis of deceased patient tissue seems to reveal the presence of the SARS-CoV-2 virus in endothelial cells [14]. However, the ACE2 receptor, which has been demonstrated to be necessary for viral entry, was detected only in pericytes of the brain, heart and pancreatic vessels and not in endothelial cells, which contradicts the possibility of active virus entry in ECs [25]. Nevertheless, transcriptomic analysis has revealed the presence of ACE2 in 20 different tissue types and in several types of endothelial cells, including the Fallopian tube, thyroid, parathyroid gland, adrenal gland, pancreas, and heart [26]. This supposition has also been supported by claims of ACE2 expression in human pulmonary artery endothelial cells. However, a deep transcriptomic analysis via scRNAseq and epigenomic data indicated that human ECs from tissues or cell lines do not express ACE2 [27]. This latter analysis is consistent with our findings because we were also unable to detect the presence of ACE2 in

all the human EC lines we tested, including human coronary endothelial cells. Thus, in our hands, ECs do not express ACE2.

Nevertheless, SARS-CoV-2 may enter ECs via an unidentified receptor. The existence of an alternative SARS-CoV-2 receptor has been recently reported [28]. For this reason, we examined whether SARS-CoV-2 could infect human endothelial cells and replicate efficiently, as well as whether the cells expressed ACE2. To more closely mimic the *in vivo* architecture of blood vessels, we utilized the vesseloid model, which we previously described [17], in our experiments.

In cells that did not express ACE2, SARS-CoV-2 was unable to enter and replicate efficiently in ECs even when it was present in a 3D vascular architecture. We then investigated whether the expression of ACE2 in ECs allows virus entry and replication. However, even under these conditions, no efficient virus replication was observed, although virus entry increased to some extent. Taken together, these findings did not support the idea of direct viral interaction with endothelial cells.

Thus, ECs are likely activated indirectly via a paracrine mechanism and/or inflammatory molecules present in the blood. It is also unlikely that the recently described alternative receptor TMEM 106B is overexpressed in ECs since this receptor was not expressed in the ECs we studied and because no efficient virus replication was found in these cells [29].

We demonstrated cross-talk between epithelial and endothelial cells, with CXCL10 playing a major role. CXCL10 was overexpressed in both epithelial cells and endothelial cells in the presence of SARS-CoV-2, which was confirmed by the protein concentration in the culture medium. In the absence of viral infection, only low levels of CXCL10 were detected in the medium. CXCL10 is known to interact with CXCR3A and B [30] [31], and a plethora of

biological functions, such as immune regulation, tumor cell invasion [32] and angiogenesis [33], have been described for this chemokine. Therefore, in parallel, we examined CXCR3 expression in ECs and epithelial cells and found that CXCR3 was indeed expressed in both cell types.

We next examined the function of CXCL10 in the activated endothelium. CXCL10-mediated disruption of EC cell junctions is dependent on VE-cadherin, which is associated with increased EC permeability. [34] Many studies have demonstrated the destabilization of VE-cad junctions during infection with SARS-CoV-2; one study even suggested that this protein could be an effective biomarker for detecting long-term COVID-19 infections [35]. This finding is supported by the observation that culture of plasma from COVID-19 patients disrupted the EC junctions of primary human pulmonary microvascular endothelial cells by affecting VE-cadherin expression [36].

Proteome analysis of the plasma of a cohort of early hospitalized patients infected with SARS-CoV-2 identified 27 proteins, which included complement factors, the coagulation system, inflammation modulators, and proinflammatory factors upstream and downstream of IL-6. CXCL10 was not detected in this study [37]. Endothelial cell damage caused by SARS-CoV-2 is the result of inflammation via an indirect mechanism. This finding is supported by studies demonstrating the adhesion of monocytes to endothelial cells during SARS-CoV-2 infection [38]. This finding is in line with the increase in monocyte migration and p-Src activation after CXCL10 stimulation shown in our study, which favors EC-monocyte/macrophage cross-talk and is consistent with the findings of other studies that highlight a role for CXCL10 during COVID-19 infection [39]. In another publication, a direct effect of SARS-CoV-2 on monocytes was proposed [40]. Monocytes were exposed to

inactivated SARS-CoV-2, and the cytokine/chemokine profile revealed significantly greater levels of IL-6, TNF-α, CXCL10, CXCL9 and CXCL11. This interaction may represent an alternative target for SARS-CoV-2 infection that contributes to cytokine/chemokine modulation in addition to the cross-talk we demonstrated in our work.

Our mechanistic hypothesis implicating CXCL10 as a major chemokine regulating epithelial-endothelial interactions during SARS-CoV-2 infection was reinforced by inhibitor studies using SCH54738 and PP2. The former inhibits the binding of CXCL10 to CXCR3 and SRC phosphorylation.

A CXCL10-independent mechanism has also been proposed. The complement system has been shown to contribute significantly to vesseloid inflammation after SARS-CoV-2 infection. Qin Z et al. [41] analyzed the effect of infection in various models, including a murine model and a primate model, and analyzed postmortem biopsies using RNAscope or electron microscopy. Their data revealed an increase in complement deposition in the lungs in murine models and inflammation. Proteomic analysis of the blood of COVID-19 patients revealed the presence of complement factor D (CFD) and abnormalities in D-dimer and fibrinogen/fibrin degradation products (FDP). These findings suggest that the complement system plays a central role in SARS-CoV-2 infections. Complement system activation could have deleterious effects after 2 to 3 weeks following infection, potentially leading to acute respiratory distress syndrome (ARDS) [42]. Finally, we investigated chemokine and cytokine expression in the blood circulation of COVID-19 patients. This analysis confirmed highly significant CXCL10 overexpression in patients with mild and severe COVID-19. This expression pattern was different from that of IL-6 or CXCL8. This highlights the potential central role of CXCL10 in COVID-19 pathogenesis. Thus, CXCL10 inhibition could constitute a

potential suitable therapeutic strategy for the treatment of COVID-19. These results were reinforced by the analysis of an independent patient cohort. Endothelial cell inflammatory markers were coexpressed with CXCL10 as well as M1 or M2 macrophages on the basis of a biased selection of monocyte/macrophage markers. Both M1 and M2 markers were coexpressed, which points to the recruitment of both M1 and M2 macrophages. This, however, does not take into account the time-dependent evolution of the disease. Indeed, the proportion of M1- or M2- recruited macrophages should vary with time, with initial M1 macrophage activation followed by M2 macrophage recruitment.

Taken together, our results are of quadruple significance. First, our findings suggest that a direct effect of SARS-CoV-2 is highly unlikely and that it favors indirect cross-talk between ECs, epithelial cells and monocytes. In our study, the infected epithelium was the prime target of infection, which triggered sequential activation of endothelial cells and monocytes. Second, in our experience, the major player in this sequential interaction is CXCL10, which is expressed in both epithelial and endothelial cells and promotes the attraction of monocytes. Third, CXCL10 is able to disrupt EC junctions, thus allowing transmigration of inflammatory cells across the endothelial cell barrier. Fourth, these effects can be inhibited by CXCR3 blockade and thus may constitute an interesting therapeutic strategy.

## Figure legends

### Figure 1: Absence of direct effects of SARS-CoV-2 on the vascular endothelium

**A)** Western blot analysis of the ACE2 protein in HUVEVs, HUAECs, HCAECs and Calu-3 cells. Tubulin was used as a control. **B)** RT–qPCR quantification of viral RNA and relative N-gene mRNA expression in media after vesseloid or Calu-3 cell SarS-CoV-2 infection for 4 hours and analysis at 3 or 4 days post infection (MOI: 1). The values were normalized to the GAPDH mRNA expression. The results are presented as the mean ± SEM (one-way ANOVA $*P < 0.05$). **C)** Relative N-gene mRNA expression in vesseloids or Calu-3 cells at 3 days post infection with SARS-CoV-2 (MOI=1). **D)** Immunofluorescence analysis of c-caspase 3 in vesseloids 3 days post infection (MOI: 1). The fluorescence intensity per nucleus was quantified. The results are presented as the means ± SEMs.

### Figure 2: The inflammatory response of ECs is mediated by epithelial-derived CXCL10

**A)** Calu-3 cells were infected with SARS-CoV-2 (MOI=1) for 4 hours and plated at the bottom of the transwell chamber before they were cocultured with vesseloids placed in the upper chamber for 3 days. **B)** Relative VCAM-1 mRNA expression in SARS-CoV-2-infected vesseloids alone or after coculture with Calu-3 cells infected with SARS-CoV-2 for 4 hours was analyzed 3 days post infection (MOI=1). The values were normalized according to tubulin mRNA expression. The results are presented as the means ± SEMs (unpaired t test; $*P < 0.05$). **C)** Heatmap of pixel density quantification from the cytokine array assay for selected chemokines and cytokines from vesseloids infected alone or after coculture with Calu-3 cells. SarS-CoV-2 was added to the culture supernatant, which was incubated for 4 hours and analyzed 3 days post infection (MOI: 1). **D)** Relative CXCL10 mRNA expression in SARS-CoV-2-

infected vesseloids alone or after coculture with infected Calu-3 cells or infected Calu-3 cells alone. SARS-CoV-2 infection was induced for 4 hours, and the data were analyzed 3 days post infection (MOI=1). The results are presented as the means ± SEMs (unpaired t test $*P < 0.05$). **E)** CXCL10 concentration in the supernatant of infected vesseloids alone or after coculture with infected Calu-3 cells or infected Calu-3 cells alone. SARS-CoV-2 infection was induced for 4 hours, after which the cells were analyzed 3 days post infection (MOI=1). The results are presented as the means ± SEMs (one-way ANOVA, $*P < 0.05$).

**Figure 3: Epithelial-derived CXCL10 opens interendothelial junctions**

**A)** HUVECs were seeded in the upper well of a transwell chamber and grown to confluency. Cells were treated with 1 µg/ml CXCL10 or 1 µg/ml VEGF-A for 1 hour after 25 µg/ml FITC-dextran was added to the upper well. Every 20 minutes, after the addition of dextran, the fluorescence in the lower compartments was measured. The results are presented as the means ± SEMs (one-way ANOVA, $*P < 0.05$). **B)** For quantification of VE-cadherin junctions, HUVECs were seeded, grown to confluency and treated for 4 hours with 1 µg/ml CXCL10 or 1 µg/ml VEGF-A and VE-cadherin, and actin was stained. The results are presented as the mean ± SEM (one-way ANOVA, $**P < 0.01$). **C)** VE-cadherin immunostaining after stimulation for 4 hours with 1 µg/ml CXCL10 or 1 µg/ml VEGF-A. Green indicates VE-cadherin, red indicates actin, and blue indicates DAPI.

**Figure 4: CXCL10 and myeloid recruitment via CXCR3 and activation of the SRC pathway**

**A)** Transwell assay of activated THP1 cells treated or not treated with 1 µg/ml CXCL10 and stained with DAPI (bleu). THP-1 cells were placed in the upper chamber, and migration was stimulated by CXCL10 in the lower chamber. The number of transmigrating cells was then counted. **B)** Quantification of (A). The results are presented as the means ± SEMs (unpaired t

test; *$P < 0.05$). **C)** Western blot analysis of p-Src, Src, p-ERK and the ERK protein. Vinculin was used as a loading control. **D)** Quantification of the data in (C). Bar graphs are presented as the means ± SEMs. **E)** THP1 cells were placed in the upper chamber, and migration was stimulated by CXCL10 in the lower chamber. The number of transmigrating cells was then counted after DAPI staining. Treatment with 1 µg/ml CXCL10 alone (blue) or in combination with 6 nM SCH or 1 nM PP2 compared to the control. The results are presented as the means ± SEMs (one-way ANOVA; *P < 0.05, **<0.01, and ***<0.001).

**Figure 5: Clinically relevant serum samples and bioinformatics data from SARS-CoV-2-infected patients**

**A)** Representation of CXCL10, CXCL8 and IL6 levels in the plasma of SARS-CoV-2-infected patients (severe or moderate) and SARS-CoV-2-noninfected patients (Bordeaux, COLCOV collection). Moderate = severe acute respiratory syndrome coronavirus 2; severe = severe acute respiratory syndrome coronavirus 2 with intensive care. The results are presented as the mean ± SEM (one-way ANOVA, P***<0.001). **B)** Heatmaps of CXCL10, F3, ICAM and CXCL8 mRNA expression in an independent patient cohort. **C)** Heatmaps of M1 or M2 mRNA expression in the independent patient cohort.

## Supplemental figures

**Supplemental Figure 1: Absence of direct infection of ECs by SARS-CoV-2**

**A)** RT–qPCR quantification of viral RNA and relative N-gene mRNA expression in media after 4 hours of SARS-CoV-2 infection in Calu-3 cells measured at 7 days post infection (MOI: 1 and MOI: 0.1). The values were normalized to the GAPDH mRNA expression. **B)** HCAEC and HUAEC RT–qPCR quantification of viral RNA and relative N-gene mRNA expression in media and cells at 3 and 6 days post infection (MOI: 1 and MOI: 0.1).

**Supplemental Figure 2: Inflammatory response in HUVECs after SARS-CoV-2 infection**

**A)** Relative ICAM-1 mRNA expression in vesseloids alone or after coculture with SARS-CoV-2 post infection (MOI: 1). The results are presented as the means ± SEMs. **B)** IL6 and CXCL8 concentrations in supernatants from vesseloids and Calu-3 cells cultured separately or in coculture for 3 days post infection (MOI: 1). The results are presented as the means ± SEMs.

**Supplemental Figure 3: CXCR3 expression in endothelial cells, monocytic THP-1 cells and Calu-3 cells**

The figure shows a Western blot probed with an anti-CXCR3 antibody for CXCR3 protein detection. Thirty μgrammes of protein were loaded in each lane. Vinculin was used as a loading control.

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**Author contributions**

L.C : Conceptualization; data curation; formal analysis; investigation; methodology; project administration; validation; roles/writing—original draft ; MLB : data curation; formal analysis, methodology ; PRP : data curation; formal analysis ; ABM : data curation; formal analysis, methodology ; IP : data curation; formal analysis, methodology ; MS : data curation; formal analysis ; NP : tool development ; MLA : contribution with ideas and general input ; FB : advice clinical ; GR : bioengineering; funding acquisition ; LA : bioengineering, funding acquisition; PN : bioengineering, intellectual input ; TM : formal analysis, methodology, investigation; methodology; AB : Conceptualization ; methodology; project administration; funding acquisition; project administration; supervision; validation; roles/writing—original draft.

**Competing interests**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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