

FINAL REPORT

Fondazione Istituto di Ricerca Virologica Oretta Bartolomei Corsi

Titolo del progetto: Caratterizzazione della Modificazione Post-Traduzionale Citrullinazione durante l'infezione da SARS-CoV-2: una nuova strategia per farmaci antivirali

Project title: Characterization of citrullination during infection with SARS-CoV-2: a new strategy for host-targeting antivirals drugs

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Introduction

Since it appeared in 2019, the SARS-CoV-2 beta-coronavirus that is responsible for the COVID-19 pandemic is a serious global threat^{1,2}. Notwithstanding progress in the understanding of SARS-CoV-2 infection and the generation of drugs^{3,4} and vaccines⁵, COVID-19 remains a public health concern. In fact, over 135,000 new cases, 2,000 new deaths, 20,000 new hospitalizations, and 500 new ICU admissions were reported worldwide between May 27 and June 23, 2024. (<https://www.who.int/emergencies/diseases/novel-coronavirus-2019/situation-reports>).

Moreover, millions of individuals worldwide are affected by the post-acute sequelae of SARS-CoV-2 infection (PASC). Persistent symptoms, complications, and COVID-19 sequelae, including pulmonary, cardiovascular, neurological, and physical effects, are characteristics of PASC, which encompasses long COVID. These symptoms can last for over a year after infection (<http://www.CDC.gov>), and the underlying mechanisms and/or biological pathways are still unclear.

Although the exact pathophysiology of SARS-CoV-2 remains unknown, viral replication causes severe inflammation and immediate lung injury. Hence, uncontrolled pulmonary inflammation is the major cause of SARS-CoV-2 deaths, with the potential to produce multiorgan failure and neurological symptoms^{1,6,7}. According to the available data, the pathogenesis of COVID-19 is primarily caused by hyperproduction of pro-inflammatory cytokines like TNF- α and IL-6, known as cytokine storm (CS), and immunothrombotic events^{8,9}.

Several studies have identified numerous vaccines⁵ and drugs^{3,4,10-12} that have been licensed for emergency use. The currently proposed treatments mainly employ two different strategies: 1) suppression of inflammation, primarily based on conventional anti-inflammatory drugs such as corticosteroids; 2) inhibition of viral replication with so-called direct-acting antiviral agents (DAAs). However, *in vitro* and *in vivo* studies have suggested that mutations in the viral genome could induce resistance to approved antiviral agents^{13,14}, suggesting the inevitable risk of drug resistance is already present. Furthermore, continuously emerging SARS-CoV-2 variants with the ability to evade the immune system pose an ongoing challenge to vaccine efficacy^{15,16}. Several biological interventions specifically targeting inflammatory cytokines and related signaling pathways have been clinically evaluated as alternatives to antivirals. Unfortunately, these treatments appear to have adverse side effects, making them unsuitable therapeutic options⁸. Hence, gaining a better understanding of the molecular basis of SARS-CoV-2 pathogenesis is critical for identifying novel targets for successful COVID-19 treatments.

In recent years, post-translational modifications (PTMs) have emerged as key factors that viruses regulate during infection to enhance their fitness. In this regard, a growing body of research suggests that PTM citrullination plays a role in the course of viral infections and viral pathogenesis¹⁷⁻²². Citrullination, or deimination, is the hydrolyzation of the guanidinium group of a peptidyl-arginine to form peptidyl-citrulline. This enzymatic conversion is catalyzed by a family of calcium-dependent enzymes known as peptidyl-arginine deiminases (PADs). To date, five mammalian PAD isoenzymes are known which, despite having high sequence homology, differ in tissue distribution, subcellular localization and substrate specificity^{23,24}. Consequently, they have different specific roles in various physiological processes including regulation of gene expression, plasticity of the central nervous system and regulation of the immune reactions²⁵⁻²⁷. The dysregulation of PADs expression and/or an aberrant profile of citrullinated protein has been observed in many inflammatory conditions and lead to several pathological states, such as: rheumatoid arthritis, multiple sclerosis, tumorigenesis, systemic lupus erythematosus, psoriasis and inflammatory bowel disease²⁸⁻³¹.

Several PAD inhibitors have been developed. The pan-PAD inhibitors, such as Cl-amidine (Cl-A) and BB-Cl-amidine (BB-Cl), can inhibit the enzymatic activity of all five isozymes^{32,33}. In order to improve the specificity of the treatment and reduce side effects, several high-specific inhibitors for the different PAD isoforms have been synthesized in recent years, such as the PAD4-specific inhibitors GSK199 and GSK484^{34,35}. Both pan-PAD and PAD4-specific inhibitors have been

tested in several *in vivo* models of inflammatory and autoimmune diseases, in which they have demonstrated high therapeutic efficacy^{34,36,37}.

We have recently shown that the beta-coronavirus HCoV-OC43 heavily relies on the PAD4 isoform for replication. Consistently, PAD4-specific inhibitors showed potent antiviral activity against that coronavirus in human fibroblasts²⁰. Intriguingly, sera from COVID-19 patients have elevated levels of PAD4-citrullinated histone 3 (H3)³⁸, a specific marker of neutrophil extracellular traps (NETs), which have been linked to thrombotic manifestations in several pathological conditions³⁹. Furthermore, increased NET formation was reported in tracheal aspirate and lung epithelial tissue, suggesting that PAD4 activity—which is necessary for the production of NETs—may play a significant role in the severity of the COVID-19 disease. Despite these evidences, the exact molecular details of how PAD4 is involved in SARS-CoV-2 pathogenesis and an accurate description of the proteome of citrullinated proteins upon infection remain to be fully elucidated.

Expanding on our previous findings, here we show that *PADI4* is induced *in vitro* by different strains of SARS-CoV-2, as well as in the lungs and the brains of SARS-CoV-2-infected mice.

Furthermore, this PAD4-specific induction is associated with a significant increase in total protein citrullination in SARS-CoV-2-infected organs compared to uninfected controls. The PAD4-specific inhibitor GSK199 consistently reduces viral particle release in the cell supernatant of infected Huh7.5 and Calu3 cells, as well as viral protein production, regardless of the viral strain used. Consistently, we show that inhibiting PAD4 *in vivo*, by the i.p. injection of GSK199, significantly reduces the viral load in the lung of infected mice and the quantity of SARS-CoV-2 infectious particles in mice's nasal swabs, and this phenotype is paralleled by a significant decrease of IL-6 expression in the lungs.

Results

SARS-CoV-2 infection modulates PAD expression in vivo and in vitro

To gain a better understanding of how SARS-CoV-2 impacts PAD expression, we first reanalyzed some previously published transcriptome and proteomic raw data. Taking advantage of a COVID-19 database (<http://www.biomedical-web.com/covid19db>), we found that the *PADI4* gene was the only PAD isoform significantly upregulated upon SARS-CoV-2 infection in a whole-blood transcriptomic study from 62 prospectively enrolled patients with COVID-19 and 24 healthy controls (Suppl Table S1). Next, since the lung is the organ that is primarily affected by SARS-CoV-2 infection, we examined the data from Wang *et al.*, who used lung samples from five COVID-19 cases and four

age-matched control individuals to perform genome-wide RNA sequencing (RNA-seq) (Suppl. Table S2). In the COVID-19 group, *PADI4*, *PADI2*, and *PADI1* genes all showed differential expression (DEGs), with *PADI4* being the most overexpressed ($\text{Log}_2\text{FC} > 3$).

To understand which cell types showed higher levels of PADI gene expression during SARS-CoV-2 infection, we used a lung data collection created by Delorey *et al.* This dataset included information from approximately 1.28 million cells and nuclei from ten studies of healthy lungs and four studies of COVID-19 patients. Each cell was assigned to one of 28 types, and two different models were used to find genes whose expression was different between COVID-19 tissues and healthy tissues. Based on snRNA-seq data, we found that *PADI4* and *PADI2* were expressed differently in two cell types: neutrophils and alveolar type 2 (AT2) cells (Figure 1A). This is of particular interest as AT2 cells are the primary site of SARS-CoV-2 replication. While it is well established that the expression of PAD enzymes can be altered in neutrophils during inflammatory responses, this has not been commonly documented in AT2 cells. This suggests that, unlike the inflammation-driven regulation seen in neutrophils, the overexpression of PADI genes in AT2 cells may be directly triggered by SARS-CoV-2 replication itself.

To define whether viral replication of different SARS-CoV-2 strains can modify *PADI4* and *PADI2* expression in infected human cell lines, total RNA was collected from 2020B.1, Delta, and Omicron infected Calu3 and Huh7.5 cells and analyzed using qRT-PCR. As shown in Figures 1B-C, Delta-infected Calu3 exhibited significantly higher levels of *PADI4* and *PADI2* than mock-infected controls starting at 24 hpi. 2020B.1 and Omicron strains were also able to significantly induce *PADI4* expression, but only at later time points and at lower levels (about 3-fold upregulation) than Delta (about 12-fold upregulation at 72 hpi). Of note, in Huh7.5 cells, infection with SARS-CoV-2 Delta and Omicron strains at 48 hpi resulted in a significant upregulation of *PADI4* expression (about a 3-fold upregulation) (Sup. Figure S1A), while *PADI2* expression wasn't affected (Sup Figure S1B). Consistent with *PADI4* upregulation, electrophoresis analysis of protein lysates obtained from Calu3 (Figure 1D, left panel) infected with SARS-CoV-2 (2020B.1 strain, MOI 0.1) and incubated with the citrulline-specific probe Rh-PG showed an increase in total protein citrullination compared to mock-infected cells at both 48 and 72 hpi. To confirm these results, we performed immunoblot analysis on the very same protein extracts using an antibody recognizing citrullinated proteins (anti-CCP). Notably, at both time points after infection, this antibody mainly recognized proteins with molecular weights consistent with those observed in the Rh-PG assay (Figure 1D right panel). As expected, only SARS-CoV-2-infected cells expressed the viral spike protein (SARS-S), confirming their effective infection. In SARS-CoV-2-infected Huh7.5 cells, both Rh-PG and anti-CCP showed a similar pattern of citrullination (Sup. Figure S1C, left and right panels, respectively).

Finally, to identify which proteins were citrullinated during infection, we employed a biotinylated citrulline-specific probe (biotin-PG) to enrich the SARS-CoV-2-associated citrullinome in Calu-3 cells using streptavidin-agarose beads (Figure 1E). We found over 706 citrullinated proteins, with over 177 showing differential citrullination in SARS-CoV-2-infected cells vs. uninfected controls. The over-citrullinated proteins belong to multiple functional classes, including RNA metabolism proteins, calcium-binding proteins, chaperones, protein-modifying enzymes, translational proteins, and transporters (Sup. Figure S1D). However, the most represented protein classes are cytoskeletal proteins and metabolite interconversion enzymes. Notably, two viral proteins, the nucleoprotein (N) and the spike protein (S), were also found to be citrullinated (Figure 1E, red dots).

SARS-CoV-2 infection affects protein citrullination by modulating PAD expression in vivo.

In order to experimentally validate these data *in vivo*, K18-hACE2 transgenic mice were infected with the SARS-CoV-2 Delta strain, and lungs and brains, along with those from mock-infected animals, were collected at 4 dpi and 6 dpi in order to assess PAD mRNA and protein expression. As shown in Figure 2A, *PADI4* was significantly upregulated in the lungs of infected mice at 4dpi compared to mock-infected controls (about 4-fold overexpression), whereas infection had only a minor effect on *PADI2* expression. Similarly, *PADI4* mRNA expression was significantly increased in brain tissues of SARS-CoV-2-infected mice, albeit to a lesser extent than in the lungs (about 2-fold upregulation), while total *PADI2* mRNA expression was unaffected by the infection (Figure 2B). Interestingly, the brains of three SARS-CoV-2-infected mice—which also displayed the highest levels of *PADI4* expression—expressed less *PADI2* than the brains of the mock-infected animals. Consistently, when we next examined the effect of SARS-CoV-2 infection on PAD protein expression from pooled tissues at 6 dpi (n=6, SARS-CoV-2-infected mice; n=4, mock-infected mice, respectively), PAD4 appeared to be increased in both the lungs and the brains upon infection (Figure 2C). PAD2 expression was barely detectable in the lungs of both SARS-CoV-2-infected and mock-infected mice, but was substantially expressed in the brains of uninfected animals, as it is generally found in the central nervous system. Surprisingly, PAD2 expression was dramatically reduced in the brains following viral infection (Figure 2C).

Finally, to validate these observations, we assessed the cellular localization and expression of PAD2 and PAD4 isozymes in samples from formalin-fixed paraffin-embedded (FFPE) tissues of SARS-CoV-2-infected tissue sections from various organs that were stained with specific murine antibodies. In the brain, regions containing large clusters of SARS-CoV-2 spike protein-positive cells consistently showed a marked reduction in PAD2 signal compared to the surrounding uninfected tissue (Figure 2D). In the lungs, however, PAD2 staining was sporadic, as expected from the

literature, and spike-positive cells showed no significant variation. In contrast, PAD4 expression was found in neutrophils and macrophages in both the brain and lungs, with a higher presence in diseased tissue, particularly near blood vessels. Furthermore, spike-positive cells in the lungs tested positive also for PAD4 staining, which was less intense than in immune cells but remained distinctly visible when compared to the complete absence of staining in spike-negative cells.

Next, to identify which proteins were citrullinated *in vivo* during infection, we used the biotin-PG probe to enrich the SARS-CoV-2-associated citrullinome in mouse lungs harvested at 6 dpi with streptavidin-agarose beads. We observed a dramatic change in overall protein citrullination in SARS-CoV-2-infected organs compared to uninfected controls (Figure 2E), with more than 110 proteins differentially citrullinated. The most represented protein classes in over-citrullinated proteins were cytoskeletal proteins and metabolite interconversion enzymes (Figure 2F), as observed in Calu-3 cells. Other classes included transfer/carrier proteins, chromatin/chromatin-binding proteins, transmembrane signal receptors, and protein-binding activity modulators. Notably, the most over-citrullinated proteins were cytoskeletal proteins from the myosin family (such as MYD1 and MYD6) and the calcium channel complex AT2A1, which is an essential regulator of cytoplasmic calcium concentration. Calcium is an essential cofactor for PAD activity, and it is well known that high levels of cytoplasmic calcium are crucial for SARS-CoV-2 replication. Citrullination of AT2A1 may disrupt its function, leading to higher cytoplasmic calcium concentrations, which both sustain SARS-CoV-2 replication and activate PADs through a positive feedback loop

Collectively, our findings indicate that SARS-CoV-2 specifically induces transcriptional activation of PAD4 in both human and murine models. Moreover, the viral infection is also associated with specific PAD2 inhibition, at least in brain mouse tissues.

PAD inhibitors block SARS-CoV-2 replication in human cells

To assess whether the enzymatic activity of PADs (particularly PAD4) plays a functional role in SARS-CoV-2 replication, and whether previous observations could be instrumental to the development of innovative antiviral drugs, we first treated infected cells with different PAD inhibitors and assessed viral plaque formation in our cell models. Thus, the SARS-CoV-2 2020B1 strain was used to infect Calu3 and Huh7.5 cells (MOI 0.1) that were treated from 1 h before infection with increasing concentrations of two pan-PAD (BB-Cl and Cl-A, 0.6–5 μ M and 25–300 μ M, respectively) and two PAD4-specific inhibitors (GSK199 and GSK484, 0.25–40 μ M and 5–20 μ M, respectively). In both cell lines, continuous exposure to PAD inhibitors resulted in a dose-dependent decrease in the amount of SARS-CoV-2 infectious viral particles compared to vehicle treatments (Figures 3A and B; Sup Figure S3A and S3B). Each inhibitor had similar half-maximal inhibitory concentrations (IC₅₀)

in the two cell lines, with the exception of BB-Cl (Table 3). We also assessed cell viability using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay in cells treated under the same conditions and found that the drugs were not cytotoxic at these concentrations (Figures 3C and 3D; Sup Figures 3C and 3D). Importantly, as shown in Table 3 and based on the calculated IC₅₀ and CC₅₀, the selectivity index (SI) of the inhibitors in SARS-CoV-2-infected Calu3 and Huh7.5 were quite similar and consistently greater than 10, with the exception of BB-Cl in Huh7.5, which was much higher (100).

Finally, we used these findings to investigate the possibility that PAD inhibition might impair the replication of two other SARS-CoV-2 variants of concern (VOCs), Delta and Omicron. Delta- and Omicron-infected Calu3 and Huh7.5 cells (MOI 0.1) were treated with BB-Cl and GSK199 at lower, non-toxic concentrations, which reduced SARS-CoV-2 2020B.1 replication by at least 90% (5 μ M and 20 μ M, respectively). As shown in Figures 3E and 3F, regardless of the viral strain employed, the PAD4-specific inhibitor GSK199 was consistently effective in reducing viral particle production. The same occurred with BB-Cl, albeit to a considerably lesser extent than GSK199.

Overall, these data suggest that PAD4 activity plays an important role in SARS-CoV-2 replication regardless of the cell model used and that PAD4 could be a suitable target for coronavirus therapy.

PAD4 inhibition reduces viral genome and protein synthesis after viral entry

Having established that PAD4 was the most dramatically impacted PAD family member in the production of new SARS-CoV-2 viral particles, we sought to determine which stage of the SARS-CoV-2 life cycle PAD inhibitors could target. We first used qRT-PCR to measure SARS-CoV-2 intracellular genome in Calu3 and Huh7.5 cells treated with BB-Cl and GSK199 (5 μ M and 20 μ M, respectively) starting 1 h before infection and infected for 24 h with 2020B.1, Delta, and Omicron (MOI 0.1). Figure 4A shows that GSK199 consistently reduced viral genome replication, regardless of strain or cell line. In particular, the decrease in Huh7.5 cells was greater than 90% when compared to mock-treated cells, whereas the effect in Calu3 cells was less pronounced yet significant. A similar profile occurred also for the treatment with BB-Cl, which was overall less effective, in particular in Calu3 cells.

Consistently, immunoblot analysis of total protein extracts from the same experiments and analyzed 48 hpi revealed a complete suppression and a dramatic downregulation of both SARS-CoV-2 Spike (S) and nucleoprotein (NP) protein expression levels in GSK199- and BB-Cl-treated Huh7.5 cells, respectively, in comparison with vehicle-treated infected cells (Figure 4B). In contrast, in Calu3 the treatment with the PAD4-specific inhibitor only led to a mild decrease in viral protein levels,

while BB-Cl effectiveness was only barely detectable in comparison with vehicle-treated infected cells for all the viral strains tested.

Since it is well known that SARS-CoV-2 uses two different entry mechanisms in Huh7.5 and Calu3 cells, namely a pH-dependent or a pH-independent pathway, respectively⁴⁰, we sought to decipher whether the differences in the inhibition performances observed in the two cell lines could be explained by a different contribution of the PAD4 enzymatic activity in the two entry routes. To test this hypothesis, we first evaluated the ability of GSK199 to limit viral entry by taking advantage of a replication-competent vesicular stomatitis virus-based model—namely, rVSV-eGFP-SARS-CoV-2- Δ S₂⁴¹—along with two cellular models paradigmatic for the two entry routes: naïve VERO-E6 or TMPRSS2-expressing VERO-E6 (pH-dependent and pH-independent, respectively). GSK199 (20 μ M) and BB-Cl (5 μ M) were tested following two different treatment schedules (Figure 4D): we added the drug 1 h before infection and either washed it away prior to viral addition (pre-treatment, Pre) or removed at 2 hpi (pre-treatment + infection, Pre⁺). In both VERO-E6-based systems, we observed no difference in the number of GFP-positive cells with either the Pre- or Pre⁺-treatment protocols in cells treated with GSK199 (Figure 4E). In contrast, using the Pre⁺-treatment procedure, BB-Cl slightly reduced the number of GFP-positive cells, indicating an off-target effect potentially inhibiting viral particle entry.

Finally, we tested the ability of GSK199 to inhibit SARS-CoV-2 viral replication even when added after viral entry and continuously kept during infection (post-treatment, Post). Regardless of the viral strain, when compared to DMSO, the PAD4 inhibitor significantly reduced viral replication (Figure 4F). Of note, compared to total treatment, the post treatment was considerably less effective in reducing SARS-CoV-2 replication only during the 2020B.1 infection. In contrast, the suppression of delta and omicron replication did not change significantly between GSK199 Total and Post treatments.

The PAD4 inhibitor GSK199 impairs SARS-CoV-2 replication in vivo

Based on the previous results, showing a convincing *in vitro* antiviral efficacy for the PAD4-specific inhibitor GSK199, we then sought to evaluate whether this activity could be confirmed *in vivo*. To the best of our knowledge, there is no data on the distribution and bioavailability of PAD inhibitors in the peripheral organs of treated mice, so we first performed an analysis to determine whether GSK199 and BB-Cl could enter the lung and brain following intraperitoneal administration. To this purpose, organs from inhibitor- and vehicle-treated mice were collected and homogenized in RIPA buffer after 7 days of daily treatment. Then, the inhibitor concentrations, expressed as [μ g of inhibitor/g of organ], were semi-quantified by LC-MS/MS. As shown in (Sup Figures 5A-D), GSK199 was

found in both the lungs and the brains of all treated animals, whereas BB-Cl was detected only in the lungs and in 5 of the 6 treated animals.

To evaluate the antiviral activity of the PAD4 inhibitor *in vivo*, K18-hACE2 transgenic mice were infected with the SARS-CoV-2 Delta strain and daily treated intraperitoneally with GSK199 (30mg/Kg), BB-Cl (1mg/Kg), or with vehicle starting from 1 day before infection (Figure 5A). At 4 dpi, when viral loads and histopathological changes were expected to be most visible, the animals (n=10/group) were sacrificed, nasal swabs were performed, and lungs and brains were collected. The organs of four animals in each group were formalin-fixed and paraffin-embedded for H&E and histopathological analyses, while the remaining animals (n=6) had their tissues processed for RNA and protein extraction. Total RNA was then used for ddPCR analysis to evaluate the absolute viral load in the lungs after treatment. As shown in Figure 5B, the GSK199-treated group had a significantly lower lung viral load than the vehicle-treated group (approximately a 100-fold reduction at 4 dpi, 6×10^4 vs. 6×10^2 , respectively). In addition, compared to mice treated with the vehicle, GSK199-treated mice had significantly fewer SARS-CoV-2 infectious particles in their nasal swabs, as demonstrated by a standard titration assay (Figure 5C). Similar effects were observed with BB-Cl treatment, although it was less effective in reducing SARS-CoV-2 replication, as previously reported in antiviral assays conducted in cell lines. Consistent with these findings, immunohistochemistry for viral N protein on lung tissues revealed a significantly reduced number of positive cells only in GSK199-treated animals compared to vehicle-treated mice (Figure 5D; extended data Table 4). Moreover, examination of hematoxylin and eosin-stained tissues revealed evidence of diffuse alveolar damage with progressive alveolar, interstitial, and blood vessel lesions characterized by edema, inflammation, and focal cytomegaly in some alveolar lining cells. All of these parameters were scored (Table 5) and, as shown in Figure 5E, the total cumulative score was reduced by treatment with both the pan-PAD or the PAD4-specific inhibitor.

Since several studies in the literature have revealed the presence of SARS-CoV-2 in the brains of mice following intranasal infection (ref), we evaluated the antiviral activity of PAD inhibitors in this tissue. As shown in Figure 5F, brain viral load showed extremely high variability in all groups. However, when the samples were categorized into two groups based on viral load, with one group exhibiting low viral load (below 10^4) and the other high viral load (above 10^6), we observed that only the samples from GSK199-treated mice were significantly more likely to fall into the low viral load group than those from vehicle-treated mice. Given that we have previously demonstrated that GSK199 may pass the blood-brain barrier, these findings suggest that the drug may be effective in inhibiting viral dissemination and replication within the brain. Immunohistochemical analyses for viral N protein (Figure 5G, Table 6) and by hematoxylin-eosin staining (Figure 5H, Table 7)

confirmed a similar pattern. Interestingly, mice treated with BB-Cl appear to be partially protected from brain infection, even though the drug is unable to reach the organ (Figure 5F-H). This suggests that treatment with BB, while not able to act at the brain level by directly inhibiting viral replication in this tissue, can prevent infection of the organ.

PAD inhibition modulates inflammation and protects against SARS-CoV-2-induced pathological changes

Several studies have shown that a cytokine and chemokine storm, characterized by an atypical release of PDGF, VEGF, IL-6, IL-8, IL-10, TNF- α , IFN- α , and IFN- β , maintains the deleterious systemic inflammatory response thought to be the primary cause of ARDS (Acute Respiratory Distress Syndrome) in COVID-19 patients. Furthermore, an impaired type I interferon response has been observed in COVID-19 followed by increased circulating levels of IL-6 and TNF- α . Additionally, Neufeld et al. (REF) found that SARS-CoV-2 infection of Calu3 cells induces a pro-inflammatory cytokine response. Based on these premises, we sought to test whether PAD inhibitors may modulate this inflammatory storm in our models.

As shown in Figure 6A, GSK199 and BB-Cl were able to significantly reduce IL-6 and TNF- α RNA expression in Calu3 cells after SARS-CoV-2 Delta strain infection. Consistently, in SARS-CoV-2-infected K18-hACE2 mice, IL-6 mRNA expression at 4 dpi was significantly reduced in the lungs and brains from GSK199-treated compared to the vehicle-treated group, while TNF- α RNA level was only slightly affected by the PAD4 inhibitor in the brains of this *in vivo* model (Figures 6B and C).

To further investigate the protective effects of PAD inhibition against the pathological changes induced by SARS-CoV-2 infection, we employed mass spectrometry analysis to examine the protein citrullinome in the lungs of infected mice at 4 dpi, comparing them to those treated with PAD inhibitors (using the same animals as in Figure 5A). In agreement with the lung citrullinome analysis at 6 dpi (Figure XX), we observed a dramatic alteration in the citrullinome of SARS-CoV-2-infected mice compared to uninfected controls, with over 270 differentially citrullinated proteins (Figure 6D). Among the most over-citrullinated proteins, we identified cytoskeletal proteins (such as MYH1) and the calcium transporter AT2A1, further confirming the pivotal role of citrullination of these proteins, irrespective of the post-infection time point. As shown in Figures 6 E and F, treatment with PAD inhibitors significantly attenuated the over-citrullination induced by the infection. Specifically, AT2A1 and MYH1 were down-citrullinated in the lungs of infected mice treated with BB-Cl and GSK199, compared to vehicle-treated controls (Figure 6E), suggesting a mock-like citrullination profile in the presence of PAD inhibitors. Notably, among the over-citrullinated proteins

during infection, we observed proteins such as FABP4, S100A6, and TTR, which have been implicated in inflammatory diseases such as rheumatoid arthritis and other autoimmune disorders, where their citrullination plays a crucial role in the dysregulation of immune responses and disease pathogenesis. Additionally, infection at 4 dpi led to an increase in the amount of citrullinated PPIA (also known as cyclophilin A), which is associated with pulmonary fibrosis. Treatment with the specific PAD4 inhibitor was capable of preventing the over-citrullination of these proteins induced by SARS-CoV-2 (Fig. 6G).

Discussion

In the last five years, SARS-CoV-2 infection has sparked significant interest in viral pathogenesis and host immune responses, particularly concerning protein modifications. Recent studies have suggested that one such modification, citrullination, may play a role in the host's response to SARS-CoV-2, although the underlying mechanisms and biological implications remain incompletely understood.

In this study, we demonstrate the significant role of PAD4 in the pathogenesis of SARS-CoV-2 infection, providing novel insights into how post-translational modifications, particularly citrullination, contribute to viral replication and the associated inflammatory response. Our findings confirm that SARS-CoV-2 infection, across multiple strains (2020B.1, Delta, and Omicron), induces a robust upregulation of PADI4 in all tested human cell lines and murine tissues, including both the lungs and brain. The upregulation of PADI4, especially in pulmonary epithelial cells (Calu-3), is of particular interest. These cells serve as the primary site of SARS-CoV-2 replication, and their increased susceptibility to elevated PADI4 expression suggests a direct effect of viral replication on PAD expression, independent of the inflammation-driven regulation observed in other immune cells. Although all three strains induced significant PADI4 upregulation, the greater fold increase observed with the Delta variant may indicate strain-specific mechanisms of viral-host interaction that could influence disease outcomes, including the potential for more severe disease manifestations. The key role of PAD4 during SARS-CoV-2 replication is further supported by the potent antiviral activity of its specific inhibitor, GSK199, which significantly reduces viral replication both *in vitro* and *in vivo*.

In our study, we observed a significant downregulation of PADI2 in the brains of SARS-CoV-2-infected mice, a phenomenon that appears to be specific to this organ, as it is not observed to the same extent in the lungs. The reduction in PAD2 expression in the brain is of particular interest because PAD2 is the isoform most highly expressed in the central nervous system (CNS), where it plays a

critical role in regulating processes such as gene expression, neuronal plasticity, and inflammation. Downregulation of PAD2 has been linked to several neuroinflammatory and neurodegenerative conditions. For example, in multiple sclerosis, reduced PAD2 activity has been associated with disease progression, as citrullination of key proteins (such as those involved in the neuronal cytoskeleton) is essential for maintaining synaptic function and reducing chronic inflammation. The downregulation of PAD2 could therefore exacerbate both inflammation and neurodegenerative damage, processes that underlie the progressive neurological disability seen in many neurodegenerative diseases and the neurological symptoms observed in COVID-19 patients.

Moreover, the data presented in this study provide new evidence suggesting that citrullination of host proteins plays a significant role during SARS-CoV-2 infection. Our results demonstrate that infection with SARS-CoV-2 induces increased citrullination of specific proteins, indicating that the virus may modulate this post-translational modification to enhance its replication and/or alter the host's immune responses. Intriguingly, one of the most over-citrullinated proteins is AT2A1, an ATPase responsible for calcium reuptake into the endoplasmic reticulum. Recent studies have shown that elevated cytoplasmic calcium concentrations in infected cells are crucial for SARS-CoV-2 replication. In this context, De Antonellis et al. demonstrated that SARS-CoV-2 inhibits AT2A1 transcription, playing a key role in promoting viral replication by maintaining high cytoplasmic calcium concentrations. Similarly, citrullination of AT2A1 could impair the function of the ATPase pump, reducing its ability to remove calcium from the cytoplasm, thereby sustaining viral replication. Moreover, since PAD enzymes are calcium-dependent, this mechanism would act in a positive feedback loop, inducing high PAD activity.

In addition to AT2A1, we identified an increase in citrullination of proteins involved in immune functions, such as inflammatory responses and interactions between immune cells and extracellular matrix proteins. This modulation could have significant implications for the pathogenesis of COVID-19. Indeed, it is known that citrullinated proteins may act as danger-associated molecular patterns (DAMPs), attracting immune cells and potentially promoting the development of chronic inflammation. An intriguing aspect emerging from our findings is that SARS-CoV-2 infection appears to induce citrullination in proteins that are specifically involved in the onset of inflammatory and autoimmune diseases (e.g., FABP4, S100A6, TTR, PPIA). These changes could contribute to both acute symptoms and long-term complications, such as Long COVID. Notably, PAD inhibitors prevent proteins over-citrullination, restoring a protein citrullination profile similar to that of healthy tissue.

Regarding the therapeutic potential of PAD inhibitors, our work demonstrates that after intraperitoneal injection, the PAD4 inhibitor GSK199 is able to cross the blood-brain barrier and act within the central nervous system. In contrast, the pan-PAD inhibitor BB-Cl-amidine remains confined to other tissues, such as the lungs, and is unable to cross the blood-brain barrier.

In conclusion, our findings highlight the critical role of citrullination in SARS-CoV-2 infection, with significant implications for immune responses and the potential onset of autoimmune diseases. Further investigation could deepen our understanding of COVID-19 pathogenesis and pave the way for novel therapeutic strategies to address the long-term complications of the infection. Notably, our study underscores the therapeutic potential of PAD4 inhibition, demonstrating its dual benefit in reducing viral replication and curbing the production of citrullinated proteins involved in the etiology of inflammatory diseases.

Materials and methods

Ethics approval statement

All experiments with SARS-CoV-2 active viral particles were performed in a BioSafety Level-3 (BSL-3) laboratory. Nasal pharyngeal swabs were collected upon approval of the Local Ethics Committee and signature of the informed consent. The Fondazione Ca' Granda, Ospedale Maggiore, Milano, Italy, protocol No. 456_2020 in May 2020.

Cell lines and viruses

African green monkey kidney Vero-E6 cells (ATCC®-1586), VERO-E6 TMPRSS2 cells (Kindly provided by John Hiscott, Pasteur Institute Rome), Calu3 cells (ATCC®- HTB-55) and Huh7.5 (RRID:CVCL_7927) cells were propagated in Dulbecco's Modified Eagle Medium (DMEM; Sigma) supplemented with 1% (v/v) penicillin/streptomycin solution (Euroclone) and heat-inactivated 10% (v/v) fetal bovine serum (FBS) (Sigma).

SARS-CoV-2 strains were isolated from positive nasal-pharyngeal swabs. The isolated SARS-CoV-2 strains belong to the B.1 lineage (SARS-CoV-2/human/ITA/Milan-UNIMI-1/2020, Gen Bank accession ID: MT748758.1), B.1.617.2 (GISAID EPI_ISL_6602889) and B.1.1.529, BA.1 (EPI_ISL_10898045). B.1 lineage carries the characteristic spike mutation D614G and is the large European lineage, the origin of which roughly corresponds to the Northern Italian outbreak in early 2020. The other two strains are parental lineages categorized as “variants of concern” (VOC) that were relevant to the epidemiological situation [<https://www.ecdc.europa.eu/en/covid-19/variants-concern>].

The replication-competent vesicular stomatitis virus rVSV-eGFP-SARS-CoV-2-SΔ21 was a kind gift from Sean P.J. Whelan (Washington University School of Medicine, USA) (doi: 10.1016/j.chom.2020.06.021).

In the in vivo experiments the SARS-CoV-2 Delta isolate used (hCoV-19/Netherlands/NH-RIVM-27142/2021_P2) was obtained from the European Virus Archive goes Global (EVAg) platform. Viral stocks were prepared by propagation in Vero E6 cells in DMEM supplemented with 2% FBS.

All experiments involving live SARS-CoV-2 were performed in accordance with the Italian Ministry of Health guidelines for Biosafety Level 3 (BSL-3) containment procedures in the approved (si dice approved??) laboratories of the Molecular Medicine Department of the University of Padova.

Reagents and treatments

The PAD inhibitors Cl-A, BB-Cl, GSK199 were purchased from Cayman Chemical (Ann Arbor). All the compounds were solubilized in DMSO according to the manufacturer's instructions. Immediately before use, the inhibitors were diluted in the culture medium to the working concentrations.

Cell viability assay

Cells were plated at 70% confluence in 96-well plates. After 24 h, cells were treated with different PAD inhibitors at the indicated concentrations or with an equal volume of the vehicle alone (DMSO). After 72 h, cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT, Sigma). Briefly, MTT solutions from the Stock (final concentration 500 µg/ml) was added to the cell medium and cells were incubated in CO₂ incubator in the dark for 2 hrs. The medium was removed and Formazan crystals formed by the cells were dissolved using 50 µl of DMSO. The absorbance was read at 570 nm using 630 nm as reference wavelength on a Victor X3 Multilabel Plate Reader (Perkin Elmer, Waltham, MA, USA; wallac 1420 work station software).

In vitro antiviral assay

Cells were cultured in 24-well plates for 1 day and then, 1 h before infection, pre-treated with PAD inhibitors or vehicle alone at the indicated concentrations. Subsequently, cells were infected with SARS-CoV-2 strains at the indicated multiplicity of infection (MOI). Following virus adsorption (2 h at 37°C) and viral inoculum removal, new medium with fresh PAD inhibitors or DMSO alone was added to the plates and kept until sample harvesting at the indicated time. The extent of SARS-CoV-2 replication was assessed by titrating the infectivity of supernatants using quantitative real-time RT-PCR plaque assay and/or plaque assay conducted as described previously (Parisi OI et al., 2021).

Quantitative real-time RT-PCR (viral load)

Absolute quantification of SARS-CoV-2 copy numbers in cell supernatants was evaluated via specific real time RT-PCR of the N1 gene, according to the protocols "Coronavirus disease (COVID-19)

technical guidance: Laboratory testing for 2019-nCoV in humans” (WHO, 2020) and “CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel” (CDC, 2020), available at: <https://www.who.int/emergencies/diseases/novel-coronavirus-2019/technical-guidance/laboratory-guidance> and <https://www.fda.gov/media/134922/download>), respectively. Briefly, the run was performed by means of AgPath-ID One-Step RT-PCR assay (ThermoFisher, Waltham, MA, USA), using the 7500 Real Time PCR system (ThermoFisher Scientific, Waltham, MA, USA). The primer and probe sequences, targeting N1 gene, have been published by CDC and WHO [2,3]. The reaction mix was conducted in a final volume of 25 μ L, containing 1 $\times$ RT-PCR buffer (2 $\times$), 0.4 μ M of each primer, 0.1 μ M of probe, 1 $\times$ RT-PCR Enzyme mix (25 $\times$), and 5 μ L of heat-inactivated cell medium. The standard curve was constructed using a serially diluted plasmid pEX-A128-nCoV_all (Eurofins, Luxemburg), containing part of the SARS-CoV-2 genome (3×10^7 – 3×10^1 copies/ μ L). The limit of detection was three copies per reaction.

Plaque assay

Vero-E6 cells were plated the day before in a 6-well plate in complete medium (7.5×10^5 /well). Briefly, after 2 h of inoculation with supernatants (500 μ L/well), the inocula were removed, and the cells were covered with a 0.3% agarose gel, dissolved in complete medium, and incubated for 48 h at 37°C with 5% CO₂. Cells were then fixed with 4% formaldehyde solution and, after agarose removal, stained with 0.5% methylene blue. Plaques were counted, and the results were expressed as plaque forming units for milliliter (PFU/mL).

RNA isolation and comparative RT-PCR

Total RNA was extracted from cell and tissue samples using the TRI Reagent solution (Sigma-Aldrich) and quantify by NanoDrop Spectrophotometers. Then, 1 μ g of total RNA were retrotranscribed using the sensiFAST cDNA synthesis kit (BIO-65054, BIOLINE) according to the manufacturer’s instructions. Comparison of mRNA expression between treated and untreated samples was performed by SYBR green-based real time RT-qPCR by a Biorad CFX96 apparatus (Stratagene, San Diego, USA) using the following primers (5’-3’): N1_SARS-CoV2_FW GACCCCAAATCAGCGAAAT, N1_SARS-CoV2_RV TCTGGTACTGCCAGTTGAATCTG, hPGK1_FW CAAGAAGTA TGCTGAGGCTGTCA, hPGK1_RV CAAATACCCCAACAGGACCA, hPAD4_FW CTA CTCTGACCAAGAAAGCC, hPAD4_RV ATTTGGACCCATAACTCGCT, hPAD2_FW ACCTCCTCAGCCTCCCC, hPAD2_RV CCTACCTCTGGACCGATGTC, hIL-6_FW ACCGGGAACGAAAGAGAAGC, hIL-6_RV CTGGCAGTTCCAGGGCTAAG, hTNF α _FW GCCAGAGGGCTGATTAGAGA, hTNF α _RV TCAGCCTCTTCTCCTTCCTG, mACTIN_FW CCCAAGGCCAACCGCGAGAAGAT, mACTIN_RV GTCCCGGCCAGCCAGGTCCAG, mPAD4_FW

CTCCATCCAAGAAGAGTACC, mPAD4_RV GAGTCTTCGTGCTTAGGGTC, mPAD2_FW TCCCCAAGCAACTCCTG, mPAD2_RV TCATGTTACCATGTTAGGG, mIL-6_FW GAGGATACCACTCCCAACAGACC, mIL-6_RV AAGTGCATCATCGTTGTTTCATACA, mTNF α _FW GAACTGGCAGAAGAGGCACT, mTNF α _RV AGGGTCTGGGCCATAGAACT

The reaction conditions were as follows: 2 min at 95°C, followed by 40 cycles of 5 sec at 95°C and 1 min at 60°C.

Droplet Digital PCR (ddPCR)

SARS-CoV-2 genomic RNA was quantified using the QX200 Droplet Digital PCR System (ddPCR, Bio-Rad, Hercules, CA, USA). One-Step RT-ddPCR Advanced Kit for Probes (Bio-Rad, #1864021) and the SARS-CoV-2 Droplet Digital PCR Kit (Bio-Rad, Hercules, CA, USA) were used following the manufacture instruction. SARS-CoV-2 quantification was expressed as a copy number/ μ g of total RNA.

Western blot analysis.

Whole-cell protein extracts were prepared with RIPA buffer, quantified by Bradford method, and subjected to immunoblotting. Briefly, an equal amount of cell extracts was fractionated by electrophoresis on SDS-polyacrylamide gels and transferred to Immobilon-P membranes (Merck Millipore, Burlington, MA, USA). After blocking with TBS (Tris buffered saline containing 0.05% Tween 20) containing 5% milk, membranes were incubated overnight at 4°C with the appropriate primary antibodies. In this study, primary antibodies used includes anti-CCP (Biorbyt, orb10262), anti-PAD2 (Proteintech, 12110-1-AP), anti-PAD4 (Proteintech,17373-1-AP), anti-actin (Sigma-Aldrich, A2066), anti-Nucleoprotein(N)_SARS-CoV-2 (GeneText, 3851), anti-Spike(S)_SARS-CoV-2 (GeneText, 1A9). Membranes were then washed and incubated for 2 h at room temperature with secondary antibodies: anti-rabbit/mouse IgG horseradish peroxidase linked species -specific whole antibody (Amersham, Merck Life Science S.r.l., Milan, Italy). Proteins were visualized using the Biorad Chemidoc Imaging System, and enhanced chemiluminescence detection kit (Thermo Fisher Scientific, Waltham, MA, Stati USA). Scanning densitometry of the bands was performed using Image Lab (version 4.6.9; Bio-Rad Laboratories S.r.l., Segrate, Italy).

Detection of citrullination with rhodamine-phenylglyoxal (Rh-PG).

Equal amounts of protein were diluted with 80% trichloroacetic acid and incubated with Rh-PG (Cayman Chemical, Ann Arbor) at the final concentration 0.1 mM for 30 minutes. The reaction was quenched with 100 mM L-citrulline, then centrifuged at 21100 xg for 10 minutes and washed with ice cold acetone and resuspended in 2X SDS loading dye for gel electrophoresis. Gels were imaged

(excitation=532 nm, emission=580 nm) using a Biorad Chemidoc Imaging System, stained with brilliant blue G-colloidal solution (Sigma-Aldrich, Milan, Italy).

Neutralization assay.

VERE E6 and VERO E6-TMPRSS2 were treated with GSK199 (20 μ M) and BB-Cl (5 μ M). After 1h of pretreatment the medium was removed and new medium without PAD inhibitors but with rVSV-SARS-CoV-2-SΔ21 at an MOI of 0.05 were added to cells in 96-well plates and incubated at 37 °C for 24 h. Subsequently, cells were fixed in 4% formaldehyde (Millipore Sigma) containing DAPI for 15 min on ice. Images were acquired using Operetta (Perkin Elmer) in both the DAPI and GFP channels to visualize nuclei and infected cells (i.e., eGFP-positive cells). Images were segmented using the following building blocks: find nuclei, find cytoplasm, calculate GFP intensity, select population (GFP+ cells). Segmentations were then analyzed by counting the GFP+ cells out of the number of nuclei, using the Harmony 4.5 software. Acquisitions were further processed to calculate the ID50—the reciprocal dilution inhibiting 50% of the infection—using the Columbus software (Perkin Elmer).

Animal studies

Transgenic B6.Cg-Tg(K18-ACE2)2PrImn/J mice were purchased from The Jackson Laboratory and bred at the Specific Pathogen-Free animal facility of IOV-IRCCS. Before the experiment, six- to 8-week-old K18-hACE2 mice were acclimatized within the Biosafety Level 3 (BSL-3) facility for 72 hours.

In the first experiment, mice were randomly distributed in four groups (n=6 each) and challenged intranasally with 10 μ l of either phosphate bufer saline (PBS) (n=6, mock-infected), or 10 μ L of 1×10^5 PFU SARS-CoV-2 Delta strain (n =12, SARS-CoV-2 infected). Throughout the experimental period, daily monitoring of body weight and physiological conditions was conducted until sacrifice at 4 or 6 days post-infection (dpi). At 4dpi organs were collected and homogenized (Milteny, gentleMACS™ Octo Dissociator with Heaters) with Trizol (Invitrogen) for RNA extraction. At 6 dpi the right lobe of the lung and the right hemisphere of the brain were fixed in 10% buffered formalin for histopathology evaluation (multiplex IHC), while the left lobe and the left hemisphere were homogenized with RIPA buffer for protein extraction.

In the second experiment, one day prior to infection (pre-treatment), BB-Cl-Amidine (1 mg/kg) or GSK199 (30 mg/kg) or the vehicle solution (PBS 10% DMSO) were injected intraperitoneally (ip). Treatments were administered daily for 5 days. At day 0, mice were intranasally infected with 10 μ L of SARS-CoV-2 Delta strain at 1×10^5 PFU. Throughout the experimental period, daily monitoring of body weight and physiological conditions was conducted until sacrifice. Euthanasia was performed 4 dpi and oropharyngeal swabs, lungs and brains were collected. The Lungs and the brains of 4

animals each groups were fixed in 10% buffered formalin for histopathology evaluation, while hole lungs and brains of 6 animals each groups were homogenized (Miltény, gentleMACS™ Octo Dissociator with Heaters) with Trizol (Invitrogen) for RNA extraction and protein extraction following the manufacture instruction.

All procedures involving the animals and their care adhered to institutional guidelines that comply with national and international laws and policies (D.L. 26/2014 and subsequent implementing circulars). The experimental protocol (Authorization n. 355/2021-PR) received approval from the Italian Ministry of Health.

SARS-CoV-2 recovery from nasopharyngeal swabs and titration

Immediately upon collection, the swabs were submerged in FBS-free DMEM and refrigerated. The medium was subsequently employed to infect Vero E6 cells (9×10^4 cells/well in a 24 wells plate) for 1 h. The infectious medium was then replaced with fresh 10% FBS DMEM. The supernatants were collected 48 h post infection and titrated. Briefly, Vero E6 cells were seeded in 24-well plates at a concentration of 9×10^4 cells per well. On the following day, serial dilutions of the viral stocks or of the tested supernatants were performed in serum-free DMEM media. Following a one-hour incubation at 37°C, the overlay media was added to the inoculum, resulting in a final concentration of 2% (v/v) FBS/DMEM media and 0.6% (v/v) methylcellulose (Merck Life Science, Cat: M0512). Subsequently, the samples were incubated at 37 °C for 48 h and fixed using 5% formaldehyde in phosphate-buffered saline (PBS) (Merck Life Science, Cat: 252549). The plaques were then visualised using a solution of crystal violet (20% ethanol, Merck Life Science, Cat: C6158).

Histology and Immunohistochemistry.

Four µm lung and brain sections were examined after staining with Haematoxylin and Eosin (HE) by a veterinary pathologist and by a board-certified veterinary pathologist. Histological features were evaluated as percentage of tissue involvement and scored blindly as follows: 0, no tissue involvement (no findings); 1, up to 25% of involved tissue (mild); 2, up to 50% of involved tissue (moderate); 3, up to 75% of involved tissue (severe); 4, up to 100% of involved tissue (massive). Finally, a total score per animal was obtained by combining cumulative scores of lesion types or anatomical compartments.

For SARS-CoV-2 antigen detection, slides were incubated with blocking reagent (5% Bovine serum albumin (BSA) for 20 min, at room temperature) followed by rabbit poloclonal antibody against SARS-CoV2 N protein (1:300 in PBS, overnight, at RT), then endogenous peroxidases were blocked by EnVision FLEX Peroxidase-Blocking Reagent (3 min, at RT). As secondary antibody was used Goat anti-Rabbit IgG (H+L) Secondary Antibody, HRP, (1:500 for 60 min, at RT), then EnVision FLEX DAB + Chromogen (1 drop/ml of substrate buffer) and EnVision FLEX Substrate buffer were

used for chromogen reaction. Tissues were examined and scored in a post-examination method of masking by a boarded experimental pathologist.

Liquid-liquid extraction of inhibitor from lung and brain homogenates

Stock DMSO solutions of both inhibitors (20 mM) were diluted in a 20 mL volumetric flask to prepare a 5000 nM working solution containing both analytes using H₂O LC-MS grade. Calibrator solutions ranging from 1-50 nM were prepared by diluting the working solution with H₂O LC-MS grade. To create a matrix-matched calibration curve and mimic analyte loss during liquid-liquid extraction, calibrators were spiked with tissue homogenate blanks and subjected to the same extraction procedure as the study samples.

The liquid-liquid extraction protocol is as follows: mix 50 μ L sample or blank matrix aliquot with 50 μ L calibrator or H₂O neat solvent for study samples, 50 μ L 10 mM NaOH, 5 μ L saturated NaCl solution, 20 μ L 5.5% w/v ZnSO₄ solution, and 400 μ L ethyl acetate (organic phase). Agitate the biphasic system for 10 minutes to maximize analyte transfer to the organic phase. Separate the emulsion using a benchtop centrifuge at 13,000 rpm for 5 minutes. Transfer 200 μ L of the organic phase to microtubes. Repeat the procedure three times to collect a total of 600 μ L of the organic phase in each microtube. Dry the extracts using an evaporator centrifuge set to 40°C under vacuum for 1 hour. Reconstitute the dried extracts with 50 μ L of ACN:H₂O (1:1) and analyze using LC-MS/MS. This procedure is optimized to enhance separation, considering the basic groups in both GSK199 and BB-Cl. Samples were stored in the Vanquish Duo UHPLC system's autosampler at 15°C during the analytical run.

Inhibitor concentrations were converted from nM to μ g of inhibitor per g of organ. The data were processed using the MetaboAnalyst web platform. All data were normalized using logarithmic transformation to achieve a normal distribution. Subsequent statistical analysis involved heatmaps to depict the abundance of the inhibitor across the study samples.

LC-HRMS Analyses

A Q-Exactive Plus UHMR Hybrid Quadrupole Orbitrap Mass Spectrometer coupled with a Vanquish Duo UHPLC system (Waltham, MA, USA) was employed for LC-MS/MS analysis. Chromatographic separations were executed on a Luna® Omega 5 μ m Polar C18 100 Å, LC 150 x 2.1 mm column (maintained at 45°C) and safeguarded by a C18 SecurityGuard (Phenomenex srl, Castel Bolognese, Italy). Aliquots (10.0 μ L) of homogenate extracts were injected and eluted with a mobile phase (flow rate 0.3 mL/min) consisting of A: H₂O 0.1% FA v/v, and B: ACN 0.1% FA v/v. The gradient elution profile was: 0.00-8.25 min [5-75% B], 8.25-9.25 min [75% B], 9.25-9.50 min

[75-5% B], 9.50-12.5 min [5% B], for a total runtime of 12.5 min including re-equilibration. Autosampler temperature was maintained at 15°C.

The eluate was introduced into the electrospray ion source, and MS data were acquired and processed using Xcalibur software. Operating conditions for the Orbitrap mass spectrometer were: ESI ionization (+/-), spray voltage 3.50 kV, capillary temperature 300°C, sheath gas flow (N₂) 45 L/min, sweep gas flow (N₂) 1 L/min, auxiliary gas flow (N₂) 10 L/min, auxiliary gas temperature 350°C, maximum spray current 100 µA, probe positioning settings: C, side-to-side position set to 0, micrometer set to 1.75. Data were acquired in Full MS-SIM (+) within the scan range 100-1500 m/z, resolution 70,000, AGC target 3e6, maximum IT 200 ms; PRM (+) settings were resolution 35,000, AGC target 1e5, maximum IT 120 ms, loop count 1, MSX count 1, isolation window 4.0 m/z. Inclusion list parameters were as follows: GSK199 [M+H⁺:433.23465 m/z], ce 28; BB-Cl [M+H⁺:460.18986 m/z], ce 28. Calibration curves were plotted using 1/x weighting linear regression via Xcalibur® software.

Citrullinome analysis by mass spectrometry: sample preparation.

Sample preparation in technical triplicates followed the procedure outlined in [42]. Equal amounts of cell lysates from each experimental group (500 µg) were diluted in buffer (100 mM HEPES pH 7.6) to a final concentration of 1 µg/µL and incubated with 20% trichloroacetic acid (TCA) and 0.5 mM biotin-PG [43] for 30 min at 37 °C. Labeled proteomes were precipitated on ice for 30 min. Samples were pelleted through tabletop centrifugation (21,100xg, 15 min) at 4 °C. The supernatants were discarded, and the pellets were washed with cold acetone (300 µL). After drying for 5 min, the pellets were resuspended in 1.2% SDS in PBS by bath sonication and heating. Samples were then transferred to 15 mL screw cap tubes and diluted in 1X PBS to a 0.2% SDS final concentration. Samples were incubated with streptavidin agarose slurry (Sigma Aldrich, 170 µL) overnight at 4 °C and for an additional 3 h at 25 °C. After discarding the flow through, the streptavidin beads were washed with 0.2% SDS in PBS (5 mL) for 10 min at 25 °C. The beads were then washed three times with 1X PBS (5 mL) and three times with water (5mL) to remove any unbound proteins. Beads were then transferred to a screw cap microcentrifuge tube and heated in 1X PBS with 500 µL 6 M urea and 10 mM DTT (65 °C, 20 min). Proteins bound to the beads were then alkylated with iodoacetamide (20 mM, 37 °C for 30 min). The beads were successively pelleted by centrifugation (1,400 x g for 3 min) and the supernatant was removed. The pellet was suspended in a premixed solution of 2 M urea, 1 mM CaCl₂ and 2 µg Trypsin Gold (Promega, Madison, USA) in PBS. These were shaken overnight at 37 °C. The supernatant was collected, and the beads were washed twice with water (50 L), each time collecting the supernatant. The fractions were combined, acidified with formic acid (5% final concentration) and stored at -20 °C until use.

Mass spectrometry

Liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) analysis was performed with an LTQ-Orbitrap Discovery mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) coupled to an Easy-nLC HPLC (Thermo Fisher Scientific, Waltham, MA, USA). Samples were pressure loaded onto a 250- μ m fused-silica capillary hand packed with 4-cm Aqua C18 reverse phase resin (Phenomenex). Samples were separated on a hand packed 100- μ m fused-silica capillary column with a 5- μ m tip packed with 10 cm Aqua C18 reverse phase resin (Phenomenex). Peptides were eluted using a 10-h gradient of 0-100% Buffer B in Buffer A (Buffer A: 95% water, 5% acetonitrile, 0.1% formic acid; Buffer B: 20% water, 80% acetonitrile, 0.1% formic acid). The flow rate through the column was set to ~400 nL/min, and the spray voltage was set to 2.5 kV. One full MS scan (FTMS) was followed by 7 data-dependent MS2 scans (ITMS) of the *n*th most abundant ions. The tandem MS data were searched by the SEQUEST algorithm using a concatenated target/decoy variant of the human and viral UniProt database. A static modification of +57.02146 on cysteine was specified to account for alkylation by iodoacetamide. SEQUEST output files were filtered using DTASelect 2.0.

Database Search.

Raw data were processed and searched using Maxquant 1.6.14 and its integrated Andromeda search engine using the Swiss-Prot human (XXXXXX) and Uniprot SARS-CoV-2 (XXXXXX). Search parameters were as follows: tryptic digestion with up to 2 missed cleavages; peptide N-terminal acetylation, methionine oxidation, N-terminal glutamine to pyroglutamate conversion were specified as variable modifications. The monoisotopic mass increment of the triplex dimethyl labels, light, medium and heavy dimethyl label at 28.0313, 32.0564 and 36.0757 Da, respectively, were set as variable modification on the peptide N-termini and lysine residues. Carbamidomethylation of cysteines was set as static modification. Main search tolerance was 6 ppm, and the first search tolerance was 50 ppm. Both the protein and peptides identification false discovery rates (FDR) were < 1%. Protein grouping, dimethyl ratio calculations and downstream statistics were performed in Scaffold Q+S 4.8.9 (Proteome Software, Portland, OR).

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Figures

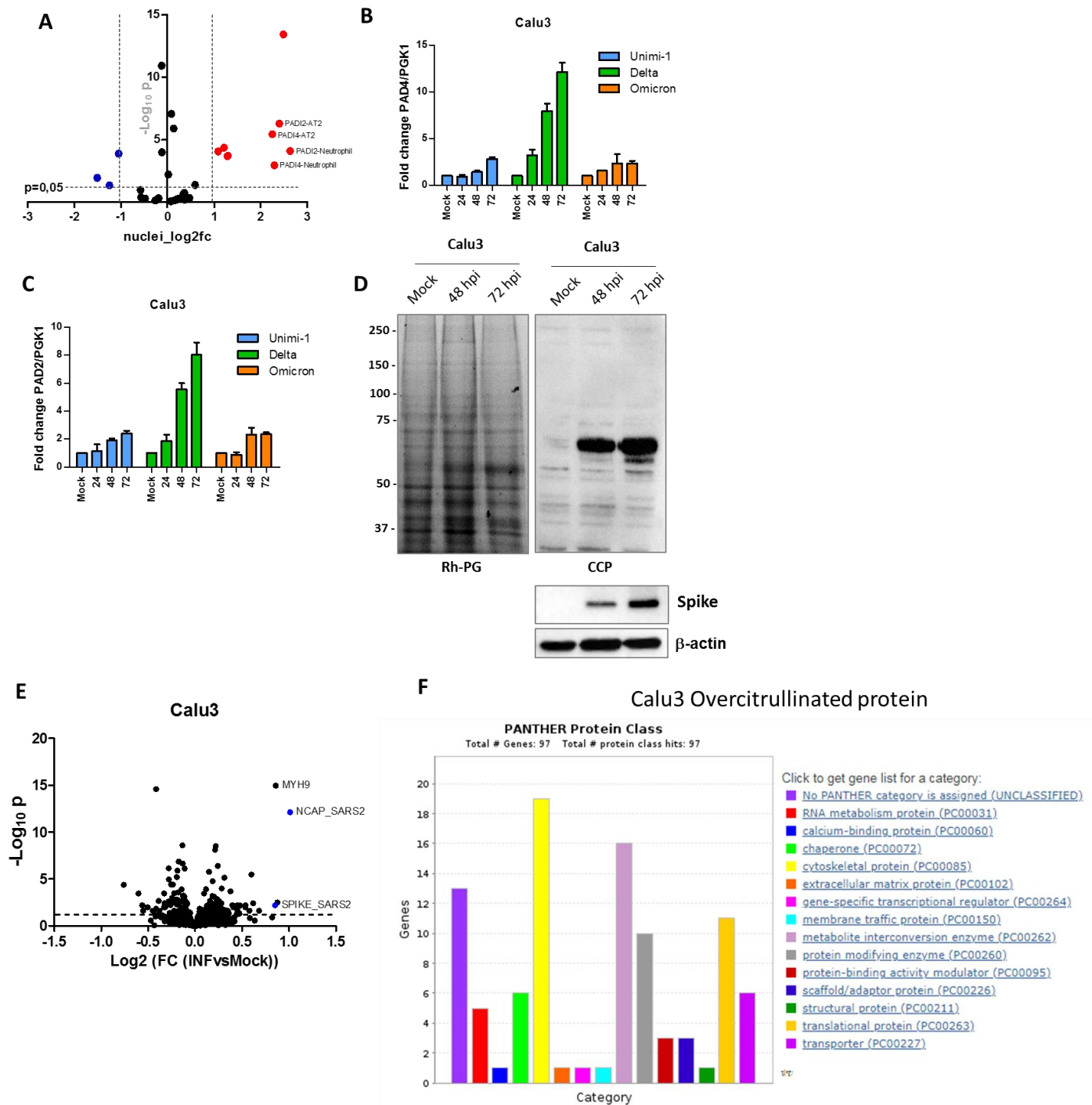
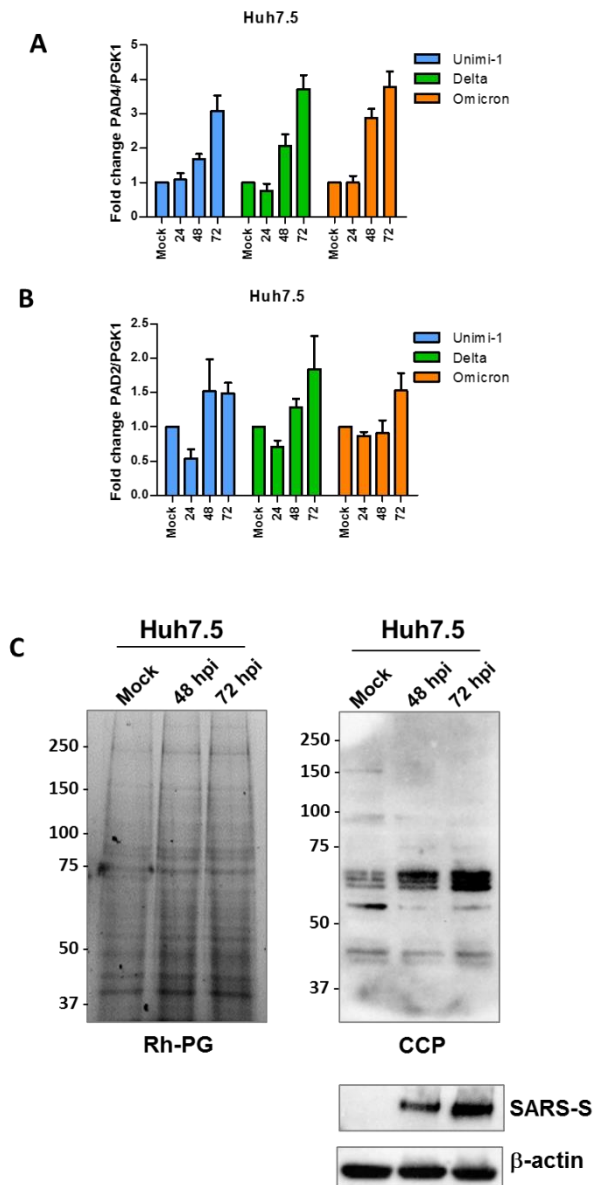


Fig. 1 PAD expression in vitro after SARS-CoV-2 infection

(A) Volcano plot of transcriptomic data from the literature; (B-C) Calu3 cells were infected with 2020B.1 (MOI 0,1), Delta (MOI 0,1) or Omicron (MOI 0,03) and PAD4 and PAD2 mRNA was assessed by RT-qPCR. Data were normalized to the housekeeping gene PGK1 and expressed as mean fold change $\pm$ SEM over mock-infected cells. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; one-way ANOVA followed by Bonferroni's post test. (D) Western blot and Rh-PG analysis of protein lysates from uninfected (mock) or SARS-CoV-2-infected Calu3 cells (MOI 0,1). One representative blot of three independent experiments is shown. (E) Volcano plot depicting the host (black dots) and viral (blue dots) citrullinated proteins of infected cells vs mock-infected cells at 48 hpi. Cell lysates were exposed to a biotin-PG to isolate citrullinated proteins on streptavidin agarose. Bound proteins

were then subjected to on-bead tryptic digestion and analyzed by LC-MS/MS—in the graph, every identified citrullinated protein corresponds to a dot. The x-axis represents the ratio of citrullination between mock and infected cells at the indicated time points, while the y-axis indicates the statistical significance. Both variables were plotted on a logarithmic scale ($n=3$). (F) PANTHER classification of overcitrullinated cellular proteins based on protein classes.



Sup. Fig. 1 PAD expression in vitro after SARS-CoV-2 infection

(A-B) Huh7.5 cells were infected with 2020B.1 (MOI 0,1), Delta (MOI 0,1) or Omicron (MOI 0,03) and PAD4 and PAD2 mRNA was assessed by RT-qPCR. Data were normalized to the housekeeping gene PGK1 and expressed as mean fold change $\pm$ SEM over mock-infected cells. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; one-way ANOVA followed by Bonferroni's post test. (C) Western blot and Rh-PG analysis of protein lysates from uninfected (mock) or SARS-CoV-2-infected Huh7.5 cells (MOI 0,1). One representative blot of three independent experiments is shown.

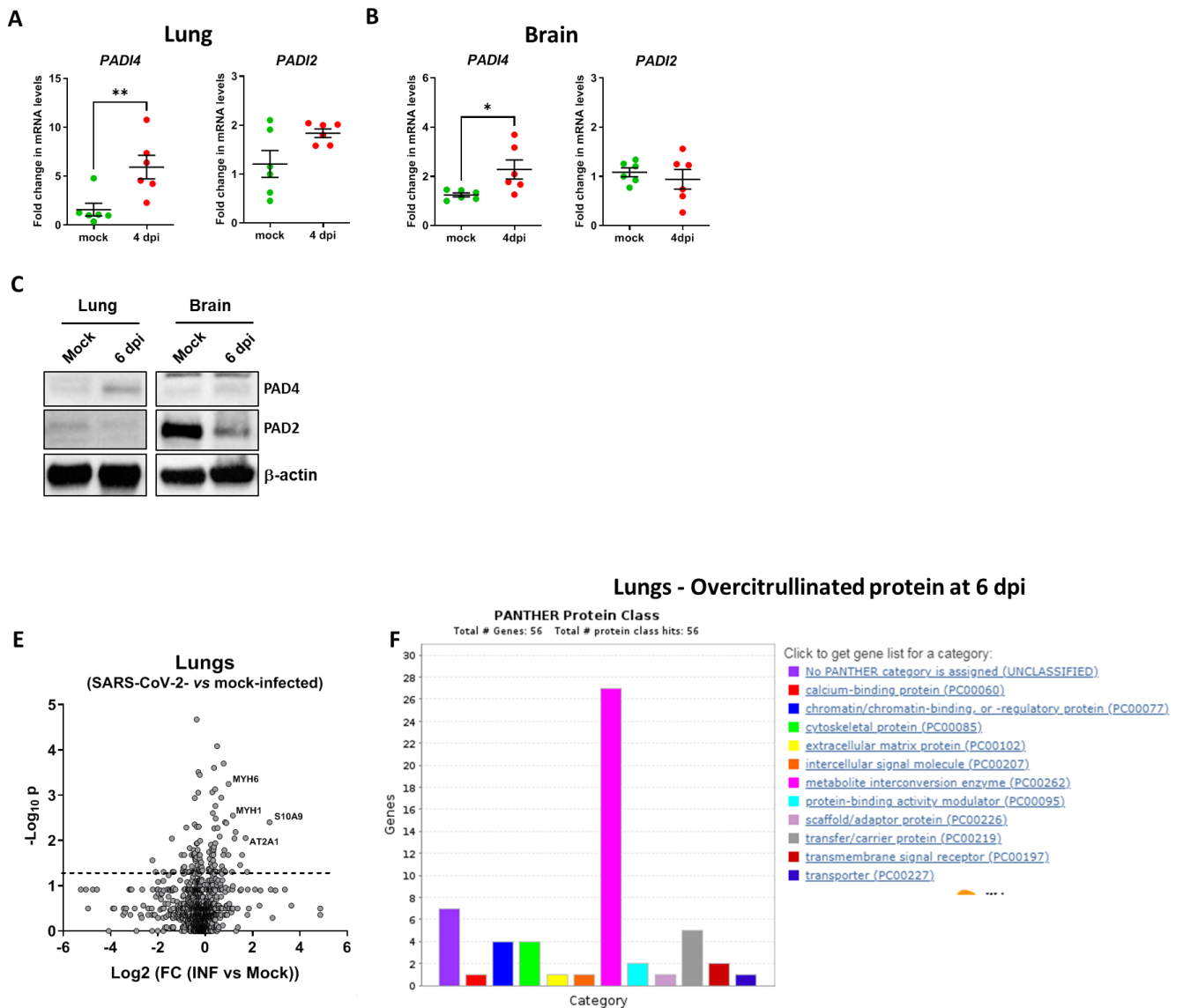


Fig. 2 SARS-CoV-2 infection affects protein citrullination by modulating PAD expression in vivo

PADI4 and PADI2 mRNA expression in mouse lungs (A-B) and in mouse brains (C-D) assessed by RT-qPCR. Each dot represents an individual mouse sample. Data were normalized to the housekeeping gene Actin and presented relative to one randomly selected non-infected sample. (E) Western blot analysis of protein lysates from pooled uninfected (mock) and SARS-CoV-2-infected (6 dpi) mouse lungs and brains. The analysis was performed using antibodies against PAD2, PAD4, and β-actin to ensure equal loading. A representative blot from three independent experiments is shown. (F)

(G) Volcano plot showing citrullinated proteins in pooled protein lysates from SARS-CoV-2-infected vs mock-infected mouse lungs at 6 dpi. Lysates were incubated with biotin-PG to isolate citrullinated proteins on streptavidin agarose, followed by on-bead tryptic digestion and LC-MS/MS analysis. Each dot represents an identified citrullinated protein. The x-axis shows the citrullination ratio between mock and infected samples, and the y-axis indicates statistical significance, both on a logarithmic scale (n=3). (H) PANTHER classification of overcitrullinated cellular proteins based on protein classes.

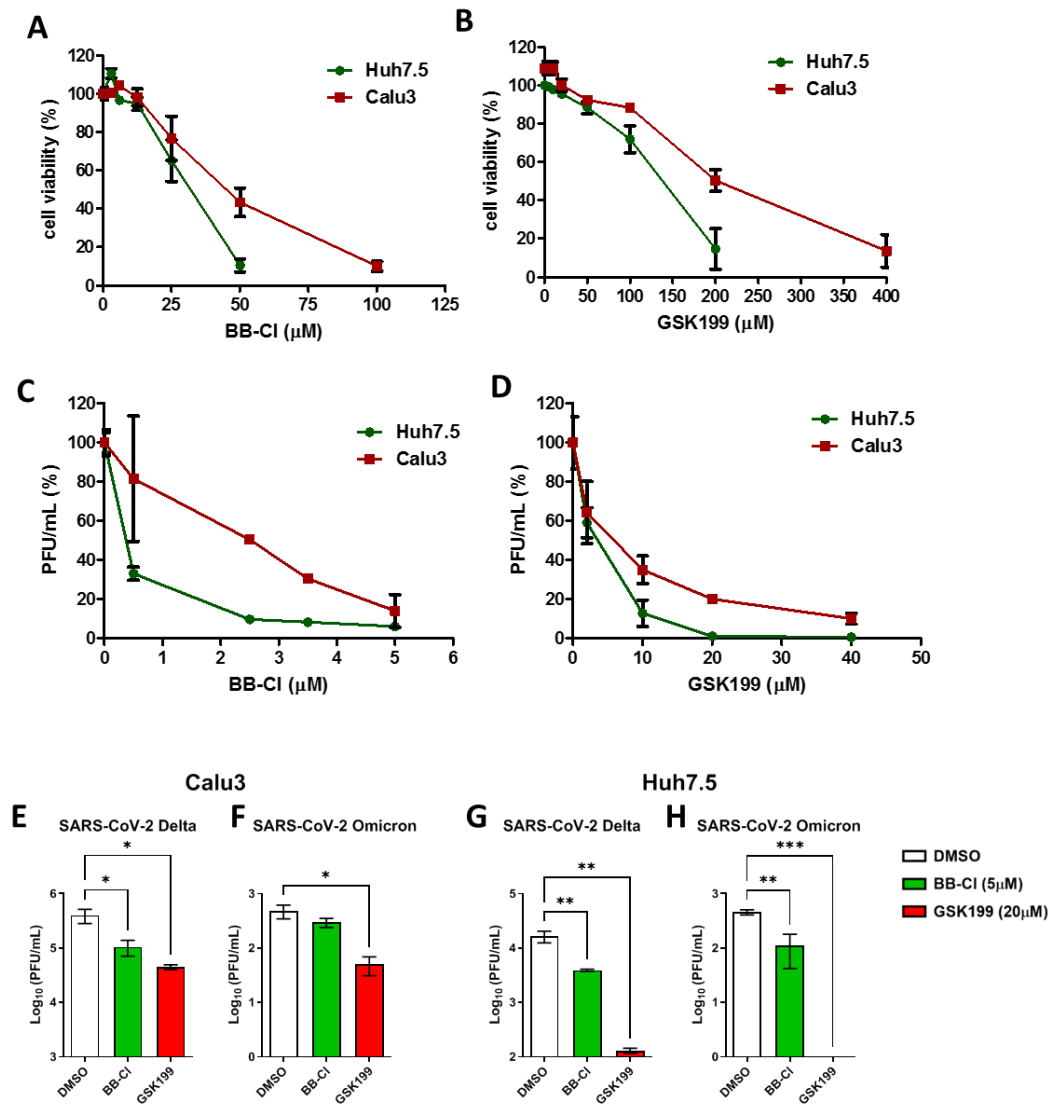


Fig. 3 in vitro antiviral activity of PAD inhibitors against SARS-CoV-2

(A-B) Uninfected Calu3 and Huh7.5 cells were treated with the indicated concentrations of BB-Cl (A) and GSK199 (B) for 72 h, and the number of viable cells was determined for each concentration using the MTT assay. Values are expressed as means $\pm$ SEM of three independent experiments. (C-D) Quantification of SARS-CoV-2 viral particle production in Calu-3 and Huh 7.5 cells treated with increasing concentrations of BB-Cl (C) or GSK199, determined by plaque assay. Results are expressed as a percentage relative to vehicle-treated cells (DMSO, marked as 0 on the graph). Data are presented as means $\pm$ SEM from three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, analyzed by one-way ANOVA followed by Bonferroni's post test. (E-F) Calu-3 cells were treated with BB-Cl (5 μ M) or GSK199 (20 μ M) one hour prior to infection with Delta or Omicron (MOI 0.1). After 24 hpi, the virus was collected and quantified by plaque assay. (G-H) Huh7.5 cells were treated with BB-Cl (5 μ M) or GSK199 (20 μ M) one hour prior to infection with Delta or Omicron (MOI 0.1). After 48 hpi, the virus was collected and quantified by plaque assay. Values are expressed as means $\pm$ SEM of three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, analyzed by t-test.

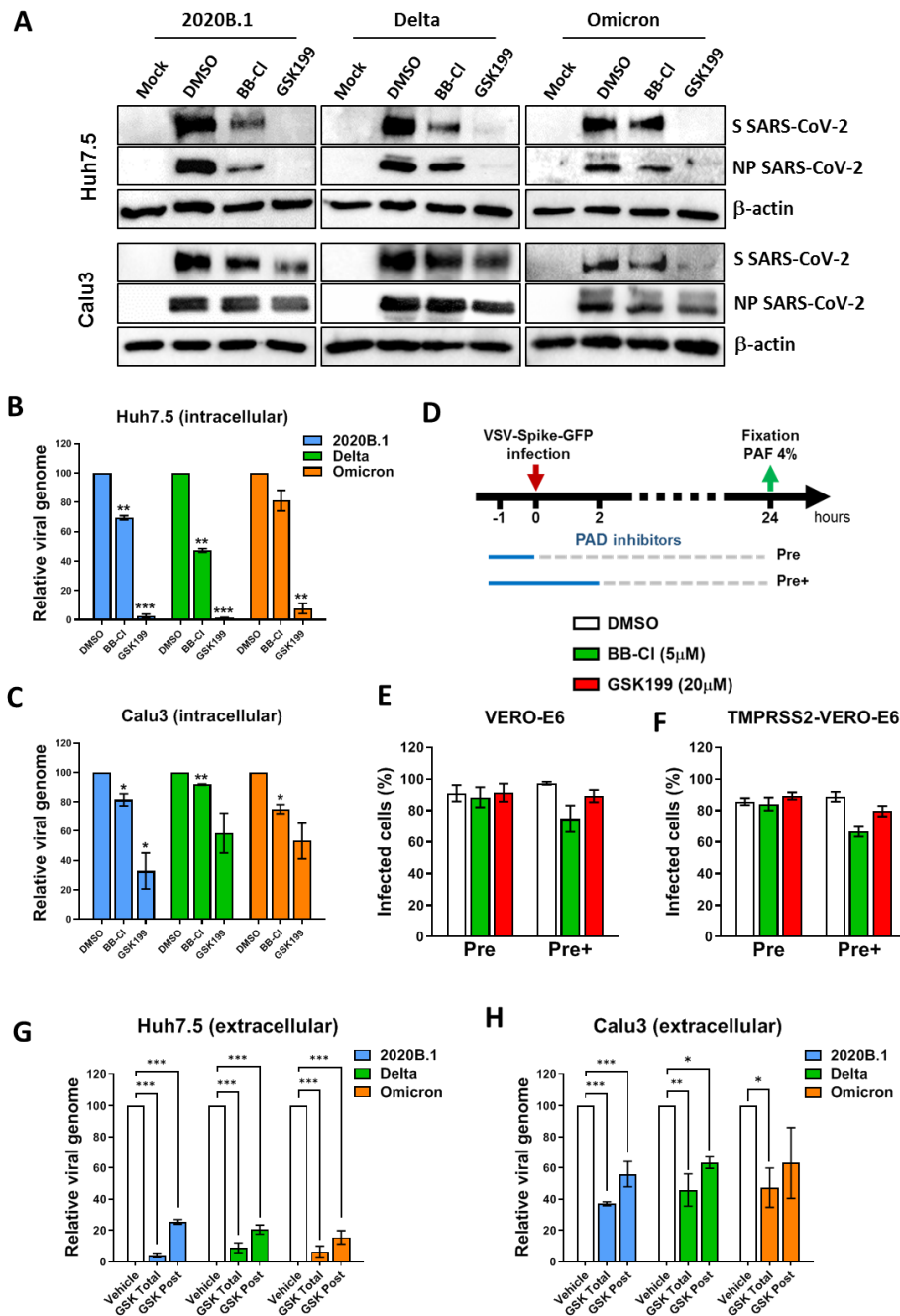


Fig. 4 PAD4 inhibition blocks SARS-CoV-2 protein and genome synthesis

(A) Western blot analysis of viral protein expression in Huh7.5 and Calu3 cells mock or SARS-CoV-2 infected (MOI 0,1) and treated with GSK199 (20 μ M), BB-CI (5 μ M), or DMSO. One representative gel of three independent experiments is shown. (B-C) RT-PCR of viral RNA in cell extracts from SARS-CoV-2-infected Huh7.5 (B) or Calu3 (C) cells (MOI 0.1) treated with GSK199 (20 μ M), BB-CI (5 μ M), or DMSO. Values are expressed as mean $\pm$ SEM of three independent experiments. (D) Graphical representation of infection and treatment patterns used for the inhibition entry assay. (E-F) Viral entry assay on VERO-E6 (E) and VERO-E6 overexpressing TMPRSS2 infected with VSV-Spike GFP (MOI 1). GFP-fluorescent infected cells were microscopically counted and the result was expressed as a percentage of the vehicle-treated cells. (G-H) RT-PCR of viral RNA released from SARS-CoV-2-infected Huh7.5 (G) or Calu3 (H) cells (MOI 0.1) treated with DMSO (vehicle) or GSK199 (20 μ M) with standard treatment (Total) or treated at 2hpi (Post). Supernatant were collected at 24hpi and the number of viral genome copies are expressed as % compared to vehicle treated cells. Values are expressed as mean $\pm$ SEM of three independent experiments.

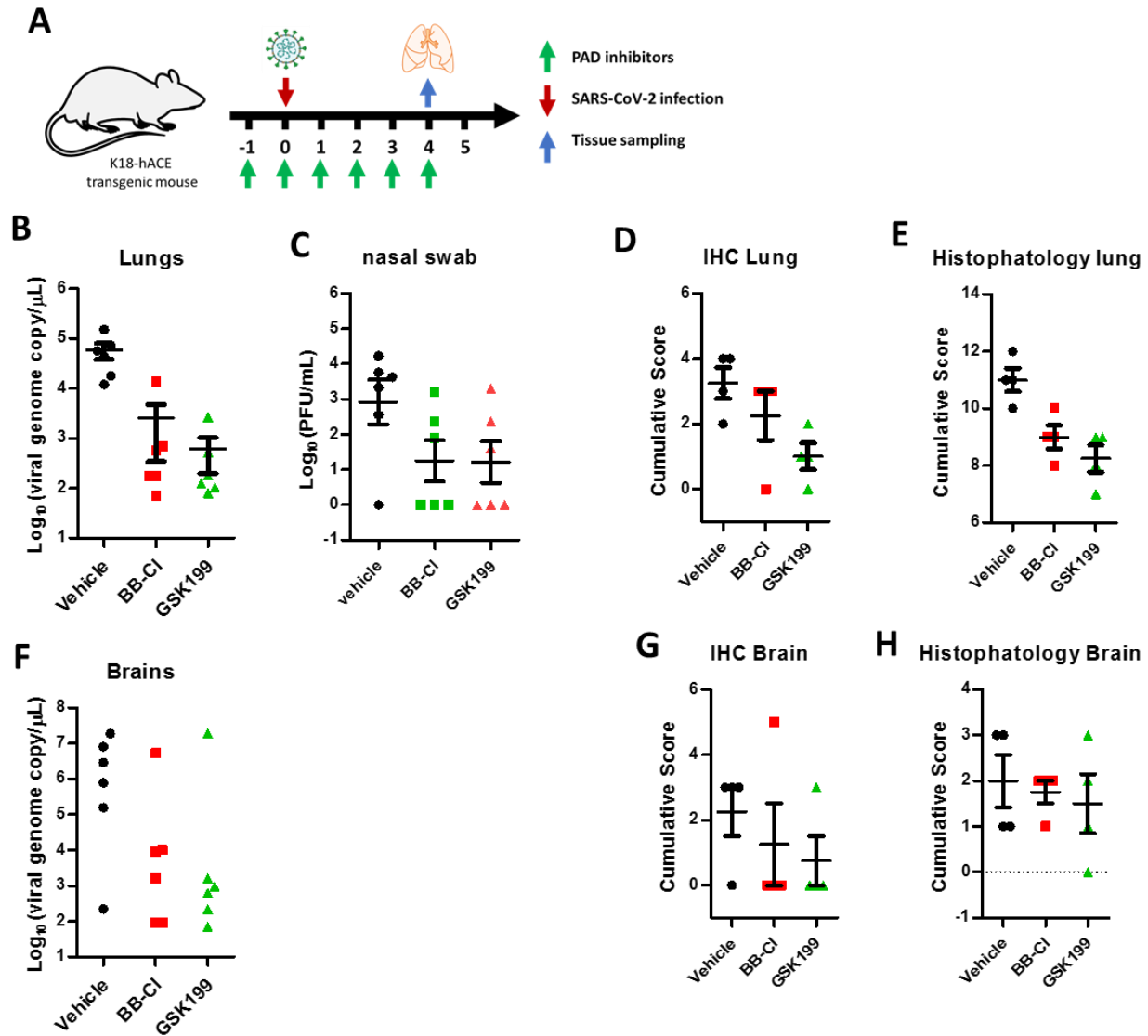
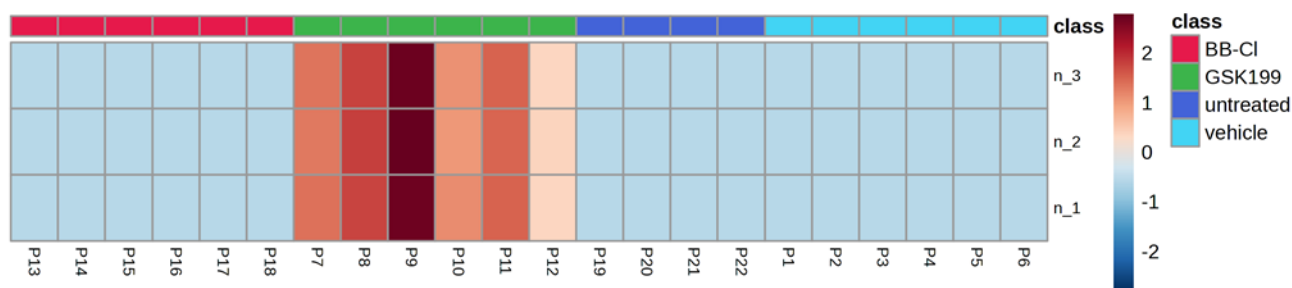


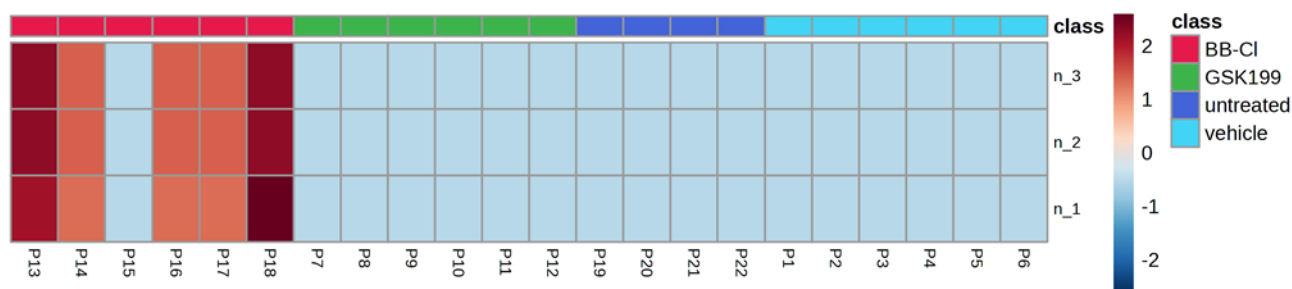
Fig. 5 in vivo antiviral activity of PAD inhibitors against SARS-CoV-2

(A) Representation of the treatment and infection scheme of K18-ACE2 transgenic mice. (B) Quantification of viral genome copies in mouse lungs (treated and infected as represented in A) by ddPCR, each dot represents an individual mouse sample. (C) Plaque assay to determine the number of infective viral particle in mouse nasal swab. (D) Graphical representation of cumulative scores from Table 4 for immunohistochemical staining in murine lungs using anti-SARS-CoV-2 nucleocapsid antibody. (D) Graphical representation of cumulative scores from Table 5 for hematoxylin-eosin staining in murine lungs, illustrating histopathological alterations classified by anatomical site and summarized as a percentage of tissue involvement. (B) Quantification of viral genome copies in mouse lungs (treated and infected as represented in A) by ddPCR, each dot represents an individual mouse sample. (G) Graphical representation of cumulative scores from Table 6 for immunohistochemical staining in murine brains using anti-SARS-CoV-2 nucleocapsid antibody. (H) Graphical representation of cumulative scores from Table 7 for hematoxylin-eosin staining in murine brains, illustrating histopathological alterations classified by anatomical site and summarized as a percentage of tissue involvement.

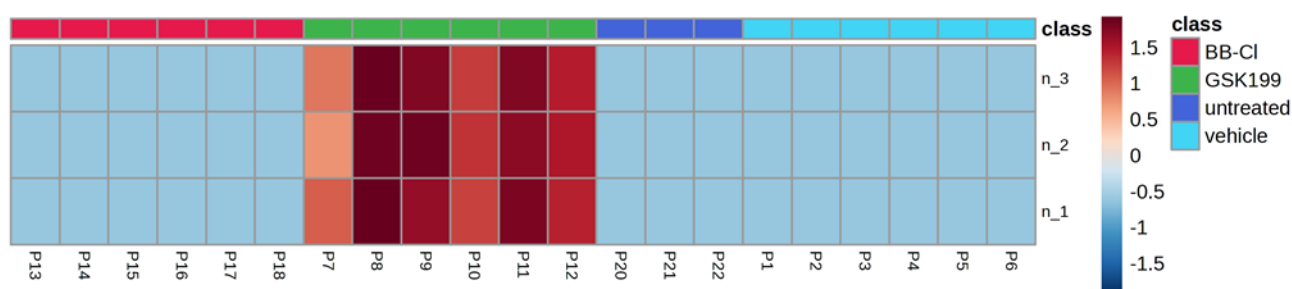
GSK199 Lungs



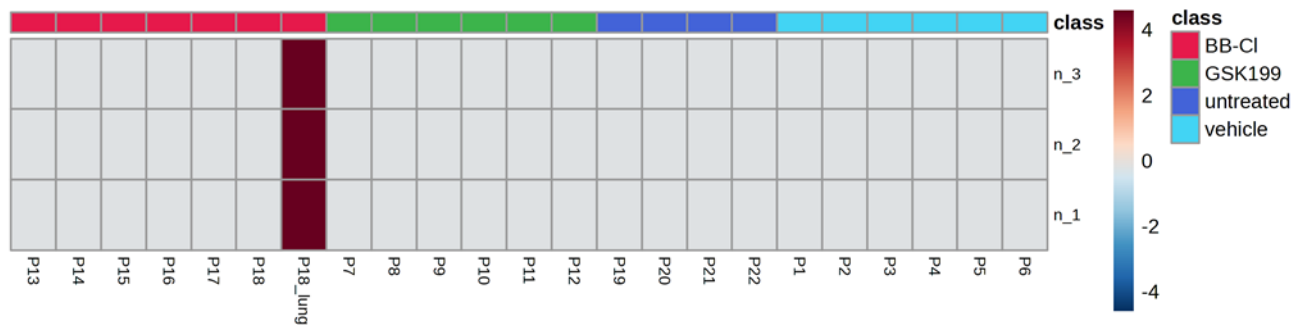
BB-CI Lungs



GSK199 Brains



BB-CI Brains



Sup. Fig. Quantification of GSK199 and BB-CI in omogenated mouse brains and lungs by LC-MS/MS.

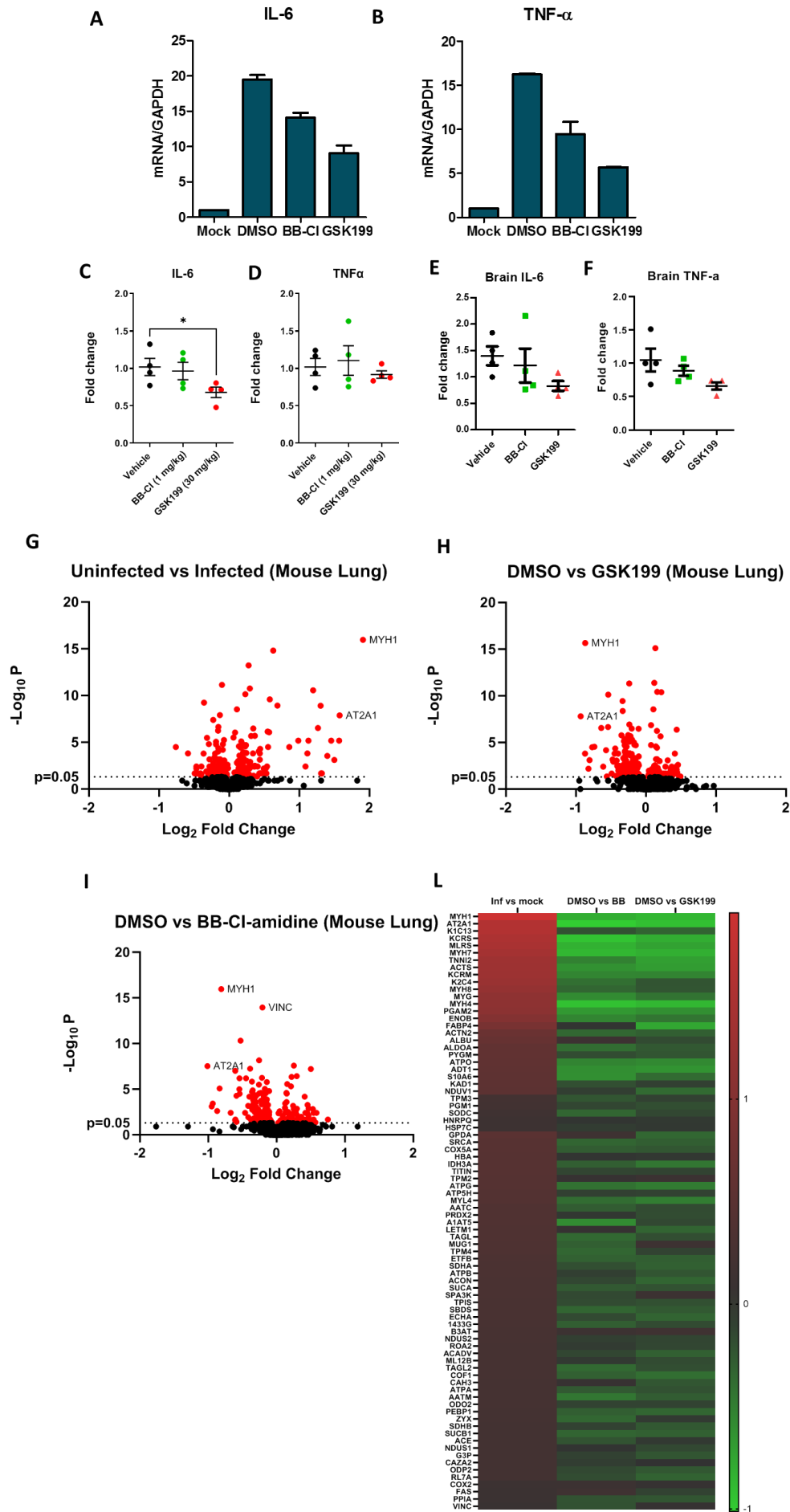


Fig. 6 PAD inhibition protects against SARS-CoV-2-induced pathological changes.

(A-B) Calu3 cells were infected with Delta (MOI 0,1) and IL6 and TNF- α mRNA was assessed by RT-qPCR. Data were normalized to the housekeeping gene PGK1 and expressed as mean fold change $\pm$ SEM over mock-infected cells. *P < 0.05, **P < 0.01, ***P < 0.001; one-way ANOVA followed by Bonferroni's post test. (C-F) IL6 and TNF- α mRNA expression in mouse lungs (C-D) and in mouse brains (E-F) assessed by RT-qPCR. Each dot represents an individual mouse sample. Data were normalized to the housekeeping gene Actin and presented relative to one randomly selected vehicle-treated sample. (G-I) Volcano plot showing citrullinated proteins in pooled protein lysates from SARS-CoV-2-infected vs mock-infected mouse lungs at 4 dpi. Lysates were incubated with biotin-PG to isolate citrullinated proteins on streptavidin agarose, followed by on-bead tryptic digestion and LC-MS/MS analysis. Each dot represents an identified citrullinated protein. The x-axis shows the citrullination ratio between mock and infected samples (G) and between infected mouse treated with vehicle (DMSO) or PAD inhibitors (GSK199 (H), BB-CI (I)) and the y-axis indicates statistical significance, both on a logarithmic scale (n=3). (L) Heatmap showing differentially citrullinated proteins identified in the lungs of mice under indicated conditions.

Tables

Table S1. mRNA expression in healthy vs. COVID19 whole bloods				
Gene symbol	p-value	Adj.p-value	Change	Log2FC
PADI4	2,518E-08	1,1002E-06	up	1,0901
PADI6	0,0001	0,0012	stable	0,4779
PADI1	0,0108	0,0343	stable	0,2387
PADI2	0,0195	0,0531	stable	0,5369
PADI3	0,0257	0,0655	stable	0,1955

Table S2. DEGs between COVID-19 and control lungs					
GeneID	Symbol	baseMean	Log2FoldChange	pvalue	padj
ENSG00000117115	PADI2	229,4182788	1,643193654	5,73E-05	0,000211
ENSG00000142623	PADI1	4,854369142	1,957246518	0,011928	0,025912
ENSG00000159339	PADI4	444,8943989	3,403236094	6,92E-19	1,82E-17

Table 3. CC50, IC50 and SI value

	CC50	IC50	SI
Huh7.5			
BB-CI	25	0.25	100
GSK199	155	5.7	27
Calu3			
BB-CI	45	2.5	18
GSK199	225	9,0	25

Treatment	Sample (lung)	BLOOD VESSELS	INTERSTITIUM	AIRWAYS/ALVEOLI			CUMULATIVE SCORE
		Vascular wall/inflammatory infiltrates	Macrophages/mononuclear cells	Type I pneumocytes	Type II pneumocytes/alveolar macrophages	Bronchial/bronchiolar epithelium	
Vehicle	#751	0	1**	0	1**	0	2
	#752	0	1**	0	2**	0	3
	#753	0	1**	0	2**	1*	4
	#754	0	1**	1**	2**	0	4
BB-Cl	#782	0	1**	0	2**	0	3
	#783	0	1**	0	2**	0	3
	#784	0	1**	1**	1**	0	3
	#785	0	0	0	0	0	0
GSK199	#790	0	0	0	0	0	0
	#791	0	1*	0	0	0	1
	#792	0	1**	0	0	0	1
	#793	0	1*	0	1**	0	2

Table 4. IHC (SARS-CoV-2 Nucleocapsid Antibody, rabbit polyclonal). Lung. Staining intensity was scored as follows: 0, not detected; 1, mild/weak; 2, moderate; 3, strong .Percentage (%) of virus Ag-positive cells: one focal immunolabelled area (*); multifocal immunolabelled areas (**).

Treatment	Sample (lung)	SUB-GROSS	BLOOD VESSELS				INTERSTITIUM					AIRWAYS/ALVEOLI					PLEURA	LUNG CUMULATIVE SCORE	
		% of lung affected	Endothelial activation/plumpling	Vasculitis/thrombosis	Perivascular edema	Vascular congestion	Neutrophilic infiltration	Lymphoplasmacytic/histiocytic infiltration	Necrosis	Haemorrhage	Fibrosis	Hyaline membranes	Alveolar edema	Pneumocytes II/alveolar macrophages	Neutrophilic infiltration	Lymphoplasmacytic/histiocytic infiltration	Hyperplasia/degeneration (necrosis) of bronchiolar/alveolar epithelium (syncytia)		Pleural involvement
Vehicle	#751	70	2	0	0	2	0	1	0	1	0	0	0	3	0	0	1	1	11
	#752	70	2	0	0	3	0	1	0	1	0	0	0	3	0	0	0	0	10
	#753	60	1	0	0	3	0	1	0	1	0	0	0	3	0	0	3	0	12
	#754	50	1	0	0	3	1	2	0	0	0	0	0	2	0	0	2	0	11
BB-Cl	#782	60	0	0	0	2	1	2	0	0	0	0	0	3	0	0	2	0	10
	#783	60	0	0	0	3	1	1	0	0	0	0	0	3	0	0	1	0	9
	#784	60	0	0	0	4	1	0	0	0	0	0	0	3	0	0	0	1	9
	#785	70	0	0	0	1	0	1	0	0	0	0	0	4	0	0	1	1	8
GSK199	#790	70	0	0	0	1	0	1	0	0	0	0	0	4	0	0	1	1	8
	#791	30	0	0	0	4	1	0	0	0	0	0	0	2	0	0	0	0	7
	#792	30	1	0	0	2	2	0	0	0	0	0	0	3	0	0	1	0	9
	#793	40	1	0	0	3	1	0	0	0	0	0	0	2	0	0	1	1	9

Table 5. Lung histopathological features classified by anatomical site and summarized in percentage of tissue involvement: 0, no tissue involvement (no findings); 1, up to 25% of involved tissue (mild); 2, up to 50% of involved tissue (moderate); 3, up to 75% of involved tissue (severe); 4, up to 100% of involved tissue (massive). Abbreviations: * (asterisk) syncytia.

Treatment	Sample (brain)	<u>BLOOD VESSELS</u>	<u>NEURONS</u>	<u>GLIA</u>	<u>WHITE MATTER</u>	<u>CUMULATIVE SCORE</u>
Vehicle	#751	0	0	0	0	0
	#752	0	2**	0	0	2
	#753	0	0	0	0	0
	#754	0	3**	2*	0	5
BB-Cl	#782	0	3*	0	0	3
	#783	0	3*	0	0	3
	#784	0	0	0	0	0
	#785	0	0	0	0	0
GSK199	#790	0	0	0	0	0
	#791	0	0	0	0	0
	#792	0	0	0	0	0
	#793	0	3**	0	0	3

Table 6. IHC (SARS-CoV-2 Nucleocapsid Antibody, rabbit polyclonal). Brain. Staining intensity was scored as follows: 0, not detected; 1, mild/weak; 2, moderate; 3, strong. Percentage (%) of virus Ag-positive cells: one focal immunolabelled area (*); multifocal immunolabelled

Treatment	Sample (brain)	<u>NEURONAL CHANGES</u>				<u>MENINGES</u>	<u>CNS CUMULATIVE SCORE</u>
		Gliosis	Perivascular cuffing	Degeneration/sp ongiosis of white matter	Neuronal degeneration/N ecrosis	Meningeal involvement	
Vehicle	#751	1	0	WM absent	0	0	1
	#752	0	0	WM absent	1	0	1
	#753	1	0	1	1	0	3
	#754	1	0	1	1	0	3
BB-Cl	#782	0	0	1	1	0	2
	#783	0	0	1	0	0	1
	#784	1	0	1	0	0	2
	#785	1	0	1	0	0	2
GSK199	#790	0	0	1	0	0	1
	#791	0	0	2	0	0	2
	#792	0	0	0	0	0	0
	#793	1	0	1	1	0	3

Table 7. Brain histopathological features classified by lesion type and summarized in percentage of tissue involvement: 0, no tissue involvement(no findings); 1, up to 25% of involved tissue (mild); 2, up to 50% of involved tissue (moderate); 3, up to 75% of involved tissue (severe); 4, up to 100% of involved tissue (massive). A cumulative score was calculated for each animal by taking the sum of all values.