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Paxillin is phosphorylated at multiple residues; however, the role of tyrosine phosphorylation of paxillin in endothelial barrier dysfunction and acute lung injury (ALI) remains unclear. We used siRNA and site-specific nonphosphorylatable mutants of paxillin to abrogate the function of paxillin to determine its role in endothelial permeability and ALI. In vitro, lipopolysaccharide (LPS) challenge of human lung microvascular endothelial cells (HLMVECs) resulted in enhanced tyrosine phosphorylation of paxillin at Y31 and Y118 with no significant change in Y181 and significant barrier dysfunction. Knockdown of paxillin with siRNA attenuated LPS-induced endothelial barrier dysfunction and destabilization of VE-cadherin. LPS-induced paxillin phosphorylation at Y31 and Y118 was mediated by c-Abl tyrosine kinase, but not by Src and focal adhesion kinase. c-Abl siRNA significantly reduced LPS-induced endothelial barrier dysfunction. Transfection of HLMVECs with paxillin Y31F, Y118F, and Y31/118F double mutants mitigated LPS-induced barrier dysfunction and VE-cadherin destabilization. In vivo, the c-Abl inhibitor AG957 attenuated LPS-induced pulmonary permeability in mice. Together, these results suggest that c-Abl mediated tyrosine phosphorylation of paxillin at Y31 and Y118 regulates LPS-mediated pulmonary vascular permeability and injury.

paxillin; c-Abl; lipopolysaccharide; tyrosine kinase; permeability; endothelial dysfunction; cytoskeleton

GRAM-NEGATIVE BACTERIA-INDUCED sepsis affects more than 750,000 patients annually in the United States (23). Acute respiratory distress syndrome (ARDS), the most severe form of acute lung injury (ALI), is characterized by endothelial hyperpermeability leading to lung inflammation and injury. Lipopolysaccharide (LPS), the bacterial endotoxin and an outer membrane component, induces proinflammatory cytokines, adhesion molecules, endothelial permeability changes, and apoptosis regulated by TLR-4 signaling via activation of Rho GTPase-dependent signaling cascade, NF-κB activation, and rearrangement of cytoskeletal proteins (15, 31). Despite recent advances in understanding the pathogenesis of ALI/ARDS, effective pharmacological therapies are not currently available and the molecular mechanisms regulating endothelial barrier dysfunction in ALI/ARDS are not completely defined.

Interactions between focal adhesions, extracellular matrix, and cytoskeletal proteins have been implicated in endothelial cell (EC) remodeling and barrier dysfunction associated with LPS- and ventilator-induced lung injury (15, 31, 48). Specific posttranslational modifications such as phosphorylation of focal adhesion proteins are also known to play a role in endothelial barrier regulation to agonists and endotoxin (8, 31). Paxillin, a focal adhesion adaptor protein of ~68 kDa, serves as an important scaffolding protein at focal adhesions by recruiting and binding to structural and signaling molecules (6, 40). Paxillin is tyrosine phosphorylated by focal adhesion kinase (FAK) and Src family kinases (5) at Y31 and Y118, which are important for paxillin redistribution and interaction with other proteins and disassembly of focal adhesion at the leading edge of migrating cells (47). Tyrosine phosphorylation of paxillin at Y31 and Y118 also activates Rho family of GTPases leading to the formation of filopodia, lamellipodia, and stress fibers, which are actin-based structures primarily localized at the leading edge of migrating cells (3, 33, 44). Paxillin has been shown to differentially regulate endothelial barrier function by growth factors via modulation of Rac-Rho signaling (4). Recent studies showed that LPS treatment is known to induce phosphorylation of paxillin in macrophages (19) and bovine pulmonary arterial ECs (7); however, little is known about the potential role of tyrosine phosphorylation of paxillin in regulating LPS-induced endothelial barrier dysfunction.

We hypothesized that tyrosine phosphorylation of paxillin at Y31 and Y118 is essential for LPS-mediated endothelial permeability changes. In the present study, we provide evidence that LPS challenge of human lung microvascular endothelial cells (HLMVECs) significantly enhanced paxillin tyrosine phosphorylation at Y31 and Y118, but not Y181, which was mediated by c-Abl but not c-Src and FAK tyrosine kinase.
Furthermore, phosphorylation of paxillin at Y31 and Y118 was critical for LPS-induced endothelial hyperpermeability because nonphosphorylatable mutants of Y31F and/or Y118F suppressed LPS-induced EC permeability. We next investigated the in vivo role of c-Abl and paxillin in LPS-induced lung injury. Inhibition of c-Abl in mouse by AG957 attenuated paxillin tyrosine phosphorylation and lung permeability, and knockdown of paxillin in mouse lung by use of paxillin siRNA significantly attenuated LPS-induced lung inflammation and injury. These results provide further insight into the role of tyrosine phosphorylation of paxillin in LPS-induced endothelial permeability.

**MATERIALS AND METHODS**

Reagents and cell culture. QuikChange II Site-Directed Mutagenesis Kit was purchased from Agilent Technologies (La Jolla, CA). Paxillin antibody (BD Transduction Laboratories, San Diego, CA); phospho Y31, Y118 paxillin, and c-Abl antibodies (Cell Signaling, Beverly, MA); phospho Y181 paxillin antibody, Src antibody, and scrambled and siRNA for paxillin and c-Abl were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-Src Y418 was procured from Sigma (St. Louis, MO). Phospho-VE-cadherin Y658 antibody, phospho-tyrosine antibody, and c-Abl inhibitor AG957 were purchased from EMD Millipore (Millipore, Temecula, CA).

RNA transfection reagent Gene Silencer was from Genlantis (San Diego, CA). PolyJet DNA in vitro transfection reagent was obtained from SigmaGen Laboratories (Rockville, MD). In vivo RNA transfection reagent jetPEI was from Polypus transfection (Illkirch, France). HLMVECs, essential basal medium (EBM-2), and growth factor kit were purchased from EMD Millipore (EMD Millipore, Temecula, CA).

Plasmid constructs. Enhanced green fluorescent protein (EGFP)-tagged wild-type paxillin and control plasmids were prepared as described previously (28). Nonphosphorylatable mutants Y31F, Y118F, Y31/118F were engineered by using a point mutation kit from Agilent Technologies (Santa Clara, CA). Briefly, a pair of mutagenic primers containing the mutated tyrosine/coding sequence (tac/ttc) for each or both sites was designed according to manufacturer’s instruction. Mutant strands were synthesized by PCR method, and PCR products were digested by 10 U/μl of DpnI restriction enzyme and 1 μl of the digested DNA were used for bacterial transformation. Generation of mutants was confirmed by DNA sequencing at Research Resource Center of University of Illinois at Chicago.

Transfection of small interfering RNA. Depletion of endogenous paxillin and c-Abl in HLMVECs was carried out by using gene-specific siRNA as described previously (12). In brief, predesigned human paxillin, c-Abl siRNA, or nonspecific/ontargeting siRNA were used to transfect HLMVECs (passage of 5–7). Before transfection, cells were starved in 2% fetal bovine serum (FBS) EBM-2 medium overnight. The next day, 50 nM scrambled, paxillin, or c-Abl siRNA complexes were prepared in Gene Silencer transfection reagent according to the manufacturer’s recommendation, cells were transfected in serum-free medium for 4 h, and the medium was replaced with fresh complete EBM-2 medium supplemented with 10% FBS and growth factors. Cells were used 72 h posttransfection.

Immunoblotting and immunoprecipitation. Immunoblotting and immunoprecipitation (IP) were performed as described previously (42). In brief, after appropriate treatments, cells were pelleted in ice-cold PBS, lysed in standard lysis buffer (Cell Signaling, Beverly, MA), and sonicated. Lysates were then centrifuged at 1,000 g for 10 min at 4°C, supernatants were collected, and protein was assayed with a BCA protein assay kit. For IP experiments, equal amounts of protein (1 mg) from each sample were precleared with control IgG conjugated to A/G agarose beads at 4°C for 1 h; supernatants were collected and incubated overnight with primary antibody conjugated to A/G agarose beads at 4°C. The next day, the samples were centrifuged at 1,000 g for 1 min in a microfuge centrifuge, and the pellet containing the agarose beads was washed three times with lysis buffer at room temperature (RT). After centrifugation at 1,000 g for 1 min, the beads were collected by removing supernatant buffer, and 40 μl of SDS sample buffer [100 mM Tris-HCl (pH 6.8), 4% SDS, 0.1% bromophenol blue, 20% glycerol, 200 mM DTT] were added to the beads and boiled. Lysates were then subjected to 10% SDS-PAGE followed by Western blotting. Proteins were detected by immunoblotting with appropriate primary antibodies and horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies. Band intensities were quantified by densitometry using Image J software.

Measurement of endothelial permeability by transendothelial electrical resistance. The endothelial permeability changes were measured by the highly sensitive biophysical assay with an electrical cell-substrate impedance sensing system (Applied Biophysics, Troy, NY) as described previously (12). Briefly, cells were grown on gold microelectrodes (10−4 cm2) in complete culture medium containing 10% FBS and growth factor supplements as described (43). Twenty-four hours prior to transendothelial electrical resistance (TER) measurements, the culture medium was replaced with EBM-2 medium containing 1% FBS. The TER was measured dynamically across the monolayer, and the effect of LPS challenge was monitored over a period of 16 h. Decrease in TER resulted from cell retraction, paracellular gap formation, rounding, or loss of adhesion. Resistance was normalized to the initial voltage and expressed as a fraction of the normalized resistance value.

Endothelial permeability measurement by FITC-dextran Transwell assay. Transwell tracer experiments with fluorescent dextran were performed with the PET track-etched membrane 24-well Transwell system (1.0 μm pore; BD). HLMVECs were transfected with scrambled RNA or siRNA for paxillin as described above. After 48 h of transfection, cells (2 × 104) were seeded on a Transwell insert membrane, and monolayers were allowed to grow to ~95% confluence. Cells were challenged with LPS (100 ng/ml) for 6 h prior to addition of FITC-dextran (3 mg/ml, 40 kDa, Sigma) to the luminal chambers, and incubation was continued for an additional 2 h. The inserts were removed and fluorescence of the medium in the lower chamber was measured at an excitation wavelength of 485 nm and emission wavelength of 530 nm by use of the Amino Bowman Series 2 Immunoassay System (2000S-039). Data were acquired with a multichannel spectrofluorometer (12).

Immunofluorescence staining and image analysis. HLMVECs grown on chamber slides (Millipore) were stimulated with vehicle or LPS (100 ng/ml) for 1–6 h. Cells were fixed and subjected to immunofluorescence staining for paxillin, VE-cadherin and F-actin. Image analysis was performed as described (43). Briefly, HLMVECs after the challenge were fixed with 3.7% formaldehyde solution in PBS for 10 min at RT, washed three times with PBS, permeabilized with 0.1% Triton X-100 in PBS for 5 min at RT, and blocked with 2% bovine serum albumin in PBS for 1 h. Incubation with antibodies of interest was performed in blocking solution at RT for 1 h followed by staining with Alexa Fluor 488-conjugated secondary antibodies (Molecular Probes, Eugene, OR). Actin filaments were stained at RT with Texas red phalloidin (Molecular Probes) for 1 h. After immunostaining, slides were examined with a Nikon TE 2000-S fluorescence microscope and Hamamatsu digital camera via a ×60 oil-immersion objective lens. Paxillin distribution at focal adhesions was quantified by using a segmentation algorithm in MetaVue software (Universal Imaging, West Chester, PA), and a minimum of 20 typical cells from each experimental condition were analyzed for paxillin distribution.

In vivo transfection of mouse lung with paxillin siRNA. The mice were housed in a pathogen-free barrier facility maintained by the University of Illinois at Chicago, Biologic Resources Laboratory. All experiments involving mice were conducted with approved protocols by the Institutional Animal Care and Use Committee of the University of Illinois at Chicago. In vivo paxillin knockdown in mouse lung was carried out by intratracheal paxillin siRNA transfection using jetPEI as a transfecting agent (1 mg/kg body wt of the mouse). Before
transfection, siRNA was diluted with 5% glucose to make a 10–15 μl solution per mouse. In parallel, jetPEI polymer at N/P ratio (nucleotide/polymer ratio) of 7 was diluted with 5% glucose in the same volume as siRNA solution. The diluted polymer was added to the diluted siRNA, vortexed gently, and incubated for 15 min at RT. Adult male C57BL/6J mice, 8–10 wk old, with average weight 20–25 g were anesthetized and intratracheally in a 20-G catheter (Penn-Century, Philadelphia, PA) and were administered 20–30 μl of siRNA working solution. Seventy-two hours after transfection, mice were challenged with vehicle or LPS for 24 h.

**Intratracheal administration of LPS or c-Abl inhibitor AG957 to mice.** Mice were anesthetized by intraperitoneal injection of ketamine (75 mg/kg) and acepromazine (1.5 mg/kg). LPS (2 mg/kg body wt, *Escherichia coli* O55:B5, dissolved in sterile water) or vehicle control (sterile water) was instilled intratracheally in a small volume (20–30 μl) using a 20-gauge catheter to scramble siRNA control, scramble siRNA LPS, siRNA paxillin control, and siRNA paxillin LPS groups of mice. In another experiment, mice were pretreated with AG957 (10 mg/kg) intratracheally 1 h before LPS treatment as described above. After 24 h of LPS treatment, mice were killed, and bronchoalveolar lavage (BAL) fluids were collected by intratracheal injection of 1 ml of sterile Hanks’ balanced salt solution followed by gentle aspiration. The lavage was used for the measurement of cell count, and protein concentration. For histopathology, lungs were flushed with 1 ml of ice-cold PBS containing 5 mM EDTA, excised, and dipped in saline; the right lobes were blotted dry, quickly snap-frozen in liquid nitrogen, and stored at −80°C for further analysis and the left lobes were fixed in 4% formalin and sectioned for hematoxylin and eosin staining.

**Assessment of pulmonary vascular leakage by Evans blue.** Evans blue dye (EBD, 30 ml/kg) was injected into the external jugular vein 2 h before termination of experiment to assess vascular leak. In brief, at the end of the experiment, thoracotomy was performed, and the right lobes were blotted dry, quickly snap-frozen in liquid nitrogen, stored at 80°C for further analysis and the left lobes were fixed in 4% formalin and sectioned for hematoxylin and eosin staining.

**Myeloperoxidase assay.** Myeloperoxidase (MPO) activity was measured in lung tissue homogenates to reflect neutrophil and macrophage parenchymal infiltration. Lung tissue was homogenized in 5 mM potassium phosphate buffer (pH 6.0) and homogenates were centrifuged (30,000 g, 30 min at 4°C); pellets were suspended in extraction buffer [50 mM potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyl trimethylammonium bromide] and subjected to three cycles of freezing and thawing. The supernatants generated (13,000 g, 15 min at 4°C) were assayed for MPO activity by kinetic readings over 3 min (200 μl sample plus 800 μl reaction buffer containing 50 mM potassium phosphate buffer, 0.167 mg/ml of O-dianisidine dihydrochloride, and 0.0006% H2O2). Absorbance was measured at 460 nm. The results are presented as MPO units per 100 milligram protein.

**Statistical analysis.** Results are expressed as means ± SE of three to five independent experiments. Statistical significance was assessed by ANOVA, followed by Bonferroni multiple-comparison post hoc tests. A value of *P* < 0.05 was considered statistically significant.

**RESULTS**

**LPS induces paxillin accumulation at focal adhesions.** LPS is known to induce cytoskeletal rearrangement; therefore, the effect of LPS on paxillin rearrangement was investigated in HLMVECs. Cells were treated with LPS (100 ng/ml) for 1, 3, and 6 h, and paxillin distribution was visualized by immunofluorescence staining. LPS challenge induced significant accumulation of paxillin at focal adhesions and stress fibers after 3 h compared with vehicle control. In LPS-treated cells, paxillin redistribution showed a pattern of enlarged focal adhesions (0.39 ± 0.18 μm²) compared with the relatively small-size focal adhesion observed in untreated control (0.11 ± 0.04 μm², *P* < 0.05) (Fig. 1A). Furthermore, the redistributed paxillin mainly flanked the two ends of stress fibers, suggesting that it serves as anchoring sites for actomyosin filaments.

** Knockdown of paxillin with siRNA attenuates LPS-induced endothelial barrier dysfunction and VE-cadherin dissociation from adherens junctions.** Having demonstrated paxillin accumulation at focal adhesions after LPS challenge, we next investigated whether paxillin plays a role in LPS-induced endothelial permeability. HLMVECs were transfected with scrambled or paxillin-specific siRNA to knock down endogenous paxillin expression, and EC permeability was assessed by measuring TER and leakage of FITC-dextran. LPS at a concentration of 100 ng/ml induced a gradual decrease in TER, suggesting increased permeability in scrambled siRNA-transfected HLMVECs (Fig. 1B), which was significant after 10 h of LPS challenge (Fig. 1E). Paxillin depletion with siRNA (Fig. 1C) significantly attenuated LPS-induced increase in EC permeability as determined by TER (Fig. 1, B and E). Similarly, downregulation of paxillin with siRNA reduced LPS-induced HLMVEC permeability as measured by FITC-dextran leakage (Fig. 1F).

The effect of paxillin on LPS-induced EC permeability was further characterized by determining disruption of VE-cadherin, a major adherens junction protein in ECs. As shown in Fig. 1G, in untreated control cells, the expression of VE-cadherin was concentrated in interendothelial junctions; however, LPS challenge induced significant dissociation of VE-cadherin from adherens junctions and downregulation of paxillin by siRNA restored VE-cadherin distribution within the adherens junctions. Taken together, these results demonstrate an essential role of paxillin in LPS-induced VE-cadherin distribution in adherens junctions and endothelial barrier dysfunction.

**LPS stimulates paxillin tyrosine phosphorylation in HLMVECs.** Earlier studies have demonstrated that LPS enhanced paxillin tyrosine phosphorylation in macrophages and bovine pulmonary arterial endothelial cells (7, 19); however, the role of tyrosine phosphorylation of paxillin on LPS-induced endothelial barrier function is not well defined. We determined whether LPS modulates paxillin tyrosine phosphorylation in HLMVECs. By using site-specific phosphor antibodies against Y31, Y118, and Y181, we found LPS treatment of HLMVECs resulted in enhanced tyrosine Y31 and Y118 phosphorylation with maximal phosphorylation seen at 1 h compared with vehicle control (1.9- and 3.2-fold, respectively, Fig. 2, A and B). In contrast to Y31 and Y118, LPS did not cause Y181 phosphorylation in HLMVECs (Fig. 2, A and B). These results suggest site-specific tyrosine phosphorylation of paxillin at Y31 and Y118 by LPS in HLMVECs.

**Role of paxillin phosphorylation at Y31 and Y118 in LPS-induced endothelial barrier dysfunction.** To further determine whether LPS-induced specific tyrosine phosphorylation at Y31 and Y118 participates in pulmonary EC barrier disruption, HLMVECs were transfected with each of the following: empty
vector, paxillin wild-type, and nonphosphorylatable single Y31F and Y118F and double-mutant Y31F and Y118F. As shown in Fig. 2, C and D, LPS-induced profound barrier disruption in cells transfected with control vector and wild-type paxillin plasmids; however, ectopic expression of Y31 and Y118 paxillin single mutants in HLMVECs partially attenuated LPS-induced barrier dysfunction, whereas transfection with the double mutant of paxillin conferred maximum protection against LPS-induced barrier dysfunction. These results further support a role for tyrosine phosphorylation of paxillin at Y31 and Y118, but not Y181, we next sought to identify the kinase(s) that mediates LPS-induced paxillin tyrosine phosphorylation. Src and FAK are two potential kinases that are known to mediate paxillin phosphorylation in different cell types (5). LPS treatment of HLMVECs resulted in increased phosphorylation of Src at Y418 (Fig. 3, A and B); however, inhibition of Src by PP2 (10 μM) showed no significant effect on paxillin Y31 and Y118 phosphorylation in response to LPS (Fig. 3, C and D). In contrast to paxillin tyrosine phosphorylation, PP2 attenuated LPS-induced FAK phosphorylation compared with cells treated with vehicle (Fig. 3, C and D). Furthermore, LPS treatment of HLMVECs did not result in increased interaction of paxillin with Src or FAK (Fig. 4A). In contrast, there was a significant

Fig. 1. Paxillin mediates LPS-induced human lung microvascular endothelial cell (HLMVEC) monolayer permeability. A: confluent HLMVEC monolayer was exposed to LPS (100 ng/ml) for varying time points as indicated followed by immunofluorescent staining of paxillin (Pxn) and F-actin. Scale bar 10 μm. B–D: HLMVECs were transfected with scrambled RNA (scRNA) or paxillin siRNA (siPxn). After 24 h, transfected cells were reseeded onto gold electrode plates or Transwell inserts and cultured for an additional 48 h to form confluent monolayer. Western blotting was performed to confirm knockdown of paxillin (C and D). Statistical analysis of the Western blot showing paxillin expression was normalized to total actin and presented as ratio of paxillin to actin. *P < 0.01, n = 5. B, E, and F: cells were then treated with LPS (100 ng/ml), and monolayer permeability was monitored by measurement of transendothelial electrical resistance (TER) (B and E) or leaked FITC-dextran from inner to outer compartment (F). *Significantly different compared with cells transfected with scrambled siRNA (P < 0.01); #significantly different compared with scrambled siRNA transfected cells challenged with LPS (P < 0.05). Knockdown of paxillin was confirmed by Western blot (C). G: immunofluorescent staining of VE-cadherin and actin was performed after LPS treatment (100 ng/ml, 6 h) in scRNA or paxillin siRNA-transfected HLMVECs. Scale bar 10 μm. Images were captured by fluorescence microscope (×60); shown are representative images of 3 independent experiments.
interaction between paxillin and c-Abl after LPS treatment compared with untreated cells (Fig. 4A). Similar to Src inhibition, transfection of HLMVECs with the FAK dominant-negative plasmid, FAK-related nonkinase (FRNK), had no effect on paxillin tyrosine phosphorylation by LPS (Fig. 4, C and D). Also, PP2 had no effect on LPS-induced paxillin accumulation at focal adhesion as indicated by immunofluorescent staining (Fig. 4E). These results show that although Src and FAK were activated by LPS, they did not mediate paxillin tyrosine phosphorylation at Y31 and Y118 in HLMVECs.

c-Abl tyrosine kinase mediates LPS-induced paxillin tyrosine phosphorylation and barrier dysfunction in HLMVECs. An earlier study has demonstrated that integrins regulate phosphorylation of paxillin by c-Abl (22), and we also showed that c-Abl mediates NADPH oxidase activation by hyperoxia in human lung ECs (34). Therefore, we investigated whether LPS-induced tyrosine phosphorylation of paxillin is dependent on c-Abl tyrosine kinase in HLMVECs. LPS stimulated phosphorylation of c-Abl at Y245 in HLMVECs (Fig. 5, A and B), indicating activation of c-Abl kinase. Downregulation of c-Abl with siRNA (Fig. 5C) resulted in ~85% loss of c-Abl expres-
c-Abl siRNA attenuates LPS-induced EC barrier dysfunction. Having established a role for c-Abl in paxillin tyrosine phosphorylation, we next determined the functional involvement of c-Abl in LPS-mediated loss in EC barrier integrity. Downregulation of c-Abl by using specific siRNA significantly reduced LPS-induced barrier disruption as indicated by restoration of TER (Fig. 5G) and inhibition of FITC-dextran leakage (Fig. 5H). These results demonstrate a key role for c-Abl in endothelial barrier dysfunction mediated by LPS.

Paxillin Y31 and Y118 mutants mitigate LPS-induced VE-cadherin destabilization in HLMVECs. VE-cadherin is a major constituent of adherens junctions and forms homophilic dimers by binding through its extracellular region to another dimmer of VE-cadherin expressed in adjacent cells (13). Tyrosine phosphorylation of VE-cadherin has often been proposed as a mechanism that controls the integrity of the adherens junctions and endothelial barrier function (9). To further study the mechanism(s) by which paxillin regulates LPS-induced barrier dysfunction, we determined the effect of LPS on VE-cadherin phosphorylation. As shown in Fig. 6, A and B, LPS stimulated VE-cadherin phosphorylation at Y658 in a time-dependent manner. To determine whether LPS-induced VE-cadherin phosphorylation is regulated by paxillin, paxillin expression was downregulated by siRNA or paxillin function was blocked by expression of paxillin mutant in ECs. As shown in Fig. 6, C and D, paxillin siRNA significantly inhibited VE-cadherin Y658 phosphorylation. Similarly, transfection of paxillin mutants Y31 and Y118 or the double mutant Y31/Y118 also blocked LPS-mediated VE-cadherin Y658 phosphorylation (Fig. 6, E and F). These results suggest that LPS-induced VE-cadherin phosphorylation is dependent on paxillin and paxillin tyrosine phosphorylation at Y31 and Y118 in HLMVECs. We next determined the role of paxillin phosphorylation on LPS-induced VE-cadherin remodeling. Taking advantage of VE-cadherin monoclonal antibody (BV6), which recognizes the extracellular domain of human VE-cadherin, we observed that LPS challenge disrupted VE-cadherin disruption at adherens junctions with increased intracellular staining in cells, which was prevented by overexpression of paxillin mutants at Y31 and Y118 (Fig. 6G). The expression of paxillin double mutant at Y31 and Y118 showed the most significant inhibitory effect on VE-cadherin disruption at adherens junctions and endocytosis. These results suggest a role for paxillin Y31 and Y118 phosphorylation by LPS in VE-cadherin disruption at adherens junctions of HLMVECs.

Inhibition of c-Abl attenuates LPS-induced paxillin phosphorylation and lung injury in mice. To further investigate the role of c-Abl in LPS-induced paxillin phosphorylation and pulmonary permeability in vivo, mice were treated intratracheally with DMSO (1:2,000 in sterile PBS, 20 μl/mouse) or AG957 (10 mg/kg) to block c-Abl activity in the lung. LPS was administered 1 h after AG957 treatment. At the end of the experiment, lung injury was evaluated by measurements of BAL cell count, protein concentration, histological analysis of lung sections, and measurements of EBD accumulation in the lung tissue. In vivo, LPS induced significant phosphorylation of paxillin at Y31 and Y118 (Fig. 7A). Inhibition of c-Abl by AG957 attenuated LPS-induced phosphorylation of paxillin at both sites (Fig. 7A). Also, LPS induced significant phosphorylation of VE-cadherin in DMSO-treated mice (Fig. 7A), which was attenuated in AG957-treated mice (Fig. 7A). In DMSO

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**Fig. 3. Src and focal adhesion kinase (FAK) activation are not required for paxillin tyrosine phosphorylation by LPS.** A: HLMVECs (~95% confluence) in 35-mm dishes were challenged with LPS (100 ng/ml) for multiple time points and total cell lysates (20 μg protein) were subjected to SDS-PAGE and Western blotting with anti-phospho-Src Y418 antibody. Shown is a representative blot from 3 independent experiments. B: Western blots were scanned and quantified by image analyzer. C: HLMVECs (95% confluence) were pretreated with PP2 (10 μM) prior to stimulation with LPS (100 ng/ml) for 2 h. Total cell lysates (20 μg protein) were subjected to SDS-PAGE and Western blotting with anti-phospho-FAK Y576, phospho-paxillin Y31, phospho-paxillin Y118, phospho-Src Y418, and respective non-phospho antibodies. Shown is a representative blot from 3 experiments. D: Western blots were scanned and quantified by image analyzer. Values are means ± SE from 3 independent experiments. *P < 0.001; #P < 0.05.
control mice, LPS instillation caused pronounced lung inflammation, as reflected by elevation of protein content, total cells in BAL fluid, and MPO activity in the lung (Fig. 7, B, C, E, and F). Compared with DMSO controls, AG957-treated mice exhibited strong protective effects. The protective effects of AG957 against LPS-induced lung vascular leak were further assessed by measurement of EBD leakage into the lung tissue. AG957 inhibited strong protective effects. The protective effects of AG957 against LPS-induced lung vascular leak were further assessed by measurement of EBD leakage into the lung tissue.

Knockdown of paxillin with siRNA in mouse lungs inhibits LPS-induced lung inflammation. Our in vitro results suggest a role for paxillin and its tyrosine phosphorylation in LPS-mediated endothelial barrier dysfunction in HLMVECs; therefore, we next examined whether paxillin mediates LPS-induced lung injury and pulmonary permeability in vivo. Paxillin in mouse lungs was downregulated by intratracheally instilling paxillin siRNA for 72 h prior to LPS challenge. As shown in Fig. 8, A–D, LPS challenge induced significant leakage of protein in scrambled RNA (scRNA)-treated mice [vehicle (Veh): 0.16 ± 0.02 mg/ml vs. LPS: 0.53 ± 0.11 mg/ml, P < 0.01] and infiltration of inflammatory cells into the alveolar space (Veh: 1.28 ± 0.21 × 10^5/ml vs. LPS: 25.02 ± 3.65 × 10^5/ml, P < 0.05); EBD (Veh): 5.81 ± 1.91 μg/ml vs. LPS: 18.63 ± 3.96 μg/ml wet lung tissue, P < 0.01; MPO activity in the lung tissue: 25.3 ± 6.2 U/mg protein vs. 138.6 ± 31.2 U/mg protein, P < 0.01). The above indicators of pulmonary leak and inflammation were significantly attenuated in paxillin knockdown lungs [siRNA + LPS: 25.02 ± 3.65 × 10^5/ml vs.
paxillin siRNA (siPxn) + LPS: 16.01 ± 3.13 × 10^5/ml, P < 0.05; protein content: nsRNA + LPS: 0.53 ± 0.11 mg/ml vs. siPxn + LPS: 0.31 ± 0.07 mg/ml, P < 0.05; EBD: nsRNA + LPS: 18.63 ± 3.96 µg/g vs. siPxn + LPS: 10.23 ± 3.21 µg/g wet lung tissue, P < 0.05; MPO activity in the lung tissue: 138.6 ± 31.2 U/mg protein vs. 63.1 ± 12.3 U/mg protein, P < 0.05]. Furthermore, histochemical analysis of paraffin-embedded mouse lungs revealed inflammatory cell recruitment and areas of alveolar hemorrhage indicative of vascular disruption in scrambled siRNA-treated mice. In contrast, there was a marked attenuation of lung injury in paxillin siRNA-treated mice (Fig. 8E). Knockdown of paxillin in mouse lungs was confirmed by Western blotting of lung tissue lysates obtained from scrambled and paxillin siRNA-administered mice (Fig. 8F). These results suggest that paxillin is an important regulator of LPS-mediated pulmonary vascular permeability in vivo.

![Graphs and images related to the text content](http://ajplung.physiology.org/)
DISCUSSION

The integrity of the pulmonary endothelium is an essential requirement for lung function preservation as movement of fluid, macromolecules, and leukocytes into the interstitium and alveolar space during ALI severely compromises oxygenation of the blood (46). This is potentially life threatening when increased hypoxemia leads to pulmonary leak, tissue edema, and multiorgan failure. Several mechanistic studies have revealed that paracellular gap formation is regulated by the balance between competing contractile forces and adhesive cell-cell and cell-matrix tethering forces, which together regulate cell shape changes (11). In addition, focal adhesions linking pulmonary ECs to underlying matrix may play an important role in defining EC remodeling and barrier integrity in healthy lungs and its failure in ALI (16, 32, 36). Although focal adhesion-associated proteins such as FAK and paxillin play a key role in focal adhesion assembly via interaction with the cytoskeleton, the mechanism(s) of endothelial barrier regulation by focal adhesion proteins remains poorly defined. The effects of LPS on pulmonary EC barrier function have been extensively studied (2), whereas the role of paxillin in LPS-induced barrier function has not been characterized. We have shown that LPS stimulates actin reorganization and paxillin accumulation at focal adhesions in HLMVECs. This study presents several novel observations that paxillin, a multidomain adapter focal adhesion protein, regulates LPS-mediated lung injury both in human lung endothelium and in mouse lungs. Furthermore, our data show tyrosine phosphorylation of paxillin at Y31 and 118, but not Y181, mediated by c-Ab1 nonreceptor tyrosine kinase activation, is important in LPS-induced EC barrier dysfunction. Additionally, tyrosine phosphorylation of paxillin-mediated destabilization of VE-cadherin at adherens junctions contributed to LPS-induced barrier dysfunction in the endothelium. The physiological relevance of the in vitro studies on the role of paxillin and tyrosine phosphorylation in barrier function were verified in an in vivo murine model of LPS-induced ALI wherein paxillin in mouse lung was knocked down by instillation of paxillin siRNA, or c-Ab1 was inhibited by AG957.

An interesting observation is that knockdown of paxillin reduced LPS-mediated endothelial barrier dysfunction in vivo and in vitro. The mechanism by which paxillin mediates this effect on endothelial barrier function is unclear. Paxillin can interact with integrins at focal adhesions, which mediate adhesion of cells to extracellular matrix proteins. Integrins themselves have no enzymatic activity but they bind to actin through paxillin. Furthermore, blockade of integrin αβ3 prevented development of lung vascular permeability in two different models of ALI: ischemia-reperfusion in rats and ventilation-induced lung injury in mice (35). Similar to their findings, in this study, blockade of paxillin prevented lung vascular permeability in a LPS-induced ALI murine model as well. Since both integrins and paxillin are focal adhesion components, both the present and earlier study (35) indicate that blockade of focal adhesions is beneficial to vascular integrity. This can be interpreted by the dynamic control between cell-matrix and cell-cell interaction. We hypothesize that weakening of cell-matrix interaction goes along with a concurrent strengthening of cell-cell contact and the overall outcome of enhanced barrier function. Since focal adhesion is a docking site for stress fibers, small focal adhesion generates less centripetal tension. This hypothesis needs further testing in other models and with a broad array of focal adhesion proteins. Of note, in fibroblasts integrins induces paxillin phosphorylation in a c-Ab1-dependent manner (22), and our results revealed that LPS-induced paxillin phosphorylation was c-Ab1 dependent and independent of Src and FAK. In this study, paxillin siRNA was delivered intratracheally to the lung, which assures reasonable siRNA delivery to the lung. However, intratracheal administration of paxillin siRNA does not ensure that knockdown is limited to the lung ECs and it is likely that other cells in the lung may be affected.

Another novel observation is the role of c-Ab1 in mediating LPS-induced endothelial permeability via phosphorylation of paxillin at Y31 and Y118 in human lung ECs. Paxillin was originally identified as a major substrate for the v-Src tyrosine kinase (14), and c-Src and FAK have been identified as key kinases responsible for paxillin tyrosine phosphorylation in response to a variety of stimuli (1, 5, 24, 27, 37, 47). Although LPS stimulates c-Src and FAK phosphorylation, knockdown of Src with siRNA or overexpression FRANK, a dominant-negative form of FAK, did not block LPS-induced tyrosine phosphorylation of paxillin in HLMVECs. However, inhibition or downregulation of c-Ab1 attenuated LPS-induced paxillin phosphorylation at Y118 in HLMVECs. Interestingly, in c-Ab1 siRNA-transfected cells, the basal level of paxillin phosphorylation at Y31 increased compared with nsRNA-transfected cells. But after LPS treatment, there was no further increase in Y31 phosphorylation, which
may be attributed to a compensatory mechanism. Downregulation of c-Abl expression may result in the upregulation of other tyrosine kinase(s), which can increase basal paxillin phosphorylation at Y31. Intratracheal administration of AG957, an inhibitor of c-Abl, attenuates LPS-induced paxillin tyrosine phosphorylation and lung injury in mice. Similar to paxillin siRNA delivery, intratracheal administration of AG957 may be inhibiting c-Abl on both endothelial and nonendothelial cells. There is evidence for the involvement of c-Abl in mediating LPS-induced tyrosine phosphorylation of paxillin. Furthermore, the proline-rich region in paxillin can provide a potential binding site for SH3 domain containing proteins such as c-Abl that could facilitate tyrosine phosphorylation of paxillin.

In our study LPS stimulated the association between paxillin and c-Abl, but not with Src or FAK. It is very likely that the SH3 and SH2 domains in c-Abl may dock with paxillin and c-Abl, but not with Src or FAK. It is very likely that the SH3 and SH2 domains in c-Abl may dock with paxillin and c-Abl, but not with Src or FAK.

Our finding that paxillin Y31F and Y118F mutants prevented LPS-induced VE-cadherin redistribution from cell periphery, VE-cadherin phosphorylation, and barrier dysfunction in HLMVECs is compelling. Paxillin tyrosine residues Y31, 118, and 181 are embedded in the SH2 domain binding site, ROS production in human lung endothelium (20).

### Fig. 6. Paxillin mediates LPS-induced VE-cadherin (VE-cad) tyrosine phosphorylation and remodeling

**A:** HLMVECs (~90% confluence) were challenged with LPS (100 ng/ml) for 0–12 h. Total cell lysates (20 μg protein) were subjected to SDS-PAGE and Western blotting with anti-phospho-VE-cadherin Y658, anti-VE-cadherin and anti-actin antibodies. Shown are representative blots from 3 experiments. **B:** blots were scanned and quantified by image analyzer. *P < 0.01, compared with vehicle control. C: HLMVECs (~60% confluence) were transfected with scRNA or paxillin siRNA (100 nM), for 72 h prior to LPS (100 ng/ml) challenge for 2 h. Total cell lysates (20 μg protein) were subjected to SDS-PAGE and Western blotting with anti-phospho-VE-cadherin Y658, anti-VE-cadherin, and anti-actin antibodies. Shown are representative blots from 3 independent experiments. **D:** blots in C were scanned and quantified by image analyzer. *P < 0.01, compared with scRNA control; #P < 0.05, compared with scRNA + LPS. **E:** HLMVECs (~60% confluence) were transfected with control or paxillin mutant plasmids, PxnY31F, PxnY118F, and PxnY31/Y118F (100 nM) for 72 h prior to LPS (100 ng/ml) challenge for 2 h. Total cell lysates (20 μg protein) were subjected to SDS-PAGE and Western blotting with anti-phospho-VE-cadherin Y658 and anti-VE-cadherin antibodies. Shown are representative blots from 3 independent experiments. **F:** blots in E were scanned and quantified by image analyzer. *P < 0.01, compared with plasmid control; #P < 0.01, compared with control plasmid + LPS; ###P < 0.05, compared with PxnY31F + LPS or PxnY118F + LPS. **G:** HLMVECs (~60% confluence) grown on glass slides were transfected with control plasmid or paxillin mutant Y31F, Y118F, or Y31F/Y118F plasmids (1 μg/ml) for 72 h. Transfected cells were challenged with vehicle or LPS (100 ng/ml) for 6 h, and images were visualized by immunofluorescence microscope (×60) after staining for VE-cadherin with VE-cadherin monoclonal antibody (BV6). Scales bar 10 μm. Shown are representative micrograph from 3 independent experiments.
Fig. 7. Inhibition of c-Abl attenuates LPS-induced lung permeability and injury. C57BL/6J mice were pretreated with DMSO (1:2,000 in sterile PBS) or AG957 (10 mg/kg) intratracheally 1 h before intratracheal LPS challenge. A: lung tissues were homogenized and subjected to SDS-PAGE and Western blotting for phosphorylation of VE-cadherin, paxillin, and c-Abl. B–E: lung permeability and injury were assessed by measurement of cell counts, and protein concentration in bronchoalveolar lavage (BAL), Evans blue accumulation in the lung tissue; MPO activity was assayed in lung tissue homogenates as described in MATERIALS AND METHODS. Values are means ± SE from 5 animals. *P < 0.01, compared with DMSO control; #P < 0.05, compared with DMSO + LPS. F: hematoxylin-eosin staining of lung sections from mice at 24 h after intratracheal instillation of 2 mg/kg of LPS. The sections are representative lung sections from 5 mice per group. Magnification ×20, scale bar, 100 μm.
which upon phosphorylation allows docking of binding partners that include FAK, GIT-2, βPIX, p190RhoGAP (29, 38, 41). Agonist-stimulated paxillin phosphorylation at Y31 and Y118 is known to enhance cell motility that can be neutralized by the expression of Y31F/Y118F mutants (39). Thus Y31 and Y118 are two key tyrosine phosphorylation sites in paxillin whose phosphorylation is induced by a variety of stimuli that induce cell motility (17, 18, 25, 26, 30, 45) and regulate barrier function. Paxillin is also known to indirectly activate Rac1 and inhibit RhoA in an Y31 and Y118 phosphorylation-dependent manner (39). Since the balance between Rac1 and RhoA plays a critical role in determining endothelial barrier function, the Y31/Y118 tyrosine phosphorylation of paxillin-dependent regulation of Rac1/RhoA activity may serve as an alternative mechanism underlying paxillin-mediated barrier regulation by LPS, which needs further investigation.

In summary, our findings presented here demonstrate an essential role for tyrosine-phosphorylated paxillin at Y31 and Y118 by c-Abl nonreceptor tyrosine kinase in LPS-induced endothelial barrier dysfunction. LPS/endotoxin mediates lung injury in animals and humans and therapeutic targeting of c-Abl and its target paxillin may provide new avenues in the management and amelioration of ALI in patients.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

P.F. and V.N. conception and design of research; P.F., P.V.U., A.L., and A.H. performed experiments; P.F., P.V.U., A.L., and A.H. analyzed data; P.F., J.G.G., R.S., and V.N. interpreted results of experiments; P.F. prepared figures;
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