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The spike protein of SARS-CoV-2 induces endothelial inflammation through integrin $\alpha 5\beta 1$ and NF- κ B

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Abstract

Vascular endothelial cells (EC) form a critical interface between blood and tissues that maintains whole-body homeostasis. In COVID-19, disruption of the EC barrier results in edema, vascular inflammation, and coagulation, the hallmarks of the severe disease. However, the mechanisms by which EC are dysregulated in COVID-19 are unclear. Here, we show that the spike protein of SARS-CoV-2 alone activates the EC inflammatory phenotype in a manner dependent on integrin $\alpha 5\beta 1$ signaling. Incubation of human umbilical vein EC with whole spike, its receptor-binding domain, or the integrin-binding tripeptide RGD induced the nuclear translocation of NF- κ B and enhanced the expression of leukocyte adhesion molecules VCAM1 and ICAM1, the adhesion of peripheral blood leukocytes, and the permeability of the monolayer. Inhibitors of integrin $\alpha 5\beta 1$ activation prevented these effects. We suggest that the spike protein, through its RGD motif in the receptor-binding domain, binds to integrin $\alpha 5\beta 1$ in EC to activate Rho GTPases, eNOS pathways, and the NF- κ B gene expression program responsible for vascular leakage and leukocyte infiltration, respectively. These findings uncover a new direct action of SARS-CoV-2 on EC dysfunction and introduce integrin $\alpha 5\beta 1$ as a promising target for treating vascular inflammation in COVID-19.

Introduction

Endothelial cells (EC) dysfunction has emerged as a major driver of COVID-19¹⁻³. During a resting state, EC maintain their barrier function by limiting vasopermeability and preventing coagulation and inflammation. However, when activated in response to damage or infection, EC produce chemoattractants, cytokines, and adhesion molecules, leading to vascular leakage, clot formation, inflammation, and leukocyte infiltration⁴. Most people with severe COVID-19 die from acute respiratory distress syndrome, pulmonary edema, cytokine storm, multiple organ failure, and disseminated intravascular coagulation², all of which reflect EC dysfunction¹⁻³. Moreover, severe cases or deaths due to COVID-19 associate with chronic endothelial damage from comorbidities such as aging, obesity, hypertension, diabetes, and cardiovascular disorders^{5,6}.

Potential mechanisms of vascular dysfunction in COVID-19 include EC death in response to SARS-CoV-2 entry and replication⁷, the binding of the spike protein of SARS-CoV-2 to the ACE2 receptor causing its downregulation and subsequent mitochondrial dysfunction⁸, the activation of the proinflammatory kallikrein-bradykinin system, and the accumulation of proinflammatory and vasoconstrictor angiotensin II². In addition, the spike protein can trigger the expression of proinflammatory cytokines and chemokines⁹, the production of toxic reactive oxygen species¹⁰, and cell death¹¹. However, the underlying mechanisms of many of these effects remain unclear.

Alternative to ACE2, integrins may function as receptors mediating SARS-CoV-2 infection. Integrins are heterodimeric transmembrane cell adhesion molecules that exert various actions on hemostasis, inflammation, and angiogenesis. The spike protein contains an integrin-binding RGD motif exposed on the surface of the receptor-binding domain^{12,13} that binds to $\beta 1$ integrins on pulmonary epithelial cells and monocytes¹⁴. In particular, blockade

of the binding of the spike protein to the integrin $\alpha 5 \beta 1$ inhibits SARS-CoV-2 infection in vitro¹¹ and in vivo¹⁵, demonstrating the therapeutic efficacy of targeting integrin $\alpha 5 \beta 1$ in COVID-19. Furthermore, fibronectin ligation of integrin $\alpha 5 \beta 1$ in EC activates the transcriptional factor NF- κ B responsible for the expression of proteins involved in inflammation and angiogenesis¹⁶. These observations prompted us to investigate whether the binding of spike to the integrin $\alpha 5 \beta 1$ in EC is sufficient to induce the endothelium proinflammatory phenotype.

Results

Spike stimulates leukocyte adhesion to EC

Inflammatory changes can be assessed by the ability of EC to attach leukocytes, a hallmark of the inflammatory process. Peripheral blood leukocytes were incubated 1 h with human umbilical vein endothelial cells (HUVEC) pretreated for 16 h with spike, the receptor-binding domain of spike, the RGD tripeptide, or TNF α as a pro-inflammatory control (Figure 1). Spike stimulated the adhesion of leukocytes to HUVEC in a dose-dependent manner with high potency ($EC_{50} = 1.6$ nM), and this stimulation paralleled the one induced by TNF α , a main inducer of EC pro-inflammatory changes¹⁷ (Figure 1a, b). In addition, the receptor-binding domain of spike and the RGD tripeptide resulted in dose-response curves very similar to the one elicited by spike ($EC_{50} = 1.8$ nM) (Figure 1c). These results show that spike alone activates the proinflammatory program in EC and suggest that the RGD sequence located in the spike receptor-binding domain is responsible for this effect.

Integrin $\alpha 5 \beta 1$ mediates spike-induced leukocyte adhesion to EC

RGD is the integrin-binding motif of several integrin ligands, including fibronectin, the main ligand of integrin $\alpha 5 \beta 1$. Fibronectin is upregulated during inflammation¹⁸ and stimulates the EC inflammatory program¹⁶. We used ELISA-based methods to confirm the binding of spike to integrin $\alpha 5 \beta 1$ ¹¹. Plates were coated with integrin $\alpha 5 \beta 1$ and incubated with spike or the receptor-binding domain of spike in the absence or presence of the RGD tripeptide or neutralizing antibodies against integrin $\alpha 5 \beta 1$ or the $\alpha 5$ integrin subunit. Spike and its receptor-binding domain bound $\alpha 5 \beta 1$ integrin with the same affinity ($K_d = 200$ pM) (Figure 2a) and, as expected, the RGD tripeptide and the anti- $\alpha 5 \beta 1$ and - $\alpha 5$ antibodies prevented this binding (Figure 2b). Because fibronectin ligation of integrin $\alpha 5 \beta 1$ induces the expression of adhesion molecules in EC¹⁶, we explored whether the binding to integrin $\alpha 5 \beta 1$ mediated the spike-induced stimulation of leukocyte attachment. The effect of anti- $\alpha 5 \beta 1$ or anti- $\alpha 5$ antibodies was tested upon spike-, the spike receptor-binding domain-, the RGD tripeptide-, or TNF α -induced stimulation of leukocyte adhesion to HUVEC (Figure 2c). Consistent with an integrin $\alpha 5 \beta 1$ -dependent effect, both antibodies blocked leukocyte adhesion in response to spike, the spike receptor-binding domain, and the RGD tripeptide. The effect of TNF α was not modified, confirming that it is integrin independent¹⁹.

Spike-induced leukocyte adhesion to EC is dependent on integrin $\alpha 5 \beta 1$ -mediated NF- κ B activation

Because integrin $\alpha 5 \beta 1$ activates NF- κ B in EC to elicit inflammation¹⁶, we asked whether the mechanism by which spike promotes leukocyte adhesion involved the activation of NF- κ B. Inactive NF- κ B is usually bound to a family of cytoplasmic inhibitory proteins, and its activation requires the degradation of these inhibitors followed by its nuclear translocation. The NF- κ B cellular distribution was studied in HUVEC using fluorescence cytochemistry and a monoclonal antibody against the p65 subunit of NF- κ B (Figure 3a). In the absence of treatment, p65 was homogeneously distributed throughout the cytoplasm of cells. Spike induced the accumulation of p65 in the cell nucleus, and this redistribution was like the one induced by TNF α . Anti- $\alpha 5$ antibodies blocked the spike-induced nuclear localization of p65, but not in response to TNF α ; whereas the NF- κ B inhibitor BAY11-7085 blocked the nuclear translocation of p65 induced by both spike and TNF α (Figure 3a). We conclude that spike activates NF- κ B through its interaction with integrin $\alpha 5 \beta 1$.

To evaluate whether the spike-induced leukocyte adhesion to EC is dependent on NF- κ B activation, we tested the effect of BAY11-7085 upon spike-, spike receptor-binding domain-, RGD tripeptide-, or TNF α -induced stimulation of leukocyte adhesion to HUVEC (Figure 3b). Inhibition of NF- κ B prevented the stimulation of leukocyte adhesion in response to all treatments (Figure 3b). Furthermore, spike elevated by 4-fold the expression of intercellular adhesion molecule 1 (ICAM1) and vascular adhesion molecule 1 (VCAM1), both of mediate the firm adhesion of leukocytes to the apical surface of EC²⁰, and the increased expression was prevented by the inhibition of NF- κ B and integrin $\alpha 5$ -immunoneutralization (Figure 3c). Altogether, our findings show that the binding of spike to integrin $\alpha 5 \beta 1$ activates the NF- κ B pathway in EC responsible for leukocyte infiltration.

Spike induces the hyperpermeability of EC monolayers via integrin $\alpha 5 \beta 1$

Besides leukocyte recruitment, $\beta 1$ integrins promote vascular permeability, another critical aspect of inflammation²¹. Treatment with spike, the receptor-binding domain of spike, and the RGD tripeptide induced a drop in the trans-endothelial electrical resistance (TEER) of the HUVEC monolayer, indicative of hyperpermeability (Figure 4a). The drop was rapid, maximal at 30 minutes, and sustained thereafter. Furthermore, immunofluorescence showed the formation of actin stress fibers, EC retraction, and inter-endothelial gaps after 30 minutes of treatment with spike (Figure 4b). Likewise, spike interfered with the peripheral distribution of CD31, an adhesion protein that maintains the junctional integrity of EC²² (Figure 4b). These observations provided direct evidence of spike-induced increase in EC permeability.

RhoA, Rac1, and Cdc42 are Rho small guanosine triphosphatases (GTPases) that control the actin cytoskeleton and regulate EC barrier function²³. Redistribution of actin stress fibers causing EC contraction involves RhoA activation²⁴, whereas Rac1 and Cdc42 induce cell spreading²⁵. Western blot analysis of HUVEC lysates incubated with spike for 30 min showed increases in RhoA and Cdc42 and a decrease in Rac1 that were blocked by the immunoneutralization of $\alpha 5$ (Figure 4c). Furthermore, endothelial nitric oxide synthase (eNOS) derived NO stimulates vasopermeability²⁶, and spike promoted the phosphorylation/activation of eNOS in HUVEC in an $\alpha 5$ -

dependent manner (Figure 4c). Finally, immunoneutralization of integrin $\alpha 5 \beta 1$ prevented spike-, spike receptor-binding domain-, and RGD tripeptide-induced reduction in TEER (Figure 4d). Taken together, these studies show that spike binding to integrin $\alpha 5 \beta 1$ regulates Rho GTPases and eNOS phosphorylation to promote EC hyperpermeability.

Volociximab and ATN-161 reduce spike-induced stimulation of EC leukocyte adhesion and permeability

Inhibitors of integrin $\alpha 5 \beta 1$ have been developed as promising therapeutics. For example, volociximab, a chimeric anti-integrin $\alpha 5 \beta 1$ monoclonal antibody, is under clinical evaluation for the treatment of cancer²⁷; and preclinical studies have evaluated the efficacy of the integrin $\alpha 5 \beta 1$ binding peptide, ATN-161, to inhibit beta-coronavirus²⁸ and SARS-CoV-2 virus infections^{11,15}. Here, we show that both volociximab and ATN-161 block the binding of spike and the spike receptor-binding domain to $\alpha 5 \beta 1$ immobilized in ELISA plates (Figure 5a); and prevent the leukocyte adhesion to HUVEC (Figure 5b) and HUVEC hyperpermeability (Figure 5c) in response to spike, spike receptor-binding domain, and RGD tripeptide.

Discussion

Accumulating evidence has defined COVID-19 as a vascular disease^{1-3,29}. Blood vessel injury causes progressive lung damage and multi-organ failure in severe COVID-19, owing to edema, intravascular coagulation, vascular inflammation, and deregulated inflammatory cell infiltration. Multiple mechanisms have been proposed for vascular dysfunction in COVID-19^{1,2,29}; however, little is known regarding the direct action of SARS-CoV-2 on EC^{10,30}.

ACE2 is the best-established host receptor for spike³¹⁻³³, although other spike cell surface receptors have been described, i.e., neuropilin-1³⁴, toll-like receptors^{9,35}, and RGD-binding integrins^{11,14}. In particular, integrin $\alpha 5 \beta 1$ is an RGD-binding integrin that, upon spike binding, mediates SARS-CoV-2 entry and infection of epithelial cells and monocytes in vitro¹¹ and increases lung viral load and inflammation in vivo¹⁵. Ligation of integrin $\alpha 5 \beta 1$ by fibronectin RGD motif activates the expression of proinflammatory genes in EC¹⁶, but the binding of spike to $\alpha 5 \beta 1$ in EC and its impact on the EC inflammatory response has not been addressed.

In this work, we show that spike binding to integrin $\alpha 5 \beta 1$ activates the inflammatory program of EC. Spike stimulated the expression of adhesion molecules ICAM1 and VCAM1 and the attachment of leukocytes to EC monolayers like TNF α , a well-known inducer of sustained EC inflammatory responses¹⁷. The fact that both the RGD tripeptide and the receptor-binding domain of spike elicited almost identical responses to those of spike, that the RGD tripeptide itself blocked spike- and spike receptor-binding domain- binding to $\alpha 5 \beta 1$, and that $\alpha 5 \beta 1$ -neutralizing antibodies prevented the spike-induced proinflammatory effect in EC indicated that spike, through its RGD motif in its receptor-binding domain, binds to integrin $\alpha 5 \beta 1$ in EC to promote inflammation. Furthermore, we show that the transcription factor NF- κ B is a primary contributor to $\alpha 5 \beta 1$ signaling in EC in response to spike. Spike and its receptor-binding domain stimulated NF- κ B nuclear translocation, and inhibitors of NF- κ B and $\alpha 5 \beta 1$

prevented the spike-induced expression of ICAM1 and VCAM1 and leukocyte adhesion to EC. These findings are consistent with a previous study showing that NF- κ B is the signaling pathway by which fibronectin ligation to $\alpha 5\beta 1$ upregulates proinflammatory genes in EC¹⁶. Also, NF- κ B is a predominant signaling molecule activated in EC by proinflammatory cytokines, such as TNF α via an integrin-independent route¹⁷.

Neutrophils, macrophages, and lung epithelial cells react to spike^{9,35} and to the envelope protein³⁶ via the activation of toll-like receptor-induced cytokine production, a mechanism underlying the cytokine storm observed in severe COVID-19³⁷. Integrin $\alpha 5\beta 1$ is widely expressed in immune cells¹⁶, and it may cooperate with toll-like receptors to boost their signaling pathways³⁸. However, human EC express few or no toll-like receptors on their surface³⁹, implying that interactions between toll receptors and integrin $\alpha 5\beta 1$ do not contribute to the proinflammatory effect of spike in the endothelium.

Excessive vasopermeability leading to edema is another hallmark of severe COVID-19. Here, we showed that spike binding to integrin $\alpha 5\beta 1$, through its RGD motif, stimulated the hyperpermeability of EC monolayers. Spike-induced hyperpermeability was mimicked by its receptor-binding domain and the RGD tripeptide and prevented by neutralizing antibodies against the $\alpha 5\beta 1$ integrin and $\alpha 5$ -integrin subunit.

$\beta 1$ -integrins are known to stabilize EC-cell junctions during development⁴⁰, but also to promote their disruption under the context of inflammation²¹. Several inflammatory agents (LPS, IL-1 β , and thrombin) signal via $\beta 1$ -integrin to promote EC permeability and contractility in vitro and vascular leakage in vivo⁴¹. Consistent with this observation, we showed that the spike signals through $\alpha 5\beta 1$ to promote the formation of stress fibers, endothelial cell retraction, and inter-endothelial gap formation. These processes are under the control of GTPases of the Rho family (RhoA, Rac, Cdc42), and spike upregulated RhoA and Cdc42 and downregulated Rac1 in EC. Rho GTPases signal downstream of integrin binding to exert both positive and negative effects on EC junctions and EC matrix adhesion depending on its context. Under inflammation, RhoA associates with loss of barrier integrity, Rac1 with maintenance, and Cdc42 with barrier stabilization and recovery⁴².

Consistent with the above findings, spike interfered with the peripheral distribution of CD31 observed in stable EC monolayers. Because CD31 exhibits adhesive properties and is concentrated mainly at junctions between adjacent cells²², its altered localization in EC is consistent with increased vasopermeability. This observation agrees with a recent report showing that spike downregulates the expression of EC junctional proteins from normal and diabetic mice, reflecting the disruption of the endothelial barrier integrity⁴³. Finally, spike stimulated the phosphorylation/activation of eNOS in EC, and eNOS-derived NO is a principal vasorelaxant and vasopermeability factor^{26,44}.

Altogether, we have shown that, by binding to $\alpha 5\beta 1$, spike changes the EC phenotype to promote vascular inflammation. Upon spike-mediated $\alpha 5\beta 1$ activation, EC lose their ability to control permeability or quiesce leukocytes, both of which are characteristics of EC dysfunction¹⁷. These findings provide insights into the

mechanisms making COVID-19 a vascular disease and encourage the development of therapeutic approaches that directly focus on $\alpha 5\beta 1$ -mediated vascular changes.

Finally, we showed that two inhibitors of $\alpha 5\beta 1$, volociximab and ATN-161, developed as promising therapeutics, blocked spike-induced leukocyte adhesion and hyperpermeability of EC. Indeed, ATN-161 has already been shown to inhibit SARS-CoV-2 virus infection in vivo¹⁵. Our findings, add EC as a target for these drugs to reduce vascular inflammation in COVID-19, suggest the therapeutic potential of agents commonly used to treat vascular diseases, and provide tools for guiding research into the molecular mechanisms mediating the pathophysiology of COVID-19.

Methods

Reagents

Recombinant SARS-CoV-2-His-Tagged spike (Active Trimer) (cat. 10549-CV) was purchased from Bio-technie (Minneapolis, MI, USA). Recombinant receptor-binding domain of spike (amino acids 319-541) flanked by the signal peptide (amino acids 1-14) and containing a 6xHis tag was produced as previously reported⁴⁵. Arg-Gly-Asp (RGD) tripeptide, the NF- κ B activation inhibitor BAY 11-7085, and ATN-161 were purchased from Sigma Aldrich (St. Louis, MO, USA), and human recombinant TNF α was from R&D Systems (Minneapolis, MN, USA). Purified human integrin $\alpha 5\beta 1$ and anti- $\alpha 5$ (MAB1956Z) and anti- $\alpha 5\beta 1$ (MAB1999) antibodies were from Merck (Kenilworth, NJ, USA). Volociximab was from Novus Biologicals (Littleton, CO, USA).

Cell culture

Human umbilical vein endothelial cells (HUVEC) were obtained as described⁴⁶. HUVEC were maintained in F12K media supplemented with 20% fetal bovine serum (FBS), 100 μ g mL⁻¹ heparin (Sigma-Aldrich), 25 μ g mL⁻¹ endothelial cell growth supplement (ECGS, Corning, NY, USA), and 100 U mL⁻¹ penicillin-streptomycin.

Leukocyte adhesion assay

HUVEC were seeded on a 96 well plate and grown to confluency. HUVEC monolayers were treated for 16 hours with TNF α , spike, the spike receptor-binding domain, and the RGD tripeptide alone or in combination with anti- $\alpha 5\beta 1$ antibodies (5 μ g mL⁻¹), anti- $\alpha 5$ antibodies (5 μ g mL⁻¹), volociximab (5 μ g mL⁻¹), or ATN-161 (500 nM) in 20% FBS F12K without heparin or ECGS. For inhibition of NF- κ B, cells were pretreated for 30 minutes with 5 μ M of BAY 11-7085. Leukocytes were prepared from whole blood collected into heparin tubes. Briefly, blood was centrifuged (300 x g for 5 minutes) and the plasma layer discarded. The remaining cell pack (~5 mL) was diluted in 45 mL of red blood cells lysis buffer containing 150 mM of NH₄Cl, 10 mM NaHCO₃, and 1.3 mM EDTA (disodium) and gently rotated for 10 minutes at RT. Leukocytes were collected by centrifugation (300 x g for 5 min), the pellet washed with cold PBS followed by centrifugation (300 x g for 5 min), and resuspended into 5 mL of warm PBS containing 5 μ g mL⁻¹ Hoechst 33342 (Thermo Fisher Scientific, Waltham, MA, USA) to stain live leukocytes.

Leukocytes were incubated in darkness for 30 minutes, washed with PBS three times by centrifugation (200 x g for 5 min each), resuspended into supplemented culture media, and taken to 10^6 cells mL^{-1} . After 16 hours of treatment, the medium of HUVEC monolayers was replaced with 100 μL of medium containing Hoechst-stained leukocytes ($\sim 100,000$ leukocytes per well) and cells incubated for 1 hour at 37°C . HUVEC monolayers were washed three times with warm PBS, and images were obtained in an inverted fluorescent microscope (Olympus IX51, Japan) and quantified using the CellProfiler software⁴⁷.

ELISA

A 96-well ELISA microplate was coated overnight at 4°C with 25 ng per well of human integrin $\alpha 5\beta 1$ diluted in PBS, and blocked for 1 hour at RT with 5% w/v nonfat dry milk in 0.1% Tween-20-PBS (PBST). After blocking, microplates were washed three times with PBST and different concentrations of spike or spike receptor-binding domain added. Anti- $\alpha 5\beta 1$ antibodies ($5 \mu\text{g mL}^{-1}$), the RGD tripeptide (500 nM), ATN-161 (500 nM), or volociximab ($5 \mu\text{g mL}^{-1}$) were added together with 100 nM of spike or spike receptor-binding domain. Dilutions were in 0.2 mg mL^{-1} BSA-PBST. Microplates were then incubated for 1 hour at RT, washed three times with PBST, incubated for 1 hour at RT with 1:1,000 of anti-spike antibody (Rabbit MAb, Sino Biological, Beijing, CN) diluted in blocking buffer, washed (three times in PBST), and incubated (1 hour at RT) with horseradish peroxidase-labeled goat anti-rabbit secondary antibodies (Thermo Fisher Scientific) diluted 1:2,500 in 50% blocking buffer. After a three-wash step, the microplates were incubated with 100 μL of an OPD substrate tablet diluted in 0.03% H_2O_2 citrate buffer for 30 minutes in darkness. The reaction was stopped with 50 μL of 3M HCl, and absorbance was measured at 490 nm.

NF- κB nuclear translocation analysis

HUVEC were seeded on 18 mm-coverslips coated with fibronectin ($1 \mu\text{g cm}^{-1}$) and placed in a 12-well plate. Cells were grown in complete media to 80% confluence and, on the day of the assay, the medium was replaced with 0.5% FBS-F12K. The NF- κB activation inhibitor, BAY 11-7085 ($5 \mu\text{M}$), or anti- $\alpha 5$ antibodies ($5 \mu\text{g mL}^{-1}$) were added 30 minutes prior to a 30-minutes incubation with spike (100 nM) or TNF α (1 nM). Cells were washed, fixed in 4% PFA for 30 minutes at RT, permeabilized for 30 minutes with 0.5% Tx-100-PBS, blocked with 0.05% Tx-100, 1% BSA, 5% normal goat serum-PBS at RT for 1 hour, and incubated overnight at 4°C in a humid chamber with 1:200 mouse monoclonal anti-NF- κB p65 antibody (Santa Cruz, CA, USA) in 0.1% Tx-100, 1% BSA-PBS. Cells were washed and incubated under darkness at RT with a goat anti-mouse secondary antibody (1:500, Alexa fluor 488, Abcam, Cambridge, UK) in 0.1% Tx-100, 1% BSA-PBS for 2 hours. Nuclear DNA was counterstained with $5 \mu\text{g mL}^{-1}$ Hoechst 33342 (Sigma-Aldrich) and the coverslips washed and mounted with Vectashield mounting medium (Vector laboratories, Burlingame, CA) and observed under fluorescence microscopy (Olympus IX51).

qPCR

HUVEC grown to 80% confluency on 6-well plates were incubated under starving conditions (0.5% FBS, F12K medium) for 30 minutes with the NF- κ B activation inhibitor, BAY 11-7085 (5 μ M) or anti- α 5 antibodies (5 μ g mL⁻¹) followed by a 4-hour incubation with 100 nM spike. RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), retrotranscribed with the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA), and quantified using Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific) in a final reaction of 10 μ L containing 20 ng of cDNA, and 0.5 μ M of each of the following human primers: VCAM1 forward (5'-gcactgggtgactttcagg-3') and reverse (5'-aacatctccgtaccatgccca-3'); ICAM1 forward (5'-gtgaccgtgaatgtgctctc-3') and reverse (5'-cctgcagtgcccattatgac-3'); and GAPDH forward (5'-gaaggtcggagtgcaacggatt-3') and reverse (5'-tgacgggtgccatggaatttg-3').

Vasopermeability assay

HUVEC were grown to confluence on a 6.5 mm transwell with a 0.4 μ m pore coated with 1 μ g cm⁻¹ fibronectin (Thermo Fisher Scientific). Trans-endothelial electrical resistance (TEER) was measured using the epithelial EVOM² Volt/Ohm meter (World Precision Instruments, Sarasota, FL, USA). The assay was performed once the TEER measurement was stable for at least two days. Cells were pretreated for 5 minutes with anti- α 5 antibodies (5 μ g mL⁻¹), anti- α 5 β 1 antibodies (5 μ g mL⁻¹), volociximab (5 μ g mL⁻¹), or 500 nM ATN-161 followed by the treatment with 100 nM spike, spike receptor-binding domain, or the RGD tripeptide. TEER measurements were made over 120 minutes.

Fluorescence cytochemistry for CD31 and F-Actin

HUVEC were seeded on 18 mm coverslips coated with fibronectin (1 μ g cm⁻¹) and placed in a 12-well plate. Cells were grown to confluence and, once the monolayer was completely formed, cells were starved (0.5% FBS) for 1 hour before the addition of 100 nM of spike for 30 minutes. Cells were washed, fixed with 4% PFA for 30 minutes, permeabilized with 0.5% Tx-100-PBS for 30 minutes, blocked with 0.1% Tx-100, 1% BSA, 5% normal goat serum-PBS for 1 hour at RT, and incubated overnight at 4°C in a humid chamber with 1:100 anti-CD31 antibody (Abcam) in 0.1% Tx-100, 1% BSA-PBS. Cells were washed and incubated in darkness with goat anti-mouse secondary antibodies (1:500, Alexa fluor 488, Abcam, Cambridge, UK) in 0.1% Tx-100, 1% BSA-PBS for 2 hours. Cells were then washed with PBS and 300 μ L of 160 nM rhodamine-phalloidin (Thermo Fisher Scientific) added to each well for 1 hour in darkness, followed by a PBS wash and nuclear DNA counterstaining with 5 μ g mL⁻¹ Hoechst 33342 (Sigma-Aldrich). Finally, the coverslips were washed, mounted, and observed under fluorescence microscopy.

RhoA/Rac1/Cdc42 analysis

HUVEC were seeded on a 6-well plate and grown in complete media. Eighty-percent confluent cells were then starved for 3 hours (0.5% FBS) and treated or not for 30 minutes with the anti- $\alpha 5$ antibodies ($5 \mu\text{g mL}^{-1}$) followed by the addition of 100 nM spike for a 30 minute-incubation. Cells were washed twice with ice-cold PBS, and the RhoA/Rac1/Cdc42 activation was evaluated using the RhoA/Rac1/Cdc42 combo activation assay kit (Abcam) according to the manufacturer's instructions. The kit is based on the binding of the active form of RhoA to the Rho-binding domain of Rhotekin and of active Rac1 or Cdc42 to the p21-binding domain of p21 activated protein kinase (PAK1). Both, Rhotekin and PAK1 binding domains are coupled to agarose beads, and the isolation and detection of the active forms of RhoA, Rac1, and Cdc42 were done under conventional bead-precipitation and western blot protocols using goat anti-mouse alkaline phosphatase secondary antibodies (Jackson ImmunoResearch, Philadelphia, PA, USA, 1:5000) and a colorimetric detection kit (BioRad, Hercules, CA, USA).

Western blot analysis of eNOS phosphorylation at Ser1177

HUVEC were seeded on a 6-well plate and grown in complete media to reach an 80% confluency. On the day of the assay, HUVEC were starved (0.5% FBS) for 3 hours and treated or not for 30 minutes with the anti- $\alpha 5$ antibody ($5 \mu\text{g mL}^{-1}$) followed by the addition of 100 nM spike for a 30 minute-incubation. Cells were washed twice with cold TBS, scraped with 200 μL RIPA buffer supplemented with 1:100 halt protease-phosphatase inhibitor cocktail (Thermo Fisher Scientific) and 5 mM of EDTA, centrifuged ($10,000 \times g$, 4°C for 10 min), and supernatants aliquoted and stored (-70°C) for western blot analysis. Forty-five μg of protein were resolved in SDS-PAGE and blotted with anti-phospho-eNOS (Ser1177) antibodies (Cell Signaling, Danvers, MA, USA, 1:250), followed by incubation with goat anti-rabbit horseradish peroxidase secondary antibodies (Jackson ImmunoResearch, 1:5000). Immunoblots were developed using the SuperSignal West Pico PLUS chemiluminescent substrate kit (Thermo Fisher Scientific) and the FluorChem E imager and gel documenter system (ProteinSimple, San Jose, CA, USA). Membranes were reblotted using antibodies against total eNOS (Cell Signaling, 1:500), goat anti-rabbit alkaline phosphatase secondary antibodies (Jackson ImmunoResearch, 1:5000), and a colorimetric detection kit (BioRad).

References

1. Teuwen, L.-A., Geldhof, V., Pasut, A. & Carmeliet, P. COVID-19: the vasculature unleashed. *Nature Reviews Immunology* (2020) doi:10.1038/s41577-020-0343-0.
2. Jin, Y. *et al.* Endothelial activation and dysfunction in COVID-19: from basic mechanisms to potential therapeutic approaches. *Signal Transduct Target Ther* **5**, 293 (2020).
3. Libby, P. & Lüscher, T. COVID-19 is, in the end, an endothelial disease. *European Heart Journal* **41**, 3038–3044 (2020).
4. Godo, S. & Shimokawa, H. Endothelial Functions. *Arterioscler Thromb Vasc Biol* **37**, e108–e114 (2017).

5. Bermejo-Martin, J. F., Martín-Fernandez, M., López-Mestanza, C., Duque, P. & Almansa, R. Shared Features of Endothelial Dysfunction between Sepsis and Its Preceding Risk Factors (Aging and Chronic Disease). *J Clin Med* **7**, (2018).
6. Li, X., Sun, X. & Carmeliet, P. Hallmarks of Endothelial Cell Metabolism in Health and Disease. *Cell Metab* **30**, 414–433 (2019).
7. Varga, Z. *et al.* Endothelial cell infection and endotheliitis in COVID-19. *The Lancet* **395**, 1417–1418 (2020).
8. Rauti, R. *et al.* Effect of SARS-CoV-2 proteins on vascular permeability. *bioRxiv* 2021.02.27.433186 (2021) doi:10.1101/2021.02.27.433186.
9. Khan, S. *et al.* SARS-CoV-2 spike protein induces inflammation via TLR2-dependent activation of the NF- κ B pathway. *bioRxiv* 2021.03.16.435700 (2021) doi:10.1101/2021.03.16.435700.
10. Meyer, K., Patra, T., Mahantesh, V. & Ray, R. SARS-CoV-2 spike protein expressing epithelial cells promotes senescence associated secretory phenotype in endothelial cells and increased inflammatory response. *bioRxiv* 2021.04.16.440215 (2021) doi:10.1101/2021.04.16.440215.
11. Beddingfield, B. J. *et al.* The Integrin Binding Peptide, ATN-161, as a Novel Therapy for SARS-CoV-2 Infection. *JACC Basic Transl Sci* 10.1016/j.jacbts.2020.10.003 (2020) doi:10.1016/j.jacbts.2020.10.003.
12. Sigrist, C. J., Bridge, A. & Le Mercier, P. A potential role for integrins in host cell entry by SARS-CoV-2. *Antiviral Research* **177**, 104759 (2020).
13. Tresoldi, I., Sangiuolo, C. F., Manzari, V. & Modesti, A. SARS-COV-2 and infectivity: Possible increase in infectivity associated to integrin motif expression. *Journal of Medical Virology* **92**, 1741–1742 (2020).
14. Park, E. J. *et al.* The Spike Glycoprotein of SARS-CoV-2 Binds to β 1 Integrins Expressed on the Surface of Lung Epithelial Cells. *Viruses* **13**, (2021).
15. Amruta, N. *et al.* In-vivo Protection from SARS-CoV-2 infection by ATN-161 in k18-hACE2 transgenic mice. *bioRxiv* 2021.05.08.443275 (2021) doi:10.1101/2021.05.08.443275.
16. Klein, S. *et al.* Alpha 5 beta 1 integrin activates an NF-kappa B-dependent program of gene expression important for angiogenesis and inflammation. *Mol Cell Biol* **22**, 5912–5922 (2002).
17. Pober, J. S. & Sessa, W. C. Evolving functions of endothelial cells in inflammation. *Nature Reviews Immunology* **7**, 803–815 (2007).
18. Al-Yafeai, Z. *et al.* Endothelial FN (Fibronectin) Deposition by α 5 β 1 Integrins Drives Atherogenic Inflammation. *Arterioscler Thromb Vasc Biol* **38**, 2601–2614 (2018).
19. Aggarwal, B. B. Signalling pathways of the TNF superfamily: a double-edged sword. *Nature Reviews Immunology* **3**, 745–756 (2003).
20. Muller, W. A. Mechanisms of transendothelial migration of leukocytes. *Circ Res* **105**, 223–230 (2009).
21. Ou, Z., Dolmatova, E., Lassègue, B. & Griendling, K. K. β 1- and β 2-integrins: central players in regulating vascular permeability and leukocyte recruitment during acute inflammation. *American Journal of Physiology-Heart and Circulatory Physiology* **320**, H734–H739 (2021).
22. Privratsky, J. R. & Newman, P. J. PECAM-1: regulator of endothelial junctional integrity. *Cell Tissue Res* **355**, 607–619 (2014).

23. Prasain, N. & Stevens, T. The actin cytoskeleton in endothelial cell phenotypes. *Microvasc Res* **77**, 53–63 (2009).
24. Campos, S. B. *et al.* Cytokine-induced F-actin reorganization in endothelial cells involves RhoA activation. *Am J Physiol Renal Physiol* **296**, F487–495 (2009).
25. Price, L. S., Leng, J., Schwartz, M. A. & Bokoch, G. M. Activation of Rac and Cdc42 by integrins mediates cell spreading. *Mol Biol Cell* **9**, 1863–1871 (1998).
26. Durán, W. N., Breslin, J. W. & Sánchez, F. A. The NO cascade, eNOS location, and microvascular permeability. *Cardiovasc Res* **87**, 254–261 (2010).
27. Besse, B. *et al.* Phase Ib safety and pharmacokinetic study of volociximab, an anti- $\alpha 5\beta 1$ integrin antibody, in combination with carboplatin and paclitaxel in advanced non-small-cell lung cancer. *Ann Oncol* **24**, 90–96 (2013).
28. Lv, X. *et al.* ATN-161 reduces virus proliferation in PHEV-infected mice by inhibiting the integrin $\alpha 5\beta 1$ -FAK signaling pathway. *Veterinary Microbiology* **233**, 147–153 (2019).
29. Siddiqi, H. K., Libby, P. & Ridker, P. M. COVID-19 - A vascular disease. *Trends Cardiovasc Med* **31**, 1–5 (2021).
30. Lei, Y. *et al.* SARS-CoV-2 Spike Protein Impairs Endothelial Function via Downregulation of ACE 2. *Circ Res* **128**, 1323–1326 (2021).
31. Hoffmann, M. *et al.* SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. *Cell* **181**, 271–280.e8 (2020).
32. Letko, M., Marzi, A. & Munster, V. Functional assessment of cell entry and receptor usage for SARS-CoV-2 and other lineage B betacoronaviruses. *Nature Microbiology* **5**, 562–569 (2020).
33. Shang, J. *et al.* Cell entry mechanisms of SARS-CoV-2. *Proc Natl Acad Sci U S A* **117**, 11727–11734 (2020).
34. Daly, J. L. *et al.* Neuropilin-1 is a host factor for SARS-CoV-2 infection. *Science* **370**, 861–865 (2020).
35. Shirato, K. & Kizaki, T. SARS-CoV-2 spike protein S1 subunit induces pro-inflammatory responses via toll-like receptor 4 signaling in murine and human macrophages. *Heliyon* **7**, e06187 (2021).
36. Zheng, M. *et al.* TLR2 senses the SARS-CoV-2 envelope protein to produce inflammatory cytokines. *Nature Immunology* (2021) doi:10.1038/s41590-021-00937-x.
37. Colunga Biancatelli, R. *et al.* The SARS-CoV-2 Spike Protein Subunit 1 induces COVID-19-like acute lung injury in K18-hACE2 transgenic mice and barrier dysfunction in human endothelial cells. *American Journal of Physiology-Lung Cellular and Molecular Physiology* (2021) doi:10.1152/ajplung.00223.2021.
38. Gianni, T. & Campadelli-Fiume, G. The epithelial $\alpha \beta 3$ -integrin boosts the MYD88-dependent TLR2 signaling in response to viral and bacterial components. *PLoS Pathog* **10**, e1004477–e1004477 (2014).
39. Flo, T. H. *et al.* Differential expression of Toll-like receptor 2 in human cells. *Journal of Leukocyte Biology* **69**, 474–481 (2001).
40. Yamamoto, H. *et al.* Integrin $\beta 1$ controls VE-cadherin localization and blood vessel stability. *Nature Communications* **6**, 6429 (2015).

41. Hakanpaa, L. *et al.* Targeting β 1-integrin inhibits vascular leakage in endotoxemia. *Proc Natl Acad Sci USA* **115**, E6467 (2018).
42. Amado-Azevedo, J., Valent, E. T. & Van Nieuw Amerongen, G. P. Regulation of the endothelial barrier function: a filum granum of cellular forces, Rho-GTPase signaling and microenvironment. *Cell Tissue Res* **355**, 557–576 (2014).
43. Raghavan, S., Kenchappa, D. B. & Leo, M. D. SARS-CoV-2 Spike Protein Induces Degradation of Junctional Proteins That Maintain Endothelial Barrier Integrity. *Frontiers in Cardiovascular Medicine* **8**, 582 (2021).
44. Teixeira, M. M., Williams, T. J. & Hellewell, P. G. Role of prostaglandins and nitric oxide in acute inflammatory reactions in guinea-pig skin. *Br J Pharmacol* **110**, 1515–1521 (1993).
45. Amanat, F. *et al.* A serological assay to detect SARS-CoV-2 seroconversion in humans. *Nature Medicine* **26**, 1033–1036 (2020).
46. Baudin, B., Bruneel, A., Bosselut, N. & Vaubourdolle, M. A protocol for isolation and culture of human umbilical vein endothelial cells. *Nat Protoc* **2**, 481–485 (2007).
47. Carpenter, A. E. *et al.* CellProfiler: image analysis software for identifying and quantifying cell phenotypes. *Genome Biol* **7**, R100 (2006).

Figures

Figure 1. Spike-induced leukocyte adhesion to EC. (a) Micrographs showing leukocytes adhered to HUVEC monolayers incubated in the absence (-) or presence of spike (10 nM) or TNFα (1 nM). Scale bar = 120 μm. Dose-response stimulation of leukocyte adhesion to HUVEC in response to spike, TNFα (b), the receptor-binding domain of spike (RBD), or the RGD tripeptide (c). Values are means ± SD, *n* = 9. Dose-response curves were fitted by least square regression analysis (*r*² > 0.93).

Figure 2. Integrin α5β1 mediates spike-induced stimulation of leukocyte adhesion to EC. (a) Binding of different doses of spike or spike receptor-binding domain (RBD) to immobilized integrin α5β1 in an ELISA-based assay. Binding curves were fitted by least square regression analysis ($r^2 > 0.97$). **(b)** Effect of the RGD tripeptide, anti-integrin α5β1 antibodies or anti-α5 antibodies on the binding of spike or RBD to immobilized integrin α5β1. * $P < 0.001$ versus basal binding in the absence of spike or RBD (-) (Two-way ANOVA, Šidák). **(c)** Leukocyte adhesion to a HUVEC monolayer in the absence (-) or presence of 100 nM spike, RBD, RGD, or 1nM TNFα, alone or together with antibodies against α5β1 integrin or the α5 integrin subunit. Values are means $\pm$ SD, $n = 9$, # $P < 0.001$ versus (-), * $P < 0.001$ versus absence of antibodies (Two-way ANOVA, Tukey).

Figure 3. Spike-induced leukocyte adhesion to EC is dependent on integrin α5β1-mediated NF-κB activation.

(a) Immunofluorescence detection of NF-κB (p65) in HUVEC incubated in the absence (-) or presence of spike (100 nM) or TNFα (1 nM) alone or in combination with anti-α5 antibodies or the NF-κB inhibitor, BAY 11-7085. Scale bar = 50 μm. **(b)** Leukocyte adhesion to HUVEC stimulated or not (-) by 1nM TNFα or 100 nM spike, spike receptor-binding domain (RBD), or the RGD tripeptide alone or together with BAY 11-7085. **(c)** Expression of intercellular adhesion molecule 1 (ICAM1) and vascular adhesion molecule 1 (VCAM1) in HUVEC incubated with or without (-) 100 nM spike, in the absence or presence of BAY 11-7085 or anti-α5 antibodies. Values are means ± SD, $n = 9$, # $P < 0.001$ versus the unstimulated control (-), * $P < 0.001$ versus absence of BAY 11-7085 or anti-α5 antibodies (Two-way ANOVA, Tukey).

Figure 4. Spike induces the hyperpermeability of EC monolayers via integrin $\alpha 5\beta 1$. (a) Changes throughout time in the transendothelial electrical resistance (Δ TEER) of HUVEC monolayers incubated without (-) or with 100 nM spike, spike receptor-binding domain (RBD), or RGD tripeptide. (b) Representative fluorescence cytochemistry of a HUVEC monolayer doubled-stained for F-actin and CD31 incubated without (-) or with 100 nM spike. Scale bar = 35 μm . (c) Representative western blot analysis of active RhoA, Rac1, Cdc42, phospho-eNOS (P-eNOS; relative to total eNOS) of lysates from HUVEC monolayers incubated without or with 100 nM spike in the absence or presence of anti- $\alpha 5$ antibodies. (d) Δ TEER of HUVEC monolayers incubated throughout 120 minutes without or with 100 nM spike, RBD, or RGD in the presence or absence of antibodies against integrin $\alpha 5\beta 1$ or the $\alpha 5$ integrin subunit. Values are means $\pm$ SD, $n = 6$.

Figure 5. Volociximab and ATN-161 reduce spike-induced stimulation of EC leukocyte adhesion and permeability. (a) Binding of 100 nM spike or spike receptor-binding domain (RBD) to immobilized integrin $\alpha 5 \beta 1$ in the absence (-) or presence of the RGD tripeptide or the integrin $\alpha 5 \beta 1$ inhibitors, volociximab ($5 \mu\text{g mL}^{-1}$) and ATN-161 (500 nM). (b) Leukocyte adhesion to HUVEC incubated without (-) or with 1 nM TNF α or 100 nM spike, RBD, or RGD, in the absence or presence of volociximab ($5 \mu\text{g mL}^{-1}$) or ATN-161 (500 nM). #P < 0.001 versus the unstimulated control (-), *P < 0.001 versus absence of integrin $\alpha 5 \beta 1$ inhibitors (Two-way ANOVA, Tukey). (c) Changes throughout time of the transendothelial electrical resistance (Δ TEER) of HUVEC monolayers incubated without (-) or with 100 nM spike in the presence or absence of volociximab ($5 \mu\text{g mL}^{-1}$) or ATN-161 (500 nM). Δ TEER values of HUVEC incubated only with volociximab or ATN-161 are also shown. Values are means $\pm$ SD, $n=6$.