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

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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/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/barrier 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 knockout mice were significantly protected from VILI, with significant reductions 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 mechanical 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 innate 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...
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 (15) 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-smMLCK. The Diff-Quik staining was 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-sm-β-actin. The cationic phospholipid A2-dioleoyl-oxypropyl-N,N,N,N-tetramethylammonium 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). Anti-nmMLCK was purchased from Sigma. The Diff-Quik stain 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 the 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 C57Bl/6 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′-GGGGCAAUUC CCCUGCAA GAU-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/DOPC, condensed 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 μl of PBS (pH 7.4; final concentration, 20 mg/ml). The cationic lipid dispersion was combined with nmMLCK siRNA (1 μg per 10 μg 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 carbonyl groups on the ACE antibody with the ane 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-μl 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
albunm IgG; Bethyl Laboratories, Montgomery, TX), washed, and blocked. After the addition of a 100-μl sample or standard serum and incubation at 37°C for 1 hour, and after three washes, 100 μl 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 μl 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 × 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). Antibodies of interest, ppMLC and pan-MLC. Proteins were detected using an enhanced chemoluminescence system (Cell Signaling).

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′-AAAGATGC TGGCTCCATTACC-3′), the MLCK target sequence 2 (5′-AAAGC CCGGACCGGACGT-3′), control sequence 1 (5′-AAAGAAG ATCGAACCAGGAA-3′), and control sequence 2 (5′-AAGAGAA CCATTAAGCGGCAAG-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 μM (1.5 μM 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 trans-endothelial 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 ± 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)/(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 (referred 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 −log10 (P value ), with P ≤ 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 ± 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 μM 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.
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.
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). 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
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 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 μM), 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.
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...
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
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 biological 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 down-regulation of genes involved in the NF-κB–regulated network (Figure E5). These results are in agreement with our previous studies, which demonstrated that nmMLCK activation resulted in NF-κB activation and NF-κB–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 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.
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 7](image-url)

**Figure 7.** Deletion of nmMLCK gene (nmMLCK<sup>−/−</sup>) 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<sup>−/−</sup> 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<sup>−/−</sup> 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.
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

**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).
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 ribonuclease 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 intra-tracheal 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 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.

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