

Steen solution protects pulmonary microvascular endothelial cells and preserves endothelial barrier after lipopolysaccharide-induced injury



Huy Q. Ta, PhD, Nicholas R. Teman, MD, Irving L. Kron, MD, Mark E. Roeser, MD, and Victor E. Laubach, PhD

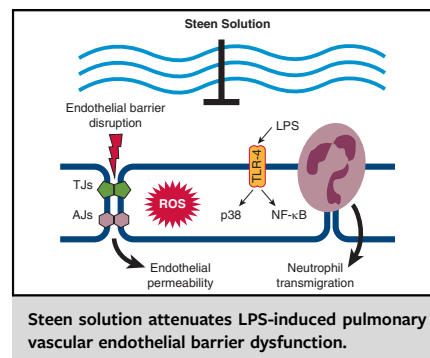
ABSTRACT

Objectives: Acute respiratory distress syndrome represents the devastating result of acute lung injury, with high mortality. Limited methods are available for rehabilitation of lungs affected by acute respiratory distress syndrome. Our laboratory has demonstrated rehabilitation of sepsis-injured lungs via normothermic ex vivo and in vivo perfusion with Steen solution (Steen). However, mechanisms responsible for the protective effects of Steen remain unclear. This study tests the hypothesis that Steen directly attenuates pulmonary endothelial barrier dysfunction and inflammation induced by lipopolysaccharide.

Methods: Primary pulmonary microvascular endothelial cells were exposed to lipopolysaccharide for 4 hours and then recovered for 8 hours in complete media (Media), Steen, or Steen followed by complete media (Steen/Media). Oxidative stress, chemokines, permeability, interendothelial junction proteins, and toll-like receptor 4-mediated pathways were assessed in pulmonary microvascular endothelial cells using standard methods.

Results: Lipopolysaccharide treatment of pulmonary microvascular endothelial cells and recovery in Media significantly induced reactive oxygen species, lipid peroxidation, expression of chemokines (eg, chemokine [C-X-C motif] ligand 1 and C-C motif chemokine ligand 2) and cell adhesion molecules (P-selectin, E-selectin, and vascular cell adhesion molecule 1), permeability, neutrophil transmigration, p38 mitogen-activated protein kinase and nuclear factor kappa B signaling, and decreased expression of tight and adherens junction proteins (zonula occludens-1, zonula occludens-2, and vascular endothelial-cadherin). All of these inflammatory pathways were significantly attenuated after recovery of pulmonary microvascular endothelial cells in Steen or Steen/Media.

Conclusions: Steen solution preserves pulmonary endothelial barrier function after lipopolysaccharide exposure by promoting an anti-inflammatory environment via attenuation of oxidative stress, toll-like receptor 4-mediated signaling, and conservation of interendothelial junctions. These protective mechanisms offer insight into the advancement of methods for in vivo lung perfusion with Steen for the treatment of severe acute respiratory distress syndrome. (J Thorac Cardiovasc Surg 2023;165:e5-20)



CENTRAL MESSAGE

Steen solution, used for isolated perfusion of injured lungs, preserves LPS-induced pulmonary endothelial barrier function by attenuating oxidative stress, TLR4 signaling, and permeability.

PERSPECTIVE

Severe ARDS lacks consistent, effective therapies. Ex vivo and in vivo lung perfusion with Steen solution shows promise in treating ARDS. We show that Steen solution preserves LPS-induced pulmonary endothelial barrier dysfunction by reducing oxidative stress, TLR4 signaling, and permeability. These results offer insight into advancement of lung perfusion methods to treat severe ARDS.

▶ Video clip is available online.

From the Department of Surgery, University of Virginia School of Medicine, Charlottesville, Va.

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Address for reprints: Victor E. Laubach, PhD, Department of Surgery, University of Virginia School of Medicine, PO Box 801359, Charlottesville, VA 22908 (E-mail: laubach@virginia.edu).

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Acute respiratory distress syndrome (ARDS) is a severe, life threatening form of acute lung injury characterized by an excessive inflammatory response with diffuse damage to vascular endothelial and alveolar epithelial barriers, resulting in hospital mortality rates as high as 40% to

Abbreviations and Acronyms

AJ	= adherens junction
ARDS	= acute respiratory distress syndrome
CAM	= cell adhesion molecule
CCL2	= C-C motif chemokine ligand 2
CXCL1	= chemokine (C-X-C motif) ligand 1
EVLP	= ex vivo lung perfusion
IVLP	= in vivo lung perfusion
KC	= keratinocytes-derived chemokine
LPS	= lipopolysaccharide
MAPK	= mitogen-activated protein kinase
MCP-1	= monocyte chemoattractant protein-1
Media	= complete media
NF- κ B	= nuclear factor κ B
PMVEC	= pulmonary microvascular endothelial cell
ROS	= reactive oxygen species
Steen	= Steen solution
TJ	= tight junction
TLR4	= toll-like receptor 4
VCAM1	= vascular cell adhesion molecule 1
VE-cadherin	= vascular endothelial-cadherin
ZO	= zonula occludens

50%.¹ The standard of care typically focuses on supportive care to allow for intrinsic organ recovery using measures such as ventilator support, medications, and extracorporeal membrane oxygenation in severe cases. There are currently no methods available to consistently treat and rehabilitate lungs affected by ARDS, and thus there is a real need for effective rehabilitative strategies.

In recent years, the development of ex vivo lung perfusion (EVLP) has enabled marginal donor lungs to be assessed prior to transplantation.² Our laboratory and others³ have demonstrated the potential of EVLP with Steen solution (Steen) to attenuate ischemia-reperfusion injury after transplant⁴ or sepsis-induced injury.⁵ Based on our studies with EVLP, we recently developed a novel technique of isolated, normothermic in vivo lung perfusion (IVLP) with Steen to attenuate lipopolysaccharide (LPS)-induced acute lung injury in swine.⁶ Here, IVLP resulted in improved oxygenation and lung compliance that coincided with reduced inflammation,^{6,7} suggesting that improved lung function after IVLP results from endothelial barrier protection to reduce edema and dampen inflammatory pathways.

Developed for the normothermic perfusion of donor lungs during EVLP, Steen is an acellular physiological solution consisting of human serum albumin, dextran 40, and electrolytes among other components. Steroids and antibiotics are usually added in a clinical setting. The precise mechanisms for the rehabilitative properties of Steen during

EVLP are unclear, and few studies have been conducted. In 2014, Carnevale and colleagues⁸ showed that Steen possesses antioxidant properties in platelets and white blood cells. More recently, Pagano and colleagues⁹ reported that Steen provides cytoprotection and free radical scavenging in endothelial cells and epithelial/basal cell lung spheroids by attenuating reactive oxygen species (ROS) after cold starvation and normothermic recovery. However, neither study addressed LPS-induced injury. In addition to direct properties of Steen components, other factors also contribute to the protective properties of Steen solution during organ perfusion such as ventilator strategies (eg, airway pressure release ventilation) and perfusion flow rates during EVLP.^{10,11}

Because ARDS frequently results from sepsis, pulmonary injury and endothelial barrier function are often studied using in vivo or in vitro models involving treatment with LPS, a major component of the outer wall of gram-negative bacteria. Thus, using an in vitro model of LPS-induced injury of pulmonary microvascular endothelial cells (PMVECs), the current study tested the hypothesis that Steen directly attenuates pulmonary endothelial barrier dysfunction and inflammation induced by LPS. Our results demonstrate that Steen preserves pulmonary endothelial barrier function after LPS treatment by promoting an anti-inflammatory environment through the attenuation of oxidative stress and preservation of interendothelial junctions (Video 1). To our knowledge, this is the first study to evaluate the protective mechanisms of Steen in a model of LPS-induced injury.

MATERIALS AND METHODS**Cell Culture and LPS Treatment**

Primary C57BL/6 murine PMVECs (Cell Biologics, Chicago, Ill) were grown in phenol red-free endothelial cell growth media (Cell



VIDEO 1. Dr Roeser introduces the significance, aims, hypothesis, methods, and results of the current study. He also discusses the clinical relevance of the study and how the results provide mechanistic insight and rationale for the continued study of the protective effects of isolated lung perfusion with Steen solution for the treatment of severe acute respiratory distress syndrome. Video available at: [https://www.jtcvs.org/article/S0022-5223\(22\)00409-3/fulltext](https://www.jtcvs.org/article/S0022-5223(22)00409-3/fulltext).

Biologics) containing 5% fetal bovine serum, 0.1% vascular endothelial growth factor, endothelial cell growth supplement, heparin, epidermal growth factor, hydrocortisone, 1% L-glutamine, and antibiotic-antimycotic solution. Primary porcine PMVECs (Cell Biologics) were grown in endothelial cell growth media with 2% fetal bovine serum, 0.1% vascular endothelial growth factor, epidermal growth factor, 1% L-glutamine, and antibiotic-antimycotic solution.

PMVECs were seeded on gelatin-coated plates or transmembrane inserts and grown to a uniform monolayer. See Figure 1 for a schematic of experimental conditions and timeline. At time 0, cells were washed with phosphate-buffered saline and exposed to 0, 1, or 100 $\mu\text{g/mL}$ LPS (*Escherichia coli* O127:B8, Sigma-Aldrich) in glucose-free, serum-free endothelial cell media for 4 hours. These LPS concentrations were based on a long history of studies utilizing an in vitro model of LPS-induced cell injury (including PMVECs¹²) as well as preliminary studies in our laboratory showing that 1 to 100 $\mu\text{g/mL}$ LPS significantly elevates markers of endothelial injury such as chemokines and permeability (some in a dose-dependent manner) with no loss in cell viability. After LPS treatment, cells were washed and recovered for 8 hours in 1 of 3 conditions: 8 hours in complete endothelial cell media (Media), 8 hours in Steen (XVivo Perfusion, Englewood, Colo), or 4 hours in Steen followed by 4 hours in complete endothelial cell media (Steen/Media). The Media condition represents the injury model without Steen intervention. The Steen condition was assessed to determine whether or not recovery in Steen alone offers protection. For some experiments (see Figures E1 and E2), cells were exposed to Perfadex solution (XVivo Perfusion) instead of Steen. The Steen/Media condition was assessed as an in vitro model of organ perfusion with Steen followed by reperfusion in blood (Media). All cultures were grown in a humidified incubator (37 °C, 5% carbon dioxide) and were washed in phosphate buffered saline after 4 hours of recovery to ensure a final, equal culture period of 4 hours in all groups.

ROS Assay

To quantify ROS, a dichlorodihydrofluorescein diacetate cellular ROS assay kit (ab113851) (Abcam) was utilized as instructed. PMVECs were grown on gelatin-coated 96-well plates, and dichlorodihydrofluorescein diacetate was added to cultures 30 minutes before the end of the recovery period. After washing, fluorescence was measured at 485/535 nm using a Tecan Infinite M Nano plate reader.

Lipid Peroxidation

To measure lipid peroxidation as an indicator of oxidative damage, a lipid peroxidation (malondialdehyde) assay kit (ab118970) (Abcam) was utilized as instructed. PMVECs were grown on gelatin-coated 60-mm dishes. After the recovery period, PMVECs were lysed in malondialdehyde lysis buffer, processed as instructed, and absorbance measured at 532 nm using a Tecan Infinite M Nano plate reader.

Measurement of Chemokines and Cell Adhesion Proteins

PMVECs were grown on gelatin-coated 24-well plates. Cell supernatant was collected at the end of the recovery period, centrifuged (1000 g, 5 minutes, 4 °C), and frozen at -80 °C. Concentration of chemokines (eg, keratinocytes-derived chemokine [KC]/chemokine [C-X-C motif] ligand 1 [CXCL1], and monocyte chemoattractant protein-1 [MCP-1]/C-C motif chemokine ligand 2 [CCL2]) and soluble cell adhesion molecules (sP-selectin, sE-selectin, and vascular cell adhesion molecule 1 [sVCAM-1]) were quantified by enzyme-linked immunosorbent assay (R&D Systems) according to the manufacturer's protocol.

Endothelial Permeability

To assess endothelial permeability, PMVECs were grown on gelatin-coated 24-transwell membrane inserts (0.4 μm pores) to form a monolayer. One hour before completion of the recovery period, 1 mg/mL 70 kDa fluorescein isothiocyanate-dextran (Sigma-Aldrich) was added to the upper well. At the conclusion of the recovery period, fluorescence in the lower chamber was measured at 490/525 nm using a Tecan Infinite M Nano plate reader.

Neutrophil Transendothelial Migration

Neutrophil transmigration assays were performed as previously described using a CytoSelect Leukocyte Transmigration Assay (Cell Biolabs Inc).¹³ Neutrophils were isolated from C57BL/6 mouse spleens using a mouse Neutrophil Isolation Kit (Miltenyi Biotec) and labeled with LeukoTracker dye. PMVECs were grown on gelatin-coated 24-transwell membrane inserts (3 μm pores) for 48 hours to form a monolayer. Following the designated recovery period, fresh LeukoTracker-labeled neutrophils were added to each transwell for 4 hours. Transmigrated neutrophils were quantified by measuring

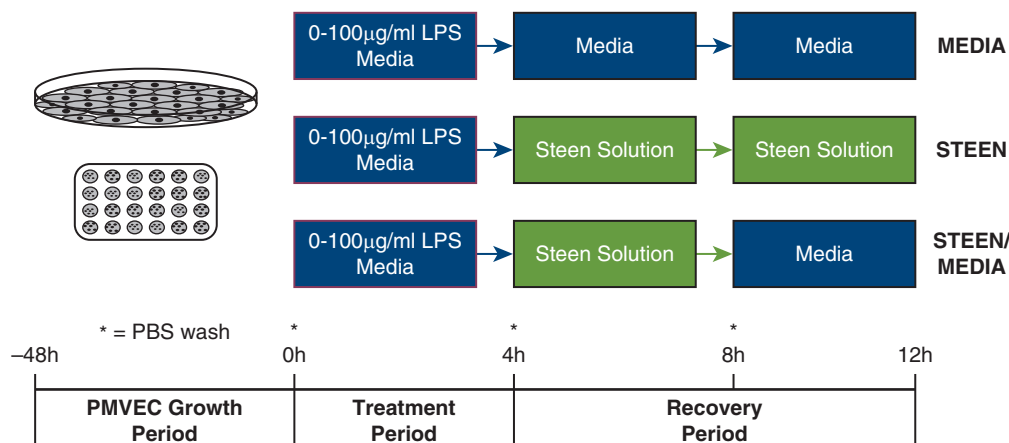


FIGURE 1. Experimental design. A total of 9 conditions were studied using pulmonary microvascular endothelial cells (PMVECs). The 3 major groups are shown horizontally. Following 48 hours of cell growth, a 4-hour treatment period started at time 0 involving exposure of PMVECs to 0, 1, or 100 $\mu\text{g/mL}$ lipopolysaccharide (LPS) in glucose-free, serum-free endothelial cell media (red outline). After washing in phosphate buffered saline (PBS), cells were then recovered in either 8 hours of complete endothelial cell media (MEDIA), 8 hours of Steen solution (STEEN), or 4 hours of STEEN followed by 4 hours of complete endothelial cell media (STEEN/MEDIA).

the fluorescence at 480/520 nm in the lower chamber using a Tecan Infinite M Nano plate reader.

Measurement of p38 Mitogen-Activated Protein Kinase and Nuclear Factor- κ B p65 Activity

Expression of total and phosphorylated p38 mitogen-activated protein kinase (MAPK) in PMVECs was assessed using a Fast Activated Cell-based ELISA (Active Motif) as instructed. To determine cell number, plates were stained with Crystal Violet for 30 minutes, washed, lysed in 1% sodium dodecyl sulfate, and absorbance was measured at 595 nm. Expression of phospho-nuclear factor (NF)- κ B p65 was quantitated using a phospho-NF- κ B p65 (S536) ELISA kit (ab279873) as instructed (Active Motif). PMVECs were grown on gelatin-coated 96-well plates. At the end of the recovery period, plates were processed as instructed, and absorbance was measured at 450 nm using a Tecan Infinite M Nano plate reader.

Immunoblotting

PMVECs were grown on gelatin-coated 60-mm plates and lysed in Pierce IP Lysis Buffer (ThermoFisher Scientific), and electrophoresis (Bolt, 4%-12% Bis-Tris Plus Gels; ThermoFisher Scientific) was performed on cell lysates (25 μ g/well). Proteins were transferred to nitrocellulose membranes (Life Technologies) using a Trans-Blot Turbo Transfer System (Bio-Rad Laboratories). Blots were blocked with 5% nonfat dry milk and incubated overnight at 4 °C with primary antibody: anti-GAPDH (Santa Cruz Biotechnology), anti-claudin-5, anti-VE-cadherin, anti-ZO-1 (ThermoFisher Scientific), or anti-ZO-2 (Cell Signaling). Following incubation with HRP-Conjugated Secondary Antibody (Santa Cruz Biotechnology), blots were imaged using an Amersham Imager 680 (GE Healthcare) and quantified using ImageJ software.

Statistical Methods

Statistical analyses were performed using Prism 9 software (GraphPad Inc). Ordinary 1-way analysis of variance was performed with post hoc Tukey's multiple comparison test. Data are presented using box-and-whisker plots where the lower and upper borders of the box represent the first and third quartiles, respectively; the middle horizontal line represents the median; and the whiskers represent the minimum and maximum data values. All data for each group are represented as individual dots on each graph. Unless indicated otherwise, for each group shown in the graphs, cells were grown in duplicate or triplicate (eg, 3 wells of a 96-well plate per condition) to obtain an average value per experiment. The experiment was then independently repeated (using separate plates on different days) for the indicated number of times whereby the average of these values is the final average for the group (condition).

RESULTS

Recovery in Steen Attenuates LPS-Induced Oxidative Stress

Oxidative stress is a component of the inflammatory response to LPS, and uninhibited activation of this response can lead to endothelial barrier dysfunction; a key aspect of ARDS.¹⁴ To determine the effect of Steen on oxidative stress in PMVECs, we measured ROS generation and lipid peroxidation as markers of oxidative stress. LPS induced dose-dependent increases in ROS levels in murine PMVECs recovered in Media, which were effectively blocked in cells recovered in Steen or Steen/Media (Figure 2, A). Recovery in Steen also reduced baseline ROS levels in untreated cells. Furthermore, similar results were observed in porcine PMVECs (Figure 3, A).

Lipid peroxidation was significantly elevated by LPS in murine PMVECs recovered in Media, which was mitigated by recovery in Steen or Steen/Media (Figure 2, B). Baseline lipid peroxidation was significantly reduced in untreated cells recovered in Steen or Steen/Media. Furthermore, similar results were observed in porcine PMVECs (Figure 3, B).

Recovery in Steen Attenuates LPS-Induced Chemokines and Cell Adhesion Molecules

Expression of chemokines and cell adhesion molecules (CAMs) play vital roles in the pathogenesis of ARDS to mediate endothelial cell activation and provoke leukocyte adhesion and transendothelial migration.¹⁵ Thus, the effect of Steen on the expression of several key chemokines (KC/CXCL1 and MCP-1/CCL2) and soluble CAMs (sP-selectin, sE-selectin, and sVCAM-1) by murine PMVECs was measured. LPS treatment induced significant production of KC/CXCL1 and MCP-1/CCL2 in a concentration-dependent manner after recovery in Media, which was blocked by recovery in Steen or Steen/Media (Figure 4, A and B). Similarly, the expression of sP-selectin, sE-selectin, and sVCAM-1 were significantly elevated by LPS-treatment after recovery in Media, which was effectively blocked by recovery in Steen or Steen/Media (Figure 4, C through E). These results indicate that Steen inhibits LPS-induced expression of chemokines and CAMs by PMVECs.

Endothelial Barrier Function is Preserved After Recovery in Steen

Permeability of murine PMVEC monolayers to fluorescein isothiocyanate-dextran was significantly elevated in a dose-dependent manner by LPS treatment and recovery in Media, which was completely blocked by recovery in Steen or Steen/Media (Figure 5, A). Baseline permeability of untreated PMVECs was also reduced by recovery in Steen or Steen/Media versus Media. Furthermore, LPS-induced permeability of porcine PMVECs was also blocked by recovery in Steen or Steen/Media (Figure 3, C).

To further assess endothelial barrier dysfunction, neutrophil transmigration through PMVEC monolayers was assessed. LPS treatment significantly induced neutrophil transmigration 3-fold after recovery in Media, which was significantly attenuated by recovery in Steen or Steen/Media (Figure 5, B). Taken together, these results demonstrate that Steen preserves endothelial barrier function after LPS treatment.

Recovery in Steen Attenuates LPS-Induced Changes in Interendothelial Junctions

Interendothelial junctions, such as tight junctions (TJs) and adherens junctions (AJs), play crucial roles in barrier integrity and barrier function, respectively. TJs are

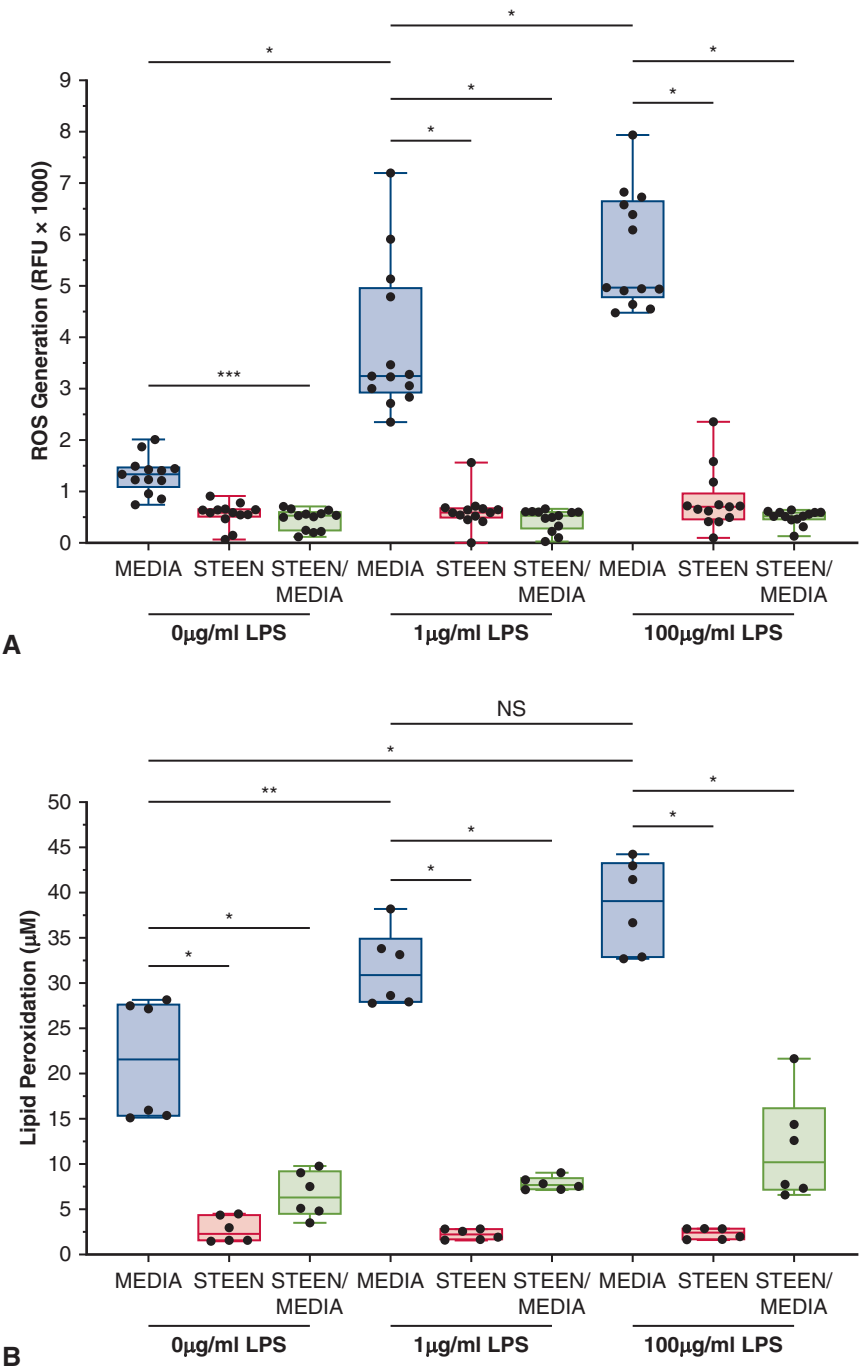


FIGURE 2. Recovery of lipopolysaccharide (LPS)-treated pulmonary microvascular endothelial cells (PMVECs) in Steen solution (STEEN) attenuates oxidative stress. A, Generation of reactive oxygen species (ROS) in PMVECs was significantly elevated after LPS treatment and recovery in complete endothelial cell media (MEDIA), which was significantly attenuated after recovery in STEEN or STEEN/MEDIA (n = 13/group). B, Similar to ROS generation, lipid peroxidation was significantly induced by LPS treatment, which was significantly attenuated after recovery in STEEN or STEEN/MEDIA (n = 6/group). Data are presented using box-and-whisker plots where the lower and upper borders of the box represent the first and third quartiles, respectively; the middle horizontal line represents the median; and the whiskers represent the minimum and maximum data values. Experiments were performed in triplicate and repeated for the indicated number of times per group. RFU, Relative fluorescence units; NS, not significant. *P < .0001. **P < .002.

composed of claudins, occludins, junctional adhesion molecules, and zonula occludens (ZO) 1 and ZO-2, and function as a selective barrier to molecules entering from the

circulation.¹⁶ VE-cadherin is a major transmembrane protein of AJs essential for maintenance of endothelial permeability.¹⁷ To determine the effects of LPS on

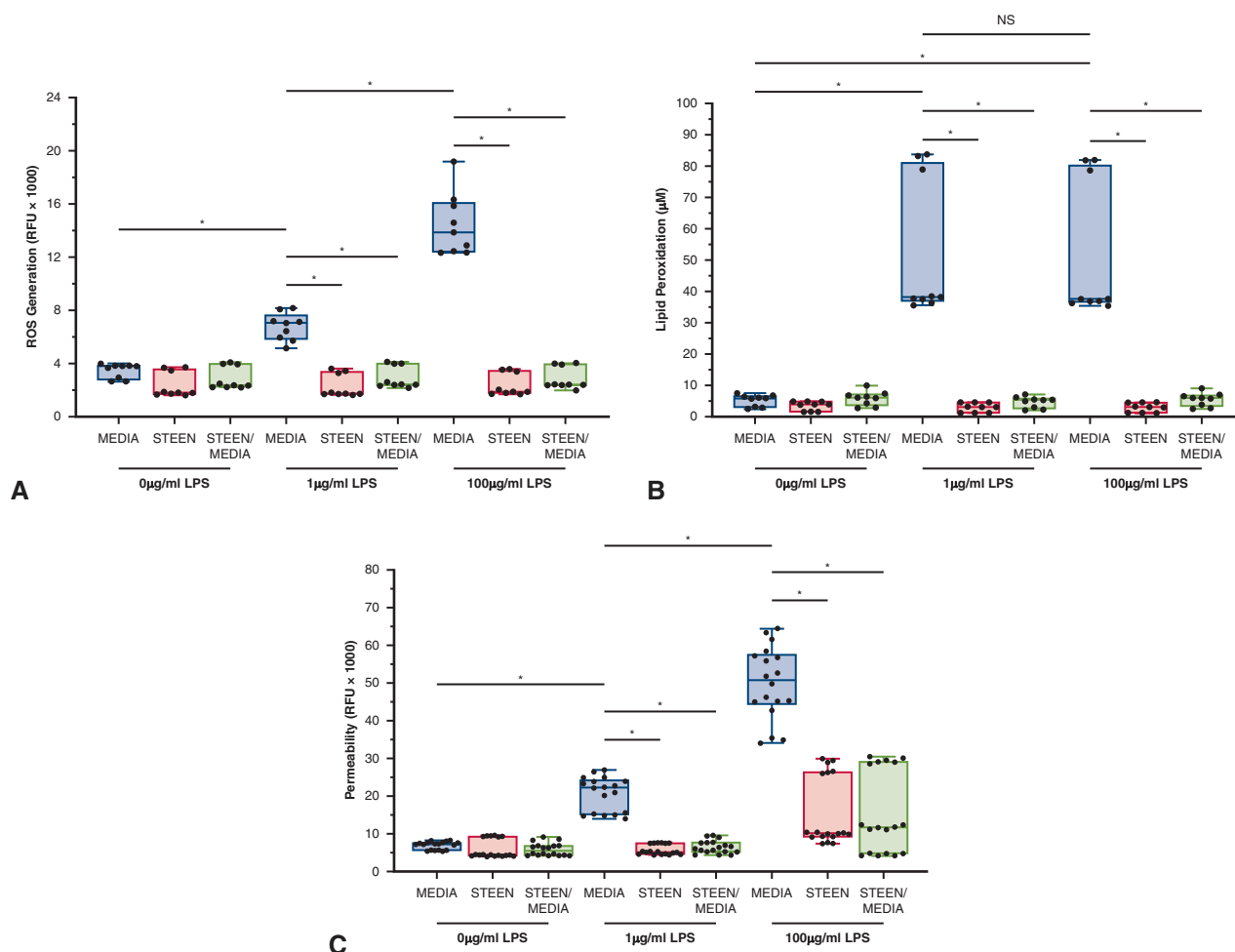


FIGURE 3. Steen solution (*STEEN*) attenuates oxidative stress and endothelial permeability following lipopolysaccharide (*LPS*) treatment of porcine pulmonary microvascular endothelial cells (PMVECs). Primary porcine PMVECs were evaluated to corroborate results obtained with murine PMVECs. Similar to results with murine PMVECs, oxidative stress as measured by reactive oxygen species (*ROS*) generation ($n = 9/\text{group}$) (A) and lipid peroxidation ($n = 9/\text{group}$) (B), as well as endothelial permeability ($n = 18/\text{group}$) (C), were significantly elevated after *LPS* treatment and recovery in complete endothelial cell media (*MEDIA*); all of which were significantly attenuated by recovery in *STEEN* or *STEEN/MEDIA*. Data are presented using box-and-whisker plots where the lower and upper borders of the box represent the first and third quartiles, respectively; the middle horizontal line represents the median; and the whiskers represent the minimum and maximum data values. Experiments were performed in triplicate and repeated for the indicated number of times per group. *RFU*, Relative fluorescence units; *NS*, not significant. $*P < .0001$.

interendothelial junctions, we assessed expression of ZO-1, ZO-2, VE-cadherin, and claudin-5 in murine PMVECs by Western blot. *LPS* treatment caused a decrease in ZO-1, ZO-2, and VE-cadherin protein levels in cells recovered in Media, whereas claudin-5 expression was increased (Figure 6). However, these *LPS*-induced changes in expression of ZO-1, ZO-2, VE-cadherin, and claudin-5 were significantly attenuated by recovery in Steen or Steen/Media (Figure 6). No significant changes in the expression of interendothelial junction proteins occludin, β -catenin, or p120 catenin were observed after *LPS* treatment (data not shown). These results suggest that Steen preserves interendothelial junctions after *LPS* exposure.

LPS-Induced Toll-Like Receptor 4 Signaling Is Dampened by Recovery in Steen

LPS is a major component of the outer wall of gram-negative bacteria that binds toll-like receptor 4 (TLR4) and activates inflammatory signaling pathways including p38 MAPK and NF- κ B.¹⁸ Although *LPS*-treatment and recovery in Media did not significantly alter total p38 protein expression in murine PMVECs, recovery in Steen or Steen/Media consistently reduced (although not significantly) total p38 levels versus Media (Figure 7, A). Phospho-p38, however, was significantly increased following *LPS* treatment and recovery in Media, which was significantly attenuated by recovery in Steen or Steen/Media (Figure 7, B).

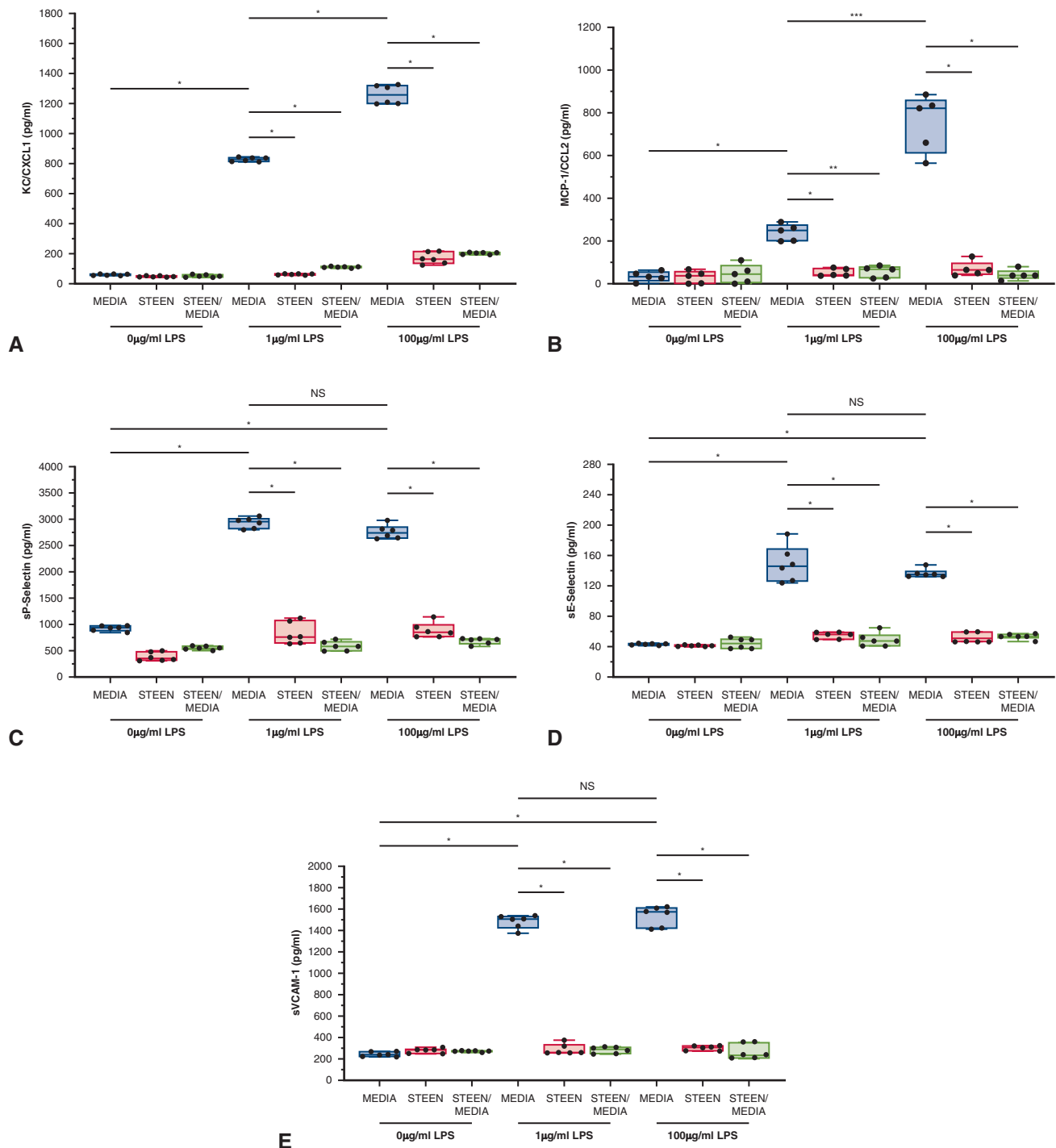


FIGURE 4. Recovery of lipopolysaccharide (LPS)-treated pulmonary microvascular endothelial cells (PMVECs) in Steen solution (STEEN) attenuates expression of proinflammatory chemokines and cell adhesion molecules. Expression of keratinocytes-derived chemokine (KC)/chemokine (C-X-C motif) ligand 1 (CXCL1) (n = 6/group) (A) and monocyte chemoattractant protein-1 (MCP-1)/C-C motif chemokine ligand 2 (CCL2) (n = 5/group) (B) were significantly elevated in LPS-treated PMVECs after recovery in complete endothelial cell media (MEDIA), which was significantly attenuated after recovery in STEEN or STEEN/MEDIA. Similar to chemokines, expression of sP-selectin (n = 6/group) (C), sE-selectin (n = 6/group) (D), and vascular cell adhesion molecule 1 (sVCAM-1) (n = 6/group) (E) were significantly elevated in LPS-treated PMVECs after recovery in MEDIA, which were significantly attenuated after recovery in STEEN or STEEN/MEDIA. Data are presented using box-and-whisker plots where the lower and upper borders of the box represent the first and third quartiles, respectively; the middle horizontal line represents the median; and the whiskers represent the minimum and maximum data values. Experiments were performed in duplicate and repeated for the indicated number of times per group. NS, Not significant. * $P < .0001$. ** $P = .0001$. *** $P = .0004$.

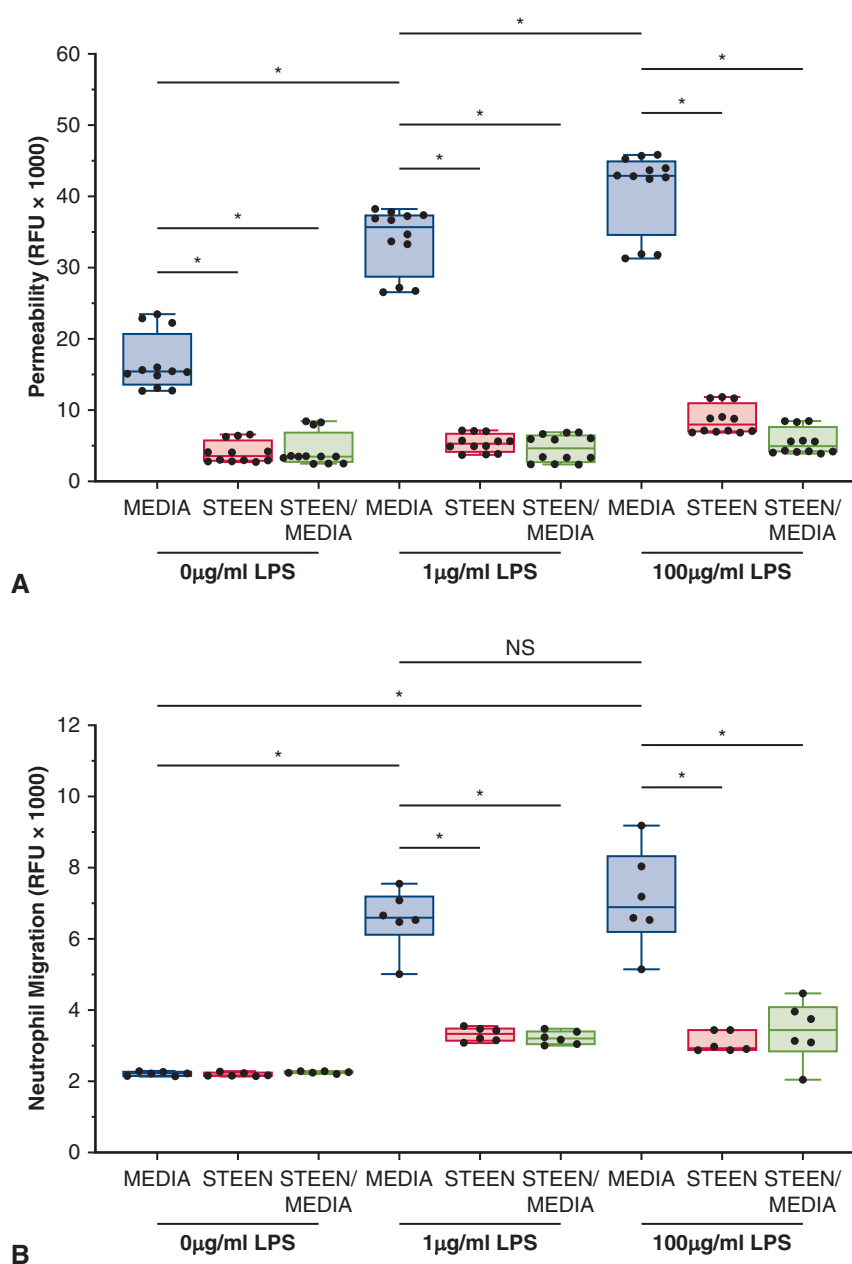


FIGURE 5. Lipopolysaccharide (LPS)-induced endothelial barrier dysfunction is attenuated by recovery of pulmonary microvascular endothelial cells (PMVECs) in Steen solution (STEEN). A, Permeability of fluorescein isothiocyanate (FITC)-dextran by PMVEC monolayers was significantly elevated after LPS treatment and recovery in complete endothelial cell media (MEDIA), which was significantly attenuated by recovery in STEEN or STEEN/MEDIA ($n = 12/\text{group}$). (B) Transmigration of neutrophils through the PMVEC monolayer was measured using a neutrophil transmigration assay ($n = 6/\text{group}$). Neutrophil transmigration was significantly elevated after LPS treatment and recovery in MEDIA, which was significantly attenuated by recovery in STEEN or STEEN/MEDIA. Data are presented using box-and-whisker plots where the lower and upper borders of the box represent the first and third quartiles, respectively; the middle horizontal line represents the median; and the whiskers represent the minimum and maximum data values. Experiments were performed in triplicate and repeated for the indicated number of times per group. RFU, Relative fluorescence units; NS, not significant. * $P < .0001$.

Phosphorylation of NF- κ B p65 was measured as an indicator of active NF- κ B signaling. LPS induced a dose-dependent increase in phospho-p65 expression in murine PMVECs recovered in MEDIA, which was greatly and significantly reduced in cells recovered in Steen or Steen/MEDIA

(Figure 7, C). Background phospho-p65 expression was significantly reduced in untreated cells after recovery in Steen or Steen/MEDIA versus MEDIA. Taken together, these results indicate that Steen attenuates TLR4-mediated p38 MAPK and NF- κ B signaling in LPS-treated PMVECs.

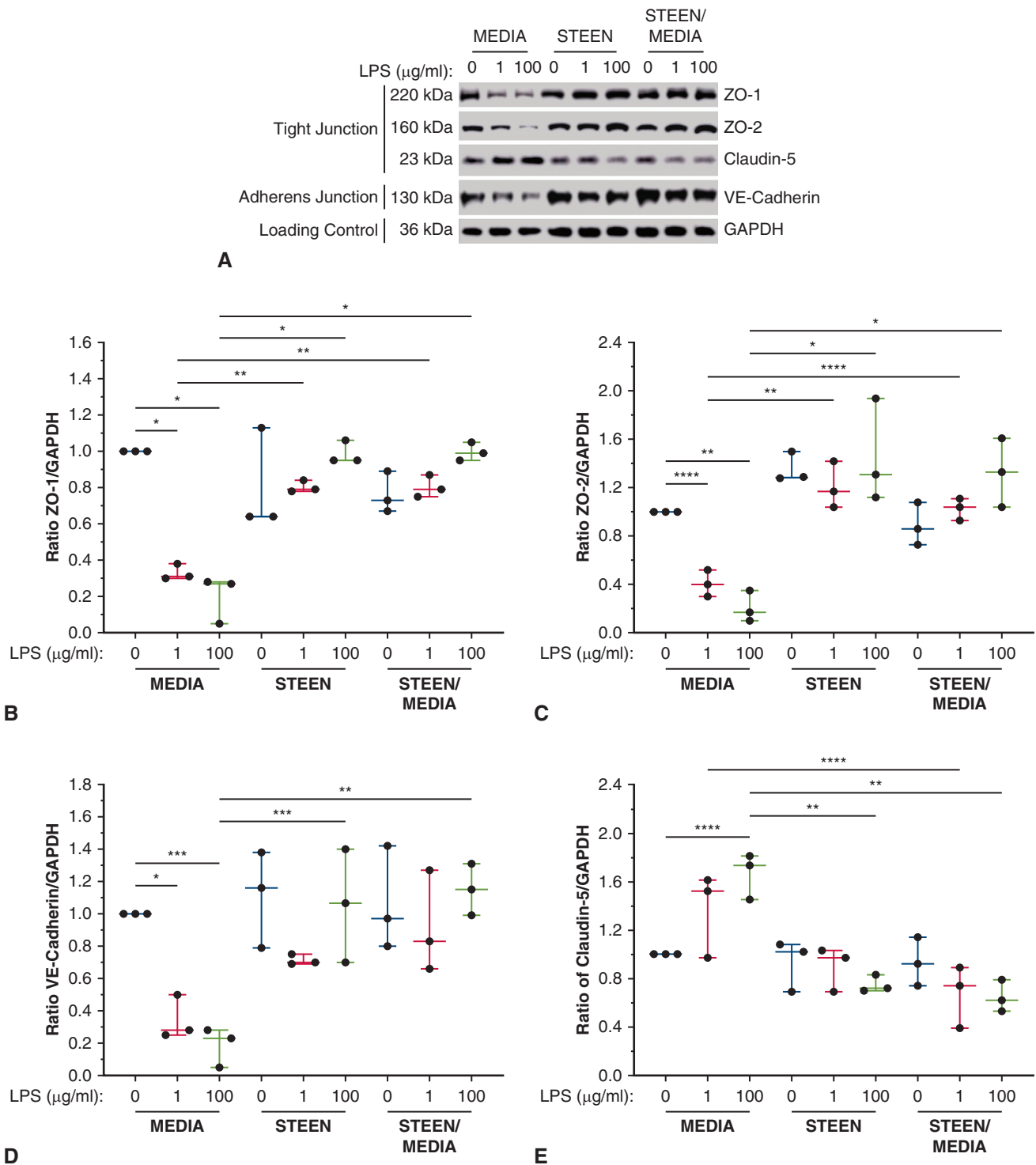


FIGURE 6. Recovery of lipopolysaccharide (LPS)-treated pulmonary microvascular endothelial cells (PMVECs) in Steen solution (STEEN) preserved interendothelial junctions. Expression of interendothelial junction proteins (zonula occludens-1 [ZO-1], zonula occludens-2 [ZO-2], vascular endothelial-cadherin [VE-cadherin], and claudin-5), as a ratio to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression, was measured by immunoblotting as described in the methods. A, Representative Western blots are shown for each interendothelial junction protein. The graphs below show quantification of Western blots normalized to the loading control GAPDH. Expression of ZO-1 (B), ZO-2 (C), and VE-cadherin (D) were significantly reduced in LPS-treated PMVECs after recovery in complete endothelial cell media (MEDIA), which was significantly attenuated by recovery in STEEN or STEEN/MEDIA. Expression of claudin-5 (E) was increased after LPS treatment, which was attenuated by recovery in STEEN or STEEN/MEDIA. All experiments were performed in triplicate (n = 3/group). **P* < .0001. ***P* < .005. ****P* < .01. *****P* < .05.

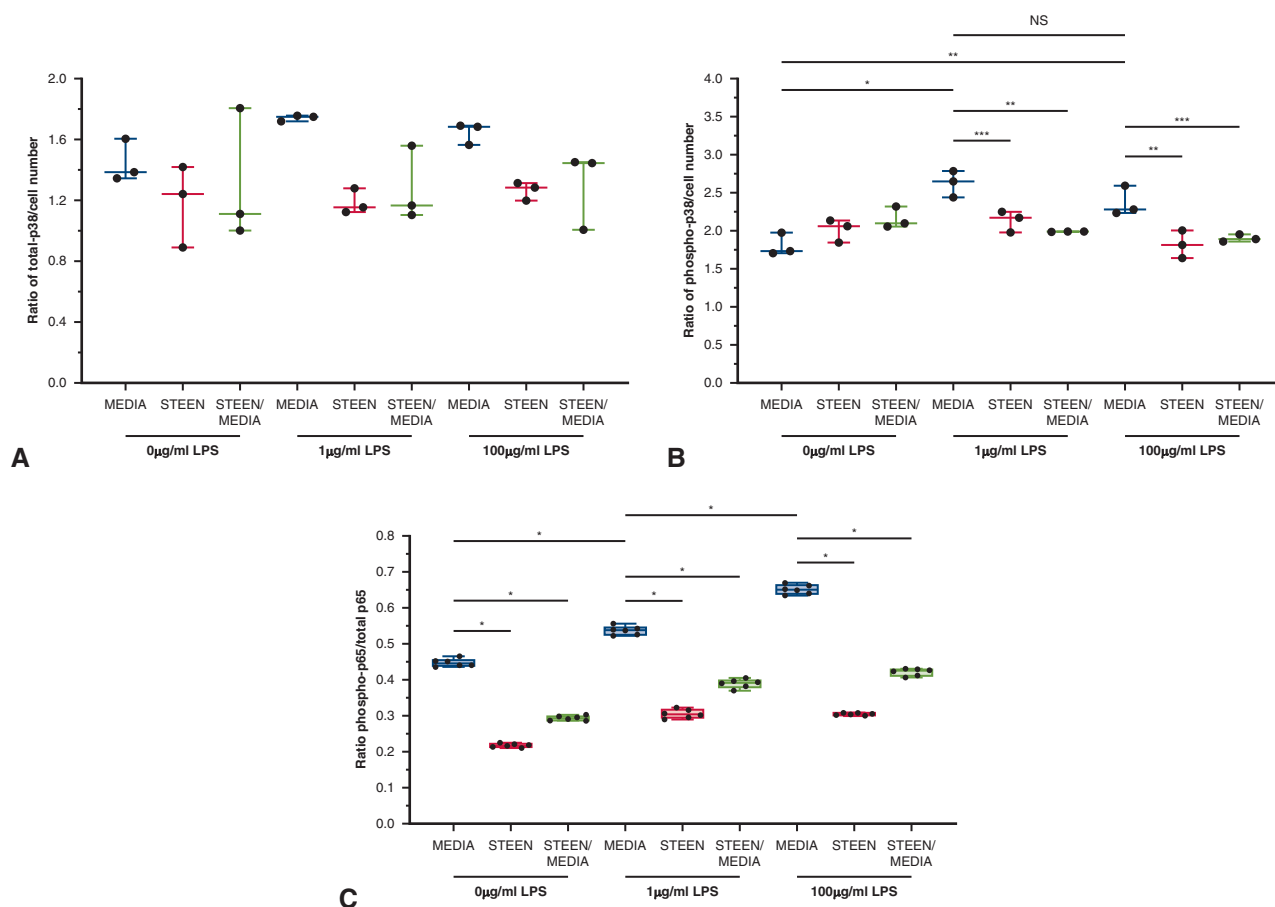


FIGURE 7. Activation of toll-like receptor 4 (TLR4)-mediated inflammatory signaling in lipopolysaccharide (LPS)-treated pulmonary microvascular endothelial cells (PMVECs) was attenuated by Steen solution (STEEN). A, LPS-treatment of PMVECs did not significantly alter total p38 mitogen-activated protein kinase (MAPK) expression ($n = 3/\text{group}$). However, phosphorylation of p38 ($n = 3/\text{group}$) (B) and nuclear factor kappa B (NF-κB) p65 ($n = 6/\text{group}$) (C) were both significantly increased following LPS treatment and recovery in complete endothelial cell media (MEDIA), which were significantly attenuated by recovery in STEEN or STEEN/MEDIA. Data are presented using box-and-whisker plots where the lower and upper borders of the box represent the first and third quartiles, respectively; the middle horizontal line represents the median; and the whiskers represent the minimum and maximum data values. NS, Not significant. * $P < .0001$. ** $P < .005$. *** $P < .02$.

Perfadex Provides Protection Similar to Steen

Perfadex solution is used for the flushing, cold static storage, and transportation of isolated donor lungs for transplantation. Perfadex, similar to Steen in many ways, is a low-potassium, dextran-based electrolyte solution. To determine if Perfadex has similar protective properties as Steen solution, we compared several outcomes after recovery of LPS-treated mouse or porcine PMVECs in Perfadex or Steen. Similar to results with Steen solution, LPS-treated murine PMVECs recovered in Perfadex for 8 hours resulted in significant attenuation of ROS generation, lipid peroxidation, and neutrophil transendothelial migration (Figure E1). Additionally, recovery of LPS-treated porcine PMVECs in Perfadex resulted in significant attenuation of ROS generation, lipid peroxidation, and endothelial permeability (Figure E2). These results suggest that significant endothelial protection in vitro can be achieved with Steen or Perfadex solutions.

DISCUSSION

ARDS entails excessive inflammatory responses with diffuse damage to both vascular endothelial and alveolar epithelial cell barriers, which greatly affects lung function.¹ We have previously demonstrated that EVLP or IVLP with Steen can recondition LPS-injured lungs.^{5,6} Steen solution is a buffered extracellular solution optimally designed to perfuse donor lungs during normothermic EVLP. A major ingredient in Steen is human albumin, the predominant protein in blood plasma, which has many functions, including maintenance of osmotic pressure.¹⁹ Dextran 40, another Steen ingredient, serves to protect the endothelium from excessive leukocyte interaction, thereby reducing endothelial damage since a major source of ROS during ARDS is infiltrating leukocytes.¹⁹ Steen also includes physiologic levels of electrolytes to stabilize endothelium, including low potassium because high potassium depolarizes membrane potential and elevates ROS.²⁰ Given these features

and the ability to transport gases, Steen functions as artificial blood designed for use as a normothermic perfusion solution for isolated lungs.

The current study focused on identifying the protective mechanisms that Steen elicits in microvascular endothelium by utilizing a model of LPS-induced injury in PMVECs. Our results demonstrate that Steen significantly attenuates LPS-induced oxidative stress and endothelial inflammation as well as endothelial barrier dysfunction and TLR4-mediated signaling (Figure 8). Thus, our data represent an initial step to support a paradigm whereby Steen solution preserves pulmonary endothelial barrier function after LPS treatment by promoting an anti-inflammatory environment through the regulation of oxidative stress and preservation of interendothelial junctions.

Oxidative stress plays a vital role in the pathogenesis of ARDS. The alveolar-capillary interface is an oxygen-rich environment, making PMVECs especially susceptible to oxidative stress, leading to endothelial barrier dysfunction and subsequent edema.²¹ Considerable evidence indicates that LPS exposure induces oxidative stress and mitochondrial dysfunction, leading to cell and tissue damage.²² Our results demonstrate that recovery of PMVECs in Steen or Steen/Media attenuates LPS-induced oxidative stress (ROS generation and lipid peroxidation). These results are supported by a recent study showing that Steen has antioxidant effects on human endothelial cells and lung spheroids by attenuating ROS production, lipid peroxidation, and cytokine production after cold starvation and normothermic recovery.⁹ Consistent with these results, we observed that recovery of LPS-treated PMVECs in Steen or Steen/Media greatly attenuated production of KC/CXCL1 and MCP-1/CCL2. In addition to umbilical vein endothelial cells⁹ and platelets and white blood cells,⁸ our results with PMVECs adds to a growing list of cells in which Steen provides protective, antioxidant effects.

Elevated ROS is known to induce expression of CAMs on endothelial cells, which are essential for immune cell attachment and infiltration.²³ Because CAMs are shed into circulation as soluble CAMs during inflammation and correlate with endothelial cell surface expression, soluble CAMs are often used as biomarkers for vascular inflammation.²⁴ Our study demonstrated that recovery of LPS-treated PMVECs in Steen or Steen/Media blocked increases in sP-selectin, sE-selectin, and sVCAM-1, suggesting that Steen suppresses expression of CAMs on PMVECs. This likely explains why we also observed significantly reduced neutrophil transmigration through LPS-treated PMVECs after recovery in Steen or Steen/Media. Permeability of small molecules (fluorescein isothiocyanate-dextran) across monolayers of LPS-treated PMVECs was also greatly inhibited by recovery in Steen or Steen/Media. Taken together, these results demonstrate that Steen

provides substantial stabilizing, anti-inflammatory effects on the endothelial barrier.

The integrity of the endothelial barrier is highly dependent on interendothelial junctions, especially TJs and AJs, and edema develops largely as a result of TJ dysfunction.²⁵ Our results confirmed reduced expression of ZO-1, ZO-2, and VE-cadherin in PMVECs after LPS treatment, which was mitigated by recovery in Steen or Steen/Media. AJs, such as VE-cadherin, are responsible for vascular stability and endothelial cell-cell adhesion, intracellular signaling, and contribute to the preservation of TJ organization.²⁶ Bogatcheva and colleagues²⁷ demonstrated LPS-induced hyperpermeability in PMVECs resulting from decreased VE-cadherin and ZO-1 expression. VE-cadherin mediates neutrophil transmigration because neutrophil-bound proteases cleave VE-cadherin to create gaps through which neutrophils migrate.²⁸ Consistent with these data, we observed an association between decreased VE-cadherin/ZO-1 expression and elevated permeability and neutrophil transmigration after LPS exposure.

Claudin-5 expression was increased in LPS-treated PMVECs, which was prevented by recovery in Steen or Steen/Media. Wang and colleagues²⁹ showed that elevated claudin-5 expression was associated with increased permeability in methanandamide-treated alveolar epithelial cells. Schlingmann and colleagues³⁰ showed that elevated claudin-5 expression in alveolar epithelial cells from alcohol-fed animals induced formation of claudin-containing TJ spikes, which correlated with greater paracellular leakage. Here, elevated permeability resulted from increased claudin-5 driving more claudin-18:claudin-5 complexes instead of claudin-18:ZO-1 interactions, which destabilized TJs. This is consistent with other studies showing that transforming growth factor- β ³¹ and NF- κ B³² induced TJ spikes and impaired epithelial barrier function. These data suggest that claudin-5 could have a dominant-negative effect on other claudins to weaken barrier function. Because we also observed a correlation between elevated claudin-5 expression and decreased endothelial barrier function in LPS-treated PMVECs, it is possible that regulation of TJ structure and stability by claudin-claudin interactions also occurs in PMVECs after LPS exposure.

LPS binds TLR4 and activates inflammatory signaling pathways such as p38 MAPK and NF- κ B.¹⁸ In response to ROS, p38 MAPK signaling mediates remodeling of the actin cytoskeleton leading to loss of endothelial barrier integrity.³³ As expected, we observed that LPS induced phosphorylation of p38 MAPK and NF- κ B p65 in PMVECs, which was attenuated by recovery in Steen or Steen/Media. Several studies have linked p38 MAPK to the regulation of NF- κ B and AP-1 signaling in endothelial cells.³⁴⁻³⁶ Ueno and colleagues³⁶ showed that nicotine increased intercellular adhesion molecule 1 and VCAM-1 expression in endothelial cells via protein kinase C- and

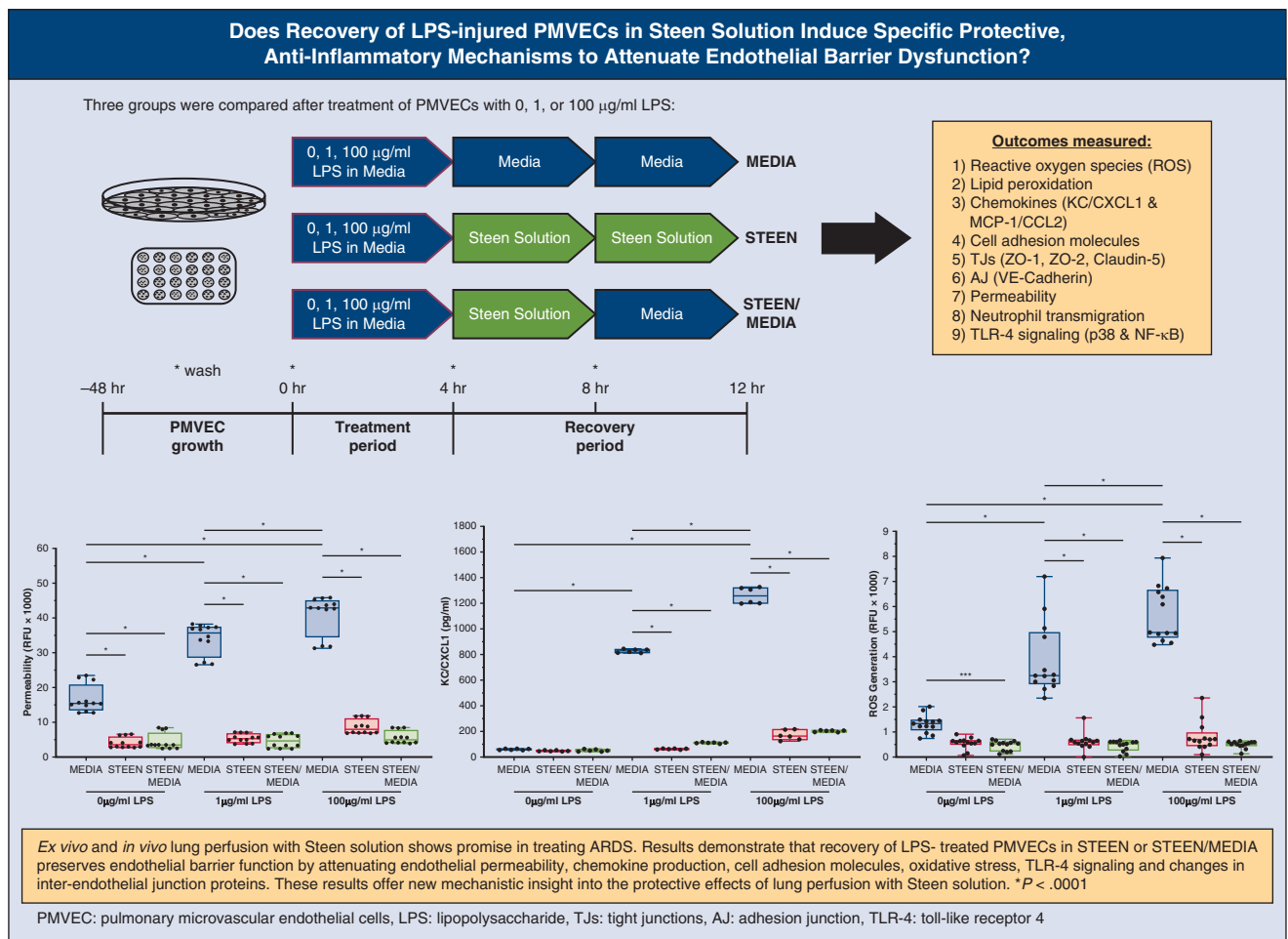


FIGURE 8. Study design and principal findings. Following exposure to 0, 1 or 100 $\mu\text{g/mL}$ LPS for 4 hours, PMVECs (murine or porcine) were recovered in 1 of 3 conditions: complete endothelial cell media (*MEDIA*) for 8 hours, Steen solution (*STEEN*) for 8 hours, or Steen solution for 4 hours followed by *MEDIA* for 4 hours. All measured outcomes of injury (shown are reactive oxygen species (*ROS*) generation, permeability, and expression of KC/CXCL1) were significantly induced by LPS after recovery in *MEDIA*. Recovery in Steen or Steen followed by *MEDIA* (*STEEN/MEDIA*) resulted in significant attenuation of all measured outcomes of PMVEC injury. These results highlight the direct protective effect of Steen solution on PMVEC barrier function. *LPS*, Lipopolysaccharide; *PMVECs*, pulmonary microvascular endothelial cell; *KC/CXCL1*, keratinocytes-derived chemokine/chemokine (C-X-C motif) ligand 1; *MCP-1/CCL2*, monocyte chemoattractant protein-1/C-C motif chemokine ligand 2; *ZO-1*, zonula occludens-1; *ZO-2*, zonula occludens-2; *AJ*, adherens junction; *VE-cadherin*, vascular endothelial-cadherin; *TLR-4*, toll-like receptor 4; *NF- κ B*, nuclear factor κ B; *RFU*, relative fluorescence units; *ARDS*, acute respiratory distress syndrome.

p38 MAPK-mediated activation of NF- κ B. Consistent with these data, we observed that LPS-induced expression of VCAM-1 by PMVECs correlated with activation of p38 MAPK and NF- κ B, suggesting that these TLR4-mediated signaling pathways regulate PMVEC inflammation, which is attenuated by Steen solution.

Our results suggest that Perfadex provides similar protection to PMVECs as Steen. These results are not surprising because both solutions were designed to protect lungs, especially by reducing edema. However, Steen was designed specifically for prolonged normothermic ex vivo perfusion of lungs and thus contains human serum albumin to provide optimal colloid osmotic pressure and dextran 40 to protect

the endothelium from excessive leucocyte interaction. Thus, our results suggest that the mechanisms of protection provided by Steen and Perfadex solutions may be similar; however, the clinical application of these solutions differ by setting (Perfadex for cold static preservation and Steen for normothermic ex vivo perfusion). Because Perfadex lacks albumin, these results also suggest that the albumin in Steen is not a significant contributor to the in vitro protective effects of Steen in PMVECs after LPS exposure.

Our study has certain limitations. The in vitro model of LPS-induced PMVEC injury is not fully representative of ARDS; however, key elements of ARDS are achieved such as vascular inflammation and endothelial barrier

dysfunction. Our study cannot rule out the possibility that some soluble factor(s) within the serum of the endothelial cell growth media is necessary for mediating the effects of LPS exposure. In addition to the endothelium, epithelial barrier dysfunction is also critical to the pathogenesis of ARDS. Although Steen directly contacts the vascular endothelium during perfusion, further studies are needed to evaluate potential protective effects of Steen on the alveolar epithelium, which may be secondary to endothelial protection. Although our study focused on the use of primary murine PMVECs, we provided evidence that our results can also be extended to porcine PMVECs. Further studies with human PMVECs are currently being investigated. Future studies will also be needed to further characterize the protective potential of Steen such as testing other cells types (eg, macrophages), examining other injury models other than LPS-induced injury, and determine whether or not the protective properties of Steen perfusion in vivo can be augmented by including therapeutic additives such as specific anti-inflammatory agents that can act on a variety of cell types beyond the endothelium.

Recent studies by our laboratory and others are beginning to demonstrate the reconditioning potential of isolated lung perfusion with Steen in the management of ARDS and acute lung injury.³ The current study provides mechanistic insight into the protective effects of Steen during EVLP or IVLP using an in vitro model of LPS-induced injury in PMVECs. We demonstrated that recovery of LPS-treated PMVECs in either Steen or Steen/Media resulted in numerous protective, anti-inflammatory effects, including reduced oxidative stress; decreased production of chemokines and cell adhesion molecules; maintenance of endothelial barrier integrity via reduced permeability, neutrophil transmigration, and preservation of interendothelial junctions; and inhibition of TLR4 signaling. Thus, our results support a paradigm wherein perfusion of injured lungs with Steen promotes an antioxidative environment in the endothelium that may preclude an inflammatory immune (cytokine) storm that ensues during ARDS resulting from sepsis, which may also apply to SARS-CoV-2 infection.

CONCLUSIONS

Collectively, this work provides mechanistic insight and rationale for the continued study of the protective effects of isolated lung perfusion with Steen for the treatment of severe ARDS. Finally, our results suggest that the primary protective effects of Steen (during normothermic EVLP or IVLP) and Perfadex (during cold storage preservation) are directed toward preservation of the microvascular endothelial barrier to prevent edema and inflammation.

Conflict of Interest Statement

The authors reported no conflicts of interest.

The *Journal* policy requires editors and reviewers to disclose conflicts of interest and to decline handling or reviewing manuscripts for which they may have a conflict of interest. The editors and reviewers of this article have no conflicts of interest.

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Key Words: Steen solution, in vivo lung perfusion, acute respiratory distress syndrome, endothelial barrier dysfunction

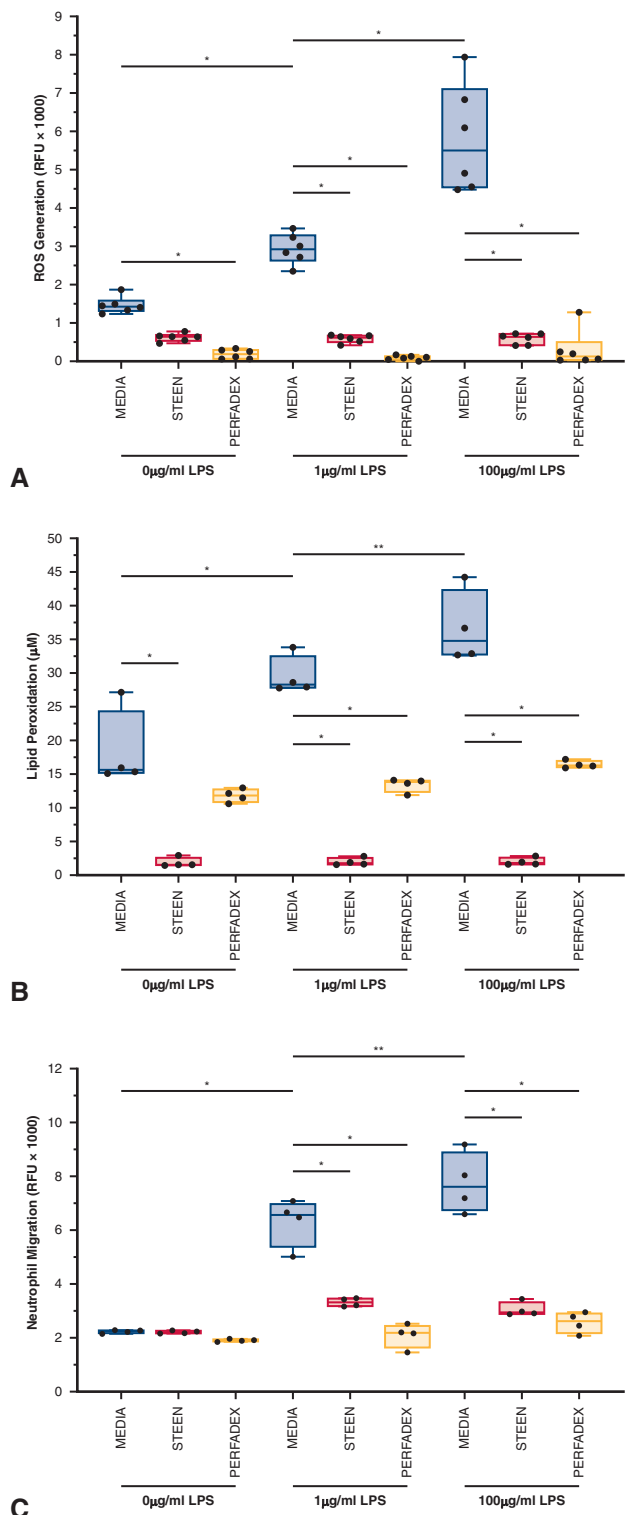


FIGURE E1. LPS-induced oxidative stress and neutrophil transmigration are attenuated in murine PMVECs recovered in Steen (*STEEN*) or Perfadex solutions (*PERFADEX*). LPS treatment and recovery of murine PMVECs in complete endothelial cell media (*MEDIA*) induced significant elevations of reactive oxygen species (*ROS*) ([A], n = 6/group), lipid peroxidation ([B], n = 6/group), and neutrophil transmigration ([C], n = 4/group), all

of which were significantly attenuated after recovery in *STEEN* or *PERFADEX*. Data are presented using box-and-whisker plots where the *lower and upper borders of the box* represent the first and third quartiles, respectively; the *middle horizontal line* represents the median; and the *whiskers* represent the minimum and maximum data values. Experiments were performed in triplicate and repeated for the indicated number of times per group. *RFU*, Relative fluorescence units; *LPS*, lipopolysaccharide. **P* < .0001. ***P* < .035.

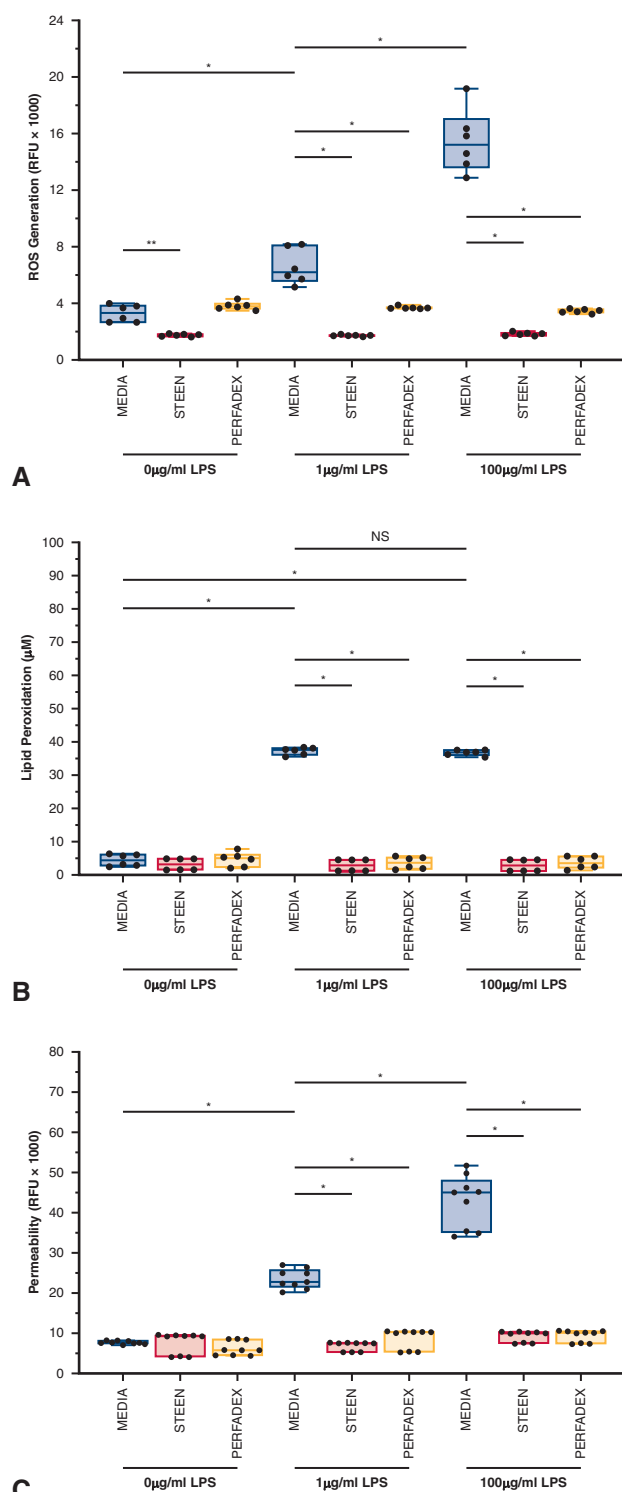


FIGURE E2. Lipopolysaccharide (LPS)-induced oxidative stress and endothelial permeability are attenuated in porcine pulmonary microvascular endothelial cells (PMVECs) recovered in Steen solution (STEEN) or Perfadex solutions (PERFADEX). LPS treatment and recovery of porcine PMVECs in complete endothelial cell media (MEDIA) induced significant elevations of reactive oxygen species (ROS) ([A], $n = 6/\text{group}$), lipid peroxidation ([B], $n = 6/\text{group}$), and endothelial permeability ([C],

$n = 9/\text{group}$), all of which were significantly attenuated after recovery in STEEN or PERFADEX. Data are presented using box-and-whisker plots where the lower and upper borders of the box represent the first and third quartiles, respectively; the middle horizontal line represents the median; and the whiskers represent the minimum and maximum data values. Experiments were performed in triplicate and repeated for the indicated number of times per group. RFU, Relative fluorescence units; NS, not significant. $*P < .0001$.