

The PTP4A3 Inhibitor K VX-053 Disrupts STAT3 and NF- κ B Signaling to Preserve Endothelial Barrier Integrity in Lipopolysaccharide-Induced Acute Lung Injury

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Additional Declarations: Competing interest reported. John S. Lazo and Elizabeth R. Sharlow are co-inventors of U.S. Patent No. 10,308,663, which describes the chemical synthesis, composition of matter and use of K VX-053 for the treatment of cancer. A provisional US patent application entitled “Composition and methods for treating/preventing lung injury” (Serial No. PCT/US2022/024927) and a European Union patent application (22788973.0) with the same title have been filed with John S. Lazo, Elizabeth R. Sharlow, John D. Catravas, Ruben M.L. Colunga Biancatelli, and Pavel A. Solopov being listed as co-inventors. John S. Lazo and Elizabeth Sharlow are co-founders of KeViRx, Inc, which has licensed the above-mentioned intellectual property from the University of Virginia, University of Pittsburgh, and Old Dominion University for further development.

Abstract

Acute Respiratory Distress Syndrome (ARDS) remains a devastating clinical condition with limited pharmacologic interventions capable of modifying disease progression. We have recently demonstrated that Protein Tyrosine Phosphatase Type IVA (PTP4A3 aka PRL3) is a key mediator of the inflammatory cascade that drives ALI following SARS-CoV-2 infection. Here, we investigated whether pharmacological inhibition of PTP4A3 with the small molecule inhibitor K VX-053 could attenuate Lipopolysaccharide (LPS)-induced acute lung injury (ALI) in C57BL/6J mice and LPS-induced endothelial barrier dysfunction in human lung microvascular endothelial cells (HLMVEC). C57BL/6J mice were intratracheally instilled with LPS (1 mg/kg) and treated with K VX-053 (10 mg/kg, intraperitoneally) beginning 1-hour post-instillation and every 24 hours thereafter for 72 hours. LPS-challenged mice exhibited significant weight loss, increased bronchoalveolar lavage fluid (BALF) white blood cell and protein concentrations, elevated lung injury scores, and impaired pulmonary mechanics. Treatment with K VX-053 attenuated weight loss, reduced BALF cytosis and proteinosis, diminished histological evidence of lung injury, and reduced the activation of key inflammatory signaling mediators, including phosphorylated STAT3 (p-STAT3), NLRP3 inflammasome, HSP70, and MMP8 in lung tissue. In HLMVEC, K VX-053 pre-treatment abolished LPS-induced phosphorylation of STAT3 and I κ B α , restored VE-Cadherin expression and phospho-Cofilin levels and preserved endothelial monolayer integrity as assessed by immunofluorescence microscopy. Genetic silencing of PTP4A3 by RNA interference protected HLMVEC barrier function against LPS, and the combination of silencing and K VX-053 was not additive, confirming that the compound acts specifically through PTP4A3. Collectively, these findings define a PTP4A3 dependent signaling axis, converging on STAT3, NF- κ B, NLRP3, and Cofilin, that governs endothelial barrier integrity in LPS induced acute lung injury, and demonstrate that its pharmacological interruption by K VX-053 attenuates pulmonary inflammation and barrier disruption, supporting the broader therapeutic potential of PTP4A3 inhibitors in ARDS and ALI.

1. Introduction

Acute Respiratory Distress Syndrome (ARDS) is a life-threatening condition characterized by diffuse alveolar damage, pulmonary edema, neutrophilic infiltration, and refractory hypoxemia, with a mortality rate that remains between 30% and 50% despite decades of research [1, 2]. The pathogenesis of ARDS is driven by an exaggerated inflammatory response that results in disruption of the alveolar-capillary barrier, massive influx of protein-rich edema fluid into the airspaces, and impaired gas exchange [3]. While the etiologies of ARDS are diverse, including pneumonia, sepsis, trauma, and aspiration, Gram-negative bacterial infections represent a leading cause of direct and indirect lung injury [4].

Lipopolysaccharide (LPS), a major structural component of the outer membrane of Gram-negative bacteria, is a potent activator of the innate immune response. Upon inhalation or systemic release, LPS binds to the Toll-like receptor 4 (TLR4) complex on alveolar macrophages, epithelial cells, and endothelial cells, triggering the activation of the NF- κ B and STAT3 signaling cascades, inflammasome assembly, and the production of pro-inflammatory cytokines including TNF α , IL-1 β , and IL-6 [5, 6]. The

ensuing "cytokine storm" recruits neutrophils into the alveolar space, promotes endothelial and epithelial barrier dysfunction, and leads to the clinical hallmarks of ARDS [7]. Intratracheal instillation of LPS in mice is among the most well-characterized and reproducible models of direct ALI, recapitulating the essential features of the human disease including neutrophilic alveolitis, increased alveolar-capillary permeability, and impaired lung mechanics [8]. Despite the clear understanding of the inflammatory pathways driving ARDS, current therapeutic interventions remain predominantly supportive. Lung-protective ventilation strategies and conservative fluid management have improved outcomes, but no pharmacologic agent has demonstrated consistent efficacy in large-scale clinical trials [9, 10]. Corticosteroids provide modest benefit in select populations, but their broad immunosuppressive effects carry significant risks [11]. The identification of novel, targetable molecular mediators that regulate the inflammatory response without suppressing the immune cells response remains a critical unmet need.

Protein Tyrosine Phosphatase Type IVA (PTP4A), also known as Phosphatase of Regenerating Liver (PRL), is a subfamily of dual-specificity protein tyrosine phosphatases that includes three members: PTP4A1, PTP4A2, and PTP4A3 [12]. Among these, PTP4A3 has emerged as a key regulator of intracellular inflammatory signaling. PTP4A3 is anchored to the inner leaflet of the plasma membrane, where it modulates the signaling of multiple receptors, including interleukins, TNF α , VEGF, and integrins [13, 14]. Functionally, PTP4A3 dephosphorylates and thereby activates multiple downstream effectors involved in inflammation, cytokine production, cytoskeletal remodeling, and cellular migration. It promotes STAT3 activation via SHP-2 repression [15], NF- κ B nuclear translocation through RAP1 dephosphorylation [16], and modulates MAPK/ERK signaling via p38 dephosphorylation [17]. In the present work we specifically interrogate which of these downstream nodes are engaged by PTP4A3 in the pulmonary endothelium during LPS challenge, in order to define the signaling architecture that governs barrier integrity. Importantly, PTP4A3 expression selectively increases under conditions of cellular stress and inflammation, suggesting that its inhibition may have limited effects on non-inflamed tissues [18, 19].

KVX-053 (also known as JMS-053) is a potent, allosteric, and reversible small molecule inhibitor of PTP4A3 with an IC₅₀ of 25 nM and a long pharmacokinetic half-life of approximately 24 hours following intraperitoneal administration [20, 21]. We have previously demonstrated that KVX-053 ameliorates ALI induced by the SARS-CoV-2 Spike Protein Subunit 1 (S1SP) in K18-hACE2 transgenic mice, by acting as a major inflammatory signaling NLRP3, I κ B α and STAT3 [18]. More recently, we also demonstrated that KVX-053 reduces live SARS-CoV-2-induced inflammation, lung injury, and clinical deterioration in K18-hACE2 mice, without affecting viral replication [22]. These findings established that PTP4A3 inhibition acts through a host-directed mechanism, modulating the inflammatory response rather than targeting the pathogen itself. Here, we hypothesized that KVX-053 may also prove beneficial in *in vitro* and *in vivo* models of LPS-induced ARDS. Human Lung Microvascular Endothelial cells (HLMVEC) were incubated with LPS and treated with different dosages of KVX-053 and similarly, C57BL/6J mice were intratracheally instilled with LPS and received treatment with KVX-053 10 mg/kg, i.p. To distinguish target specific effects from potential off-target actions of the compound, we additionally silenced PTP4A3 by RNA interference and monitored endothelial barrier function in real time. Our findings

broaden the therapeutic potential of PTP4A3 inhibitors in the post-pandemic era as a cause-agnostic and host-directed intervention for ARDS and ALI, and define the STAT3, NF- κ B, and Cofilin dependent signaling through which PTP4A3 governs endothelial barrier integrity.

2. Methods

2.1 Materials

KVX-053 was acquired from KeViRx, Inc. at a purity level > 98% (Lazo *et al.*, Biomolecules, 2021). LPS from *Escherichia coli* O111:B4. RIPA buffer and protease inhibitor cocktail were obtained from Sigma-Aldrich Corporation (St. Louis, MO, USA), the BCA Protein assay kit from Pierce Co. (Rockford, IL, USA), EDTA and Western blot membranes from GE Healthcare (Chicago, IL, USA). For SDS-PAGE, ProtoGel (30% acrylamide mix) and TEMED were from National Diagnostics (Atlanta, GA, USA), Tris-HCl buffer from Teknova (Hollister, CA, USA), 10% SDS and ammonium persulfate from ThermoFisher Scientific (Waltham, MA, USA), and Protein Dual Color Standards and Tricine Sample Buffer from Bio-Rad Laboratories (Hercules, CA, USA). All antibodies were purchased from reputable commercial sources with published immunospecificity data. For Western blotting, the following antibodies were used: p-STAT3 (#9145), STAT3 (#9139), NLRP3 (#15101), p-I κ B α (#2859), I κ B α (#4814), p-ERK (#4370), ERK (#4695), p-Cofilin (#3313), Cofilin (#5175), and HSP70 (#4873) were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA); p-HSP90 (#PA5-104782) was from ThermoFisher Scientific; MMP8 (#ab81286) was from Abcam (Cambridge, MA, USA); VE-Cadherin (#sc-9989), PTP4A3 was from () and β -actin (#A5441) were from Sigma-Aldrich Corporation. Secondary IRDye 800CW goat anti-rabbit (926-32211) and IRDye 680RD goat anti-mouse (926-68070) were obtained from LI-COR Biosciences (Lincoln, NE, USA). For immunofluorescence, Alexa Fluor 488-conjugated VE-Cadherin antibody, Alexa Fluor 594-Phalloidin (F-actin), and DAPI were obtained from ThermoFisher Scientific.

2.2 Cell Culture

In-house harvested primary human lung microvascular endothelial cells (HLMVEC) were maintained in MDEM media supplemented with 10% fetal bovine serum (FBS) and antibiotics/antimycotics as described previously [23]. Cells were maintained at 37°C with 5% CO₂ and used between passages 4 and 8.

2.3 In Vitro LPS Challenge and KVX-053 Treatment

For Western blot experiments, HLMVEC were grown to confluence in 100 mm plates. Cells were pretreated with KVX-053 (12.5 μ M or 25 μ M) or vehicle (10% DMSO) for 4 hours, followed by stimulation with LPS (20 nM) for 3 hours. At the end of the experiment, cells were washed with ice-cold PBS, scraped with cell lifters and lysed in RIPA and Protease inhibitor cocktail mixture (1:100). Cell lysates were sonicated 3 times for 2 seconds with 1 minute in between and gently rotated for 10 minutes at 4°C. Cell lysates were then spun at 11,000 $\times$ g and supernatant transferred, the protein content assessed by BCA assay and normalized samples were mixed in a 1:1 ratio with tricine buffer.

For immunofluorescence experiments, HLMVEC were grown on glass coverslips in 24-well plates. Cells were pretreated with K VX-053 (12.5 μ M) or vehicle for 4 hours, followed by LPS (20 nM) challenge for 3 hours. HLMVEC grown on glass coverslips were fixed with 4% paraformaldehyde for 15 minutes at room temperature, permeabilized with 0.1% Triton X-100 for 10 minutes, and blocked with 5% BSA for 1 hour. Cells were incubated with anti-VE-Cadherin antibody followed by Alexa Fluor 488-conjugated secondary antibody. F-actin was visualized with Alexa Fluor 594-conjugated phalloidin, and nuclei were counterstained with DAPI. Coverslips were mounted and imaged on a fluorescence microscope. Images were acquired at identical exposure settings across all conditions.

2.4 PTP4A3 gene silencing

Twenty-four hours after seeding, cells were forward transfected with Silencer Select siRNAs (ThermoFisher Scientific) using Lipofectamine RNAiMAX. Transfection complexes were prepared in Opti-MEM I and delivered at 25 μ L per well to reach a final siRNA concentration of 9.1 nM. The conditions comprised untransfected cells, a lipofectamine only control, a non-targeting negative control siRNA (#4390843), a GAPDH positive control siRNA (#4390849), and three independent PTP4A3 targeting siRNAs (s22005, s22006, s22007). Silencing efficiency was verified at 72 hours after transfection at both the transcript and protein level. For transcript quantification, total RNA was extracted from transfected HLMVEC, reverse transcribed, and PTP4A3 expression was measured by RT-qPCR using GAPDH as the internal reference gene and expressed as fold of untreated control. For protein confirmation, whole cell lysates were resolved by SDS PAGE and probed for PTP4A3 with β -actin as the loading control.

2.5 Real-time barrier measurement by ECIS

Real time endothelial barrier function was assessed by Electric Cell substrate Impedance Sensing (ECIS, Applied BioPhysics, Troy, NY, USA) using 96W10 + arrays. HLMVEC were seeded at confluence in 250 μ L EGM-2 MV per well and continuous resistance recording was initiated. Twenty-four hours after seeding, cells were forward transfected, as described in section 2.4 with Silencer Select siRNAs (ThermoFisher Scientific), treated with K VX-053 (0.5 μ M) or LPS (100 ng/mL). At 70 hours post transfection, K VX-053 or vehicle was added, and at 72 hours cells were challenged with LPS or vehicle. Barrier resistance was recorded continuously throughout the experiment and normalized to baseline for each well.

2.6 Animals

All animal experiments were carried out in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee (IACUC) at Old Dominion University. Male C57BL/6J mice (8–10 weeks old, #00064 Jackson Laboratories, Bar Harbor, ME, USA) were used for all *in vivo* experiments. Mice were housed in specific pathogen-free conditions with a 12-hour light/dark cycle and free access to food and water. Animals were given 48 hours for acclimatization prior to the experiments.

2.7 LPS Intratracheal Instillation

Mice were anesthetized with ketamine (60 mg/kg) and xylazine (6 mg/kg) and placed in a supine position on a heating pad. A small (~ 1 cm) incision was made in the neck, the salivary glands isolated and trachea visualized. Then, the tongue was gently retracted, and a 20-24G catheter was inserted into the trachea and LPS (1 mg/kg in 50 μ L sterile saline) or an equal volume of sterile saline (vehicle) was instilled directly into the lungs. The presence of the catheter in the trachea was confirmed visually through the neck incision. After instillation, mice were maintained in an upright position and 100 μ L of air were pushed into the lungs to ensure distal and homogeneous distribution of the instillate. Neck incisions were closed with Vetbond, and additional oxygen was given to mice during their recovery period and progressively weaned from 100% to 21%. Mice were continuously monitored during their recovery and hourly for the next 4 hours.

2.8 Experimental Groups

Mice were randomly assigned to the following experimental groups:

- **Saline** (n = 6–8): mice intratracheally instilled with 50 μ L sterile saline.
- **LPS + Vehicle** (n = 6–8): mice intratracheally instilled with LPS 1 mg/kg and treated with vehicle (30% Captisol, 40% PEG400, 30% PBS) intraperitoneally every 24 hours beginning 1-hour post-instillation.
- **LPS + KVX-053** (n = 6–8): mice intratracheally instilled with LPS 1 mg/kg and treated with KVX-053 10 mg/kg i.p. beginning 1-hour post-instillation and every 24 hours thereafter.

In all groups, mice were monitored daily for weight changes and clinical signs of distress. Mice were euthanized at 72 hours post-LPS instillation.

2.9 Bronchoalveolar Lavage Fluid (BALF)

At the end of the experimental period (72 hours), mice were euthanized with an overdose of Pentobarbital (100mg/kg i.p.), and bronchoalveolar lavage fluid (BALF) was collected. A 20G catheter was inserted into the trachea, and the lungs were lavaged with 1 mL of sterile PBS. The recovered BALF was centrifuged at 300 $\times$ g for 10 minutes at 4°C. The cell pellet was resuspended in PBS, and total white blood cell (WBC) counts were determined by hemocytometer. The cell-free supernatant was aliquoted and stored at – 80°C for protein quantification. BALF total protein concentration was determined using the BCA Protein Assay Kit according to the manufacturer's instructions.

2.10 Pulmonary Function Testing

Pulmonary mechanics were assessed at 72 hours post-LPS instillation using the Flexivent system (SCIREQ, Montreal, QC, Canada). Mice were anesthetized with pentobarbital (50-75mg mg/kg, i.p.), tracheostomized with an 18G metal cannula, and connected to the ventilator. Mice were mechanically ventilated at a tidal volume of 10 mL/kg, a respiratory rate of 150 breaths/min, and a positive end-expiratory pressure (PEEP) of 3 cm H₂O. After two deep inflations to standardize lung volume history, baseline measurements of total respiratory system resistance (Rrs), elastance (Ers), Newtonian

resistance (Rn), tissue damping (G), and tissue elastance (H) were recorded. Methacholine challenge was performed by nebulizing increasing concentrations of methacholine (0, 6.25 and 12.5 mg/mL) for dose-response assessment. Pressure-volume (PV) loops were generated using a stepwise quasi-static inflation-deflation maneuver from 0 to 35 cm H₂O.

2.11 Histopathology

At the end of the experimental period, lungs were inflated and fixed with 10% formaldehyde solution through the trachea at a pressure of 15 cm H₂O and placed in 10% formaldehyde for 72 hours before processing. Mid-transverse slices were made from the formalin-fixed lungs and were embedded in paraffin. Sections (5 µm thick) were prepared from the blocks and stained with hematoxylin and eosin (H&E). Following the 2011 American Thoracic Society (ATS) report [8], ten randomly selected fields from each slide were examined for signs of acute lung injury, including (1) presence of neutrophils in the alveolar or interstitial spaces, (2) hyaline membranes, (3) proteinaceous debris filling the airspaces, and (4) alveolar septal thickening. Lung sections were examined under 20× magnification and each parameter scored either 0, 1, or 2 depending on the severity of injury. Weighted parameters were then averaged for the number of fields and plotted as previously described [24]. The observer was blinded to treatment assignment.

2.12 Tissue Collection and Western Blotting

Immediately after euthanasia, the chest was opened, blood was aspirated from the heart through the right ventricle, and the pulmonary circulation was flushed with sterile PBS containing EDTA. Lungs were dissected, snap-frozen in liquid nitrogen, and stored at – 80°C for subsequent processing. Proteins from lung tissue homogenates were extracted by sonication (50% amplitude, three times for 10 seconds) in ice-cold RIPA buffer with added protease inhibitor cocktail (100:1). Protein lysates were gently mixed for 3 hours at 4°C and centrifuged twice at 14,000 × g for 10 minutes. Equal amounts of protein were mixed with Tricine Sample Buffer 1:1, boiled for 5 minutes, and separated on a 10–12% polyacrylamide SDS gel by electrophoresis, transferred to nitrocellulose membranes, incubated with the appropriate primary antibody overnight at 4°C, followed by incubation with the secondary antibody for 1 hour at room temperature, and detected by digital fluorescence imaging (LI-COR Odyssey CLx, Dallas, TX, USA). β-actin was used as the loading control. ImageJ software v.1.8.0 was used to perform densitometric quantification of the bands (Accessed 10 January 2026). Membranes were stripped in stripping buffer for 20 minutes, blocked, and incubated with other primary and secondary antibodies.

2.13 RNA extraction and RT-qPCR

Total RNA was extracted from snap-frozen right lung tissue using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. RNA concentration and purity were assessed spectrophotometrically (NanoDrop 2000, Thermo Fisher Scientific), and only samples with an A260/A280 ratio ≥ 1.8 were used. Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative real-time PCR was performed on a StepOne Plus RT-PCR system (Applied Biosystems)

using PowerUp SYBR Green Master Mix (Applied Biosystems). Pre-validated primer pairs for murine interleukin-6 (IL-6), tumor necrosis factor alpha (TNF α), elastin microfibril interface-located protein 1 (Emilin-1), and the housekeeping gene β -actin were used. Relative mRNA expression was calculated using the $2^{-\Delta\Delta C_t}$ method, with β -actin as the internal reference gene, and data were expressed as fold change relative to the saline control group. All reactions were run in triplicates and melt curve analysis was performed to confirm the specificity of each amplicon.

2.14 Statistical Analysis

Statistical analysis was performed using GraphPad Prism Software (version 10.4, GraphPad Software, San Diego, CA, USA). Data are presented as mean $\pm$ SEM. Statistical significance was set at $p < 0.05$, determined by one-way or two-way analysis of variance (ANOVA) followed by appropriate post hoc tests (Tukey's or Bonferroni's multiple comparison test), or by repeated-measures two-way ANOVA for weight changes over time.

3. Results

3.1 K VX-053 attenuates alveolar and interstitial inflammation

Mice receiving LPS (1 mg/kg) exhibited severe and progressive weight loss reaching approximately 20% reduction from baseline by 72 hours ($p < 0.0001$ vs. saline), while treatment with K VX-053 (10 mg/kg/day i.p.) partially attenuated this decline (Fig. 1A). Intratracheal LPS provoked a marked increase in BALF total protein concentration at 72 hours ($p < 0.0001$ vs. saline), indicative of alveolar-capillary barrier disruption, and K VX-053 treatment significantly reduced BALF protein compared to LPS alone ($p < 0.05$) (Fig. 1B). K VX-053 was also able to reduce the BALF total white blood cell concentration compared to mice instilled with LPS and receiving vehicle (Fig. 1C). Differential cell counts revealed that the reduction in cellularity was primarily driven by a significant decrease in neutrophils and monocytes (Fig. 1D). Histological examination of H&E-stained lung sections confirmed that LPS produced severe ALI characterized by dense alveolar and interstitial neutrophilic infiltration, proteinaceous debris, and alveolar septal thickening, while K VX-053-treated mice showed an intermediate phenotype with reduced but still appreciable inflammation (Fig. 1E). The Lung Injury Score was markedly elevated in LPS-challenged mice, and K VX-053 significantly reduced this score, though it remained above saline levels (Fig. 1F). RT-qPCR analysis of lung homogenates demonstrated that LPS significantly upregulated IL-6 and TNF α and that K VX-053 strongly reduced the LPS-induced increase in inflammatory cytokine production (Fig. 1G, H). Notably, LPS instillation resulted in a marked suppression of Emilin-1 mRNA, that was restored above baseline during treatment with K VX-053 (Fig. 1I).

3.2 K VX-053 Suppresses STAT3, NF- κ B, and NLRP3 Signaling in LPS Challenged Lungs

LPS significantly increased STAT3 phosphorylation, and K VX-053 treatment completely prevented this activation, returning p-STAT3 to baseline levels (Fig. 2A). LPS induced a robust upregulation of NLRP3 inflammasome components, strongly modulated by treatment with K VX-053 (Fig. 2B). LPS increased the p-I κ B α /I κ B α ratio indicative of NF- κ B pathway activation, and K VX-053 treatment reduced this ratio toward baseline (Fig. 2C). MMP8 expression was not significantly altered by LPS alone compared to saline; however, K VX-053 treatment significantly reduced MMP8 levels below both saline and LPS groups, suggesting a direct or indirect effect of PTP4A3 inhibition on neutrophil collagenase expression or degranulation (Fig. 2D). Furthermore, K VX-053 reduced the LPS-induced increase to HSP70 (Fig. 2E), while there were no differences in ERK activation (*i.e.*, phosphorylation) across all groups (Fig. 2F).

3.2 K VX-053 Preserves Pulmonary Mechanics and Lung Compliance

Pulmonary function was assessed at 72 hours using the flexiVent forced oscillation technique. Pressure-volume (PV) loops demonstrated a downward and rightward shift in LPS-challenged mice compared to saline controls, indicating reduced lung compliance, while K VX-053-treated mice showed partial preservation of the PV relationship (Fig. 3A). Accordingly, K VX-053 improved the LPS-induced reduction in static and dynamic Compliances (C_{st}, C_{rs}) (Fig. 3B, F). Total respiratory system resistance (R_{rs}) was not significantly different among groups (Fig. 3C). Elastance (E_{rs}) was significantly increased by LPS, with K VX-053-treated mice showing intermediate values not significantly different from saline (Fig. 3D). Newtonian resistance (R_n) was not significantly altered by LPS or K VX-053 (Fig. 3E). We then performed a methacholine challenges revealing an overall hyperresponsiveness in the LPS-instilled mice, which were significant only for Elastance (E_{rs}) and tissue damping (G) (Fig. 3I, J). K VX-053 treatments were able to block the bronchial hyperresponsiveness and keep pulmonary baselines indistinguishable from saline-instilled controls (Fig. 3G-K).

3.3 K VX-053 restores lung endothelial barrier dysfunction

To determine whether the *in vivo* protective effects were mediated in part through direct actions on pulmonary endothelial cells, HLMVEC were challenged with LPS (20 nM) in the presence or absence of K VX-053 pre-treatment (12.5 μ M, 4 hours). LPS dramatically increased STAT3 phosphorylation, and K VX-053 completely abolished this response (Fig. 4A). LPS significantly increased VE-Cadherin protein expression, likely reflecting compensatory upregulation in the setting of junctional disruption, mechanism that was normalized by K VX-053 treatment (Fig. 4B). LPS significantly reduced p-Cofilin levels, restored by pretreatment (Fig. 4C). ERK phosphorylation was not significantly altered by LPS or K VX-053 (Fig. 4D). Confocal immunofluorescence imaging of HLMVEC stained for DAPI (nuclei), F-actin (phalloidin), and VE-Cadherin confirmed that K VX-053 pre-treatment largely prevented LPS-induced actin stress fiber formation, redistribution of F-actin from the cortical ring to transcellular stress fibers, and disruption of VE-Cadherin continuity with the appearance of intercellular gaps (Fig. 4E).

3.4 Genetic silencing of PTP4A3 protects HLMVEC barrier function

To confirm that the endothelial protection conferred by K VX-053 is mediated specifically through PTP4A3, rather than through an off-target action of the compound, we silenced PTP4A3 by RNA interference in HLMVEC and monitored barrier function in real time by Electric Cell substrate Impedance Sensing (ECIS, Applied Biophysics). Cells were transfected with one of three independent PTP4A3 targeting siRNAs (#cat s22005, s22006, s22007), a non-targeting negative control siRNA, or a GAPDH positive control siRNA, alongside untransfected and lipofectamine only conditions. Additionally, a PTP4A3 siRNA was selected to interrogate the interaction between genetic silencing, K VX-053, and LPS challenge (Fig. 5A). LPS produced a marked and progressive fall in transendothelial resistance in negative control transfected monolayers, indicative of barrier disruption. Silencing of PTP4A3 siRNA2 preserved barrier resistance following LPS challenge to a degree comparable to that achieved by K VX-053, and the combination of PTP4A3 silencing with K VX-053 did not produce an additive protection, consistent with both interventions acting through the same molecular target. Knockdown was confirmed at 72 hours at the transcript level, with the three PTP4A3 targeting siRNAs markedly reducing PTP4A3 mRNA relative to control conditions (Fig. 5B).

4. Discussion

In the present study, we demonstrate that pharmacological and genetic inhibition of PTP4A3 disrupts STAT3 and NF- κ B dependent signaling and thereby preserves endothelial barrier integrity and attenuates LPS induced acute lung injury in C57BL/6J mice and in human lung microvascular endothelial cells, extending our previous observations in SARS-CoV-2-driven ALI models [18, 22]. These results suggest that PTP4A3 inhibition represents a cause-agnostic, host-directed therapeutic strategy for ALI/ARDS. The intratracheal LPS model is a well-established system for studying direct ALI [8], and in our hands produced robust systemic and pulmonary injury at 72 hours, including severe weight loss, elevated BALF cellularity and proteinosis, histological diffuse alveolar damage, and impaired lung compliance. K VX-053 treatment significantly reduced BALF protein concentrations, total WBC counts, and neutrophil and monocyte infiltration, without significantly altering alveolar macrophage or lymphocyte counts, suggesting a selective anti-inflammatory effect targeting the acute innate effector response rather than broadly suppressing resident immune populations (Fig. 1). This selectivity is translationally relevant, as global immunosuppression remains a significant concern with current anti-inflammatory agents in the critical care setting [25]. K VX-053 successfully prevented LPS-induced STAT3 phosphorylation in both lung tissue and HLMVEC. STAT3 is a critical transcription factor in the amplification of the inflammatory response during ARDS, and PTP4A3 promotes STAT3 activation through suppression of SHP-2, a key negative regulator of the STAT3 pathway [26–28]. Consistent with STAT3 blockade, K VX-053 significantly reduced IL-6 mRNA expression. TNF α mRNA, while elevated by LPS, was not significantly reduced by K VX-053, likely reflecting the predominant role of NF- κ B, rather than STAT3, in driving TNF α transcription [29]. Indeed, the p-I κ B α /I κ B α ratio showed a reduction during K VX-053 treatment that did not reach

statistical significance, consistent with a partial effect on the NF- κ B axis (Fig. 2). The significant reduction in NLRP3 expression by K VX-053 aligns with our prior findings [18] and supports the concept that PTP4A3 participates in inflammasome priming through STAT3- and NF- κ B-dependent transcriptional upregulation [30, 31]. HSP70 normalization by K VX-053 reflects reduced cellular stress in parallel with the improved histological findings [32], while the reduction of MMP8 below control levels is particularly noteworthy. MMP8 is a major effector of neutrophil-mediated tissue destruction in ALI [33], and its reduction likely confirms both the diminished neutrophil recruitment and the decreased degranulation.

K VX-053 significantly preserved both static and dynamic compliance, restoring both to values not significantly different from saline controls, while resistance parameters were unaffected, consistent with the predominantly parenchymal rather than airway nature of this injury (Fig. 3). In HLMVEC, K VX-053 completely abolished LPS-induced STAT3 phosphorylation, restored phospho-Cofilin to its inactive state, preventing Cofilin-mediated actin depolymerization and stress fiber formation [34], and ultimately normalized VE-Cadherin expression, with immunofluorescence confirming preserved cortical actin architecture and junctional continuity (Fig. 4). Critically, genetic silencing of PTP4A3 confirmed the barrier protection provided by K VX-053, and the two interventions were not additive when combined, providing direct genetic evidence that the endothelial protection is PTP4A3 dependent rather than an off-target property of the small molecule (Fig. 5). These endothelial findings provide a direct mechanistic basis for the reduced alveolar-capillary permeability observed in vivo. A novel and intriguing finding of this study is the marked deregulation of Emilin-1 mRNA in lung tissue following LPS-induced ALI, to our knowledge, the first such report in experimental ALI. Emilin-1 is a secreted ECM glycoprotein essential for elastic fiber assembly, vascular homeostasis, and regulation of TGF- β bioavailability [35, 36]. Emilin-1-deficient mice develop arterial hypertension, increased vascular stiffness, and reduced vessel compliance [37]. We hypothesize that Emilin-1 upregulation, by K VX-053, represents a reparative mechanism aimed at reconstituting the damaged elastic fiber network, potentially serving as a counterregulatory brake on excessive TGF- β activity during the fibroproliferative phase of ALI [38]. Indeed, while PTP4A3 interacts with integrin signaling pathways [39], and Emilin-1 is a ligand for $\alpha 4\beta 1$ integrin [40], the coordinated effects of PTP4A3 inhibition on MMP8 suppression and preserved Emilin-1 expression could collectively favor matrix preservation over degradation during ALI resolution, a critical consideration given that ARDS survivors frequently develop persistent pulmonary dysfunction [41, 42]. The overall signaling architecture through which PTP4A3 inhibition preserves the endothelial barrier and resolves inflammation is summarized in Fig. 6.

However, our hypothesis is preliminary and needs additional and detailed investigation to sustain the mechanistic interface of PTP4A3 inhibition and Emilin protein (*i.e.*, emilins 1–3) upregulation. Our manuscript has several limitations, including the use of a single dose of K VX-053 and a single LPS concentration, the absence of dose-response or delayed treatment studies, and the lack of comprehensive multiplex cytokine profiling in BALF. The genetic knockdown for PTP4A3 significantly advances the relevance of K VX-053 effects, thus strongly supporting PTP4A3 as a druggable mediator of pulmonary inflammation and barrier dysfunction. The demonstration that K VX-053 is efficacious in an

LPS-driven model, in addition to SARS-CoV-2-driven models, provides compelling evidence that PTP4A3 inhibition targets a fundamental host inflammatory mechanism shared across diverse etiologies of ARDS. Future studies should focus on optimizing dosing strategies, exploring delayed treatment windows, validating the Emilin-1 findings at a protein level, and testing the efficacy of K VX-053 in additional preclinical models of ALI/ARDS.

Declarations

Author contribution

J.S.L., E.R.S., R.M.L.C.B. and J.D.C. conceived and designed research; R.M.L.C.B., P.A.S., D.E.A., A.E.Y., M.G.T.S., and T.D. performed experiments; R.M.L.C.B., P.A.S., T.D., A.E.Y., M.G.T.S., and D.E.A. and J.D.C. analyzed data; J.S.L., E.R.S. and J.D.C. interpreted results of experiments; R.M.L.C.B., P.A.S., prepared figures; R.M.L.C.B. drafted the manuscript; E.R.S., J.S.L., and J.D.C. edited and revised manuscript.

Consent for Publication:

All authors approved the final version of the manuscript and provided their consent for its publication.

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethical approval

All animal studies were approved by the Old Dominion University Institutional Animal Care and Use Committee (IACUC) and adhered to the principles of animal experimentation as published by the American Physiological Society. Studies were carried out in Biosafety Level 2 (BSL-2) and Animal Biosafety Level 2 (ABSL-2) facilities at the Frank Reidy Research Center for Bioelectrics in Norfolk, Virginia.

Informed Consent

Not applicable.

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Disclosures and conflict of interests

John S. Lazo and Elizabeth R. Sharlow are co-inventors of U.S. Patent No. 10,308,663, which describes the chemical synthesis, composition of matter and use of KVX-053 for the treatment of cancer. A provisional US patent application entitled “Composition and methods for treating/preventing lung injury” (Serial No. PCT/US2022/024927) and a European Union patent application (22788973.0) with the same title have been filed with John S. Lazo, Elizabeth R. Sharlow, John D. Catravas, Ruben M.L. Colunga Biancatelli, and Pavel A. Solopov being listed as co-inventors. John S. Lazo and Elizabeth Sharlow are co-founders of KeViRx, Inc, which has licensed the above-mentioned intellectual property from the University of Virginia, University of Pittsburgh, and Old Dominion University for further development.

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Figures

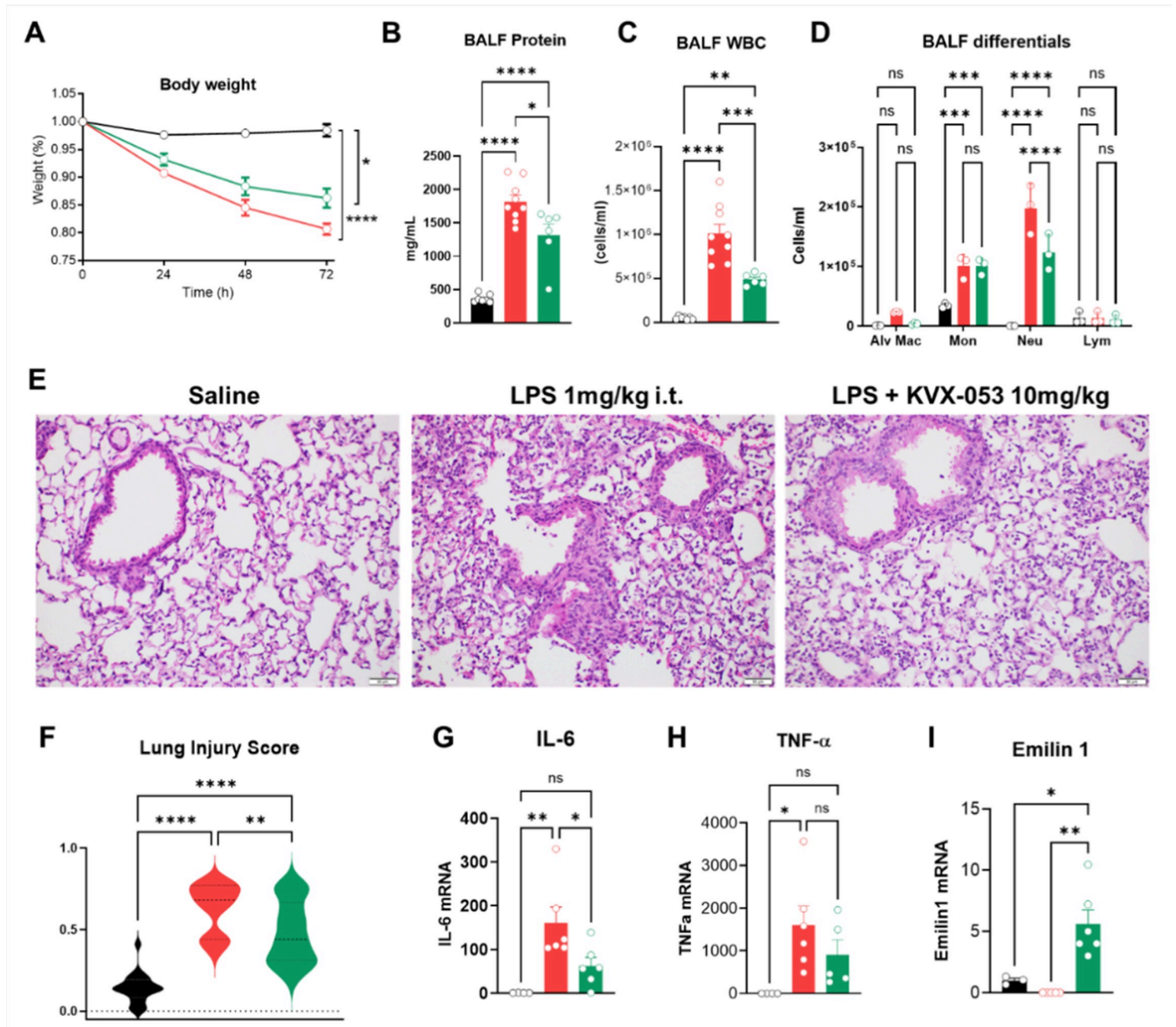


Figure 1

KVX-053 attenuates LPS-induced pulmonary injury and modulates inflammatory and extracellular matrix gene expression. C57BL/6J mice received intratracheal instillation of saline (black), LPS 1 mg/kg (red), or LPS followed by KVX-053 10 mg/kg i.p. every 24 hours (green). **(A)** Body weight expressed as percentage of initial weight over 72 hours. **(B)** BALF total protein concentration at 72 hours, **(C)** BALF total white blood cell (WBC) counts at 72 hours and **(D)** BALF differential cell counts at 72 hours: alveolar macrophages (*Alv Mac*), monocytes (*Mon*), neutrophils (*Neu*), and lymphocytes (*Lym*). **(E)** Representative H&E-stained lung sections at 72 hours from saline (*left*), LPS (*center*), and LPS + KVX-053 (*right*) groups. **(F)** Quantification of Lung Injury Score according to the ATS scoring system. **(G)** IL-6 mRNA expression in lung homogenates at 72 hours by RT-qPCR. **(H)** TNFα mRNA expression. **(I)** Emilin-1 mRNA expression. Data are presented as mean ± SEM. n = 6-8 per group. *:p < 0.05; **:p < 0.01; ***:p < 0.001; ****:p <

0.0001 by two-way repeated-measures ANOVA with Tukey's post hoc test (A) or one-way ANOVA with Tukey's post hoc test (B, C, F–I) or two-way ANOVA with Šídák's post hoc test (D). ns = not significant.

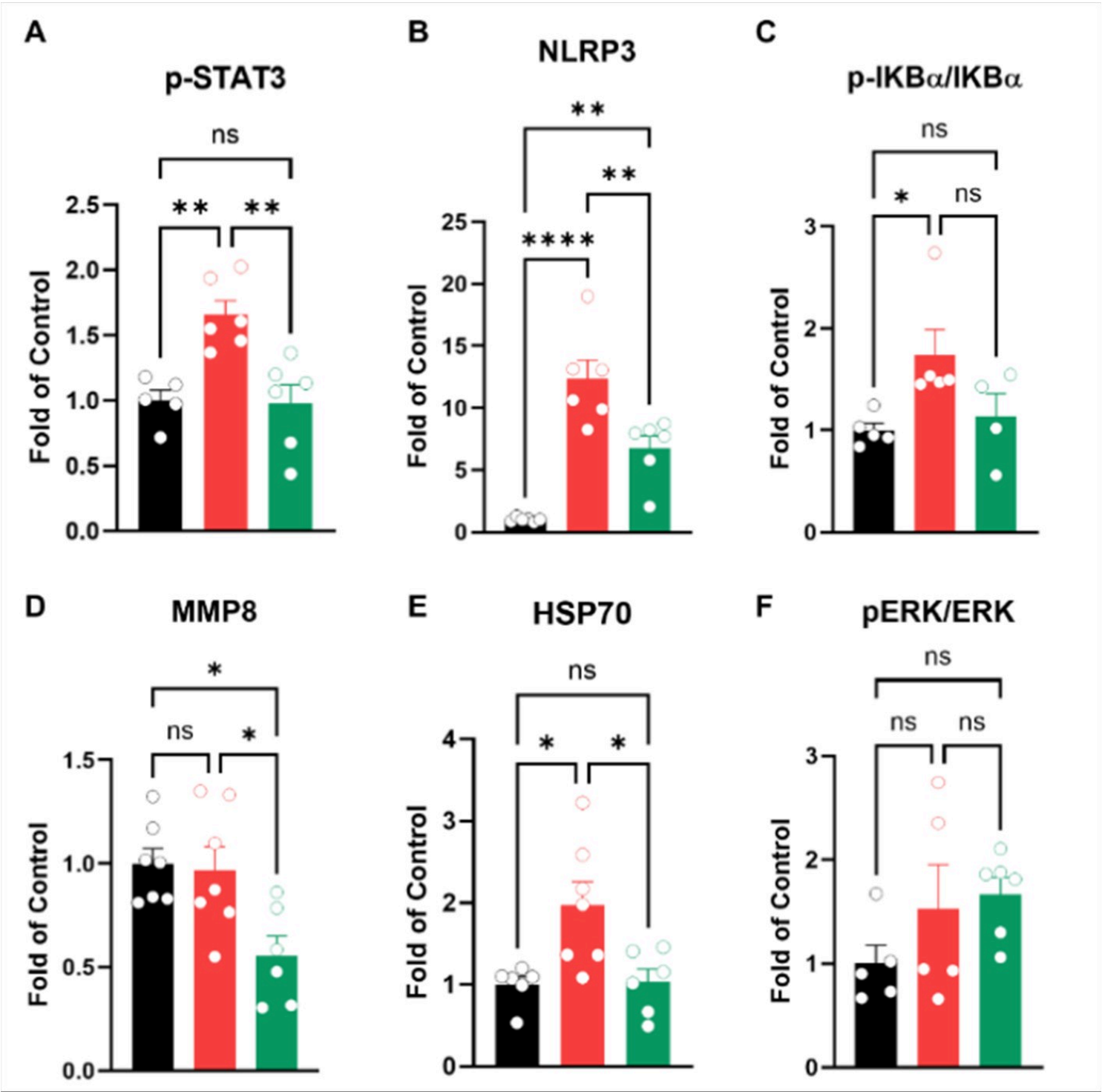


Figure 2

KVX-053 modulates inflammatory and stress signaling mediators in LPS-challenged lungs. Western blot densitometric analysis of lung homogenates from C57BL/6J mice at 72 hours post-instillation. (A) Phospho-STAT3/STAT3; (B) NLRP3; (C) Phospho-IkB α /IkB α ; (D) MMP8; (E) HSP70; and (F) Phospho-ERK/ERK. Black bars = saline; red bars = LPS; green bars = LPS + KVX-053. All values normalized to β -

actin and expressed as fold of control. Data are presented as mean $\pm$ SEM. n = 6-8 per group. *:p < 0.05; **:p < 0.01; ****:p < 0.0001 by one-way ANOVA with Tukey's post hoc test. ns = not significant.

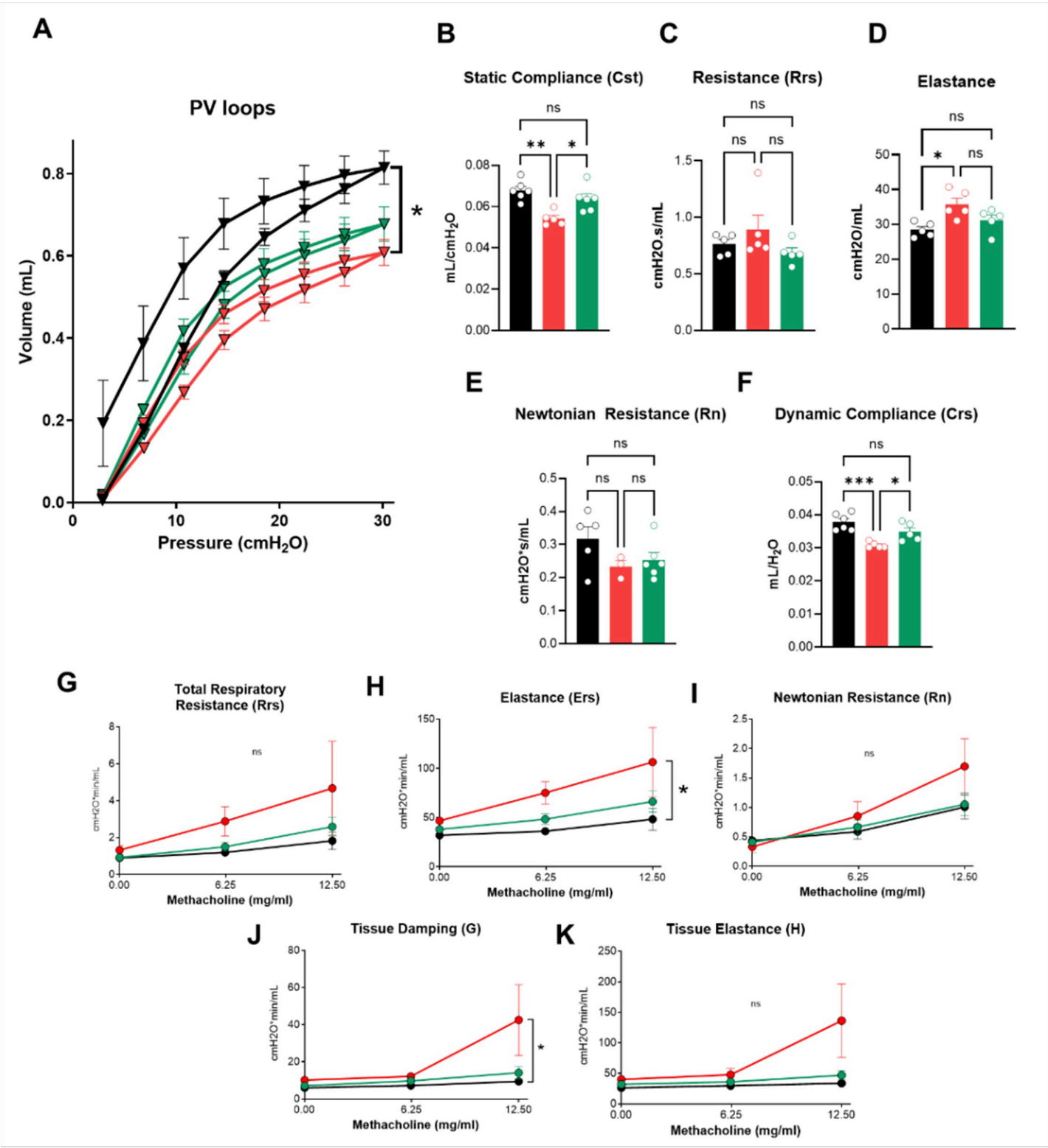


Figure 3

KVX-053 preserves pulmonary mechanics following LPS-induced acute lung injury. Pulmonary function assessed by flexiVent (SCIREQ) forced oscillation technique at 72 hours in C57BL/6J mice instilled with

saline (black), LPS 1 mg/kg (red), or LPS + K VX-053 10 mg/kg (green). **(A)** Pressure-volume (PV) loops; **(B)** Static compliance (Cst); **(C)** Total Respiratory Resistance (Rrs); **(D)** Elastance (Ers); **(E)** Newtonian resistance (Rn); and **(F)** Dynamic compliance (Crs). **(G–K)** Methacholine dose-response curves (0, 6.25, 12.5 mg/mL) for total respiratory resistance (Rrs, **G**), elastance (Ers, **H**), Newtonian resistance (Rn, **I**), tissue damping (G, **J**), and tissue elastance (H, **K**). Data are presented as mean ± SEM. n = 5–7 per group. *p < 0.05; **p < 0.01; ***p < 0.001 by one-way ANOVA with Tukey's post hoc test (B–F) or two-way repeated-measures ANOVA with Tukey's post hoc test (A, G–K). ns = not significant.

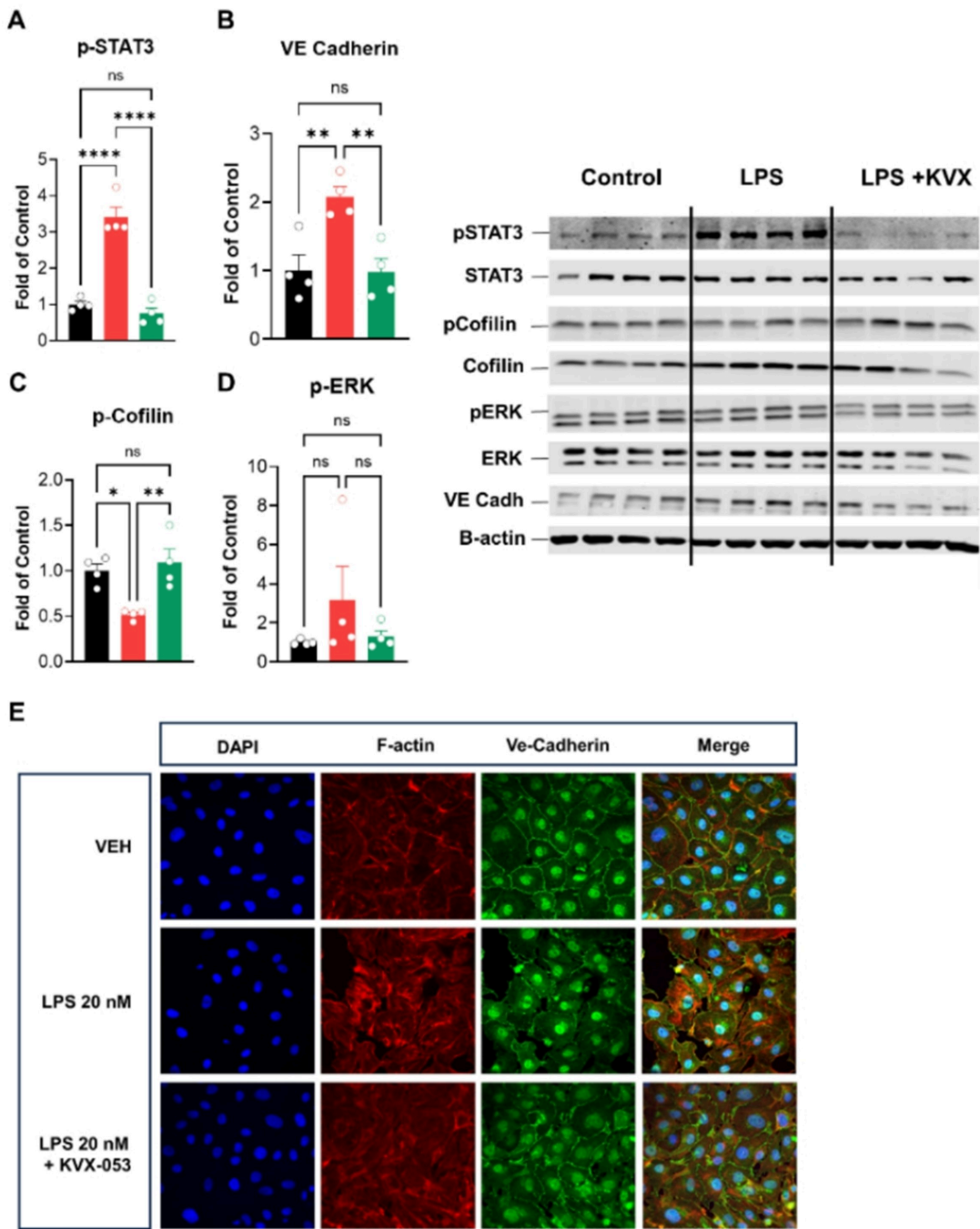


Figure 4

KVX-053 abolishes LPS-induced inflammation, barrier dysfunction and preserves cytoskeletal architecture in HLMVEC.

HLMVEC were pretreated with KVX-053 (12.5 μ M) or vehicle for 4 hours, followed by LPS (20 nM) for 3 hours. **(A)** Western blot densitometric analysis for phospho-STAT3/STAT3, **(B)** VE-Cadherin protein expression, **(C)** Phospho-Cofilin/Cofilin, and **(D)** Phospho-ERK/ERK.

Representative Western blots are shown to the right of the quantification panels. Black bars = vehicle control; red bars = LPS; green bars = LPS + KVX-053. Values normalized to β -actin (or respective total protein) and expressed as fold of housekeeping β actin and as fold of control. **(E)** Representative immunofluorescence images of HLMVEC stained for DAPI (*blue*), F-actin (*red*, phalloidin), VE-Cadherin (*green*), and merged images under vehicle (VEH), LPS (20 nM), and LPS + KVX-053 (12.5 μ M) conditions. Data are presented as mean $\pm$ SEM. n = 3–5 per group. *:p < 0.05; **:p < 0.01; ****:p < 0.0001 by one-way ANOVA with Tukey's post hoc test. ns = not significant.

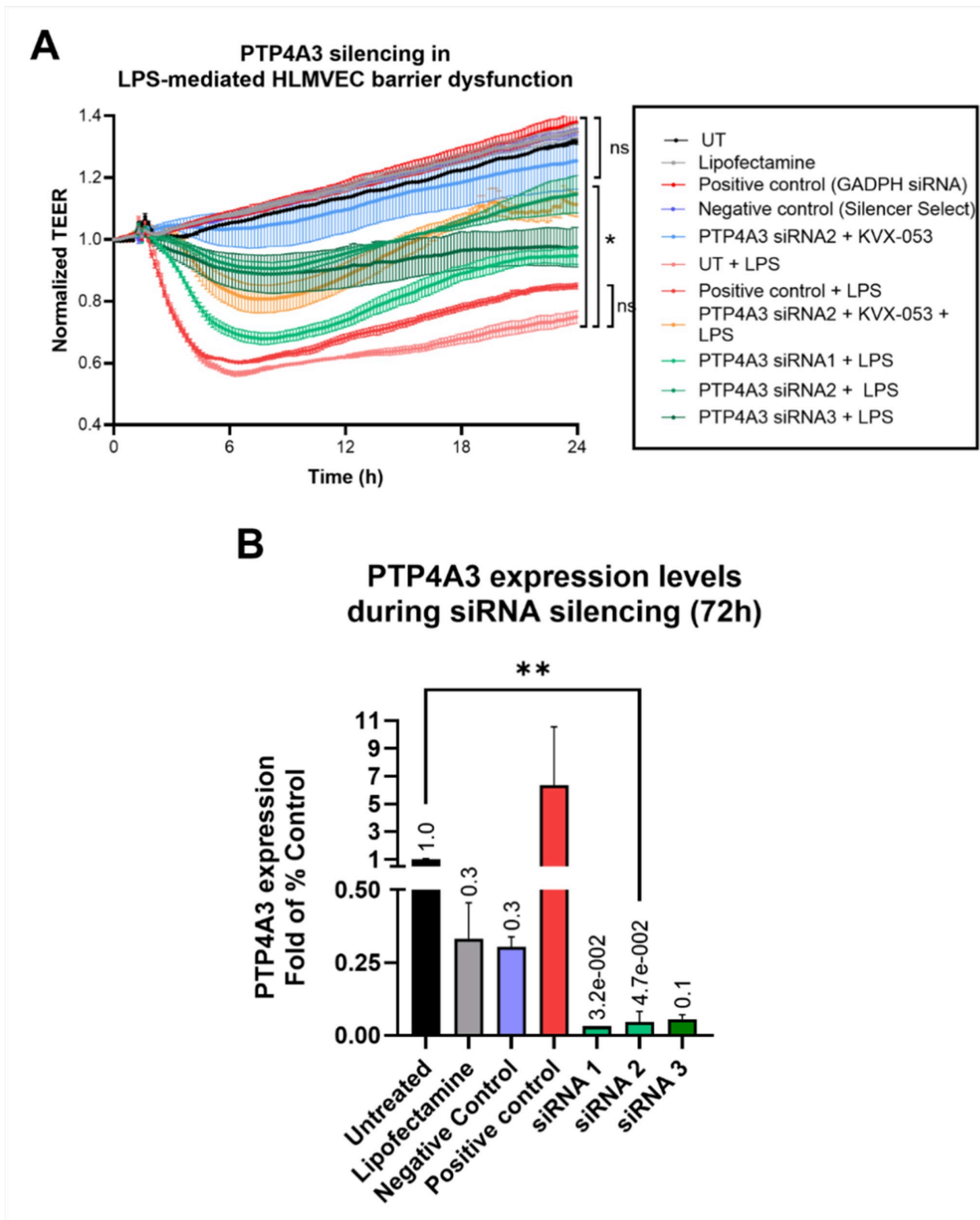


Figure 5

Genetic silencing of PTP4A3 reproduces the barrier protective effect of KVX-053 in LPS challenged HLMVEC. Pre seeded HLMVEC were monitored in real time by Electric Cell substrate Impedance Sensing (ECIS, 96W10+ array, Applied BioPhysics) and forward transfected 24 hours after seeding with a non-targeting negative control siRNA (Silencer Select), a GAPDH positive control siRNA, or one of three independent PTP4A3 targeting siRNAs (siRNA1, s22005; siRNA2, s22006; siRNA3, s22007), at a final

concentration of approximately 9.1 nM. Untransfected and lipofectamine only served as additional controls. Additionally, a PTP4A3 siRNA was pretreated with KVVX-053 (0.5 μ M) at 70 hours and challenged with LPS (100 ng/mL) at 72 hours, alongside untreated and LPS only conditions. (A) Normalized transendothelial electrical resistance (TEER) over 24 hours for the indicated conditions. (B) PTP4A3 mRNA expression at 72 hours after transfection, quantified by RT-qPCR and expressed as fold of untreated control. Data are presented as mean $\pm$ SEM. n = 4 per condition. *:p < 0.05; **:p < 0.01; ***:p < 0.001; ****:p < 0.0001 by one-way or two-way ANOVA with Tukey's post hoc test. ns = not significant.

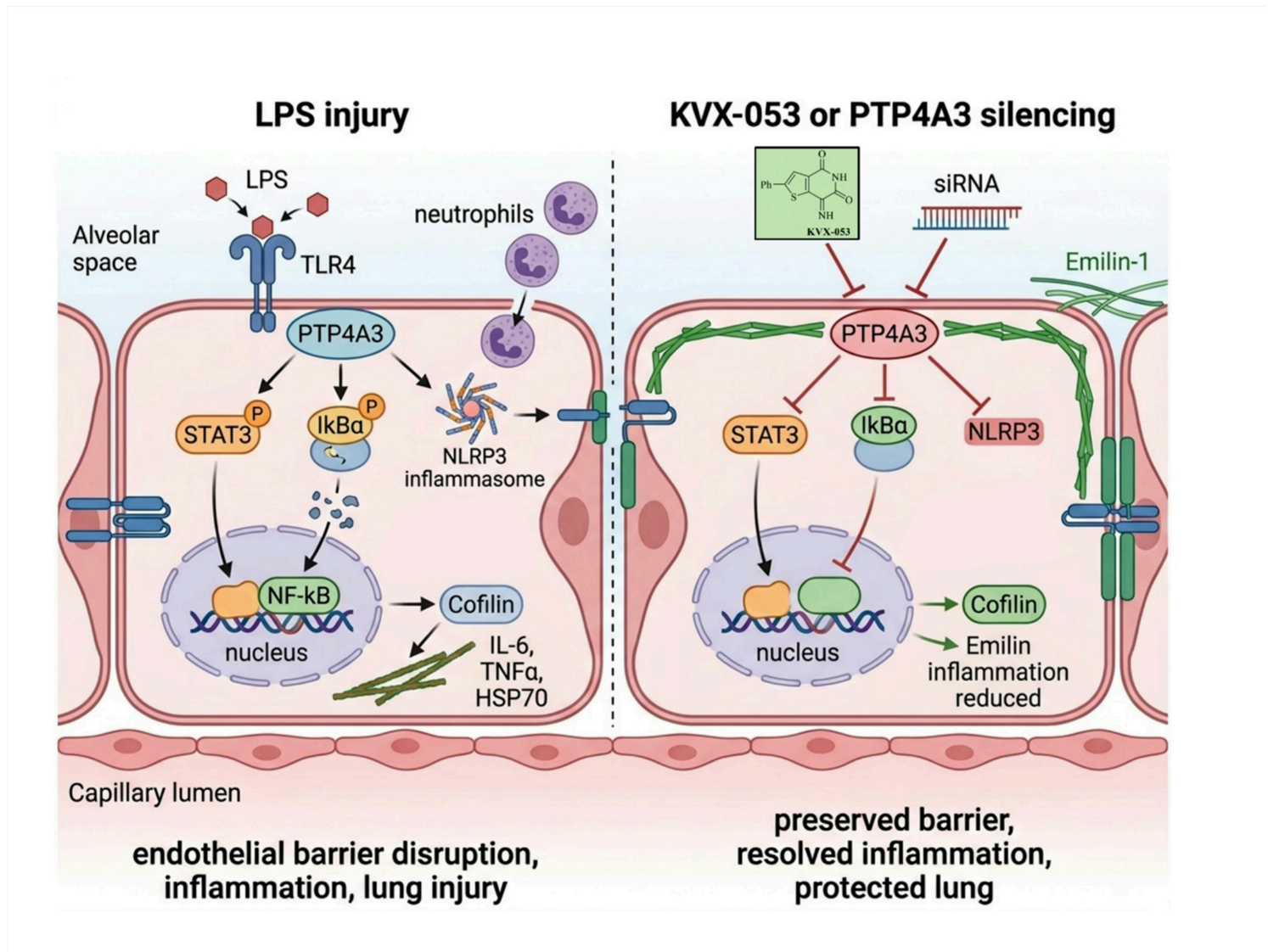


Figure 6

Proposed mechanism through which PTP4A3 inhibition preserves the endothelial barrier and resolves inflammation in LPS induced acute lung injury. Schematic contrasting the injured state (left) with the state produced by pharmacological or genetic inhibition of PTP4A3 (right). During LPS injury, LPS engages the Toll like receptor 4 (TLR4) complex at the alveolar surface and activates PTP4A3, which drives phosphorylation of STAT3 and I κ B α , assembly of the NLRP3 inflammasome, and induction of HSP70 and MMP8. Nuclear translocation of STAT3 and NF- κ B promotes transcription of IL-6, TNF α , and

HSP70, while Cofilin mediated actin remodeling and neutrophil recruitment contribute to cytoskeletal disruption, endothelial barrier failure, and lung injury. Inhibition of PTP4A3 by K VX-053, or its genetic silencing by siRNA, interrupts this signaling axis, reducing STAT3 and NF- κ B activation, NLRP3, HSP70, and MMP8, restoring the inactive phospho-Cofilin state, and preserving Emilin-1 in the extracellular matrix. The net result is a preserved endothelial barrier, resolved inflammation, and a protected lung. Created with OpenArt.

Supplementary Files

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