Non-Muscle Myosin Light Chain Kinase Isoform Is a Viable Molecular Target in Acute Inflammatory Lung Injury

Tamara Mirzapoiazova¹, Jaideep Moitra¹, Liliana Moreno-Vinasco^{2,6}, Saad Sammani^{2,6}, Jerry R. Turner³, Eddie T. Chiang^{2,6}, Carrie Evenoski^{2,6}, Ting Wang^{2,6}, Patrick A. Singleton^{2,6}, Yong Huang^{1,4}, Yves A. Lussier^{1,4}, D. Martin Watterson⁵, Steven M. Dudek^{2,6}, and Joe G. N. Garcia^{2,6}

¹Department of Medicine, University of Chicago; ²Section of Pulmonary, Critical Care, Sleep, and Allergy, University of Illinois at Chicago; ³Department of Pathology; ⁴Section of Genetic Medicine, University of Chicago; ⁵Northwestern Medical School; and ⁶Institute for Personalized and Respiratory Medicine, University of Illinois at Chicago, Chicago Illinois

Acute lung injury (ALI) and mechanical ventilator-induced lung injury (VILI), major causes of acute respiratory failure with elevated morbidity and mortality, are characterized by significant pulmonary inflammation and alveolar/vascular barrier dysfunction. Previous studies highlighted the role of the non-muscle myosin light chain kinase isoform (nmMLCK) as an essential element of the inflammatory response, with variants in the *MYLK* gene that contribute to ALI susceptibility. To define nmMLCK involvement further in acute inflammatory syndromes, we used two murine models of inflammatory lung injury, induced by either an intratracheal administration of lipopolysaccharide (LPS model) or mechanical ventilation with increased tidal volumes (the VILI model). Intravenous delivery of the membrane-permeant MLC kinase peptide inhibitor, PIK, produced a dose-dependent attenuation of both LPS-induced lung inflammation and VILI (~50% reductions in alveolar/vascular permeability and leukocyte influx). Intravenous injections of nmMLCK silencing RNA, either directly or as cargo within angiotensin-converting enzyme (ACE) antibody-conjugated liposomes (to target the pulmonary vasculature selectively), decreased nmMLCK lung expression (~70% reduction) and significantly attenuated LPS-induced and VILI-induced lung inflammation (~40% reduction in bronchoalveolar lavage protein). Compared with wild-type mice, nmMLCK knock-out mice were significantly protected from VILI, with significant reductions in VILI-induced gene expression in biological pathways such as *nrf2*-mediated oxidative stress, coagulation, *p53*-signaling, leukocyte extravasation, and IL-6-signaling. These studies validate nmMLCK as an attractive target for ameliorating the adverse effects of dysregulated lung inflammation.

Keywords: endotoxin/lipopolysaccharide; nmMLCK; mice; lung injury; endothelial barrier

The disruption of lung epithelial and vascular endothelial barriers is a consequence of profound lung inflammation, and results in capillary leakage with protein-rich alveolar edema, a defining feature of acute lung injury (ALI). Acute lung injury occurs in critically ill patients, producing elevated mortality and morbidity. It commonly arises from etiologies such as trauma, sepsis, and pneumonia (1). The profound alveolar flooding observed in ALI produces respiratory failure requiring mechan-

ical ventilation, which unfortunately also contributes to *de novo* lung injury, a process known as ventilator-induced lung injury (VILI) (2). Like ALI, VILI is also associated with increasing vascular permeability, alveolar edema, and increases in the expression of proinflammatory cytokine (3).

Current concepts of vascular permeability and the formation and resolution of alveolar edema indicate that these processes reflect the loss of integrity of both endothelial and alveolar epithelial cellular barriers via intimate involvement with the cytoskeleton. Activation of the cytoskeletal apparatus results in a loss of barrier integrity and the amplification of inflammatory processes, with increased plasma protein influx and the diapedesis of inflammatory cells. Polymorphonuclear leukocytes (PMNs) were strongly implicated in the disruption of the vascular cellular barrier and in the development of pulmonary edema and alveolar flooding. The activation of PMNs results in an excessive release of cytotoxic products capable of damaging lung tissue, including the vascular endothelium. Inflammatory agents promote the recruitment and activation of PMNs at sites of lung injury (4). We showed that the non-muscle myosin light chain kinase isoform (nmMLCK) is centrally involved in driving rearrangement of the cytoskeleton, which regulates vascular endothelial barrier function, angiogenesis, endothelial cell apoptosis, and leukocytic diapedesis (5–8). The nmMLCK enzyme phosphorylates the myosin light chain (MLC), leading to cell contraction and disruption of the vascular endothelial barrier, indicating a potential role of nmMLCK in regulating vascular permeability (6). Our *in vitro* work implicated a mechanistic role for nmMLCK in edemagenic agonist-induced endothelial cell permeability (8), as well as in alveolar and gastrointestinal epithelial barrier dysfunction (9, 10). Consistent with these observations, mice with a targeted deletion of nmMLCK exhibited *in vivo* protection from lipopolysaccharide (LPS)-induced ALI and VILI (11). In addition, we recently generated genetically engineered mice that overexpressed nmMLCK targeted to the vascular endothelium, resulting in a marked exaggeration of LPS-induced and VILI-induced lung injury in a sex-specific and age-specific manner (12). Finally, previous case-control association studies also highlighted the capacity of genetic variants (single-nucleotide polymorphisms, or SNPs) within *MYLK*, the gene encoding nmMLCK, in contributing to the susceptibility to sepsis and ALI, as well as to severe asthma in African Americans (13–15).

In the present study, we sought to confirm murine and human *in vivo* and *in vitro* studies that implicated nmMLCK as an attractive molecular target in ALI and VILI. Using two complementary approaches (an MLCK inhibitory oligopeptide known as PIK, and MLCK silencing RNA [siRNA]), we investigated these therapeutic strategies that focus on nmMLCK activity to reduce ALI inflammation and decrease alveolar and vascular permeability and lung inflammation. Furthermore, microarray analyses of lung RNA, obtained from VILI-exposed

(Received in original form June 7, 2009 and in final form November 24, 2009)

This study was supported by grants HL058064 and HL91889 (to J.G.N.G.) from the National Heart, Lung and Blood Institute of the National Institutes of Health.

Correspondence and requests for reprints should be addressed to Joe G. N. Garcia, M.D., Institute for Personalized and Respiratory Medicine, University of Illinois at Chicago, 1705 Polk # 305, Chicago IL 60612. E-mail: jggarcia@uic.edu

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Cell Mol Biol Vol 44, pp 40–52, 2011

Originally Published in Press as DOI: 10.1165/rcmb.2009-0197OC on February 5, 2010

Internet address: www.atsjournals.org

nmMLCK knockout (KO) mice, revealed significant reductions in the expression of unique pathways (e.g., nrf2-mediated oxidative stress, coagulation, and p53-signaling pathways) and in alterations of previously noted (16) VILI-associated pathways (leukocyte extravasation–signaling pathways, IL-10–signaling pathways, and IL-6–signaling pathways). Heat-map analyses identified 38 significantly dysregulated VILI-mediated genes with decreased expression that were normalized in VILI-exposed nmMLCK KO mice, as well as four VILI-mediated, significantly dysregulated genes whose expression was diminished in nmMLCK KO mice. Together, these analyses implicate the involvement of nmMLCK in pathobiologic processes directly relevant to ALI and VILI. Moreover, these analyses convincingly validate nmMLCK as a relevant target to ameliorate dysregulated lung inflammation with peptide inhibitors, and the role of siRNAs as feasible and effective therapeutic strategies.

MATERIALS AND METHODS

Cell Culture and Reagents

Human pulmonary microvascular endothelial cells (ECs) were obtained from Cambrex Corp. (Walkersville, MD), and cultured as described previously (17) in EC basal medium–2 complete media (Cambrex Corp.) at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. Endothelial cells at passages 6–10 were used for experimentation. Unless otherwise specified, reagents were obtained from Sigma (St. Louis, MO), including LPS, mouse pan-MLC antibody, and mouse anti- β -actin. The cationic phospholipid *N*-[1-(dioleoyloxy)propyl]-*N,N,N*,*N*-trimethylammonium chloride (DOTAP) and the neutral phospholipid dioleoylphosphatidylethanolamine (DOPE) were purchased from Avanti Polar Lipids (Alabaster, AL). Mouse angiotensin-converting enzyme (ACE) antibody was obtained from R&D Systems (Minneapolis, MN). Reagents for SDS-PAGE were purchased from Bio-Rad (Hercules, CA). Immobilon-P transfer membrane was purchased from Millipore Corp. (Bedford, MA). Rabbit anti-ppMLC antibody was purchased from Cell Signaling (Beverly, MA), rabbit anti-nmMLCK antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and mouse anti-smMLCK was purchased from Sigma. The Diff-Quik stain set was obtained from Dade Behring, Inc. (Newark, DE). We synthesized PIK as described previously (18).

Animal Housing and Procedures

All animal care and treatment procedures were approved by University of Chicago Institutional Animal Care and Use Committee. The mice (C57BL/6, weighing 20–25 g) were handled according to the *National Institutes of Health Guide for the Care and Use of Laboratory Animals*, and were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were housed until the time of experiments in their cages, with access to food and water in a temperature-controlled room with a 12-hour dark/light cycle. For experiments performed in intact animals, male C57BL/6 mice (aged 8–10 weeks) were anesthetized with intraperitoneal ketamine (150 mg/kg) and acetylpromazine (15 mg/kg), according to the approved protocol. The generation and phenotype of the nmMLCK^{-/-} mice were previously described (19).

Model of LPS-Induced Murine Acute Lung Injury

We used a previously described experimental model of LPS-induced lung injury (20), with an intratracheal administration of *Escherichia coli* LPS solution (2.5 mg/kg) or sterile saline via a 20-gauge catheter. Simultaneously, mice received either various concentrations of PIK (0.075–0.25 mg) or PBS as vehicle via intravenous injection (into the internal jugular vein). In specific experiments, we addressed the potential of PIK to reverse LPS-induced lung injury, with PIK administered intravenously via the internal jugular vein (3 hours after LPS). The animals were allowed to recover for 24 hours, and both bronchoalveolar lavage (BAL) fluid and lungs were collected and stored at –70°C for evaluations of lung injury.

Model of Ventilator-Induced Murine Lung Injury

In separate experiments, we used a second model of inflammatory lung injury with increased mechanical stress-mediated lung injury via mechanical ventilation, as previously described (12). Two different tidal volumes were designed to produce VILI challenge in C57BL6 mice and nmMLCK KO mice. Wild-type control (nmMLCK^{+/+}) or nmMLCK^{-/-} KO mice were weighed, and groups of mice were randomly allocated to a spontaneously breathing group (SB), or anesthetized and connected to a ventilator (Harvard Apparatus, Boston, MA) in room air, with a tidal volume of 40 ml/kg, 65 breaths per minute, and a positive expiratory pressure (PEEP) of 0 cm H₂O for 4 hours. Normal saline (0.2 ml) was given via intraperitoneal injection at the onset of ventilator use and after 2 hours to all ventilated mice. Peak inspiratory pressures were monitored continuously. Similarly, C57BL6 mice were randomly allocated to an SB group, or anesthetized and connected to a Harvard Apparatus ventilator in room air, with a tidal volume of 30 ml/kg, 75 breaths per minute, and a PEEP of 0 cm H₂O for 4 hours. Mice were treated with 0.25 mg of PIK intravenously, 18 hours before VILI challenge.

In Vivo nmMLCK siRNA Design

For *in vivo* silencing, custom-designed siRNAs targeting murine nmMLCK (NM_021524) were synthesized with siSTABLE modification by Dharmacon (Lafayette, CO) (sequence: 5'-GGGCGAUUCU CCUGCAA GAUU-3'). Mice were pretreated intratracheally with nmMLCK siRNA (5 mg/kg) for 3 days. Before being killed, animals received 2.5 mg/kg LPS intratracheally via a 20-gauge catheter, and were allowed to recover for 18 hours, or ventilated with high tidal volume (40 ml/kg) for 4 hours. The BAL fluid and lungs were extracted for biological analyses.

ACE Antibody-Conjugated Liposome Delivery of nmMLCK siRNA

Liposomes were generated using a 1:1 molar ratio of DOTAP/DOPE, dissolved in chloroform to a concentration 10 mg/ml. The solvent was evaporated in a water bath set at 50°C under nitrogen. The resulting dry lipid film was immediately suspended in 100 μ l of PBS (pH 7.4; final concentration, 20 mg/ml). The cationic lipid dispersion was combined with nmMLCK siRNA (1 μ g per 10 μ g lipid) in a glass container. The liposome/siRNA mixture was sonicated to clarity in a water bath sonicator (Fisher Scientific, Itasca, IL). For the ACE antibody, primary amines were blocked with sulfo-N-hydroxysuccinimide (NHS) acetate in PBS (pH 7.4), and incubated for 1 hour at room temperature. The solution was then filtered with a 30-kD Ultrafree-MC filter (Millipore, Danvers, MA) and adjusted to a final concentration of 0.2 mg/ml. The modified ACE antibody was crosslinked to liposomes containing nmMLCK siRNA by covalently linking the carboxyl groups on the ACE antibody with the amine groups on liposomes, using 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) reagent (Pierce, Rockford, IL) as described in the manufacturer's protocol. Labeled liposomes were purified by dialysis in 20 kD Slide-A-Lyzer (Thermo Scientific) against a 1,000-fold excess volume of sterile PBS (pH 7.4) overnight. A 100- μ l aliquot of sterile ACE-conjugated liposomes (containing 10 mg/kg nmMLCK siRNA) was injected intravenously into the internal jugular vein, and exposed to VILI on Day 5 after the injection.

BAL Protein and Cellular Analysis in Murine Lungs

Mice underwent BAL of both lungs with Hanks' balanced salt solution (1 ml/mouse), and the recovered BAL fluid was used in assays as previously described (20). The BAL fluid was used to measure total protein according to the manufacturer's manual (BCA Protein Assay Kit; Bio-Rad). Cell pellets were examined for total number of white blood cells and cell differential analyses, using cytocentrifugation and Diff-Quik staining.

Tissue and BAL Albumin Concentration

To determine the concentration of albumin present in either BAL fluid or murine lungs, assays were performed in 96-well plastic plates (Nalge Nunc A/S, Roskilde, Denmark) coated with capture antibody (mouse

albumin IgG; Bethyl Laboratories, Montgomery, TX), washed, and blocked. After the addition of a 100- μ l sample or standard serum and incubation at 37°C for 1 hour, and after three washes, 100 μ l of goat anti-mouse albumin antibody (horseradish peroxidase-conjugated) were transferred to each well, as previously described (21). Finally, substrate 3,3',5,5'-tetramethylbenzidine solution was added for 10 minutes, and the reaction was stopped by adding 100 μ l 2 M H₂SO₄. The absorbance at 450 nm was read on a microplate reader (Molecular Devices, Sunnyvale, CA).

Assessment of Myeloperoxidase Activity in Murine Lungs

Myeloperoxidase activity (MPO) was isolated and measured in snap-frozen right lungs, as previously described (22). The right lung was homogenized in 1 ml of 50 mM potassium phosphate (pH 6.0) with 0.5% hexadecyltrimethylammonium bromide. The resulting homogenate was sonicated and then centrifuged at 12,000 $\times$ g for 15 minutes. The supernatant was mixed 1:30 with assay buffer (100 mM potassium phosphate, pH 6.0, 0.005% H₂O₂, and 0.168 mg/ml o-dianisidine hydrochloride) and read at 490 nm. MPO units were calculated as the change in absorbance over time.

BAL Cytokine Concentrations

Bio-Plex cytokine assays (Bio-Rad) were used to measure levels of TNF- α , IL-6, or chemokine (C-X-C motif) ligand 1 (CXCL1) in mouse lung BAL fluids, assayed according to the manufacturer's recommended protocol (Bio-Rad) and as previously described (21).

Western Blot Analysis

The perfused lungs were minced and homogenized. The proteins were solubilized in tissue lysis reagent (Pierce), and were separated by SDS-PAGE. After separation, the proteins were transferred to nitrocellulose membrane, and immunoreactivity was assessed with the antibodies of interest, ppMLC and pan-MLC. Proteins were detected using an enhanced chemoluminescence system (Cell Signaling). Integrated densities of bands were quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

In Vitro siRNA Design

The siRNA sequence targeting human MLCK was generated using mRNA sequences from Gen-Bank (gi, 7,239,695). Sequences containing less than 50% glycine/cysteine content were chosen if available. Specific target sequences were then aligned to the human genome database in a basic local alignment search tool (BLAST) search, to eliminate sequences with significant homology to other human genes. Control sequences were also aligned to the human genome database, in a BLAST search to determine there was no significant homology to any known human gene. For each mRNA (or control), two targets were identified. Specifically, the MLCK target sequence 1 (5'-AAGATGCTGGCTCCCATTACC-3'), the MLCK target sequence 2 (5'-AAAGCCGGACACAGGGACAGT-3'), control sequence 1 (5'-AAGAGAAATCGAAACCGAAAA-3'), and control sequence 2 (5'-AAGAACC CAATTAAGCGCAAG-3') were used. Sense and antisense oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). For construction of the siRNA, a transcription-based kit was used (Silencer siRNA Construction Kit; Ambion, Austin, TX). Endothelial cells were then transfected with siRNA, using siPORTamine (Ambion) as transfection reagent, according to the manufacturer's protocol. Cells (~40% confluent) were serum-starved for 1 hour, followed by incubation with 3 μ M (1.5 μ M of each siRNA) of target siRNA (or control siRNA, or no siRNA) for 6 hours in serum-free media. Media with serum were then added (1% serum final concentration) for 42 hours before biochemical experiments or functional assays were performed.

Measurement of Transendothelial Electrical Resistance in Endothelium

Human lung endothelia were grown to confluence on evaporated gold microelectrodes in polycarbonate wells, and measurements of transendothelial electrical resistance (TER) were performed, using an electrical cell-substrate impedance sensing system from Applied Biophysics (Troy, NY), as previously described in detail (21). The values of

TER from each microelectrode were pooled at discrete time points, and plotted versus time as the mean $\pm$ SE.

Microarray Hybridization and Analysis

We extracted total RNA from frozen lungs with a combined protocol using TRIzol reagent (Invitrogen, Carlsbad, CA) and the RNeasy kit (Qiagen, Valencia, CA), as previously described (23). For each group, lung RNA was prepared ($n = 3$ mice), and chip quality, Affymetrix "present" or "absent" calls, normalization, and probe set intensity were analyzed and computed using Bioconductor software (GeneChip Robust Multiarray Averaging (GCRMA) ([\(http://www.Bioconductor.org\)](http://www.Bioconductor.org)) (24), package) (24). All RNA samples and chips adopted in this study passed established quality criteria (data not shown). All the microarray data generated were submitted to the National Center for Biotechnology Information's Gene Expression Omnibus repository (GSE14525). To identify differentially expressed genes, a two-group comparison was performed, using a significance analysis of microarrays (SAM) (25). Only the probe sets present (as determined by the Affymetrix "present" call) in all three replicates of at least one group in the two-group comparison were used for data analyses. The gene-filtering parameters and results are summarized in Table E1 in the online supplement.

Identification of Gene Ontology Categories via Ingenuity Pathway Analysis

Functional gene expression profiles were represented by the biological processes in the Gene Ontology (GO) database (26), where the number of dysregulated genes in each GO category was compared with the entire murine genome (Mouse 432 2.0 chip arrays and protocols, Affymetrix, Santa Clara, CA) to determine the significance of the GO category. Onto-Express analysis was performed (27), using hypergeometric distribution as the default statistical method, followed by a Benjamini-Hochberg false discovery rate correction (28). Differentially regulated genes were uploaded into Ingenuity Pathway Analysis software (<http://www.ingenuity.com>), a web-delivered application that uses the Ingenuity Pathways Knowledge Base (IPKB), containing a large quantity of individually modeled relationships between gene objects (e.g., genes, mRNAs, or proteins) for the dynamic generation of significant regulatory and signaling networks or pathways. The submitted genes, as mapped to the corresponding gene objects in the IPKB, are called "focus genes." The significance of a canonical pathway is controlled by P value, which is calculated using a right-tailed (referring to the overrepresented pathway) Fisher exact test for 2×2 contingency tables, by comparing the number of "focus" genes that participate in a given pathway, relative to the total number of occurrences of those genes in all pathways stored in the IPKB. The significance threshold of a canonical pathway is set at 1.3, which is derived by $-\log_{10}(P \text{ value})$, with $P \leq 0.05$.

Statistical Analysis

Except where noted, results were analyzed using standard one-way ANOVA. Groups were compared using the Newman-Keuls test. Differences between groups were considered statistically significant when $P < 0.05$. Results are expressed as mean $\pm$ SE. The number of animals for each experimental group ranged from 3–6 mice.

RESULTS

In Vitro and In Vivo Effects of PIK on LPS-Stimulated MLC Phosphorylation and Vascular Barrier Disruption

We tested the potential beneficial effects of the membrane-permeant inhibitor of MLC kinase activity (PIK), which reduces concentrations of MLC phosphorylation (29). Using human lung endothelial cells, we observed that 250 μ M PIK inhibited MLC kinase activity and MLC phosphorylation (Figure 1A, *inset*), as well as LPS-induced reductions in TER, an indicator of endothelial monolayer integrity (Figure 1A). The PIK alone caused rapid increases in TER that were sustained for 18 hours, and that promoted a partial restoration of LPS-induced declines in TER.

Figure 1. PIK attenuates LPS-induced vascular permeability and lung injury *in vitro* and *in vivo*. (A) Human lung endothelia were plated on gold microelectrodes, cultured to confluence, and challenged with agonist, as indicated. Measurements of TER were recorded, and the data depicted are representative of three independent experiments. Shown are the responses to LPS (100 ng/ml), PIK (250 μM), or a combination of the two agents. Lipopolysaccharide elicits significant and sustained decreases in TER. PIK alone caused a prolonged, significant initial increase in TER, and restored TER values in LPS-challenged cells. *Inset*: Example of MLC phosphorylation after PIK/LPS treatment. *Veh*, vehicle. (B) Dose-dependent inhibition of MLC phosphorylation by PIK after LPS challenge. The phosphorylation of MLC was detected in mouse lungs after LPS/PIK treatment, as described in MATERIALS AND METHODS. *Inset*: PIK alone (0.25 mg/mouse) did not alter basal levels of MLC phosphorylation. Representative Western blot with quantification shows dose-dependent decreases of MLC phosphorylation (0.075–0.25 mg PIK per mouse) compared with LPS. Values are presented as ratios of densitometry units of ppMLC to total MLC. (C and D) Assessments of BAL albumin and tissue albumin concentrations after LPS/PIK. Lipopolysaccharide induced increases in albumin leakage from the vascular space into surrounding lung tissues and BAL, which were significantly reduced in PIK-treated mice. *Significant differences between LPS and LPS/PIK groups. #Significant differences in vehicle versus LPS-challenged mice. Overall, the concentration of albumin in BAL fluids was reduced in a dose-dependent manner by PIK.

The intravenous administration of PIK produced significant dose-dependent decreases in the MLC phosphorylation induced by LPS (2.5 mg LPS/kg) in mouse lungs (Figure 1B, *inset*), similar to the *in vitro* effects, as confirmed by the quantification of Western blots (Figure 1B). The intratracheal administration of LPS (24 hours) produced inflammatory responses characteristic of ALI, with increased microvascular leakage, as evidenced by increases in BAL albumin concentrations and lung tissue albumin concentrations. The intravenous injection of PIK at the optimally effective concentration (0.25 mg/mouse) attenuated LPS-mediated pulmonary hyperpermeability, with significant decreases in BAL and lung albumin and BAL protein concentrations (markers of pulmonary capillary leakage) (Figures 1C and 1D and Figure E1). The PIK alone did not alter basal total BAL protein or BAL albumin concentrations, and increasing the concentration of PIK above 0.25 mg/mouse did not produce greater protection against LPS-induced lung injury (data not shown).

In Vivo Effects of PIK on LPS-Induced Lung Inflammatory Indices

We next assessed lung tissue MPO activity, a measure of leukocyte accumulation and infiltration in the lung parenchyma of LPS-treated animals. Compared with control mice, LPS caused a significant increase in BAL white blood cells (WBCs), whereas the intravenous delivery of PIK significantly reduced the LPS-induced accumulation of WBCs in BAL fluid (Figure 2A) and decreased lung MPO activity (Figure 2B). Because the systemic effects of LPS with increased microvascular leakage were linked to the release of inflammatory cytokines (TNF-α and IL-6) from activated PMNs and macrophages, we analyzed the effects of PIK on LPS-mediated cytokine production. Intratracheal challenge with LPS produced large increases in the BAL concentrations of TNF-α, CXCL1, and IL-6, which were significantly reduced in PIK-treated, LPS-challenged mice

Figure 2. PIK reduces LPS-induced accumulation of WBCs in BAL. (A) LPS-mediated cell infiltration into lung tissue was reduced in a dose-dependent manner by intravenous administration of PIK. *Significant differences in LPS versus LPS/PIK. Con, control; Veh, vehicle. (B) Activities of MPO were elevated in lung tissue from LPS/vehicle groups, but decreased in LPS/PIK-treated mice (0.15 PIK/LPS versus LPS, $*P < 0.01$; 0.25 PIK/LPS versus LPS, $*P < 0.01$). (C) Effects of PIK on BAL cytokine levels in C57BL/6 mice after challenge with LPS. Concentrations of TNF- α , IL-6, and CXCL1 in BAL were significantly reduced compared with LPS alone. *Significant reduction resulting from PIK. (D) Histologic assessment of PIK's effects on LPS-induced lung inflammation. Lungs were analyzed for interstitial edema and inflammatory cell infiltration. Paraffin-embedded hematoxylin-and-eosin-stained sections were prepared by standard techniques. Sagittal sections through central and peripheral areas of each lobe were obtained, to represent histologic changes in the entire lung ($n = 3$ from each treatment group). Quantitative analyses of lung inflammation, using parameters of severity (score, 0–3), focal/diffuse inflammation (score, 0–3), and parenchyma/airway inflammation (score, 0–3), demonstrated fewer pathologic changes in LPS-challenged mice receiving PIK. *Significant difference produced by PIK. #Significant difference produced by LPS compared with vehicle. PIK alone did not induce inflammation. (E) Hematoxylin-and-eosin staining of lung sections indicated that LPS-induced edema and leukocyte infiltration were reversed by administration of PIK (0.25 mg/mouse).

(Figure 2C). Histologic analyses revealed a lung injury score of 7.3 ± 0.3 in LPS-challenged mice, which was significantly decreased by PIK treatment (4.3 ± 0.3 ; $P = 0.003$) (Figure 2D) and highlighted by reductions in the architectural distortion of lung alveoli (thickness and neutrophil infiltration) and alveolar and tissue edema (Figure 2E). Furthermore, PIK treatment 3 hours after LPS significantly attenuated the BAL protein elevation and BAL neutrophil invasion (Figures 3B and 3C). This ability of PIK to rescue established ALI was also demonstrated *in vitro*, because PIK administered 3 hours after LPS successfully restored microvascular endothelial cell barrier function (Figure 3A). Taken together, these studies indicate that PIK-mediated reductions in MLCK enzymatic activity and subsequent MLC phosphorylation significantly attenuated LPS-induced inflammatory lung injury. The protective effects of PIK were retained during both simultaneous treatment with PIK and LPS, and PIK rescue after challenge with LPS.

Effect of Alterations in nmMLCK Lung Expression (siRNA) on LPS-Induced Inflammatory Lung Injury

We next used siRNA to reduce nmMLCK expression, to investigate the further role of nmMLCK in vascular barrier regulation *in vitro* and *in vivo*. Initial *in vitro* studies demonstrated that human lung ECs that were transfected with nmMLCK-specific siRNA abolished nmMLCK expression (Figure 4A, inset), as well as LPS-mediated reductions in TER compared with scrambled siRNA (Figure 4A).

We next performed *in vivo* experiments designed to decrease the murine expression of nmMLCK via an intratracheal delivery of siRNA. The administration of nmMLCK siRNA reduced the expression of protein detected by Western blots in mouse lung homogenates by 80%, compared with control mice (Figure 4B). To assess the effects of nmMLCK silencing on lung microvascular leakage (BAL protein), nmMLCK-specific

Figure 3. (A) Endothelial cells were cultured to confluence on gold microelectrodes and exposed to LPS (100 ng/ml), as indicated. Transendothelial electrical resistance was recorded for 18 hours of PIK (250 μ M), administered 3 hours after LPS challenge, resulting in an increase of TER to initial basal level in LPS-challenged cells. (B) Lipopolysaccharide-mediated protein accumulation into mouse BAL fluid was reduced by intravenous injection of PIK (0.25 mg/mouse), 3 hours after LPS challenge. *Significant difference. (C) The BAL neutrophil count was sufficiently decreased in the LPS/PIK group, compared with LPS alone. *Significant difference.

siRNA was intratracheally injected (single dose, 3 days before LPS challenge) into mice. Silencing of the nmMLCK isoform resulted in a significant attenuation of LPS-induced microvascular leakage, as indicated by a 36% reduction in BAL protein concentration (Figure 4C). Delivery of the nmMLCK siRNA produced significant decreases in BAL cells (Figure E2A), and particularly in the LPS-induced accumulation of neutrophils (Figure E2B), with BAL PMNs decreased 40% by nmMLCK siRNA compared with control samples (Figure 4D).

Effects of PIK and Altered nmMLCK Expression on Murine VILI

To extend the applicability of targeting nmMLCK in acute inflammatory lung injury, we used a second model of inflammatory lung injury produced by the increased mechanical stress associated with mechanical ventilation at high tidal volumes

(30 or 40 ml/kg). Mice treated with mechanical ventilation at 40 ml/kg exhibited increased concentrations of BAL protein and albumin, and BAL and lung tissue neutrophil infiltration. The intratracheal administration of nmMLCK siRNA did not alter the expression of the lung smooth muscle isoform in ALI and VILI models (Figure E4), but significantly attenuated concentrations of BAL protein and albumin (Figures 5A and 5B). The administration of PIK in VILI-challenged mice (high tidal volume, 30 ml/kg) to inhibit nmMLCK activity did not decrease concentrations of BAL protein and albumin (data not shown), but significantly attenuated BAL neutrophil infiltration (Figure 5C). Similarly, we analyzed hematoxylin-and-eosin-stained lung tissue sample images, to determine the fold change in neutrophil infiltration after challenge with VILI (Figure E3), and we observed that the administration of PIK significantly reduced neutrophil migration into lung tissue (Figure 5D).

Figure 4. *In vitro* and *in vivo* silencing of nmMLCK expression attenuated LPS-induced endothelial barrier dysfunction. (A) Endothelial cells were cultured to confluence on gold microelectrodes and exposed to LPS (100 ng/ml) as indicated, with transendothelial electrical resistance (TER) recorded for 20 hours. Lipopolysaccharide elicited a significant, sustained decrease in TER, with the nmMLCK siRNA abolishing the disruptive effect of LPS. Data are expressed as mean $\pm$ SE. (B) The siRNA specific for mouse nmMLCK was directly injected as described in MATERIALS AND METHODS. Western blots of nmMLCK from mouse lung lysates show decreased nmMLCK expression ($\sim 80\%$) between the control/LPS group and the siRNA/LPS group, 72 hours after silencing. *Inset:* The decreased expression of nmMLCK in ECs after treatment with siRNA. *Significant difference between control group and siMLCK or siMLCK/LPS group. #Significant difference between LPS group and siMLCK or siMLCK/LPS group. The β -actin blot confirms equal protein loading. The blot shown is representative of at least of three experiments. (C) Mouse lungs with combined siMLCK/LPS challenge produced evidence for decreased epithelial-endothelial permeability, as reflected by BAL protein concentrations, whereas LPS or siLuc/LPS treatment exhibited significantly increased BAL protein. Lipopolysaccharide-induced elevation of BAL protein was significantly reduced by nmMLCK siRNA. *#Significant decreases compared with LPS or siLuc/LPS. (D) The increase in number of BAL neutrophils (PMNs) in LPS-challenged mice. The BAL neutrophil counts were significantly decreased in the siMLCK/LPS group, compared with LPS or LPS/siLuc mice. *#Significant decreases ($P < 0.05$).

Targeted Lung Delivery of nmMLCK siRNA within ACE Antibody-Conjugated Liposomes in the Murine VILI Model

Both the PIK and nmMLCK siRNA strategies to reduce inflammatory lung injury, targeting the nmMLCK isoform, have potentially widespread off-target effects on circulating leukocytes and on the vasculature of other organ beds. To focus on the role of nmMLCK expression in the lung vasculature, we generated ACE antibody-conjugated liposomes that encapsulate nmMLCK siRNA as a cargo to reduce nmMLCK expression selectively in the lung vasculature. Immunohistochemical imaging demonstrated a visual abatement of nmMLCK expression in lung vessels, but not in the renal vasculature, suggesting the specific targeting of siRNA to the lung (Figures 6A and 6B). Western blotting of mouse lung homogenates from ACE-nmMLCK-treated mice demonstrated reduced nmMLCK expression in the lung tissue of both SB and VILI-exposed animals

(Figure 6C). Importantly, and in agreement with the inhibition of nmMLCK activity, ACE-liposome nmMLCK silencing attenuated the vascular leakage in ventilator-induced lung injury, as indicated by the significant decrease in total concentrations of BAL protein (Figure 6D) after exposure to mechanical stress associated with high tidal volume mechanical ventilation.

Physiologic and Genomic Response of nmMLCK^{-/-} KO Mice to Ventilator-Induced Lung Injury

For a further understanding of the role of nmMLCK in ALI, we exposed genetically engineered nmMLCK KO mice to severe challenge with VILI (4 hours, 40 ml/kg). These nmMLCK KO mice (retaining the smooth muscle MLCK isoform) were markedly less susceptible to VILI, with significant reductions in BAL protein and albumin concentrations compared with VILI-treated wild-type (WT) mice (Figures 7A and 7B). Genome-wide gene expression in pulmonary tissues was next

Figure 5. Effects of PIK and nmMLCK silencing on murine VILI. *Veh*, vehicle; *Spont. Breathing*, spontaneously breathing. (A) The concentration of BAL protein was significantly elevated in all VILI-treated groups compared with SB groups. In addition, when mice were treated with nmMLCK siRNA and exposed to VILI, the siMLCK/VILI group had a significantly lower concentration of BAL protein. ***,#Significant differences. (B) Evaluation of BAL albumin concentration showed that mice receiving nmMLCK siRNA had significantly lower concentrations of albumin compared with mice exposed only to mechanical ventilation with vehicle or control siRNA. ***,#Significant differences. (C) Increased BAL neutrophil invasion, associated with VILI, was significantly attenuated after administration of PIK. *Significant difference between VILI and VILI/PIK group. #Significant difference between VILI versus vehicle or PIK alone. (D) Administration of PIK significantly reduced the fold change in neutrophil migration in lung tissue. *Significant difference between the VILI and VILI/PIK groups. #Significant difference between VILI versus vehicle or PIK alone.

analyzed according to the Affymetrix microarray in four experimental groups: WT control, VILI-exposed WT, nmMLCK^{-/-} mice, and VILI-exposed nmMLCK^{-/-} mice. The lists of differentially expressed genes generated using SAM software are summarized in Table E1. Ventilator-induced lung injury induced a greater number of up-regulated genes in WT animals (Gene List 1, 163 up-regulated genes versus 122 down-regulated genes), whereas nmMLCK^{-/-} mice displayed a greater number of down-regulated genes (Gene List 2, 138 up-regulated genes versus 220 down-regulated genes), indicating that the nmMLCK^{-/-} mouse phenotype attenuated VILI-mediated gene expression in lung tissues (*see online supplement for Gene List 1 and Gene List 2*).

We next explored the global gene expression pattern in VILI-exposed, WT, and nmMLCK^{-/-} mice. The expression profile of the overlapping genes in Gene Lists 1 and 3 are displayed in Figure 7C. Thirty (Clusters A and C) of the 33 overlapping genes dysregulated in WT-VILI-exposed mice were recovered at approximately normal levels of expression in nmMLCK^{-/-} mice exposed to VILI. Furthermore, heat-map analysis identified 38 VILI-mediated, significantly dysregulated

genes with decreased expression that were normalized in VILI-exposed nmMLCK^{-/-} mice. The dysregulated genes were submitted to Ingenuity Pathway Analysis software to identify related canonical pathways. Interestingly, despite a greater number of VILI-dysregulated genes in nmMLCK^{-/-} mice (358 genes in Gene List 2) compared with WT mice (285 genes in Gene List 1), less dysregulation occurred in the canonical pathways in nmMLCK^{-/-} mice, as determined by *P* values of the Fisher exact test (Figure 8A). After exposure to VILI, nmMLCK KO mice exhibited significantly reduced expression of the genes involved in NRF2-mediated oxidative stress response, in coagulation system responses, in p53 signaling pathways, and in genes involved in previously described VILI-associated pathways (16), including leukocyte extravasation, IL-10, and IL-6 (acute-phase reactants). The VILI-dysregulated genes involved in the leukocyte extravasation signaling pathway are depicted in Figure 8B, and include the immunoglobulin superfamily member Jam2, which is expressed in the junction of high endothelial venules and plays a role in facilitating lymphocyte transmigration (30). The non-receptor protein-tyrosine-kinase Fer is highly expressed in macrophages and

Figure 6. Attenuation of VILI by intravenously administered ACE antibody-conjugated liposomes (ACE-Lipo), with nmMLCK siRNA as cargo. (A) Immunofluorescence analysis of lung tissue (*in vivo* targeting of microvascular endothelial cells). The ACE antibody (ab)-conjugated liposomes (containing siRNA against nmMLCK) were injected into the jugular vein, to deliver nmMLCK siRNA specifically to lung vessels. After 5 days, tissues were assessed by examining sections under a fluorescence microscope, revealing that pulmonary vessels from mice treated with ACE antibody-conjugated liposomes exhibited reduced nmMLCK expression (red), compared with vessels from control mice. The vascular lumen is identified by vessel staining with endothelial cell-specific von Willebrand Factor antibody (green). (B) Immunohistochemical analysis of mouse kidney tissues from mice challenged with ACE antibody-conjugated liposomes (containing siRNA against nmMLCK) indicates no changes in nmMLCK expression within the renal vasculature, compared with control mice. (C) Liposomal delivery of nmMLCK siRNA decreases nmMLCK expression in mouse lung homogenates. A representative Western blot is shown (representing at least three experiments). (D) Graphic representation of BAL protein indicates that ACE antibody-conjugated liposome delivery significantly reduces VILI-induced lung injury. *[#]Significant differences ($P < 0.05$). *Spont. Breathing*, spontaneously breathing.

plays a role in regulating the cytoskeletal rearrangements and inside-out signaling that accompany receptor-ligand, cell-matrix, and cell-cell interactions (31). Coagulation cascade genes are also significantly downregulated in response to nmMLCK KO mice, providing novel evidence for a possible role of nmMLCK in regulating blood hemostasis. Although the nature of this regulation is unknown, importantly, nmMLCK KO mice exhibited extended bleeding times compared with WT control mice (Figure 8C).

The downregulation of genes in VILI-challenged nmMLCK^{-/-} KO animals may be mechanistically related to the reduced vascular leakage and alveolitis observed after the inhibition of nmMLCK or in nmMLCK^{-/-} KO mice (32). Similar to the findings in canonical pathway analyses, GO analyses revealed a VILI-induced deregulation of four biolog-

ical processes only in WT mice, but not in nmMLCK^{-/-} mice (Table E2).

Finally, additional exploration of the impact of the nmMLCK^{-/-} genotype on VILI responses (when we compared the gene expression of nmMLCK^{-/-} mice exposed to VILI with similarly exposed WT mice; Table E1, Gene List 3) identified a downregulation of genes involved in the NF- κ B-regulated network (Figure E5). These results are in agreement with our previous studies, which demonstrated that nmMLCK activation resulted in NF- κ B activation and NF- κ B-dependent increases in the expression of genes related to cell survival and inflammation via a TNF-mediated pathway (33). Together, these findings reveal that in addition to the important physiologic involvement of nmMLCK in inflammatory lung injury, nmMLCK^{-/-} KO mice are genetically programmed to exhibit reductions in the in-

Figure 7. Deletion of nmMLCK gene (nmMLCK^{-/-}) results in protection from severe murine ventilator-induced lung injury. *Spont. Breathing*, spontaneously breathing. (A) In contrast to increase in BAL protein induced by VILI in WT mice, this effect was largely abrogated in nmMLCK^{-/-} mice ($n = 6$ animals per group). (B) Ventilator-induced lung injury induces increased lung microvascular permeability, as reflected by BAL albumin, compared with SB mice. In addition, nmMLCK^{-/-} mice are less susceptible to VILI, and exhibit significantly reduced BAL albumin concentrations, compared with WT mice exposed to VILI. (C) Gene expression pattern of differentially expressed genes in VILI-challenged WT mice and nmMLCK KO mice. Gene identity is shown at the right of each row. The overlap of Gene Lists 1 and 3 was classified by dChip (<http://biosun1.harvard.edu/complab/dchip/>). Red, white, and blue indicate expression above, at, and below mean level, respectively.

flammation and gene dysregulation induced by VILI, compared with WT mice.

DISCUSSION

Lung alveolar and vascular barrier regulation are functionally complex processes intrinsically linked to the major pathobiology observed in ALI and VILI: refractory hypoxemia and inflammation, increased vascular permeability, and alveolar flooding (5, 34, 35). We previously demonstrated that the endothelial cell regulation of the trafficking of inflammatory cells into the lung parenchyma and airways requires the essential participation of the key multifunctional effector, nmMLCK, a critical actin-binding protein and driver of actin cytoskeletal rearrangement (6, 36). Neutrophil–endothelial interactions directly increase nmMLCK activity, and modulate neutrophil transmigration in response to leukotriene B₄ (7). The nmMLCK isoform is also directly involved in regulating the semiselective pulmonary vascular barrier that exists between the blood and the lung interstitial space, and is thus an essential participant in inflammatory lung vascular permeability and pathobiology. The physiologic consequences of nmMLCK

activation by contractile agonists involve a ratcheting of actin and myosin bonds catalyzed by MLC phosphorylation, resulting in the development of tension, the formation of paracellular gaps, and vascular barrier dysfunction (7, 34, 37). The nmMLCK isoform also exerts important and specific functional consequences in both the lung and gastrointestinal epithelium, with the MLCK-specific regulation of TNF-mediated epithelial tight junction permeability (38). Our previous studies demonstrated that MLCK-dependent MLC phosphorylation resulted in tight junction reorganization, the internalization of occludin and barrier dysfunction in epithelial cells, with MLCK inhibition restoring tight junction integrity and barrier function (38). Furthermore, LPS-induced alveolar flooding and profound vascular leakage *in vivo* (34, 35, 39) are accompanied by increases in MLC phosphorylation (36, 40) and a reorganization of the actomyosin cytoskeleton (41). Finally, our previous studies highlighted the role of genetic variants of the *MYLK* gene encoding MLCK, which contribute to ALI susceptibility and severity. Several case–control studies confirmed *MYLK* as a candidate gene in sepsis-associated and trauma-associated ALI (14). Consistent with the highly multifunctional nature of the *MYLK* gene product in inflammation, we recently identified

Figure 8. Ingenuity Pathways Analysis of VILI-dysregulated genes in WT and nmMLCK^{-/-} mice. (A) Significant canonical pathways were enriched with dysregulated genes in WT-VILI (blue) and KO-VILI animals (red). (B) Fold changes of dysregulated genes, induced by WT-VILI or KO-VILI in the leukocyte extravasation signaling pathway. All genes shown were identified by SAM software, using criteria of fold change > 3 and false discovery rate < 1% (Table E1). (C) Bleeding times (in seconds) were analyzed in genetically engineered nmMLCK KO and WT mice. The nmMLCK^{-/-} mice exhibited significantly prolonged bleeding times (1.5-fold longer).

MYLK coding SNPs as risk variants in three different asthmatic populations of African descent (i.e., Chicago Collaborative Study on the Genetics of Asthma [CSGA], Baltimore CSGA, and Barbados) (13, 14, 42). Together, these studies dramatically highlight the importance of elucidating the functional role of nmMLCK in barrier regulation and inflammatory lung injury.

Here, we sought to confirm these *in vivo* and *in vitro* murine and human studies, and further implicate nmMLCK as an attractive molecular target in ALI and VILI, using complementary approaches to reduce nmMLCK activity in two well-described murine models of inflammatory lung injury (LPS and VILI). We recently demonstrated that a novel membrane-permeant MLC kinase oligopeptide inhibitor (known as PIK) inhibits intracellular MLC kinase and regulates the paracellular permeability of epithelial monolayers *in vitro* (29). As a potential therapeutic agent effective in targeting intracellular MLCK, PIK inhibits MLCK by serving as a competitive inhibitor, binding to the catalytic domain of MLCK and preventing interactions with the MLC substrate. Small inhibitory peptides have a key advantage because they can be engineered with high

specificity, with efficacy at lower concentrations of the inhibitor. Conversely, a disadvantage of small molecule inhibitors is their susceptibility to degradation, thereby potentially requiring multiple administrations or additional modifications. Because PIK is very effective at correcting or preventing mild cellular barrier dysfunction, we evaluated PIK as a possible pharmacologic reagent to control the disruption of semipermeable endothelial barriers through the inhibition of MLCK activity in inflammatory lung injuries. Direct, intravenous PIK injections produced dose-dependent attenuations of LPS-induced lung inflammation when delivered intravenously, with approximately 50% reduction in alveolar/vascular permeability, increases of cytokine, and leukocyte influx accompanied by histologic evidence of the attenuation of endothelial and epithelial injury. These results, consistent with studies *in vitro*, studies in isolated lung preparations, and models of LPS-induced lung injury (35, 36), demonstrate the critical role of MLCK in ALI and the feasibility of attenuating vascular permeability via nmMLCK inhibition. The administration of PIK was beneficial only in decreasing neutrophil invasion to the BAL fluid and lung tissue in the

mouse VILI model. In this model, PIK may inhibit neutrophil-mediated nmMLCK activation and the subsequent formation of paracellular gaps that facilitates neutrophil invasion (7).

The modulation of nmMLCK expression and activity via RNA interference (siRNAs) is a promising biological approach to attenuating gene expression and optimizing assessments of the therapeutic feasibility of potential molecular targets in ALI. The inhibition of RNA is a naturally occurring mechanism for regulating gene expression, as observed in several model organisms, and is mediated by double-stranded RNA (43, 44). Currently, multiple siRNA therapeutic strategies, targeting genes such as *VEGF* and ribonucleotide reductase (45), are undergoing clinical trials. Although RNA interference techniques allow for the regulation of gene expression via exogenous agents while using endogenous machinery with considerable freedom in molecular design, siRNA requires specific delivery strategies. We used nmMLCK siRNA (5 mg/kg), either directly injected intravenously, or administered as cargo within ACE antibody-conjugated liposomes (to target the pulmonary vasculature selectively), as a second strategy to ameliorate ALI and VILI inflammatory injury (12, 16, 19, 46). The delivery of nmMLCK siRNA by either strategy decreased nmMLCK lung expression (~70% reduction) and significantly attenuated LPS-induced lung inflammation as well as VILI, with approximately 40% reduction in BAL protein consistent with improved endothelial-epithelial barriers and considerable attenuation of VILI-induced lung injury (i.e., decreases of leukocyte infiltration into lung tissue). Although the intratracheal injection of naked siRNA results in less efficient gene targeting to cells of interest, and therefore carries a higher likelihood of producing nonspecific effects (47), the strong agreement in results obtained from naked and targeted siRNA delivery suggests that naked siRNA is sufficient to silence nmMLCK effectively in mouse lung endothelial cells, with negligible nonspecific effects.

Wainwright and colleagues (19) demonstrated a decreased susceptibility of the endothelium to injury after LPS treatment in nmMLCK KO mice, and proposed nmMLCK as a possible therapeutic target against ALI. We extended these findings, and described a significant role for gender and age in the severity of lung injury, with genetically engineered mice overexpressing nmMLCK in the vasculature exhibiting a greater inflammatory response and augmented vascular barrier dysfunction (12). To enhance our understanding of the physiology and importance of nmMLCK in lung barrier regulation, we performed expression profiling of the lung genes obtained from nmMLCK KO mice for microarray assays after exposure to VILI. Ventilator-induced lung injury-treated nmMLCK^{-/-} mice demonstrated fewer up-regulated than down-regulated genes (138 versus 220, respectively) compared with WT mice. Further, nmMLCK KO mice exhibited physiologic protection from lung injury. Common canonical pathways that were deregulated in both the VILI-exposed WT and nmMLCK groups included leukocyte extravasation, IL-10, and IL-6, whereas novel deregulated pathways induced by VILI only in KO mice included nuclear factor erythroid 2-related factor 2 (NRF2)-mediated oxidative stress responses, coagulation genes, and p53-signaling pathways, as well as the NF- κ B network. The finding that NF- κ B genes were significantly affected by deletion of the nmMLCK allele supports our previous finding that nmMLCK is directly involved in the NF- κ B-dependent transcription of inflammatory and survival genes (33).

The finding of dysregulation of the procoagulant pathway is of considerable interest, because ALI is often characterized by increased procoagulant activity and an inhibition of fibrinolysis (48). In addition, activated protein C is an anticoagulant and

barrier-regulatory agent effective in severe sepsis, substantiating a link between coagulation, vascular permeability, and inflammation (49). Moreover, leukocyte extravasation and IL-6 signaling pathways were also implicated in ALI pathology (16), and nmMLCK KO mice exhibited a significantly decreased expression of coagulation system genes, providing further evidence for the importance of hemostatic imbalance in the development of ALI. Although the role of nmMLCK in regulating coagulation remains to be elucidated (and is currently being explored), our *in vivo* results support a direct effect of nmMLCK on murine coagulation pathways, because longer bleeding times were evident in nmMLCK KO mice compared with WT mice. Identifying the mechanisms of the effects of nmMLCK on hemostasis may provide robust therapeutic targets in the treatment of ALI (50).

In conclusion, these studies extend a number of previous studies and demonstrate that the inhibition of nmMLCK activity, using either genetic or pharmacologic strategies, reduces acute lung injury, microvascular leakage, and lung edema via enhancement of the vascular endothelial barrier. The clinical validation of the contributions of the non-muscle isoform of MLCK in vascular restoration during lung injury requires further studies of nmMLCK, an attractive candidate gene and molecular target in sepsis-associated ALI and VILI.

Author Disclosure: D.M.W. received a sponsored grant from the National Institutes of Health (\$100,000 or more). J.R.T. has patents received or pending from Unity Pharmaceuticals (the overall license fee to the University of Chicago was less than \$30,000) and from the University of Chicago Tech for a patent application for PIK (with financial benefits through the license). J.R.T. also received sponsored grants from the National Institutes of Health (\$100,000 or more) and the Department of Defense (\$100,000 or more). Y.A.L., in affiliation with Columbia University, has three patents pending: (1) a Provisional Patent Application: Methods for Extracting Phenotypic Information from the Literature via Natural Language Processing; (2) US Patent 20060074991, System and Method for Generating an Amalgamated Database, and Clinigene International Patent Application Number PCT/US03/35470; and (3) Terminological Mapping, a Continuation-in-Part of International Patent Application Number PCT/US03/35470. Y.A.L.'s spouse has a patent with the Memorial Sloan-Kettering Cancer Center (US Patent 7528116, Kinase Suppressor of Ras Inactivation for Therapy of Ras Mediated Tumorigenesis). None of the other authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

References

1. Lazar V, Garcia JG. A single human myosin light chain kinase gene (*MLCK*; *MYLK*) transcribes multiple nonmuscle isoforms. *Genomics* 1999;57:256-267.
2. Dreyfuss D, Saumon G. Ventilator-induced lung injury: lessons from experimental studies. *Am J Respir Crit Care Med* 1998;157:294-323.
3. Ma SF, Grigoryev DN, Taylor AD, Nonas S, Sammani S, Ye SQ, Garcia JG. Bioinformatic identification of novel early stress response genes in rodent models of lung injury. *Am J Physiol* 2005;289:L468-L477.
4. Chopra M, Reuben JS, Sharma AC. Acute lung injury: apoptosis and signaling mechanisms. *Exp Biol Med* 2009;234:361-371.
5. Petrache I, Verin AD, Crow MT, Birukova A, Liu F, Garcia JG. Differential effect of MLC kinase in TNF-alpha-induced endothelial cell apoptosis and barrier dysfunction. *Am J Physiol* 2001;280:L1168-L1178.
6. Dudek SM, Garcia JG. Cytoskeletal regulation of pulmonary vascular permeability. *J Appl Physiol* 2001;91:1487-1500.
7. Garcia JG, Verin AD, Hereniyova M, English D. Adherent neutrophils activate endothelial myosin light chain kinase: role in transendothelial migration. *J Appl Physiol* 1998;84:1817-1821.
8. Garcia JG, Davis HW, Patterson CE. Regulation of endothelial cell gap formation and barrier dysfunction: role of myosin light chain phosphorylation. *J Cell Physiol* 1995;163:510-522.
9. Kawkitinarong K, Linz-McGillem L, Birukov KG, Garcia JG. Differential regulation of human lung epithelial and endothelial barrier function by thrombin. *Am J Respir Cell Mol Biol* 2004;31:517-527.
10. Nusrat A, Turner JR, Madara JL. Molecular physiology and pathophysiology of tight junctions. IV. Regulation of tight junctions by

- extracellular stimuli: nutrients, cytokines, and immune cells. *Am J Physiol Gastrointest Liver Physiol* 2000;279:G851–G857.
11. Gao L, Grant A, Halder I, Brower R, Sevransky J, Maloney JP, Moss M, Shanholtz C, Yates CR, Meduri GU, *et al.* Novel polymorphisms in the myosin light chain kinase gene confer risk for acute lung injury. *Am J Respir Cell Mol Biol* 2006;34:487–495.
 12. Moitra J, Evenoski C, Sammani S, Wadgaonkar R, Turner JR, Ma SF, Garcia JG. A transgenic mouse with vascular endothelial overexpression of the non-muscle myosin light chain kinase-2 isoform is susceptible to inflammatory lung injury: role of sexual dimorphism and age. *Transl Res* 2008;151:141–153.
 13. Flores C, Ma SF, Maresco K, Ober C, Garcia JG. A variant of the myosin light chain kinase gene is associated with severe asthma in african americans. *Genet Epidemiol* 2007;31:296–305.
 14. Gao L, Grant A, Chi P, Gao P, Stockton M, Watson H, Hansel N, Diette G, Dunston G, Mathias RA, *et al.* Myosin light chain kinase (MYLK) variants that confer increased risk of sepsis and acute lung injury are associated with asthma and associated phenotypes [abstract]. Annual Meeting of the American Academy of Allergy, Asthma and Immunology, 2007.
 15. Desai AA, Hysi P, Garcia JG. Integrating genomic and clinical medicine: searching for susceptibility genes in complex lung diseases. *Transl Res* 2008;151:181–193.
 16. Hong SB, Huang Y, Moreno-Vinasco L, Sammani S, Moitra J, Barnard JW, Ma SF, Mirzapioazova T, Evenoski C, Reeves RR, *et al.* Essential role of pre-B-cell colony enhancing factor in ventilator-induced lung injury. *Am J Respir Crit Care Med* 2008;178:605–617.
 17. Garcia JG, Liu F, Verin AD, Birukova A, Dechert MA, Gerthoffer WT, Bamburg JR, English D. Sphingosine 1-phosphate promotes endothelial cell barrier integrity by Edg-dependent cytoskeletal rearrangement. *J Clin Invest* 2001;108:689–701.
 18. Owens SE, Graham WV, Siccardi D, Turner JR, Mrsny RJ. A strategy to identify stable membrane-permeant peptide inhibitors of myosin light chain kinase. *Pharm Res* 2005;22:703–709.
 19. Wainwright MS, Rossi J, Schavocky J, Crawford S, Steinhorn D, Valenza AV, Zasadzki M, Shirinsky V, Jia Y, Haiech J, *et al.* Protein kinase involved in lung injury susceptibility: evidence from enzyme isoform genetic knockout and *in vivo* inhibitor treatment. *Proc Natl Acad Sci USA* 2003;100:6233–6238.
 20. Peng X, Hassoun PM, Sammani S, McVerry BJ, Burne MJ, Rabb H, Pearce D, Tudor RM, Garcia JG. Protective effects of sphingosine 1-phosphate in murine endotoxin-induced inflammatory lung injury. *Am J Respir Crit Care Med* 2004;169:1245–1251.
 21. Meyer NJ, Huang Y, Singleton PA, Sammani S, Moitra J, Evenoski CL, Husain AN, Mitra S, Moreno-Vinasco L, Jacobson JR, *et al.* *GADD45a* is a novel candidate gene in inflammatory lung injury via influences on Akt signaling. *FASEB J* 2009;3:1325–1337.
 22. Peng X, Sammani S, McVerry BJ, Hassoun PM, Garcia JG. Sphingosine 1-phosphate reduces lung vascular permeability in a murine model of LPS-mediated acute lung injury. *Am J Respir Crit Care Med* 2003;167: A778.
 23. Simon BA, Easley RB, Grigoryev DN, Ma SF, Ye SQ, Lavoie T, Tudor RM, Garcia JG. Microarray analysis of regional cellular responses to local mechanical stress in acute lung injury. *Am J Physiol* 2006;291: L851–L861.
 24. Wu Z, Irizarry R, Gentleman R, Martinez-Murillo F, Spencer F. A model-based background adjustment for oligonucleotide expression arrays. *J Am Stat Assoc* 2004;99:909–917.
 25. Tusher VG, Tibshirani R, Chu G. Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci USA* 2001;98:5116–5121.
 26. Consortium. The Gene Ontology (GO) Project in 2006. *Nucleic Acids Res* 2006;34:D322–D326.
 27. Draghici S, Khatri P, Bhavsar P, Shah A, Krawetz SA, Tainsky MA. Onto-tools, the toolkit of the modern biologist: onto-express, onto-compare, onto-design and onto-translate. *Nucleic Acids Res* 2003;31:3775–3781.
 28. Benjamini YHY. On the adaptive control of the false discovery rate in multiple testing with independent statistics. *J Educ Behav Stat* 2000; 25:60–83.
 29. Zolotarevsky Y, Hecht G, Koutsouris A, Gonzalez DE, Quan C, Tom J, Mrsny RJ, Turner JR. A membrane-permeant peptide that inhibits MLC kinase restores barrier function in *in vitro* models of intestinal disease. *Gastroenterology* 2002;123:163–172.
 30. Aurand-Lions M, Johnson-Leger C, Lamagna C, Ozaki H, Kita T, Imhof BA. Junctional adhesion molecules and interendothelial junctions. *Cells Tissues Organs* 2002;172:152–160.
 31. Greer P. Closing in on the biological functions of Fps/Fes and Fer. *Nat Rev Mol Cell Biol* 2002;3:278–289.
 32. Garcia JG, Lazar V, Gilbert-McClain LI, Gallagher PJ, Verin AD. Myosin light chain kinase in endothelium: molecular cloning and regulation. *Am J Respir Cell Mol Biol* 1997;16:489–494.
 33. Wadgaonkar R, Linz-McGille L, Zaiman AL, Garcia JG. Endothelial cell myosin light chain kinase (MLCK) regulates TNFalpha-induced NFkappaB activity. *J Cell Biochem* 2005;94:351–364.
 34. Goldblum SE, Hennig B, Jay M, Yoneda K, McClain CJ. Tumor necrosis factor alpha-induced pulmonary vascular endothelial injury. *Infect Immun* 1989;57:1218–1226.
 35. Hirano S, Rees RS, Yancy SL, Welsh MJ, Remick DG, Yamada T, Hata J, Gilmont RR. Endothelial barrier dysfunction caused by LPS correlates with phosphorylation of HSP27 *in vivo*. *Cell Biol Toxicol* 2004;20:1–14.
 36. Essler M, Staddon JM, Weber PC, Aepfelbacher M. Cyclic AMP blocks bacterial lipopolysaccharide-induced myosin light chain phosphorylation in endothelial cells through inhibition of Rho/Rho kinase signaling. *J Immunol* 2000;164:6543–6549.
 37. Cui Y, English DK, Siddiqui RA, Heranyiova M, Garcia JG. Activation of endothelial cell phospholipase D by migrating neutrophils. *J Invest Med* 1997;45:388–393.
 38. Clayburgh DR, Musch MW, Leitges M, Fu YX, Turner JR. Coordinated epithelial NHE3 inhibition and barrier dysfunction are required for TNF-mediated diarrhea *in vivo*. *J Clin Invest* 2006;116:2682–2694.
 39. Penn MS, Chisolm GM. Relation between lipopolysaccharide-induced endothelial cell injury and entry of macromolecules into the rat aorta *in vivo*. *Circ Res* 1991;68:1259–1269.
 40. Moriez R, Salvador-Cartier C, Theodorou V, Fioramonti J, Eutamene H, Bueno L. Myosin light chain kinase is involved in lipopolysaccharide-induced disruption of colonic epithelial barrier and bacterial translocation in rats. *Am J Pathol* 2005;167:1071–1079.
 41. Jacobson JR, Dudek SM, Singleton PA, Kolosova IA, Verin AD, Garcia JG. Endothelial cell barrier enhancement by ATP is mediated by the small GTPase Rac and cortactin. *Am J Physiol* 2006;291:L289–L295.
 42. Ma SF, Moreno-Vinasco L, Moitra J, Wang T, Hysi P, Flores C, Dudek SM, Nicolae DL, Ober C, Watterson MA, *et al.* Non-muscle myosin light chain kinase is a novel molecular target in murine and human asthma with MYLK polymorphisms conferring ethnic-specific asthma susceptibility. *J Invest Med* 2008;56:594 (Abstract).
 43. Hammond SM, Caudy AA, Hannon GJ. Post-transcriptional gene silencing by double-stranded RNA. *Nat Rev* 2001;2:110–119.
 44. Tuschl T. RNA interference and small interfering RNAs. *ChemBioChem* 2001;2:239–245.
 45. Castanotto D, Rossi JJ. The promises and pitfalls of RNA-interference-based therapeutics. *Nature* 2009;457:426–433.
 46. Gao L, Flores C, Fan-Ma S, Miller EJ, Moitra J, Moreno L, Wadgaonkar R, Simon B, Brower R, Sevransky J, *et al.* Macrophage migration inhibitory factor in acute lung injury: expression, biomarker, and associations. *Transl Res* 2007;150:18–29.
 47. Lingor P, Michel U, Scholl U, Bahr M, Kugler S. Transfection of “naked” siRNA results in endosomal uptake and metabolic impairment in cultured neurons. *Biochem Biophys Res Commun* 2004;315:1126–1133.
 48. Idell S, Gonzalez K, Bradford H, MacArthur CK, Fein AM, Maunder RJ, Garcia JG, Griffith DE, Weiland J, Martin TR, *et al.* Procoagulant activity in bronchoalveolar lavage in the adult respiratory distress syndrome: contribution of tissue factor associated with factor VII. *Am Rev Respir Dis* 1987;136:1466–1474.
 49. Finigan JH, Dudek SM, Singleton PA, Chiang ET, Jacobson JR, Camp SM, Ye SQ, Garcia JG. Activated protein C mediates novel lung endothelial barrier enhancement: role of sphingosine 1-phosphate receptor transactivation. *J Biol Chem* 2005;280:17286–17293.
 50. van der Poll T. Tissue factor as an initiator of coagulation and inflammation in the lung. *Crit Care* 2008;12:S3.