

The SARS-CoV-2 main protease Mpro causes microvascular brain pathology by cleaving NEMO in brain endothelial cells

Markus Schwaninger (✉ markus.schwaninger@uni-luebeck.de)

University of Lübeck <https://orcid.org/0000-0002-4510-9718>

Josephine Lampe

University of Lübeck

Jan Wenzel

University of Lübeck

Helge Müller-Fielitz

University of Lübeck <https://orcid.org/0000-0003-2815-4426>

Kristin Müller

University of Lübeck

Raphael Schuster

University of Lübeck

Linlin Zhang

University of Lübeck

Marietta Zille

University of Lübeck <https://orcid.org/0000-0002-0609-8956>

Markus Krohn

University of Lübeck

Ümit Özorhan

University of Lübeck <https://orcid.org/0000-0002-7661-2818>

Yun Jiang

University of Luebeck <https://orcid.org/0000-0002-2886-7373>

Vanessa Neve

University of Lübeck

Anke Fähnrich

University of Lübeck

Sonja Binder

University of Lübeck

Hauke Busch

University of Lübeck

Ludovic Collin

Roche Innovation Center Basel

Roberto Villasenor

Roche Innovation Center Basel

Olaf Jöhren

University of Lübeck

Hermann Altmeyen

University Medical Center Hamburg <https://orcid.org/0000-0001-9439-6533>

Manolis Pasparakis

University of Cologne <https://orcid.org/0000-0002-9870-0966>

Ruben Nogueiras

Department of Physiology, CIMUS, University of Santiago de Compostela-Instituto de Investigación Sanitaria, Santiago de Compostela, 15782, Spain <https://orcid.org/0000-0002-9976-9930>

Vincent Prevot

Inserm <https://orcid.org/0000-0001-7185-3615>

Rolf Hilgenfeld

University of Lübeck

Markus Glatzel

University Medical Center Hamburg-Eppendorf <https://orcid.org/0000-0002-7720-8817>

Fabian Ott

University of Lübeck

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Abstract

Several lines of evidence suggest that neurological symptoms in COVID-19 patients are partially due to damage to small vessels. However, the potential mechanisms are unclear. Here, we show that brain endothelial cells express SARS-CoV-2 receptors. The main protease of SARS-CoV-2 (M^{pro}) cleaves NEMO, the essential modulator of NF- κ B signaling. By ablating NEMO, M^{pro} induces the death of human brain endothelial cells and a microvascular pathology in mice that is similar to what we find in the brain of COVID-19 patients. Importantly, the inhibition of receptor-interacting protein kinase (RIPK) 3, a mediator of regulated cell death, blocks the vessel rarefaction and disruption of the blood-brain barrier due to NEMO ablation. Our data suggest RIPK as a therapeutic target to treat the neuropathology of COVID-19.

Introduction

Starting in December 2019, the newly discovered coronavirus SARS-CoV-2 has caused the coronavirus disease 2019 (COVID-19)¹⁻³. Within a few months, COVID-19 has developed into a pandemic with millions of people infected worldwide and a high death load⁴. Symptoms typically originate from the respiratory tract. However, many patients also suffer from the involvement of other organ systems that is not secondary to respiratory failure or the severe systemic inflammation due to pneumonia. A considerable proportion of patients, up to 84% of those with severe COVID-19, show neurological signs and symptoms including anosmia, epileptic seizures, strokes, loss of consciousness, and confusion⁵⁻⁹. Typically, COVID-19 can present with the clinical picture of encephalopathy⁸. The detection of SARS-CoV-2 viral genomes in the brain and cerebrospinal fluid (CSF) of some patients has supported the notion that SARS-CoV-2 gains access to the brain¹⁰⁻¹². Viral RNA has been found in blood and virus-like particles or viral proteins in brain endothelial cells^{10,13,14}, suggesting that SARS-CoV-2 reaches the brain by a hematogenous route. In line with a vascular infection, endothelial cells in other organs have been identified as targets of SARS-CoV-2 infection^{15,16}. In COVID-19 patients, magnetic resonance imaging detected lesions that are compatible with a microvascular pathology¹⁷⁻²¹. Autopsy studies have confirmed this interpretation^{17,22-24}. However, the mechanisms that lead to small vessel disease in COVID-19 are still unclear.

The pathogenicity of viruses depends on their ability to manipulate host cells. There is evidence that SARS-CoV-2 proteins interact with the cellular machinery in various ways, including the cleavage of host proteins by viral proteases^{25,26}. The SARS-CoV-2 genome encodes two viral proteases that are responsible for processing the viral polyproteins into the individual components of the replication and transcription complexes. We found that one of them, the main protease of SARS-CoV-2 M^{pro} (also called Nsp5 or 3CL^{pro})²⁷, cleaves the host protein NF-kappa-B essential modulator (NEMO). NEMO is involved in signaling cascades that regulate the transcription of numerous genes, including the anti-viral type I interferons and other immune genes²⁸. Beyond gene regulation, NEMO modulates cell survival and prevents apoptosis and necroptosis²⁹. The ablation of NEMO in brain endothelial cells induced microvascular pathology in mice that was reminiscent of what we observed in brains of COVID-19

patients. Of note, the widespread death of endothelial cells, rarefaction of capillaries, disruption of the blood-brain barrier (BBB), and neuroinflammation due to NEMO ablation were prevented by deleting receptor-interacting protein kinase 3 (*Ripk3*), a protein kinase that is essential for regulated cell death. These data suggest RIPK3 inhibitors as a therapeutic option to interfere with the neurological consequences of COVID-19.

Results

Brain endothelial cells express SARS-CoV-2 receptors

To approach the question of how SARS-CoV-2 can infect brain endothelial cells, we determined the expression of membrane receptors and enzymes that are known to facilitate the entry of SARS-CoV-2 in host cells, namely angiotensin-converting enzyme 2 (ACE2), neuropilin-1 (Nrp1), basigin (Bsg), CD209A, Cd209b, Cd209c, Cd209d, and *Tmprss2*^{9,14,30-32}. When analyzing isolated mouse brain cells by single-cell RNA sequencing (sc-RNA-seq), we identified 20 cell clusters, including two endothelial clusters (Extended data Fig. 1a,b). Some cells in the endothelial cell cluster 2 expressed *Ace2*, albeit at a lower level than pericytes (Fig. 1a). Imaging confirmed that ACE2 was expressed in cerebral microvessels but co-staining of endothelial and pericytic markers and high-resolution microscopy demonstrated that ACE2 was mainly localized in pericytes (Fig. 1a). In contrast, we observed high levels of *Nrp1* and *Bsg* expression in endothelial cells by sc-RNA-seq and immunostaining (Fig. 1b,c), but no *Cd209a-d* or *Tmprss2* (Extended data Fig. 1c-e). In summary, NRP1, BSG, and possibly ACE2 are potential receptors mediating the infection of brain endothelial cells by SARS-CoV-2.

M^{Pro} cleaves NEMO

SARS-CoV-2-infected cells produce specific viral proteins. A strong immune response in COVID-19 patients implies that the viral main protease M^{Pro} is expressed at a high level³³. With respect to potential host substrates of M^{Pro}, we speculated that it could be a successful strategy for SARS-CoV-2 to cleave NEMO as an essential component of the anti-viral immune response³⁴. Indeed, purified M^{Pro} cleaved recombinant human NEMO as well as human and mouse NEMO in extracts of brain endothelial or HEK293T cells in a dose- and time-dependent manner (Fig. 2a,b; Extended data Fig. 2, Extended data Fig. 3a,b). Importantly, M^{Pro} cleaved NEMO also in intact brain endothelial cells (Fig. 2c). When co-expressed with NEMO-2A-GFP that self-processes to NEMO-2A and GFP, HA-tagged M^{Pro} completely neutralized NEMO-2A in human hCMEC/D3 cells (Fig. 2c), although the GFP signal persisted (Extended data Fig. 3c), indicating that M^{Pro} degraded NEMO.

In vitro, M^{Pro} produced several NEMO fragments (Fig. 2a, Extended data Fig. 2). Trypsin digestion of M^{Pro}-treated NEMO followed by mass spectrometry of the generated peptides showed that cleavage occurred at Q83, Q205, Q231, Q304, and Q313 (Fig. 2d-e, Extended data Fig. 4). The cleavage sites that we have

identified (Extended data Fig. 4k) resemble other known recognition sequences of M^{Pro} (reference²⁷). We verified the cleavage at Q231 by using synthetic peptides as substrates corresponding to both the human and mouse NEMO sequence (Fig. 2f, Extended data Fig. 5a-c). With the human NEMO sequence at Q231, the apparent catalytic efficiency (about $43 \text{ s}^{-1} \text{ M}^{-1}$) was in the range that has been reported for the cleavage site between Nsp4 and Nsp5 (Extended data Fig. 5d)³⁵. In keeping with the central role of Q in the recognition sequence, the mutation Q231A in the human NEMO sequence prevented cleavage by M^{Pro} (Fig. 2f).

NEMO is an essential component of the canonical pathway leading to the activation of NF- κ B by inflammatory factors such as interleukin (IL)-1 β . Supporting the functional relevance of NEMO cleavage, M^{Pro} blocked NF- κ B activation. When expressed in human brain endothelial hCMEC/D3 cells, M^{Pro} prevented the nuclear translocation of the NF- κ B subunit p65, reflecting its activation in response to IL-1 β (Fig. 3a). M^{Pro} also abolished the stimulation of NF- κ B-mediated gene transcription by IL-1 β , which we investigated in hCMEC/D3 cells and mouse brain endothelial bEnd.3 cells using luciferase reporter gene assays (Fig. 3b,c). Thus, we obtained unequivocal evidence that M^{Pro} cleaves and thereby inactivates NEMO.

M^{Pro}-induced damage mimics microvascular pathology in COVID-19 patients

As NEMO is required for the integrity of some but not all cell types²⁹, the question arose whether the M^{Pro}-mediated cleavage of NEMO compromises endothelial survival. To test whether M^{Pro} induces endothelial cell death, we transfected hCMEC/D3 cells with a plasmid encoding for M^{Pro}-HA and treated the cells with tumor necrosis factor (TNF) to model the elevated TNF serum concentrations in COVID-19 patients³⁶. M^{Pro}-expressing cells were more often positive for the cell death marker TUNEL, especially when exposed to TNF (Fig. 3d), demonstrating that M^{Pro} promotes endothelial cell death.

To explore the function of M^{Pro} *in vivo*, we employed the vector AAV-BR1 that selectively targets brain endothelial cells when injected intravenously³⁷. After administering the control vector AAV-BR1-GFP, about 10% of cerebral capillaries expressed GFP (Fig. 3e,f). When mice had received AAV-BR1-M^{Pro} two weeks before, we observed a decreased vascular density and an increased number of empty basement membrane tubes in their brains (Fig. 3g,h; Extended data Fig. 6). These so-called string vessels have been reported as a sign of microvascular pathology³⁸. They appeared as thin structures with a typical diameter of 0.5–1 μm . Double staining of the vascular basement membrane marker collagen IV and the endothelial marker CD31 revealed that they did not contain endothelial cells.

Importantly, we also found string vessels in the brain of patients that had died with a SARS-CoV-2 infection (Fig. 3i). As in mice, string vessels appeared as thin basement membrane tubes. In the frontal

cortex of six patients with SARS-CoV-2 infection, the density of string vessels was higher than in six age- and sex-matched control patients (Fig. 3i, Extended data Table 1).

Capillaries are at risk from ablating NEMO

To confirm that the vascular pathology induced by M^{Pro} is linked to NEMO ablation, we used a mouse model of inducible *Nemo* deletion in brain endothelial cells (*Nemo*^{beKO})³⁹. Similar to the M^{Pro}-mediated cleavage of NEMO, its genetic ablation led to numerous string vessels in the brain (Fig. 4a,b; Extended data Fig. 7a). STED microscopy showed that string vessels are tube-like structures with a similar morphology in humans and *Nemo*^{beKO} mice (Fig. 4b, Extended data Fig. 7a). They were deficient of endothelial cells. Our interpretation of string vessels as a sign of ongoing endothelial demise is based on the following observations. NEMO inactivation induced endothelial cell death as detected by staining for active caspase 3 or the TUNEL reaction (Fig. 4c,d). The death of endothelial cells typically occurred in vessel segments adjacent to string vessels (Fig. 4c,d). Most of them were observed in place of higher-order capillaries (Fig. 4e, Extended data Fig. 7b,c), suggesting that capillaries are particularly susceptible to NEMO deficiency. Consequently, small-diameter vessels were predominantly lost and mice developed patchy cerebral hypoxia (Fig. 4f,g). Finally, NEMO ablation led to a significant vessel rarefaction in the brain (Fig. 5a).

The loss of endothelial cells induced by NEMO deficiency also affected other cell types in the neurovascular unit. While the overall coverage of vessels by pericytes was slightly lowered (Fig. 4h), the number of microglia increased and they exhibited an activated morphology (Fig. 4i). The astrogliosis marker GFAP was strongly upregulated, indicating an inflammatory response (Fig. 4j).

RIPK3 mediates microvascular pathology induced by NEMO ablation

Microglia orchestrate the neuroinflammatory response in the brain, including the activation of astrocytes⁴⁰. Therefore, we tested their role in the microvascular pathology induced by NEMO deficiency. However, ablating microglia by administering the CSF-1R antagonist PLX5622 did neither prevent the formation of string vessels nor the activation of astrocytes (Extended data Fig. 8).

To develop alternative therapeutic options, we considered previous reports that NEMO blocks apoptosis or necroptosis in epithelial cells²⁹. In *Nemo*^{beKO} mice, we inactivated the Fas-associated death domain protein (FADD), a component of apoptosis signaling, and RIPK3, a kinase central for both necroptosis and apoptosis (Extended data Fig. 9a). Unexpectedly, FADD deficiency did not ameliorate the consequences of *Nemo* deletion but significantly enhanced the damage induced by NEMO ablation (Extended data Fig. 9b-f). The deletion of only *Fadd* in brain endothelial cells led to the formation of string vessels and a disruption of the BBB as shown by the extravasation of IgG and albumin as well as brain edema

(Extended data Fig. 9b-d). Consequently, *Nemo*^{beKO}*Fadd*^{beKO} mice died within 9 days after tamoxifen injection inducing recombination and knockout (Extended data Fig. 9e).

In contrast, RIPK3 deficiency, which by itself did not affect the cerebral microvasculature, prevented the formation of string vessels and the rarefaction of cerebral vessels due to NEMO ablation (Fig. 5a). Probably as a response to the vessel rarefaction, NEMO deficiency stimulated endothelial proliferation indicating angiogenesis, which has been described in COVID-19 patients before¹⁵. Notably, *Ripk3* deletion abrogated endothelial proliferation (Fig. 5a). *Ripk3* deletion also normalized survival, brain weight, and body weight of mice with a NEMO deficiency in brain endothelial cells and reduced the extravasation of IgG and albumin into the parenchyma, showing that disruption of the BBB was mitigated (Fig. 5b,c; Extended data Fig. 9d,f). Consequentially, NEMO ablation did not activate microglia or astrocytes in the absence of RIPK3 (Fig. 5d).

To explore the mechanisms of BBB protection by RIPK3 deficiency, we quantified the levels of the tight junction protein occludin. NEMO deficiency led to interruptions and the disintegration of occludin⁺ tight junctions, which was prevented by RIPK3 deficiency (Fig. 5e). In addition to endothelial tight junctions, the BBB is characterized by a low rate of transcytosis in cerebral capillaries. The increased IgG extravasation in *Nemo*^{beKO} mice was associated with a lower number of IgG-filled vesicles in brain endothelial cells (Fig. 5f). While this may seem counterintuitive, we have observed a similar reduction of IgG-filled vesicles despite increased overall IgG extravasation in *Pdgfr*^{ret/ret} mice, demonstrating that the detected population of IgG-filled vesicles limit IgG permeation across the BBB^{41,42}. RIPK3 deficiency did not counteract the effect of NEMO ablation on IgG transcytosis (Fig. 5f). Therefore, we conclude that RIPK3 deficiency improves the BBB tightness of *Nemo*^{beKO} mice mainly by preventing endothelial cell death and rescuing tight junctions. Overall, these data suggest that inhibitors of RIPK3 signaling may protect against the microvascular pathology induced by M^{pro} and SARS-CoV-2 infection.

Discussion

In many COVID-19 patients, neurological symptoms occur during the acute disease and possibly also as a late consequence⁴³. Here, we show microvascular pathology in the brain of COVID-19 patients and propose a mechanism by which SARS-CoV-2 infection compromises brain endothelial function, damages the BBB, and reduces CNS perfusion.

Our data demonstrate that M^{pro} of SARS-CoV-2 cleaves host cell NEMO with high efficiency. In infected cells, M^{pro} is located in the cytosol and nucleus, where NEMO is also present^{44,45}. Cleavage by M^{pro} inactivated NEMO. This may benefit the virus by preventing the induction of anti-viral type I interferons that depend on NEMO²⁸. Indeed, levels of type I interferons are low or absent in the peripheral blood of COVID-19 patients^{46,47}. Cleaving NEMO is also a strategy used by other viruses⁴⁸⁻⁵⁰. However, the tropism of SARS-CoV-2 is likely to limit NEMO inactivation to specific cell types. Accordingly, some NF- κ B-dependent cytokines, such as TNF and IL-6, are highly upregulated, indicating that the cells of origin have

escaped NEMO inactivation⁴⁷. In contrast, several studies report evidence that SARS-CoV-2 infects endothelial cells^{10,14,15,51}. In support, we find the SARS-CoV-2 receptors Nrp1, Bsg, and Ace2 expressed in brain endothelial cells. In addition to its central role in immunity, NEMO supports the survival of some but not all cell types²⁹. While neurons, glia, and endothelial cells of peripheral vessels seem to resist NEMO deficiency or are even protected by it against inflammatory stimuli^{52,53}, the survival of other cells, including brain endothelial cells, is supported by NEMO. Our data suggest that, in COVID-19, brain endothelial cells are at disproportionate risk when being infected by SARS-CoV-2 because of their dependence on NEMO activity for survival.

Cleavage of NEMO by M^{pro} mimics the rare genetic disease *incontinentia pigmenti* that is caused by inactivating mutations in the *NEMO (IKBKG)* gene. In the latter condition, patients suffer from a mix of neurological symptoms, such as encephalopathy, stroke, and seizures that resemble neurological manifestations of COVID-19^{54,55}. The absence of NEMO induced a loss of endothelial cells and microvascular pathology. Subsequently, patchy hypoxia developed in the brain and the BBB became leaky. In parenchymal cells, a prominent upregulation of GFAP indicated the activation of astrocytes, in line with the finding that GFAP concentrations are elevated in the blood of COVID-19 patients⁵⁶. An increased BBB permeability and astrocyte activation may cause epileptic seizures in COVID-19 patients as in *incontinentia pigmenti*^{54,55,57}.

M^{pro}-mediated damage of brain endothelial cells suggests that inhibitors of M^{pro} (reference²⁷) may prevent neurological complications of the SARS-CoV-2 infection. Another therapeutic approach may arise from the observation that deletion of *Ripk3* profoundly improved the microvascular pathology. RIPK1 and RIPK3 inhibitors are available and have already entered phases of clinical testing⁵⁸⁻⁶⁰ suggesting therapeutic options for COVID-19 as well as for *incontinentia pigmenti*.

Declarations

Competing interests

L.C. and R.V. are under paid employment by Hoffman La Roche.

Author contributions

J.L., J.W., H.M.F., and M.S. designed the study. J.L., J.W., H.M.F., K.M., R.S., M.Z., M.K., Y.J., Ü.Ö., V.N., A.F., F.O., S.B., H.B., L.C., R.V., and O.J. performed experiments and analyzed the data. L.C., R.V., O.J., M.P., R.H., M.G., R.N., and V.P. provided tools, samples and conceptual support. J.L., J.W., H.M.F., R.N., V.P. and M.S. drafted the article. All authors revised the manuscript for important intellectual content.

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