

Research Article

# Genetic and epigenetic regulation of the non-muscle myosin light chain kinase isoform by lung inflammatory factors and mechanical stress

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**Rationale:** The myosin light chain kinase gene, *MYLK*, encodes three proteins via unique promoters, including the non-muscle isoform of myosin light chain kinase (nmMLCK), a cytoskeletal protein centrally involved in regulation of vascular integrity. As *MYLK* coding SNPs are associated with severe inflammatory disorders (asthma, acute respiratory distress syndrome (ARDS)), we explored clinically relevant inflammatory stimuli and promoter SNPs in nmMLCK promoter regulation. **Methods:** Full-length or serially deleted *MYLK* luciferase reporter promoter activities were measured in human lung endothelial cells (ECs). SNP-containing non-muscle *MYLK* (nm*MYLK*) DNA fragments were generated and nm*MYLK* promoter binding by transcription factors (TFs) detected by protein–DNA electrophoretic mobility shift assay (EMSA). Promoter demethylation was evaluated by 5-aza-2'-deoxycytidine (5-Aza). A preclinical mouse model of lipopolysaccharide (LPS)-induced acute lung injury (ALI) was utilized for nmMLCK validation. **Results:** Lung EC levels of nmMLCK were significantly increased in LPS-challenged mice and LPS, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), 18% cyclic stretch (CS) and 5-Aza each significantly up-regulated EC nm*MYLK* promoter activities. EC exposure to FG-4592, a prolyl hydroxylase inhibitor that increases hypoxia-inducible factor (HIF) expression, increased nm*MYLK* promoter activity, confirmed by HIF1 $\alpha$ /HIF2 $\alpha$  silencing. nm*MYLK* promoter deletion studies identified distal inhibitory and proximal enhancing promoter regions as well as mechanical stretch-, LPS- and TNF $\alpha$ -inducible regions. Insertion of ARDS-associated SNPs (rs2700408, rs11714297) significantly increased nm*MYLK* promoter activity via increased transcription binding (glial cells missing homolog 1 (GCM1) and intestine-specific homeobox (ISX), respectively). Finally, the *MYLK* rs78755744 SNP (–261G/A), residing within a nm*MYLK* CpG island, significantly attenuated 5-Aza-induced promoter activity. **Conclusion:** These findings indicate nm*MYLK* transcriptional regulation by clinically relevant inflammatory factors and ARDS-associated nm*MYLK* promoter variants are consistent with nmMLCK as a therapeutic target in severe inflammatory disorders.

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## Introduction

The pulmonary vascular endothelium serves as a semi-selective barrier between circulating blood and surrounding tissues, with endothelial cell (EC) integrity critical to tissue and organ function. The SARS-CoV-2/COVID-19 pandemic with unprecedented cases of COVID-induced acute respiratory distress syndrome (ARDS) has dramatically highlighted the role of EC loss of barrier integrity in ARDS

pathobiology with vascular leakage and multiple vital organ failure driving ARDS mortality [1]. Disruption of vascular barrier integrity induced by inflammatory agonists, such as lipopolysaccharide (LPS), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), eNAMPT and IL-1 $\beta$ , and by excessive mechanical stress produced by mechanical ventilation, leads to hypoxemia, increased multi-organ failure and potential ARDS mortality [2–4].

The pathobiological mechanisms producing increased vascular permeability in severe SARS-Co-V-2 infection are incompletely understood but undoubtedly involve robust activation of the EC cytoskeleton, well recognized as critical to vascular barrier regulation and repair [5]. Inflammatory cell or mediator-induced activation of vascular barrier-disruptive signaling pathways, in combination with increases in reactive oxygen species (ROS), result in enhanced EC contractility, loosening of inter-endothelial junctions, formation of paracellular gaps, and development of profound vascular leakage and organ edema [5]. In addition, several cytoskeletal target genes harbor variants which contribute to the genetic basis of well-recognized health disparities in ARDS subjects of African descent [6–10].

The non-muscle isoform of myosin light chain kinase (nmMLCK), encoded by the *MYLK* gene, is essential to cytoskeletal regulation of EC–matrix and cell–cell adhesion, vascular integrity and permeability, key pathophysiological roles in lung inflammatory diseases [9,11–13]. In addition to EC barrier regulation, the pleiotropic nmMLCK is centrally involved in angiogenesis [14], EC apoptosis [15] and leukocytic diapedesis [16]. *MYLK* is a cytoskeletal target gene whose coding variants (SNPs) also contribute to the genetic basis for observed ARDS health disparities in severe sepsis-induced ARDS and severe asthma [9,17] and major trauma-induced ARDS/acute lung injury (ALI) [7] in African Americans (AAs) and/or European Americans (EAs). In severe asthma AA and Spanish population cohorts, two *MYLK* variants were identified as significantly associated with susceptibility to severe asthma [17–19]. Non-muscle *MYLK* (nm*MYLK*)-coding SNPs have been shown to directly alter EC barrier integrity and to delay vascular recovery from inflammation-induced permeability [20]. nmMLCK is robustly activated by biophysical forces, such as excessive mechanical stress, that are key to ventilator-induced lung injury (VILI) [13]. nmMLCK knockout mice were significantly protected from mechanical ventilation-induced lung injury and exhibited decreased oxidative stress, coagulation, p53 signaling, leukocyte extravasation and IL-6 signaling [13]. nmMLCK also modulates radiation-induced lung injury [21], murine asthmatic inflammation [22] and is significantly linked to human asthma severity and exacerbation status [23].

Despite these studies highlighting the critical involvement of the non-muscle MLCK isoform, nmMLCK, in acute inflammatory processes, much less information is available regarding the mechanistic regulation of the nm*MYLK* promoter activity by inflammatory factors. We previously demonstrated epigenetic 3'UTR regulation of nm*MYLK* expression in human lung EC by specific miRNAs in response to excessive mechanical stress, LPS and TNF- $\alpha$  [24]. The nm*MYLK* promoter is also triggered by the VEGF signaling pathway with critical involvement of the Sp1 transcription factor (TF) [25]. Recently we demonstrated novel regulation of nm*MYLK* transcriptional activity by the redox-sensitive TF, NRF2, via an antioxidant response elements (AREs) that results in transcriptional repression of nm*MYLK* expression [26].

The current study extends these prior reports to demonstrate inhibitory and enhancing nm*MYLK* promoter regions and significant regulation by ARDS-relevant inflammatory stimuli: LPS, TNF- $\alpha$ , and excessive mechanical stress (mimicking VILI). Furthermore, the nm*MYLK* promoter is under strong influence by hypoxia-inducible transcription factors (HIFs), HIF-1 $\alpha$  and HIF-2 $\alpha$ , and by ARDS-associated SNPs that alter specific TF binding and promoter DNA methylation. These findings are consistent with nm*MYLK*'s contribution to inflammatory disease susceptibility with nmMLCK representing an attractive molecular target in complex lung disorders such as ARDS given the continued absence of FDA-approved ARDS pharmacotherapies [27].

## Methods

### Cell culture, cyclic stretch, and reagents

Human pulmonary artery ECs were obtained from Lonza (Walkersville, MD) and cultured as described previously [28,29] in the manufacturer's recommended endothelial growth medium-2 (EGM-2). Cells were grown at 37°C in a 5% CO<sub>2</sub> incubator, and passages 6–9 were used for experiments with media changed 1 day before experimentation. For cyclic stretch (CS) studies, ECs were plated on Bioflex collagen I type cell culture plates (FlexCell International, Hillsborough, NC) and stimulated for 4 h at 18% CS as previously described [30] on the FlexCell FX-5000 System (FlexCell International), mimicking high tidal volume ventilation. For demethylation studies, ECs were treated with 5-aza-2'-deoxycytidine (5-Aza) (Sigma–Aldrich, St. Louis MO) for 24 h at indicated concentrations to inhibit DNA methyltransferase enzymes as described previously [31].

**Table 1 Biotinylated/nonbiotinylated oligonucleotide probes for EMSA**

Probe names	Sequences (5' to 3')
rs2700408A-biotin	\ 5Biosg \ CTCTTCAGCTGGCCCAAATTGGTTTCTGTTA
rs2700408G-biotin	\ 5Biosg \ CTCTTCAGCTGGCCCGAATTGGTTTCTGTTA
rs2700408del-biotin	\ 5Biosg \ CTCTTCAGCTGGCCCAAATTGGTTTCTGTTA
Consensus-GCM1	CTCTTCAGCTGGCCCGCATTGGTTTCTGTTA
rs11714297T-biotin	\ 5Biosg \ TGTGGACATCCGAATTGGCAATGACATGAAT
rs11714297C-biotin	\ 5Biosg \ TGTGGACATCCGAATCGGCAATGACATGAAT
Consensus-ISX	TGTGGACATCCTAATTGGCAATGACATGAAT

Abbreviations: GCM1, glial cells missing homolog 1; ISX, intestine-specific homeobox.

## Gene cloning, mutagenesis, and 5'-deletion mutations

Gene cloning, mutagenesis, and luciferase activity assays were performed as previously described [32]. Briefly, 2512-bp DNA fragments of the nmMYLK promoter region (−2412 bp to +104 bp from the transcription start site (TSS)) were synthesized by GenScript (Piscataway, NJ 08854) based on sequence NM\_053025 and confirmed by sequencing. The DNA were modified by site-directed mutagenesis to generate fragments containing minor alleles at these loci. All allele-containing fragments were fused to a pGL3-basic reporter vector (Promega, Madison, WI). To further study the putative *cis*-elements, a series of deletion mutants (every 300 bp) sparing the TSSs were constructed by PCR amplification. The DNA fragments containing the truncated region were inserted into the XhoI and MluI sites of pGL3, and the relevant regions of the final constructs were confirmed by sequencing.

## Luciferase reporter gene assays

All constructs were transfected into EC, where a plasmid containing the *Renilla* luciferase gene (pRL-TK) was co-transfected as a control. Transfected cells were exposed to indicated concentrations of LPS and TNF- $\alpha$ , or to 18% CS, and then lysed in passive lysis buffer. Luciferase activity was measured by Dual-Luciferase Assay Kits using the GloMax-Multi Detection System (Promega). Relative activities were expressed as the ratio of firefly luciferase in pGL3 to *Renilla* luciferase in pRL-TK (RLU). Five independent transfections and duplicate luciferase assays were performed for each condition.

## Electrophoretic mobility shift assays

Electrophoretic mobility shift assays (EMSAs) for DNA TF binding was detected as previously described [33]. Briefly, nuclear extracts from HeLa cells (Promega, Madison, WI) were used as the source of TFs. Prediction of TF-binding sites and effects of SNPs were performed by positional weight matrices search using Genomatix MatInspector 8.4 (<http://www.genomatix.de/>) analysis. Biotinylated oligonucleotide probes (Table 1), containing TF-binding motifs of the partial MYLK DNA containing rs2700408 and rs11714297 alleles, were utilized for the detection of nuclear protein complexes bound to the oligonucleotides by using a Light Shift Chemiluminescent EMSA kit (Thermo Scientific, Rockford, IL). Nuclear protein (5  $\mu$ g) was incubated with 20 fmol of the biotin-labeled oligonucleotide, with or without a 10- to 50-fold molar excess (0.2–1 pmol) of unlabeled oligonucleotide. After electrophoresis, the DNA–protein complex on Hybond-N+ membrane was detected by horseradish peroxidase and electrochemiluminescence.

## Preclinical model of LPS-induced lung injury and immunohistochemistry

All experiments were approved by the Animal Care and Use Committee at the University of Arizona. Murine model of LPS-induced ALI was re-established as previous described [34,35]. Briefly, 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, intubated and intratracheally injected with LPS 1 mg/kg or PBS. Mice were sacrificed after 18 h and the lungs excised and embedded for Hematoxylin and Eosin staining. Immunohistochemistry was performed on paraffin-embedded sections utilizing anti-nmMLCK antibodies (sc-365352, Santa Cruz Biotechnology, Texas, U.S.A.), which are specific for mouse, rat and human N-terminus of MYLK, at 1:50 dilution, and biotinylated secondary antibody, as previous described [34,35].

## Statistical analysis

The ANOVA test was used for comparison of luciferase activities among different constructs. Otherwise, the Student's *t* test was used, and the results were expressed as means  $\pm$  SEM. Statistical significance was defined at  $P < 0.05$  in all tests.

## Results

### Lung inflammatory factors increase nmMYLK promoter activity

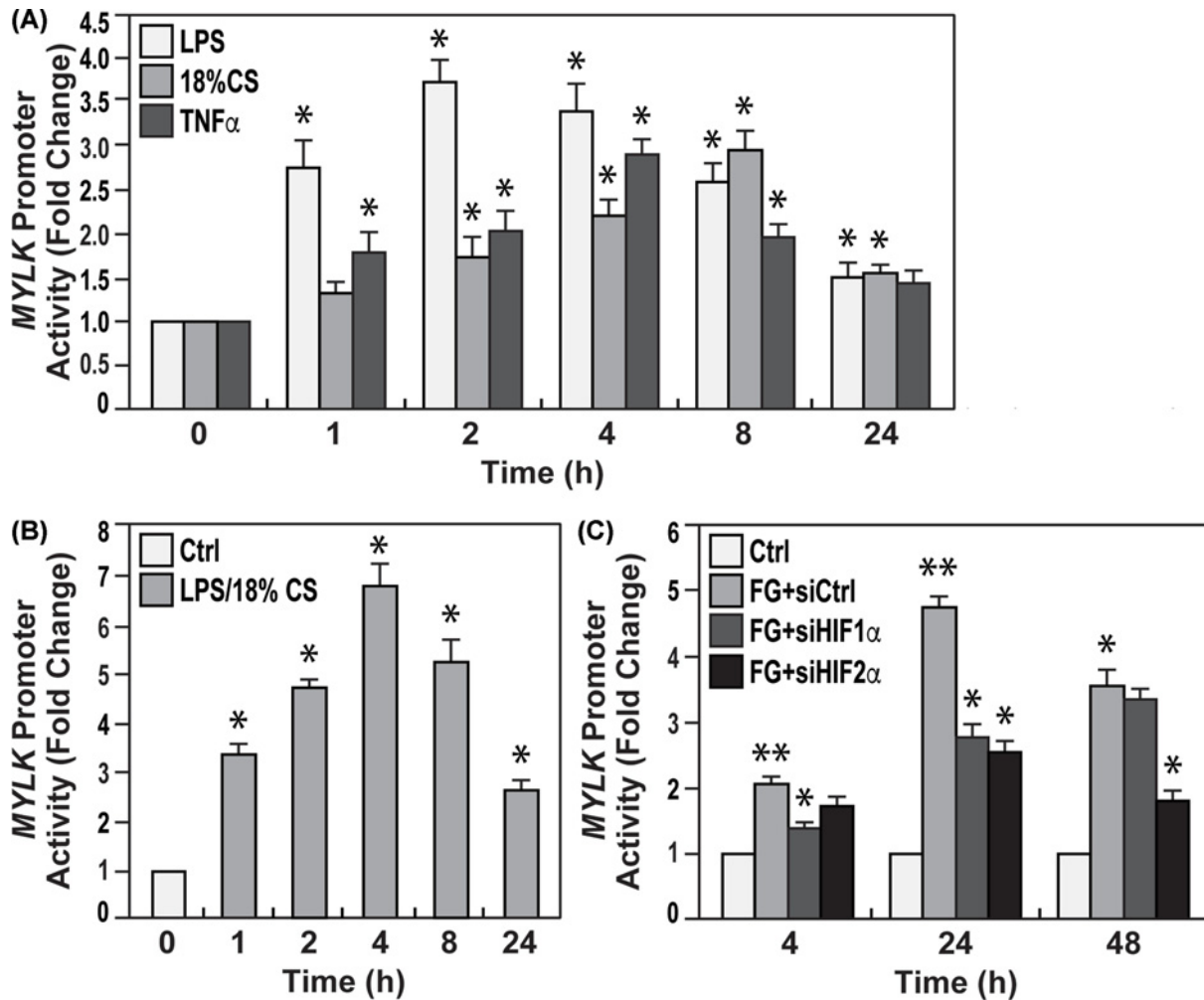
In addition to LPSs and TNF- $\alpha$ , excessive mechanical stress from mechanical ventilation also contributes to the development of severe inflammatory lung injury and vascular leakage driving the severity of ARDS/VILI [36]. Based on calculations, high tidal volume ventilation resulting in  $\sim 40$ – $50\%$  increases in lung alveolar surface is reflected *in vitro* by exposure to 18% CS [30,37]. We explored the effects of these inflammatory agonists on nmMYLK gene transcription activity in human lung ECs via transfection with the full length 2.5 kb nmMYLK promoter in firefly luciferase reporter pGL3, and co-transfection with the *Renilla* luciferase reporter phRL-TK as internal controls. nmMYLK promoter activity was measured by dual-luciferase activity assay after exposure to LPS (100 ng/ml), TNF- $\alpha$  (10 ng/ml), or 18% CS for 1, 2, 4, 8, or 24 h. Each inflammatory stimulus independently induced significant and sustained increases in nmMYLK promoter activity (Figure 1). The extrinsic inflammatory factor, LPS, significantly elevated nmMYLK promoter activity beginning at 1 h (2.8-fold), peaking at 2 h (3.7-fold), and gradually declining by 24 h (Figure 1A). In contrast, EC exposure to 18% CS, used as an *in vitro* model to mimic high tidal volume mechanical ventilation, the root cause of VILI and potent inflammatory stimulus [38–40], augmented nmMYLK promoter activity beginning at 2 h and reached maximal increases in nmMYLK promoter activity at 8 h (3-fold). The intrinsic inflammatory factor, TNF- $\alpha$ , increased nmMYLK promoter activity at 1 h peaking at 4 h (2.9-fold) (Figure 1A). We further detected significant effects of the combined LPS and 18% CS exposure on nmMYLK promoter activities. Compared with controls, nmMYLK promoter activity was significantly increased by combined LPS and 18% CS exposure at 1 and 2 h (3.4- and 4.7-fold), peaking at 4 and 8 h (6.8- and 5.2-fold), with sustained increase at 24 h (2.6-fold) (Figure 1B). Together, these data indicate strong up-regulation of nmMYLK promoter activity in ECs by inflammatory stimuli relevant to human ARDS and VILI.

### Up-regulation of the nmMYLK promoter activity by hypoxia-inducible factors

Profound hypoxia is the hallmark of ARDS pathophysiology. HIFs, HIF-1 $\alpha$  and HIF-2 $\alpha$ , are TFs activated by exposure to hypoxia with nuclear binding to HIF-response elements (HREs). Both hypoxia and the HIF prolylhydroxylase (PHD) inhibitor, FG-4592, block the degradation of HIF-1 $\alpha$  and HIF-2 $\alpha$  to increase intracellular protein levels. Human ECs were co-transfected with nmMYLK promoter reporter with scrambled siRNA (siCtrl), siRNA for HIF-1 $\alpha$  and HIF-2 $\alpha$ , then treated with vehicle or PHD inhibitor FG-4592 (100 nM) for 4, 24 and 48 h, followed by measurements of luciferase activity. FG-4592 significantly increased nmMYLK promoter activities at 4 and 24 h (\*\* $P < 0.01$  vs. vehicle) with significantly attenuation by HIF-1 $\alpha$  and HIF-2 $\alpha$  silencing at 24 and 48 h (\* $P < 0.01$  vs. FG-4592 with siCtrl) (Figure 1C). Thus, HIF-1 $\alpha$  and HIF-2 $\alpha$  contribute to temporal regulation of nmMYLK promoter activities.

### Identification of nmMYLK inhibitory and enhancer regions

We next defined the core promoter region required for nmMYLK responses to important ARDS-relevant inflammatory factors. Serial nested deletion mutations (every 300 bp) sparing TSSs, generated eight truncated nmMYLK promoters from full-length nmMYLK promoter with luciferase reporter. We next assessed nmMYLK luciferase reporter basal promoter activities and their response to LPS, 18% CS, and TNF- $\alpha$  (Figure 2A). These studies revealed that activities of full-length and truncated nmMYLK promoter from  $-1512$  to  $-2412$  bp (upstream of TSS) were associated with decreased basal nmMYLK promoter activity ( $\sim 50\%$  reduction), compared with activity of  $-1512$  bp promoter ( $P < 0.05$ ) (Figure 2A), whereas truncation of the nmMYLK promoter from  $-312$  to  $-1512$  bp increased promoter activity ( $\sim 60\%$  increase) (Figure 2A). These results suggest that the  $-2412$  to  $-1512$  bp region represents an inhibitory region basally influenced by factors which suppress promoter activity. In contrast, the  $-1512$  to TSS region appears essential for core nmMYLK promoter activity.



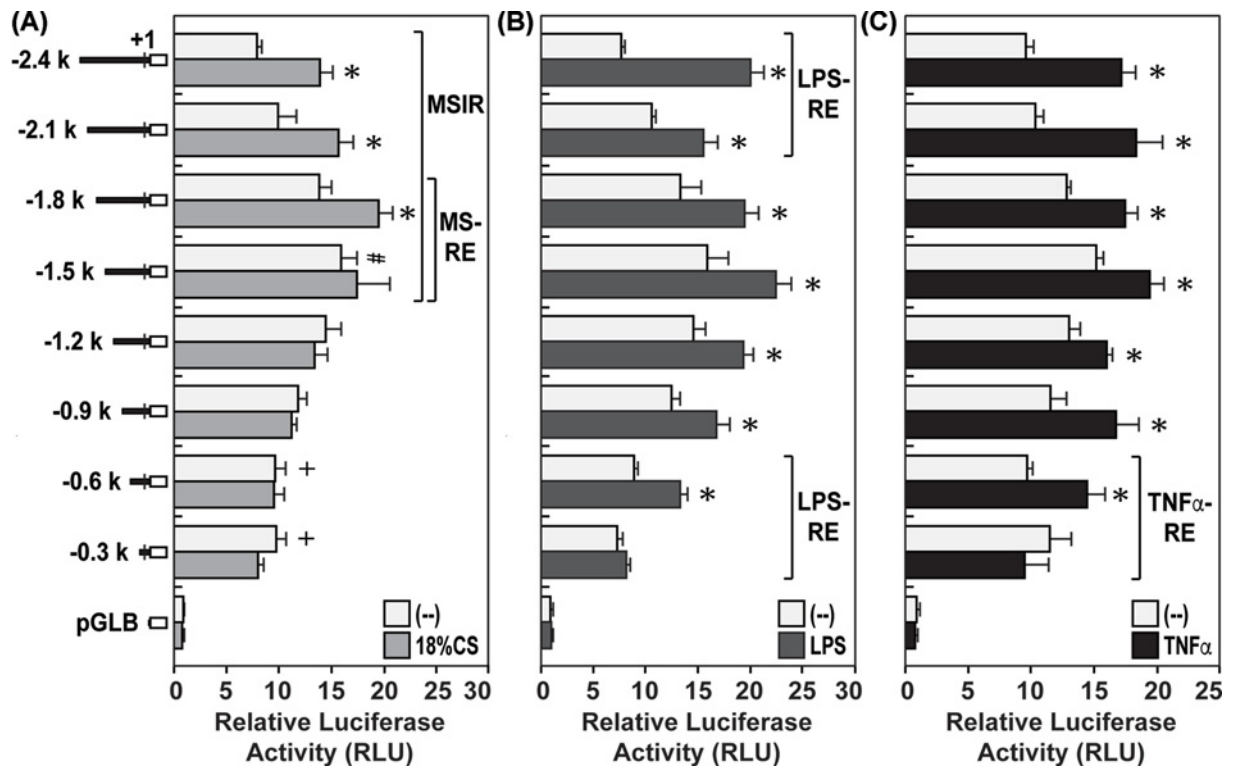
**Figure 1. Lung inflammatory factors increase nmMYLK promoter activities**

Full-length *MYLK* promoter (2.5 kb) was constructed into upstream of firefly luciferase gene in pGL3-basic. Human pulmonary artery ECs were transfected by full-length *MYLK* promoter construct along with *Renilla* luciferase reporter phRL-TK as normalization control vector. **(A)** nmMYLK promoter constructs transfected ECs were exposed to LPS (100 ng/ml), TNF- $\alpha$  (10 ng/ml), or 18% CS for 1, 2, 4, 8, and 24 h, and luciferase activity measured using the Dual Luciferase Assay System (Promega). The bar graph represents normalized relative luciferase activity by vehicle-treated control ( $*P < 0.05$  vs. control,  $n = 4$  each group). Compared with controls, LPS, TNF- $\alpha$  (10 ng/ml), or 18% CS all significantly increased nmMYLK promoter activities in time-dependent manner. **(B)** nmMYLK promoter constructs transfected ECs were exposed to the combination of LPS (100 ng/ml) and 18% CS for 1, 2, 4, 8, and 24 h, and luciferase activity measured. Compared with controls, the combination of LPS and 18% CS significantly increased nmMYLK promoter activities at all time points ( $*P < 0.05$  vs. control,  $n = 6$  each group). **(C)** nmMYLK promoter activities were significantly increased by HIF-1/2 $\alpha$ . Human ECs were co-transfected with nmMYLK promoter reporter with scramble siRNA (siCtrl), siRNA for HIF-1 $\alpha$  or HIF-2 $\alpha$ , then treated with vehicle or HIF prolylhydroxylase (PHD) inhibitor FG-4592 (100 mM) for 4, 24, and 48 h, and luciferase activity was measured. FG-4592 significantly increased *MYLK* promoter activities ( $**P < 0.01$  vs. vehicle). Effects of FG-4592 were significantly attenuated by siHIF-1 $\alpha$  at 4 and 24 h, decreased by HIF2 $\alpha$  at 24 and 48 h ( $*P < 0.01$  vs. FG-4592 with siCtrl) ( $n = 4$  each).

## Identification of a mechanical stress-inducible region in the nmMYLK promoter

We next analyzed nmMYLK luciferase reporter promoter activation in response to 18% CS using a nested deleted promoter with varying DNA length in ECs (Figure 2A). ECs transfected with identical series of nmMYLK promoter fragments were next exposed to 18% CS or static conditions (8 h) with exposure to 18% CS increasing nmMYLK promoter activity in the presence of the -2412 to -1812 region (~80–100% increase) (Figure 2A). The 18% CS-mediated





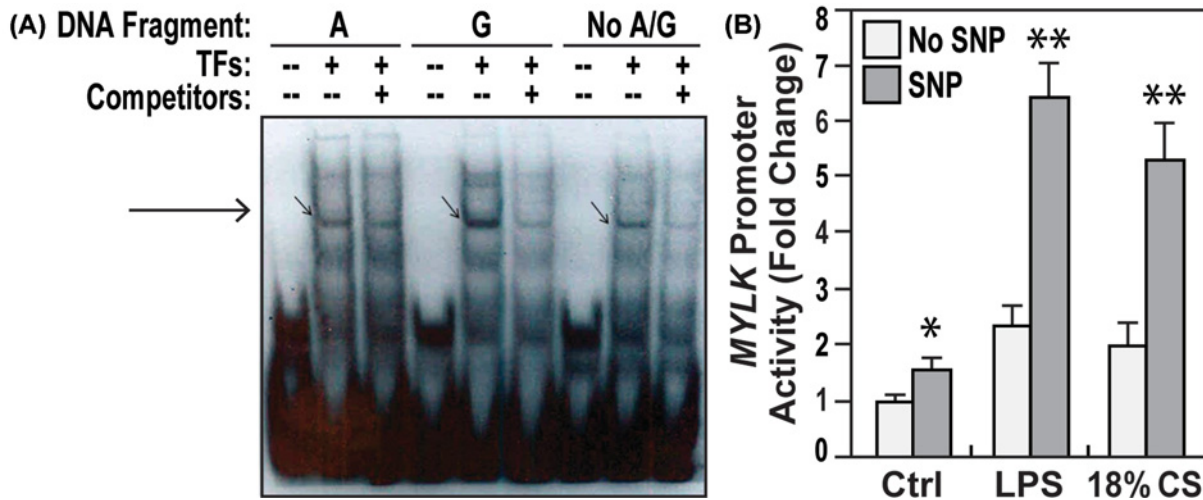
**Figure 2. Functional analysis of nmMYLK promoter regions responsive to LPS, TNF- $\alpha$ , and 18% CS**

(A) ECs were co-transfected with serially deleted nmMYLK promoter reporter along with phRL-TK and treated with control vehicle or LPS, TNF- $\alpha$ , 18% CS for 4 h, and luciferase activity was measured using the Dual Luciferase Assay System (Promega). The bar graph represents ratio of firefly to *Renilla* luciferase activity and normalized by luciferase activity in pGL3-basic. MYLK promoter basic activity in full-growth medium and a region sensitive to 18% CS was identified using a series of nested deletion constructs in conjunction with luciferase reporter activity assays ( $n=4$  each,  $*P<0.05$  vs. vehicle). Mechanical stress-inducible region (MSIR) and region for mechanical response elements (MS-RE) were identified. (B) ECs transfected by serial nmMYLK-promoter reporter constructs and treated with control vehicle or LPS for 4 h, and luciferase activity was measured. nmMYLK promoter regions sensitive to LPS (LPS-RE) were identified ( $*P<0.05$  vs. vehicle,  $n=4$  each). (C) ECs transfected by serial MYLK-promoter reporter constructs and treated with control vehicle or TNF- $\alpha$  (10 ng/ml) for 4 h, and luciferase activity was measured. nmMYLK promoter regions sensitive to TNF- $\alpha$  (TNF $\alpha$ -RE) were identified ( $*P<0.05$  vs. vehicle, # $P<0.05$  vs. -2.4k(-), + $P<0.05$  vs. -1.5k(-),  $n=4$  each).

increase in nmMYLK promoter activity was abolished by truncation of the promoter from -1812 to -1512 (Figure 2A), highly suggestive of a mechanical stress-inducible region (MSIR) at -2412 to -1512 which contains critical nmMYLK promoter mechanical stress-responsive elements.

## Identification of promoter region-specific nmMYLK responses to inflammatory factors

We next analyzed nmMYLK luciferase reporter promoter activation in ECs in response to LPS using the nested deleted promoter constructs (Figure 2B). ECs transfected with identical series of nmMYLK promoter fragments were exposed to LPS 100 ng/ml (4 h) which increased nmMYLK promoter activity (~200%) in the presence of the -2412 to -2112 region, and in the presence of the -1812 to -612 region (~100%) ( $*P<0.05$  both vs. vehicle). LPS-mediated increases were abolished by truncation of the nmMYLK promoter from -612 to -312 (Figure 2B). Similarly, we analyzed nmMYLK luciferase reporter promoter activation in response to TNF- $\alpha$  (10 ng/ml, 4 h) using nested deleted promoters in ECs with the TNF $\alpha$ -mediated increase promoter activity abolished by truncation of the nmMYLK promoter from -612 to -312 (Figure 2C). Together, these results indicate two bacterial endotoxin-inducible regions (-2412 to -2112 and -612 to -312) and a TNF $\alpha$ -inducible region (-612 to -312) which are capable of rapid induction of nmMYLK promoter activities as part of an integrated early inflammatory response.



**Figure 3. Influence of the ARDS-associated 5'UTR variant rs2700408 on nmMYLK promoter activities**

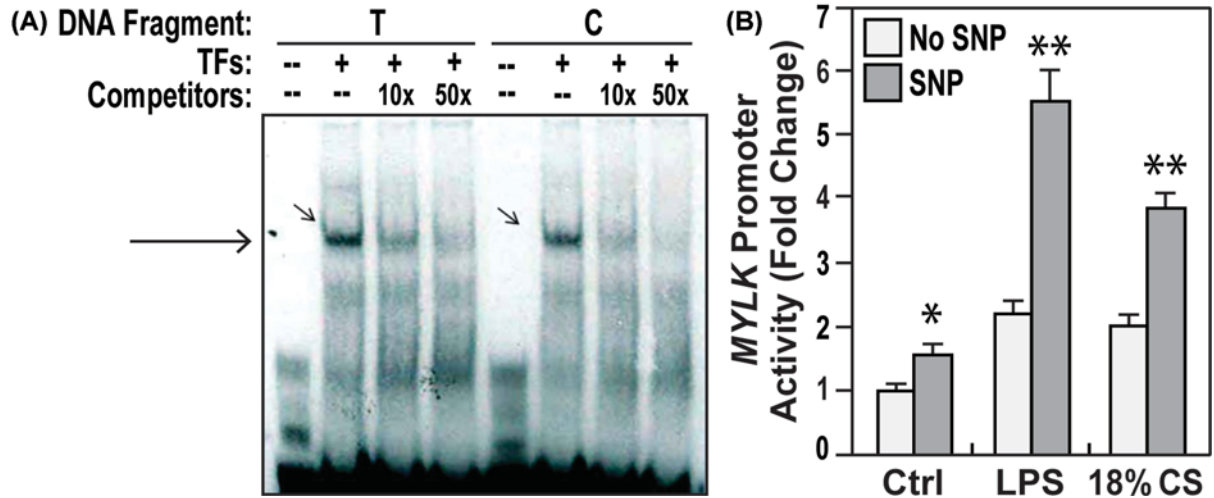
In our recent sequencing studies, rs2700408 was associated with sepsis induced-ARDS/ALI in AAs [41]. (A) Rs2700408A/G interrupted nmMYLK 5'UTR binding to TF GCM1. EMSA was utilized for studying TF protein–nmMYLK DNA interactions. Nuclear extract from HeLa cells for the TF pool was incubated with biotin-labeled MYLK DNA fragments containing rs2700408 A or G alleles, competed by an unlabeled DNA fragment containing GCM1 sequence. After electrophoresis, the DNA–protein complex was detected by HRP and ECL. Compared with rs2700408A, rs2700408G significantly increased GCM1 binding to MYLK DNA. (B) HPAECs were transfected with MYLK promoter-exon2 in pGL3 with rs2700408A or rs2700408G. Rs2700408G significantly increased promoter activities of MYLK, especially in response to LPS and 18% CS (\* $P < 0.05$  vs. rs2700408A, \*\* $P < 0.01$  vs. LPS/No SNP and 18% CS/No SNP,  $n = 4$  each).

## Regulation of nmMYLK promoter activities by ARDS-associated nmMYLK variants with altered TF binding

Recent MYLK sequencing identified an association of the MYLK SNP rs2700408 with increased susceptibility to development of sepsis induced-ARDS in AAs [41]. Rs2700408 (A/G) is a 5'UTR variant on second exon of nmMYLK with *in silico* analyses revealing the A to G SNP to likely increase nmMYLK binding affinity to the TF, glial cells missing homolog 1 (GCM1). Utilizing a protein–DNA EMSA, we found that both DNA fragments containing rs2700408A and rs2700408G bind to GCM1, however, the strength of binding is greater with rs2700408G, indicating that this ARDS risk allele significantly influences GCM1 affinity for nmMYLK compared with the non-risk allele rs2700408A (Figure 3A).

We next subcloned and inserted the nmMYLK second exon containing either rs2700408A or rs2700408G into the full-length nmMYLK promoter with luciferase reporter in pGL3. The constructs were co-transfected with pRL-TK into HPAEC and exposed to LPS and 18% CS, followed by dual luciferase activity measurements. Basal promoter activities of the nmMYLK promoter containing rs2700408G were significantly increased compared with nmMYLK promoter containing rs2700408A ( $P < 0.05$ ). EC exposure to either LPS or 18% CS also confirmed the further significantly enhanced nmMYLK promoter activities by rs2700408G compared with rs2700408A ( $P < 0.05$  each) (Figure 3B).

We previously reported that the nmMYLK promoter 5'UTR intronic variant SNP rs11714297(C/T) was associated increased susceptibility of sepsis induced-ARDS in EAs [9] (OR: 2.08; 95% CI: 1.09–3.99,  $P = 0.02$ ). We again utilized *in silico* analyses to explore potential rs11714297C/T binding alterations and determined that the TF intestine-specific homeobox (ISX) likely confers increased nmMYLK binding affinity at this site in the presence of the rs11714297C/T SNP. By protein–DNA EMSA, we found that both DNA fragment containing rs11714297T and rs2700408C bound to ISX with greater level of ISX bound to DNA with rs11714297T suggesting higher affinity to ISX compared with the non-risk allele rs11714297C (Figure 4A). As with the s2700408A/G studies, insertion of the rs11714297C/T-containing intron produced significantly increased basal and LPS- or 18% CS-induced nmMYLK promoter activity compared with intron-rs11714297C ( $P < 0.05$ , respectively) (Figure 4B).



**Figure 4. Influence of ARDS-associated intronic variant rs11714297C/T on nmMYLK promoter activities**

Rs11714297C/T was associated with ARDS in EA [9]. (A) Rs11714297C/T interrupted MYLK 5'UTR binding to TF ISX. EMSA was utilized for studying TF protein–MYLK DNA interactions. Nuclear extract from HeLa cells for the TF pool was incubated with biotin-labeled MYLK DNA fragments containing rs11714297 C or T alleles, competed by an unlabeled DNA fragment containing ISX sequence. After electrophoresis, the DNA–protein complex was detected by HRP and ECL. Compared with rs11714297C, rs11714297T significantly increased ISX binding to MYLK DNA. (B) HPAECs were transfected with nmMYLK promoter–Luc–intron with rs11714297C or rs11714297T, co-transfected with pRL-TK. Thirty-six hours later, ECs were exposed to LPS 100 ng/ml for 4 h or 18% CS for 8 h. The relative luciferase activities were measured and normalized by controls. Rs11714297T significantly increased promoter activities of MYLK, especially in response to LPS and 18% CS (\* $P < 0.05$  vs. rs11714297C, \*\* $P < 0.01$  vs. LPS/No SNP and 18% CS/No SNP,  $n = 4$  each).

## Influence of DNA demethylation and SNP-CpG disruption on nmMYLK promoter activities

We next addressed epigenetic regulation of the nmMYLK promoter activity by exposing EC, transfected with full-length and truncated nmMYLK promoters, to the demethylation agent, 5-Aza, which significantly increased nmMYLK promoter activities ( $P < 0.05$ , compared with vehicle). The methylation span across 2.4-kb promoter, especially proximal promoter (0.3 kb) with CpG island (from –2412 to –312 bp,  $P < 0.05$  each) (Figure 5). *In silico* analyses suggested that SNP rs78755744 (–261G/A) may disrupt a CpG site within the 0.3 kb nmMYLK core promoter potentially altering responses to methylation. We utilized site-directed mutagenesis to produce wild-type and mutated promoter containing rs78755744A within the truncated –0.3 kb MYLK promoter. We noted that the 5-Aza-induced increases in nmMYLK promoter activity noted observed with the wild-type promoter containing –261G was significantly attenuated by introduction of –261A ( $P < 0.05$ ) (Figure 5). These studies indicate strong epigenetic regulation of nmMYLK promoter activities, with further influence by nmMYLK SNPs.

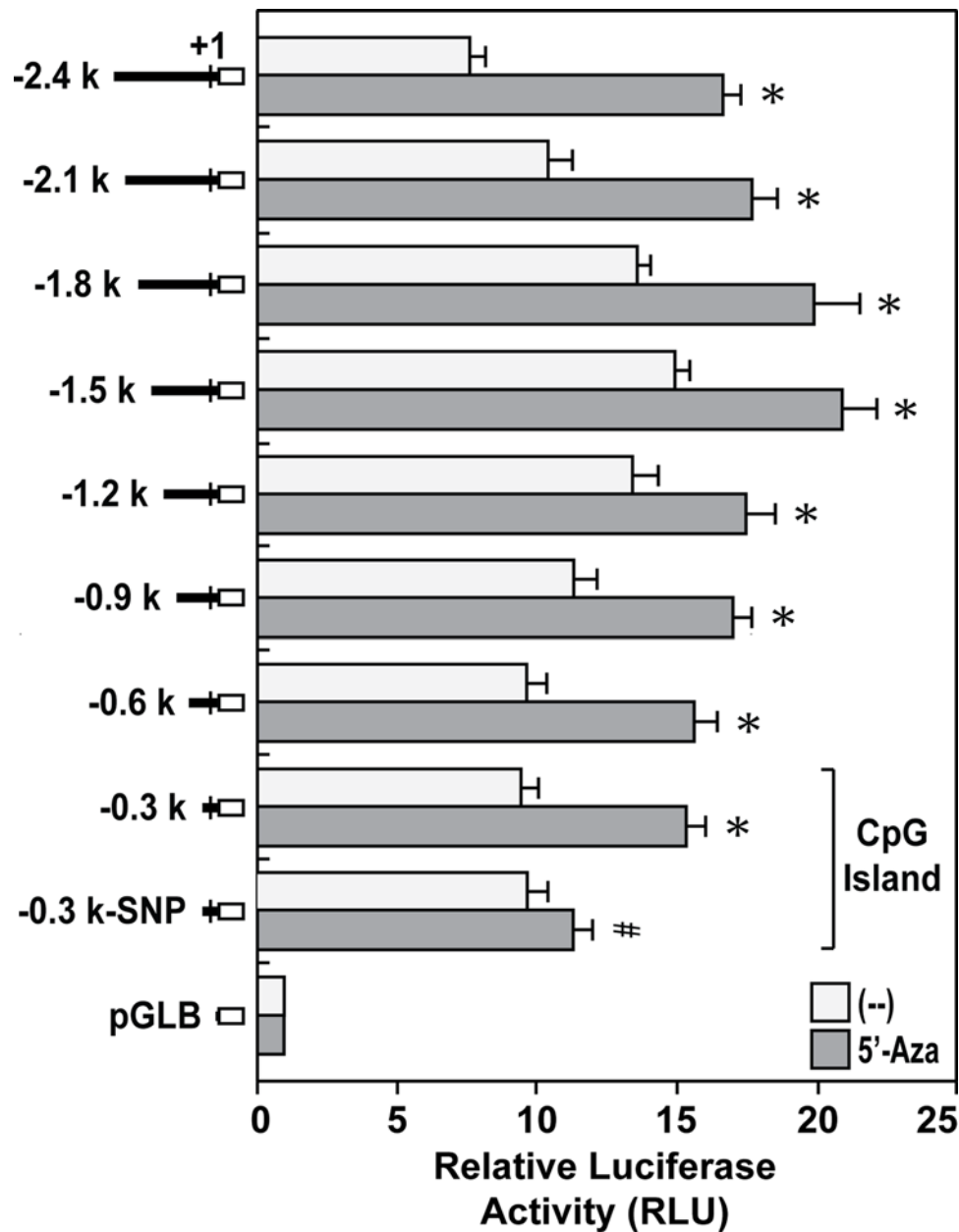
## nmMLCK protein expression is significantly increased in a preclinical murine ARDS model

We explored the levels of nmMLCK protein expression in murine lungs from LPS-induced murine ALI. Robust increases in nmMLCK protein levels were detected in LPS-challenged lungs, including enhanced expression in lung microvascular endothelium (Figure 6). These data validate the hypothesis that inflammatory factors enhance nmMYLK promoter activities both *in vitro* and *in vivo*.

## Discussion

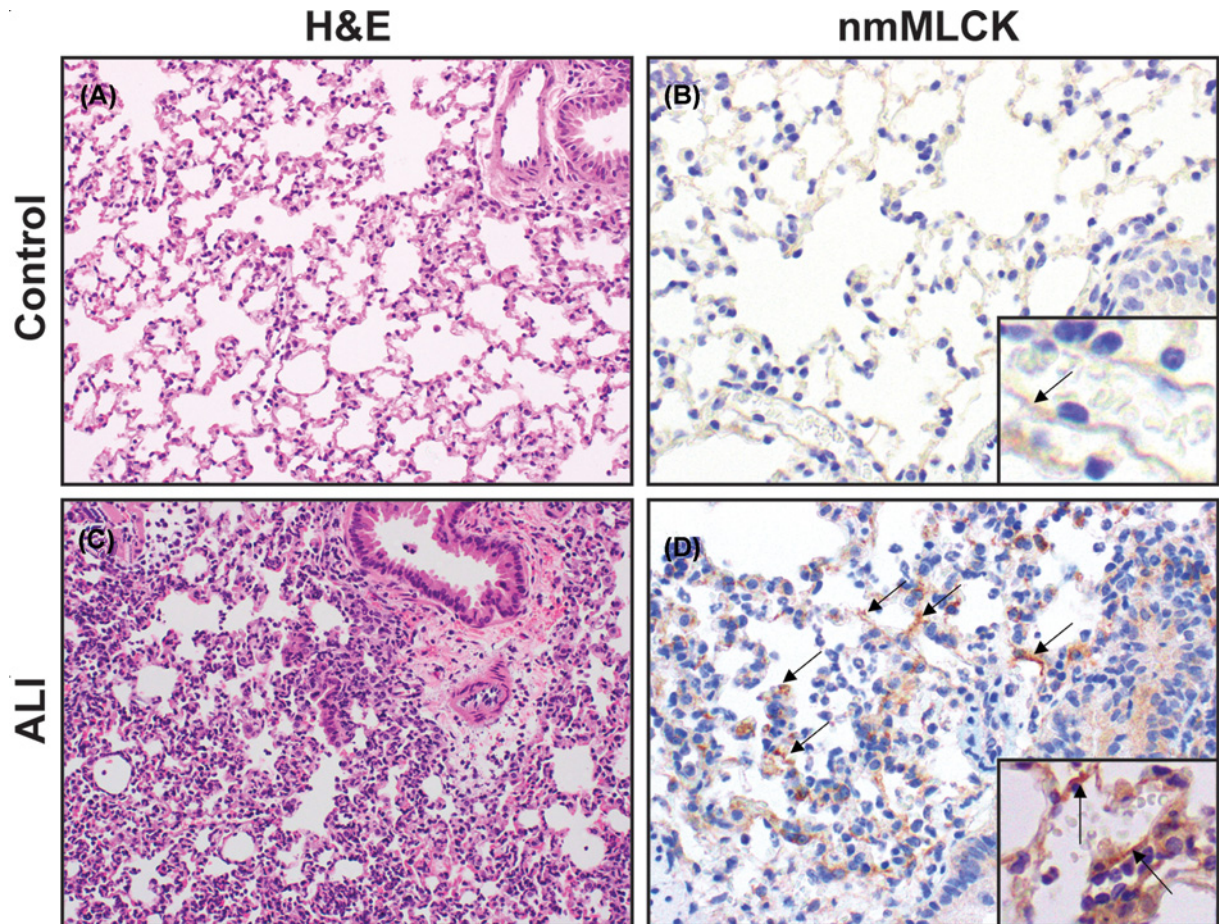
The incidence of ARDS among non-survivors of COVID-19 is 90% [42]. Despite improved understanding of the pathophysiology of ARDS, the underlying mechanisms for the injurious effects of inflammatory processes in the setting of ARDS remain unclear, and effective pharmacotherapies have not yet emerged. Our laboratory was the first to identify the human non-muscle MLCK isoform (nmMLCK) diseases [11], and nmMLCK splice variants [43,44] to





**Figure 5. Demethylation significantly increases nmMYLK transcription/promoter activities: influenced by SNP**

Human pulmonary ECs were co-transfected by full-length nmMYLK promoter constructs in pGL3 and renilla luciferase reporter in phRL-TK. MYLK promoter constructs transfected ECs were exposed to 5-Aza for 24 h and the luciferase activity was measured using the Dual Luciferase Assay System (Promega). The bar graph represents normalized relative luciferase activity by vehicle-treated control. Compared with controls, 5-Aza significantly increased MYLK promoter activities (\* $P < 0.05$  vs. control,  $n = 4$  each). Demethylation by 5-Aza significantly increased MYLK promoter activities. The methylation span across 2.4-kb promoter, especially proximal promoter (0.3 kb) with CpG island. Mutated nmMYLK promoters containing SNP rs78755744 (–261G/A) were generated from wild-type 0.3 kb nmMYLK promoter in pGL3-basic by site-directed mutagenesis. Wild-type (–261G) or mutated (–261A) nmMYLK promoters were transfected to HPAEC and ECs exposed to 5-Aza for 24 h. The SNP rs78755744 (–261G) significantly attenuated nmMYLK promoter response to 5-Aza, compared with rs78755744 (–261A) (\* $P < 0.05$  vs. (–261A)/5-Aza, # $P < 0.05$  vs. –0.3k/5'-Aza,  $n = 4$  each).



**Figure 6. Increased nmMLCK protein expression in a preclinical murine ARDS model**

A well-established preclinical murine model of ARDS was utilized to assess nmMLCK protein expression. Mice were intratracheally injected with LPS 1 mg/kg ( $n=3$ ) or PBS (controls) ( $n=3$ ). After 18 h, mice were harvested, and lung tissue sections stained by Hematoxylin and Eosin with immunohistochemistry studies (IHC) performed with antibodies for nmMLCK proteins, which are specific for mouse, rat and human N-terminus of MYLK (sc-365352, Santa Cruz Biotechnology). (A,B) Control lungs from mice challenged with intratracheal PBS administration show normal H & E staining and histology (A) and weak IHC staining for nmMLCK (B). (C,D) H & E staining of lung tissues harvested from mice exposed to intratracheal LPS for 18 h, show significant acute lung inflammation and injury with interstitial and intra-alveolar neutrophil infiltration and fibrin deposition (C). IHC staining of LPS-exposed lung tissues also exhibited significant increases in nmMLCK immunoreactivity (D) including in lung ECs (arrows). (A,C) ( $\times 200$ ); (B,D) ( $\times 400$ ).

demonstrate nmMLCK as a critical lung cytoskeletal effector protein intimately involved in regulation of inflammatory processes observed in ARDS and VILI [7,9,13], including increased lung vascular permeability.

In the present study, we explored the underlying molecular mechanisms involved in regulation of *MYLK*, the gene coding nmMLCK, in the settings of ARDS- and VILI-related inflammatory processes. Through serial progressive 5' to 3' unidirectional deletions and promoter activity assays, we detected a distal negative regulatory promoter region (−2512 to −1512 bp), and a proximal enhancer promoter region (−1512 to −312 bp) that is highly conserved across vertebrate species, suggesting a common *MYLK* regulatory mechanism. We now demonstrate significant regulation of *MYLK* transcription activities by extrinsic and intrinsic inflammatory factors (LPS, TNF- $\alpha$ , mechanical stress, HIs). These data are consistent with our previous epigenetic 3'UTR-focused studies indicating early elevations in nmMYLK mRNA levels in ECs following that LPS, TNF- $\alpha$ , and 18% CS which reached maximum levels at 24 h [24]. We now complement these data with 5'UTR interrogation which revealed specific and distinct regions of the nmMYLK promoter critical to inflammatory factor regulation including two LPS-responsive and a single TNF $\alpha$ -responsive promoter region. While we did not identify the exact TFs involved in nmMYLK regulation, an important limitation of the present study, these LPS- and TNF $\alpha$ -responsive promoter regions contain *in silico* binding sequences for known

TFs involved in LPS and TNF transcriptional regulation in EC including NF $\kappa$ B [45–47], AP-1 [48,49], JAK-STAT, and JNK stress kinase pathways [50]. TNF- $\alpha$  also induced EC responses involving Sp-1 in human microvascular EC [51] similar to what we have previously reported with VEGF-induced nmMYLK up-regulation [25].

Given the essential contribution of ventilator-induced excessive mechanical stress to ARDS mortality [52], the identification of an MSIR critical for MYLK responses to 18% CS provides a mechanistic link between increased vascular permeability and VILI while corroborating additional genetic [25] and epigenetic mechanisms of MYLK regulation [24]. Our previous studies in EC identified significant contributions of the STAT family of TFs, STAT5a/b and STAT3, in regulating mechanical stress-induced transcriptional activity of two critical damage-associated molecular pattern proteins or DAMPs and key ARDS/VILI inflammatory therapeutic targets, NAMPT [4,29,53–55], and HMGB1 [56]. TF STAT response elements are also located in nmMYLK promoter –1812 to –1512 bp region by *in silico* analysis. Other MSIRs were also reported to match TF AP-1 or NF $\kappa$ B specific-binding sites on target genes in ECs. Cyclic strain induces endothelin-1 (ET-1) expression by increasing recruitment of AP-1 to specific ET-1 promoter region for AP-1 binding [57]. Continuous mechanical stretch induces IL-6 secretion from ECs, most likely through interaction of activated TF NF $\kappa$ B with IL6 gene [58].

As VILI from excessive mechanical stress is a key contributor to ARDS inflammatory burden and mortality, we confirmed the synergistic effects of combined LPS and 18% CS exposure on increasing nmMYLK promoter activities which remained sustained for up to 24 h.

Hypoxemia is a critical pathological hallmark of ARDS/ALI and via HIFs, HIF-1 $\alpha$  and HIF-2 $\alpha$ , hypoxia is a potent stimulus for amplification of ARDS inflammatory cascades [59–62]. Both HIF-1 $\alpha$  and HIF-2 $\alpha$  are multifunctional and have been implicated as contributing to ARDS severity. While HIF-1 $\alpha$  drives LPS-induced IL-1 $\beta$  [63], TNF, IL-12, and VEGF expression [64], HIF1 $\alpha$  has been postulated to reduce VILI via A2B receptor regulation [65,66]. We have shown HIF-2 $\alpha$ , and to a lesser extent HIF-1 $\alpha$ , are involved in transcriptional regulation of the ARDS/VILI DAMP, eNAMPT, [64,67,68]. Our current studies demonstrate that HIF-1 $\alpha$  and HIF-2 $\alpha$  accumulation (elicited by PHD2 inhibition) to also significantly increase nmMYLK promoter activities with HIF-1 $\alpha$  induction more rapid and HIF-2 $\alpha$  induction more delayed. HIF1 $\alpha$ /2 $\alpha$  may contribute to ARDS in part by regulation of nmMLCK expression while HIF1 $\alpha$  may potentially herald resolution of ARDS/ALI [69] via purinergic signaling pathways [70,71].

Our study has also addressed the involvement of two nmMYLK promoter SNPs associated with risk of developing ARDS [9] to alter TF binding to the nmMYLK promoter resulting in significantly increased nmMYLK promoter activity in response to inflammatory factors, including excess mechanical stress. Previous studies suggest aberrant DNA methylation of lung tissues may be involved in the pathophysiology of LPS-induced ALI/ARDS [72] and we have previously demonstrated excessive mechanical stress and LPS to reduce NAMPT promoter DNA methylation to increased gene transcription [31]. Our current studies with nmMYLK, another inflammatory target, indicates epigenetic regulation of the nmMYLK promoter via demethylation that significantly enhances promoter activities. A genetic variant that interrupts a CpG site within a nmMYLK CpG island (0.3 kb proximal promoter to TSS) significantly attenuates demethylation-mediated enhancement of MYLK promoter activity. These studies are consistent with our previous report linking epigenetic regulation of nmMYLK to ARDS risk [73].

In summary, we examined mechanisms of molecular, genetic and epigenetic regulation of nmMYLK gene under lung inflammatory conditions relative to ARDS/VILI pathobiology. These studies add to our previous preclinical studies utilizing genetically engineered nmMLCK<sup>-/-</sup> KO mice [13,22] and human genetic studies [7,9]. Increased levels of nmMLCK protein were detected in lung tissues and microvascular endothelium in a preclinical murine model of ARDS confirming the clinical relevance of the effects of extrinsic inflammatory factors (LPS), intrinsic inflammatory factors (TNF), and hypoxemia on enhancing nmMYLK promoter/transcription activities *in vitro*. Limitations of the present study include the absence of exact characterization of the specific inflammation-related TFs involved in nmMYLK regulation such NF $\kappa$ B, AP-1, STAT5, and HIF1 $\alpha$ /2 $\alpha$ . However, despite these limitations, our results, indicating strong up-regulation of nmMYLK promoter activity in ECs by the inflammatory stimuli LPS and TNF- $\alpha$  and by exposure to excess mechanical stress, further confirm nmMYLK and its coding protein, nmMLCK, as attractive therapeutic targets to significantly attenuate inflammation-induced vascular permeability and the severity of inflammatory lung injury including ARDS and VILI [9,13,19,74,75].

## Clinical perspectives

- The genetic and epigenetic factors that influence the regulation of the nmMYLK promoter encoding nmMLCK, an important cytoskeletal protein and contributor to the severity of asthma and the ARDS, are unknown.



- Extrinsic and intrinsic inflammatory factors, such as cytokines, excessive mechanical stress, hypoxia, and DNA demethylation were each found to significantly increase nmMYLK promoter activity with significant influence of ARDS-associated SNPs via altered TF binding.
- nmMLCK expression is increased by highly clinically relevant inflammatory factors thereby contributing to inflammatory disease severity and representing a potential therapeutic strategy.

## Data Availability

All supporting data are included within the main article and its supplementary files.

## Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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## CRedit Author Contribution

**Xiaoguang Sun:** Conceptualization, Resources, Data curation, Software, Formal analysis, Supervision, Validation, Investigation, Visualization, Methodology, Writing—original draft, Project administration, Writing—review and editing. **Belinda L. Sun:** Validation, Visualization, Writing—original draft, Writing—review and editing. **Saad Sammani:** Investigation. **Tadeo Bermudez:** Investigation. **Steven M. Dudek:** Writing—review and editing. **Sara M. Camp:** Writing—review and editing. **Joe G.N. Garcia:** Conceptualization, Funding acquisition, Writing—review and editing.

## Abbreviations

AA, African American; ALI, acute lung injury; ARDS, acute respiratory distress syndrome; ARE, antioxidant response element; CI, confidence interval; CS, cyclic stretch; EA, European American; EC, endothelial cell; EMSA, electrophoretic mobility shift assay; eNAMPT, extracellular nicotinamide phosphoribosyltransferase; FDA, The Food and Drug Administration; GCM1, glial cells missing homolog 1; HIF, hypoxia-inducible factor; ISX, intestine-specific homeobox; LPS, lipopolysaccharide; MSIR, mechanical stress-inducible region; nmMLCK, non-muscle isoform of myosin light chain kinase; nmMYLK, non-muscle MYLK; OR, odds ratio; RLU, relative light unit; SNP, single nucleotide polymorphism; TF, transcription factor; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; TSS, transcription start site; VEGF, vascular endothelial growth factor; VILI, ventilator-induced lung injury; 5-Aza, 5-aza-2'-deoxycytidine.

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