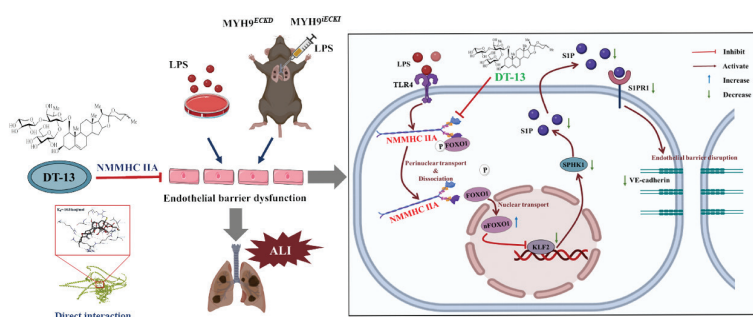


DT-13 ameliorates LPS-induced acute lung injury via targeting NMMHC IIA to modulate the FOXO1/KLF2/SPHK1 pathway

Graphical abstract



Highlights

- NMMHC IIA drives FOXO1 nuclear translocation to suppress KLF2/SPHK1 signaling and disrupt endothelial barrier function.
- DT-13 alleviates LPS-induced acute lung injury by directly targeting non-muscle myosin heavy chain IIA (NMMHC IIA).
- Pharmacologic inhibition of NMMHC IIA by DT-13 restores the FOXO1/KLF2/SPHK1 axis, reducing LPS-induced pulmonary vascular leakage and inflammation.

Authors

Jiazhi Zhang, Jiahui Tang, Huifen Ma, Ziqian Pan, Mengxue Xiao, Jianhao Zhou, Ling Zhang, Shuaishuai Gong, Fang Li, Boyang Yu, Yuanyuan Zhang and Junping Kou

Correspondence

boyangyu59@163.com (B. Yu);
yuanyuanzhang@cpu.edu.cn (Y. Zhang);
junpingkou@cpu.edu.cn (J. Kou)

In brief

This study reveals that NMMHC IIA disrupts endothelial barrier function in lung injury by promoting FOXO1 nuclear translocation to inhibit KLF2/SPHK1 signaling. The natural compound DT-13 directly targets NMMHC IIA to restore this axis, reducing vascular leakage and inflammation, thereby identifying a key pathogenic mechanism and a promising therapeutic strategy for acute lung injury.

Research Article

DT-13 ameliorates LPS-induced acute lung injury via targeting NMMHC IIA to modulate the FOXO1/KLF2/SPHK1 pathway

Jiazhi Zhang^{a,1}, Jiahui Tang^{a,1}, Huifen Ma^a, Ziqian Pan^a, Mengxue Xiao^a, Jianhao Zhou^a, Ling Zhang^a, Shuaishuai Gong^a, Fang Li^a, Boyang Yu^{a,*}, Yuanyuan Zhang^{a,*} and Junping Kou^{a,*}

^aState Key Laboratory of Natural Medicines, Jiangsu Key Laboratory of TCM Evaluation and Translational Research, Department of Pharmacology of Chinese Materia Medica, School of Traditional Chinese Pharmacy, China Pharmaceutical University, Nanjing 211198, China

¹Jiazhi Zhang and Jiahui Tang contributed equally to this work.

*Correspondence: boyangyu_59@163.com (B. Yu); yuanyuanzhang@cpu.edu.cn (Y. Zhang); junpingkou@cpu.edu.cn (J. Kou)

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ABSTRACT

The pathogenesis of acute lung injury (ALI) and the severe form of ALI, acute respiratory distress syndrome (ARDS), is incompletely understood. We aimed to determine the mechanism of action for non-muscle myosin heavy-chain IIA (NMMHC IIA) and the NMMHC IIA targeting compound in the context of lipopolysaccharide (LPS)-induced pulmonary endothelial barrier dysfunction associated with ALI. Endothelial-specific monoallelic knockout of NMMHC IIA alleviated ALI and reversed alterations in sphingosine-1-phosphate (S1P), a serum metabolite. Inhibition of NMMHC IIA upregulated SPHK1, a key S1P-synthesizing enzyme, and the SPHK1 transcriptional regulator, KLF2. NMMHC IIA directly interacted with FOXO1 in LPS-treated endothelial cells to promote FOXO1 nuclear translocation. Knockdown of MYH9 or FOXO1 restored barrier integrity by activating the KLF2/SPHK1 pathway. Endothelial NMMHC IIA knockdown promoted FOXO1 dephosphorylation and KLF2/SPHK1 activation *in vivo*, which increased serum S1P levels; NMMHC IIA overexpression exerted opposite effects. Furthermore, DT-13, a steroidal sapogenin derived from *Liriope muscari*, was confirmed to bind to NMMHC IIA via the cellular thermal shift assay (CETSA) and microscale thermophoresis (MST) assay. DT-13 attenuated LPS-induced endothelial barrier disruption by targeting NMMHC IIA and mediating the FOXO1/KLF2/SPHK1 axis. The findings herein elucidate a new mechanism underlying ALI pathogenesis and suggest promising therapeutic strategies.

Keywords: DT-13, non-muscle myosin IIA, acute lung injury, metabolomic, FOXO1/KLF2/SPHK1 pathway

1. INTRODUCTION

Acute lung injury (ALI) and the severe phase of ALI, acute respiratory distress syndrome (ARDS), are acute hypoxemic respiratory failure syndromes that are characterized by diffuse pulmonary infiltration, heightened capillary permeability, compromised gas exchange, and pulmonary inflammation. Despite ongoing randomized trials, effective therapeutic modalities for ALI/ARDS remain elusive with optimized mechanical ventilation and fluid therapy emerging as the primary interventions associated with improved clinical outcomes [1, 2]. Despite extensive research into the pathologic mechanisms underlying ALI, there are no effective drug treatments for ALI that significantly reduce mortality and improve patient quality of life with mortality rates still

reaching 30–40% [3–6]. Furthermore, co-morbidities in patients with ARDS remain a major contributor to the limited effectiveness of current treatments [7]. Thus, it is essential to further elucidate the pathophysiologic mechanisms underlying ALI and develop effective therapeutic strategies.

Disruption of the pulmonary endothelial barrier integrity results in increased vascular permeability, ensuing pulmonary edema, and substantial sequestration of inflammatory cells. Such derangements compromise the critical process of air-blood exchange, precipitating hypoxemia and respiratory failure, which ultimately culminates in ARDS [8, 9]. Therefore, protecting the functional integrity of the pulmonary vascular endothelial barrier is an important therapeutic strategy for the prevention and management of ALI.

Non-muscle myosin heavy chain IIA (NMMHC IIA), which is encoded by the *MYH9* gene, is a member of the myosin superfamily that regulates essential physiologic processes, such as cell migration, adhesion, vesicular transport, and signal transduction [10]. NMMHC IIA has an important role in various disease processes by regulating vascular endothelial function. In addition, NMMHC IIA affects endothelial cell secretion of hemophilic factors and modulates tissue factor expression, thereby impinging upon thrombotic processes [11]. Moreover, NMMHC IIA has emerged as a central mediator in ischemia-reperfusion-induced cerebral endothelial barrier disruption, modulating Hippo and PPAR γ signaling pathways [12]. The N-terminal and head functional regions of NMMHC IIA directly interact with toll-like receptor 4 (TLR4). Stimulation of lung endothelial cells with lipopolysaccharide (LPS) leads to dissociation of NMMHC IIA from TLR4, resulting in disruption of the lung endothelial barrier integrity and development of ALI [13]. Nevertheless, the precise molecular mechanisms underlying the resulting alterations in NMMHC IIA after dissociation from TLR4 require further elucidation.

DT-13, a steroidal saponin isolated from *Liriope muscari*, exhibits various pharmacologic effects, including anti-tumorigenic, immunomodulatory, anti-inflammatory, and endothelial barrier-protective activities [14, 15]. NMMHC IIA is a potential target of DT-13, through which NMMHC IIA mitigates tumor necrosis factor- α (TNF- α)-induced endothelial hyperpermeability by modulating NMMHC IIA and Src/PI3K/Akt-mediated signaling pathways [16]. Therefore, we speculated that DT-13 mitigates LPS-induced pulmonary vascular endothelial barrier dysfunction by modulating NMMHC IIA.

In the present study the NMMHC IIA regulatory mechanism in ALI after dissociation from TLR4 was investigated first and the therapeutic effects of DT-13 on LPS-induced pulmonary endothelial barrier dysfunction during ALI was subsequently evaluated.

2. METHODS AND MATERIALS

2.1 Reagents and antibodies

LPS (L2880) and dexamethasone [DEX] (D1756) were obtained from Sigma-Aldrich (St. Louis, MO, USA). 3,4,5-dimethylthiazolyl-2,5-diphenyltetrazolium bromide [MTT] (M8180) was purchased from Solarbio Science & Technology Co., Ltd. (Beijing, China). Anti-CD31 (AF3628) was purchased from R&D Systems (Minneapolis, MN, USA). Anti-MYH9 (11128-1-AP) was purchased from Proteintech (Chicago, IL, USA). Anti-SPHK1 (sc-365401), anti-FOXO1 (sc-374427), anti-S1PR1 (sc-48356), and anti-KLF2 (sc-166238) were obtained from Santa Cruz Biotechnology, Inc. (TX, USA). Anti-VE-cadherin (ab33168) and anti-albumin were provided by Abcam (Cambridge, MA, USA). Anti-phospho-FOXO1 family (9461) was from Cell Signaling Technology (Boston, MA, USA).

2.2 Extraction and isolation of DT-13

DT-13 was isolated, as previously described, and identified as 25(R, S)-ruscogenin-1-O- $[\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 2)] $[\beta$ -D-xylopyranosyl-(1 $\rightarrow$ 3)]- β -D-fucopyranoside by comparison of physical data (^1H NMR, ^{13}C NMR, MS) with published values. The purity of DT-13 was shown to be 98.5% using HPLC-ELSD assays, as previously reported [17].

2.3 Animal experiments

MYH9^{fl/+}, *Tek-Cre* and *MYH9^{+/+}*, *Tek-Cre*, NMMHC IIA-induced endothelial-specific overexpression (*MYH9^{IECKI}*), and control mice (18–22 g) were obtained from Nanjing Biomedical Research Institute of Nanjing University (Certificate number: SCXK Jiangsu 2018-0008) [18]. C57BL/6 male mice (18–22 g) were provided by the Experimental Animal Center of Yangzhou University (Certificate number: SCXK 2020-0002). The animals were fed at a standardized temperature of $22 \pm 2^\circ\text{C}$ and relative humidity of $60\% \pm 2\%$. The mice were randomly divided into different groups after 3 d of adjustable feeding. The mice in the ALI group were challenged with LPS ($5 \text{ mg}\cdot\text{kg}^{-1}$) by intratracheal instillation for 6 h after inhalation anesthesia with isoflurane [13], while sterile saline was used in the control group. DT-13 and DEX were administrated orally 1 h before LPS treatment. DEX, which is clinically used to treat ALI, serves as a positive control in this study [19]. All mice were euthanized after LPS induction and lung tissues were collected for the subsequent experiments. All animal experimental procedures were carried out according to the National Institutes of Health guidelines and the protocols were approved by the Animal Ethics Committee of China Pharmaceutical University (certificate no. 2021-10-022).

2.4 Histopathologic analysis

Lung tissues fixed in 4% paraformaldehyde were embedded in paraffin, cut into 4- μm sections, and stained with hematoxylin and eosin (H&E). Pathologic images were examined using an optical microscope (Nikon, Japan) and the extent of the injury was scored by pathologists. The histopathologic analysis was independently performed four times. There were six mice per group in each experiment.

2.5 Lung wet:dry weight ratio

Lung tissues were weighed immediately after removal as the wet weight, then placed into a 60°C oven for 48 h. The dried lung tissues were reweighed as the dry weight and the wet:dry weight ratios were calculated. The lung wet:dry weight ratio was independently detected two times. There were six mice per group in each experiment.

2.6 Lung Evans blue (EB)-albumin leakage

Mice were injected intravenously with EB-albumin ($50 \text{ mg}\cdot\text{kg}^{-1}$) via the tail vein 4 h after LPS exposure and the

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right ventricle was perfused 2 h later with PBS containing 5 mM EDTA-2Na to remove the intravascular dye from the lung. Lung tissues were collected, flash-frozen in liquid nitrogen, and homogenized in a 10-fold volume of formamide. The homogenate was incubated at 60°C for 18 h. The absorption of EB in the supernatant was measured at 620 nm after centrifugation at 5000 × g for 30 min and the EB content was measured with a standard curve. Lung EB-albumin leakage was measured two times. There were six mice per group in each experiment.

2.7 Bronchoalveolar lavage fluid (BALF) cell count

The BALF was centrifuged (1500 rpm) at 4°C for 10 min and the precipitate was resuspended in 500 × g of PBS. The number of white blood cells (WBC) and neutrophils (NEU) was quantified using a blood analyzer (ADVIA 2120; Siemens, Germany). The BALF cell count was performed in a single independent experiment. There were six mice per group in each experiment.

2.8 Immunofluorescence assays

The lung tissue frozen sections (10-μm thick) were fixed with 4% formaldehyde and permeabilized with 0.1% Triton X-100 (P0096; Beyotime Biotechnology, Shanghai, China), then blocked with 5% bovine serum albumin [BSA] (B265994, Aladdin, Shanghai, China) and probed with the following primary antibodies overnight at 4°C: CD31 (1:100 dilution, R&D Systems, Minneapolis, MN, USA); VE-cadherin (1:100 dilution; Abcam, Cambridge, MA, USA); and MYH9 (1:200 dilution; Proteintech, Rosemont, IL, USA). The next day the following corresponding fluorescence-conjugated secondary antibodies were used: Alexa Fluor 594-donkey anti-goat IgG (A11058; Thermo Fisher Scientific, Waltham, MA, USA); Alexa Fluor 488-goat anti-mouse IgG (A21202; Thermo Fisher Scientific); and Alexa Fluor 488-donkey anti-rabbit IgG (A21206, 1:500 dilution, Thermo Fisher Scientific). Fluorescent images were visualized using confocal laser scanning microscopy [CLSM] LSM700; Zeiss, Germany). Immunofluorescence assays were independently repeated three times. There were six mice per group in each repetition.

2.9 Cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from the Shanghai Institute of Cell Biology (Chinese Academy of Sciences, Shanghai, China). HUVECs were grown in RPMI-1640 medium (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum [FBS] (ScienCell, CA, USA), 100 U·mL⁻¹ of penicillin, 100 μg·mL⁻¹ of streptomycin, and 2.0 g·L⁻¹ of sodium bicarbonate. Cells were maintained at 37°C with 95% humidity and 5% CO₂.

2.10 Trans-endothelial electrical resistance (TEER) and EB-albumin leakage assays

HUVECs were seeded on Transwell inserts (0.4 μm pore, 6.5 mm diameter; Millipore, USA) for 7 d. The TEER of the monolayer was also measured daily with a Millicell-ERS voltohmmeter (Millipore). Resistance values of multiple Transwell inserts of the experimental group were measured sequentially; the mean resistance value was expressed in the common unit (Ω·cm²) after subtraction of the value of a blank cell-free filter. The TEER of the monolayers was recorded when a stable resistance reading was achieved with triplicate measurements that were obtained for each Transwell. DT-13 (0.01–1 μM) was added to the upper chamber for 1 h and 5 μg·mL⁻¹ of LPS was added for 6 h [13]. The inner chamber fluid was changed to 200 μL of EB-albumin solution as the tracer and the outer chamber fluid was changed to 600 μL of 4% BSA solution and incubated in an incubator for 1 h. The extracellular fluid was collected, 200 μL was added per well of a 96-well plate, the absorbance at 620 nm was measured, and the content of EB-albumin leakage was calculated according to the EB curve. TEER measurements were independently repeated five times and EB-albumin leakage assays were independently repeated three times. There were six cell cultures per group in each repetition. Each cell culture was derived from an independent passage or thawing event.

2.11 siRNA transfection

MYH9 siRNA and control non-specific siRNA were purchased from Biomics Biotechnologies Co. (Nantong, China). Transfection of cells with the siRNAs was performed using HiFectPlus Transfection Reagent (Siyifan, China) according to the manufacturer's instructions. Briefly, the endothelial cells were plated on a 60-mm dish at a concentration of 5 × 10⁵ cells/dish. After reaching 50% confluence, the cells were transfected with the siRNA for 48 h and treated with DT-13 (1 μM) for 1 h followed by a 6-h treatment with LPS (5 μg·mL⁻¹) at 37°C. The MYH9 siRNA sequences were as follows: forward, 5'-GAGGCAAUGAUCACUGACUdTdT-3'; and reverse, 5'-AGUCAGUGAUCUUGCCUCdTdT-3'. The KLF2 siRNA sequences were as follows: forward, 5'-GGUAUU UAUUGGACCCAGAdTdT-3'; and reverse, 5'-UCUGGGUCCAAUAAUACCDdTdT-3'. The FOXO1 siRNA sequences were as follows: 5'-GAGGUAUGAGUCAGU AUAA dTdT-3'; and reverse, 5'-UUAUACUGACUCAUACC UC dTdT-3'.

2.12 Western blot analysis

The total protein extracted from HUVECs or lung tissues was quantified using a BCA protein assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Equal amounts of total protein (40 μg) in each group were separated by SDS-PAGE gels and transferred to PVDF membranes, then the membranes were blocked in 5% BSA for 2 h and incubated overnight at 4°C with

the following primary antibodies: VE-cadherin (1:1,000 dilution, Abcam); SPHK1, FOXO1, S1PR1, and KLF2 (1:500 dilution; Santa Cruz Biotechnology, Inc.); and MYH9 (1:8,000 dilution; Proteintech). The membranes were incubated with HRP anti-conjugated secondary antibodies for 1.5 h at room temperature after washing three times with TBST. The bands were detected using an ECL kit (Vazyme Biotechnology, Nanjing, China) and analyzed with Image Lab™ software. Western blot analysis was performed for four independent *in vivo* experiments. Six mice per group were used in each experiment. Western blot analysis was independently repeated six times for *in vitro* experiments. Six cell cultures per group were used for each repetition. Each cell culture was derived from an independent passage or thawing event.

2.13 Cellular thermal shift assay (CETSA)

HUVECs cultured in 10-cm petri dishes were collected and cleaved by repeated freezing and thawing in liquid nitrogen and the supernatant-containing protein was collected by centrifugation. The separated supernatant was treated with DMSO or DT-13 (5 $\mu\text{L}\cdot\text{mL}^{-1}$) at room temperature for 30 min. Each sample was divided into 10 parts after the reaction and heated for 3 min in the PCR cycle at the following temperatures: 40°C; 43°C; 46°C; 49°C; 52°C; 55°C; 58°C; 61°C; 64°C; and 67°C. Then, the sample was centrifuged at $20,000 \times g$ for 20 min and the same amount of supernatant was collected into a 1.5-mL EP tube. The sample was heated at 95°C for 10 min after adding an appropriate amount of loading buffer. The western blot method was used for detection after cooling. CETSA was performed in a single independent experiment.

2.14 Isothermal dose–response Fingerprint (ITDRF)_{CETSA}

Different concentrations of DT-13 (0, 1.25, 2.5, 5, 10, 20, and 40 μM) were added to isolated HUVEC cell lysates and incubated at room temperature for 30 min. Then, the sample was heated in a PCR circulator at 58°C for 3 min and centrifuged at $20,000 \times g$ for 20 min to remove the precipitation. The sample was heated at 95°C for 10 min after adding an appropriate amount of loading buffer. Western blot was used for detection and analysis after cooling. ITDRF_{CETSA} was performed in a single independent experiment.

2.15 Microscale thermophoresis (MST) assay

NMMHC IIA was divided into four major domains according to the MYH9 cDNA sequence analysis to further confirm the specific domain of NMMHC IIA upon which DT-13 acts: N terminal (aa. 29-69); head domain (aa. 83-764); IQ motif (aa. 775-835); and tail domain (aa. 842-1921). HEK293T cells were transfected with expression plasmids for GFP-tagged NMMHC IIA domains and the lysate was collected as an assay buffer for MST

experiments. A Nano Temper Monolith Instrument (NT.115; Nano Temper Technologies, München, Germany) was used for measuring thermophoresis. Samples were loaded into Monolith NT.115Pico MST standard-treated capillaries (Nano Temper Technologies) and measured using a Monolith NT.115Pico and MO.Control software (Nano-Temper Technologies) at 37°C.

2.16 Molecular docking

The chemical structure of DT-13 ingredients can be downloaded from the ChemSpider website (<http://www.chemspider.com/>). The X-ray 3D crystal structure of the NMMHC IIA protein was obtained from the AlphaFold2 database (<https://www.alphafold.ebi.ac.uk/>). The crystal structure of the protein and monomer results of GXNI components were imported into the Autodock software client to analyze and display the interaction relationship between DT-13 and NMMHC IIA and display the 3D interaction diagram.

2.17 Statistical analysis

All data are presented as the mean $\pm$ standard deviation (SD) and analyzed with GraphPad Prism software (version 8.0). Statistical significance was calculated with one-way analysis of variance (ANOVA) followed by Dunnett's test. Differences were considered significant at a *P* value < 0.05.

3. RESULTS

3.1 NMMHC IIA mediates LPS-induced reduction of endothelial S1P release via the KLF2/SPHK1 pathway

An LPS-induced ALI model was established in endothelial-specific NMMHC IIA monoallelic knockout (*MYH9^{ECKD}*) mice to determine the downstream molecular mechanism by which NMMHC IIA contributes to LPS-induced pulmonary endothelial barrier dysfunction with an NMMHC IIA knockdown efficiency of approximately 50% (Figure S1A). The alterations in serum metabolites of LPS-induced ALI mice was first analyzed after endothelial-specific allelic knockout of NMMHC IIA through untargeted metabolomics analysis. The hierarchical clustering heatmaps provided a more intuitive visualization of the metabolite changes. Glyceraldehyde, L-leucine, sphingosine-1-phosphate, and dihomolinolenic acid levels in the serum were significantly altered in LPS-induced ALI. However, endothelial-specific allelic knockout of NMMHC IIA reversed these changes (Figure 1A, B). Numerous metabolites were altered by the endothelial-specific allelic knockout of NMMHC IIA. Gene Ontology enrichment analysis of the related regulatory enzymes was performed using Metascape (<https://www.metascape.org/>) and revealed that the sphingolipid metabolic process, glycerolipid metabolic process, and metabolism of ketone bodies were regulated by endothelial-specific allelic knockout of NMMHC IIA (Figure 1C, D).

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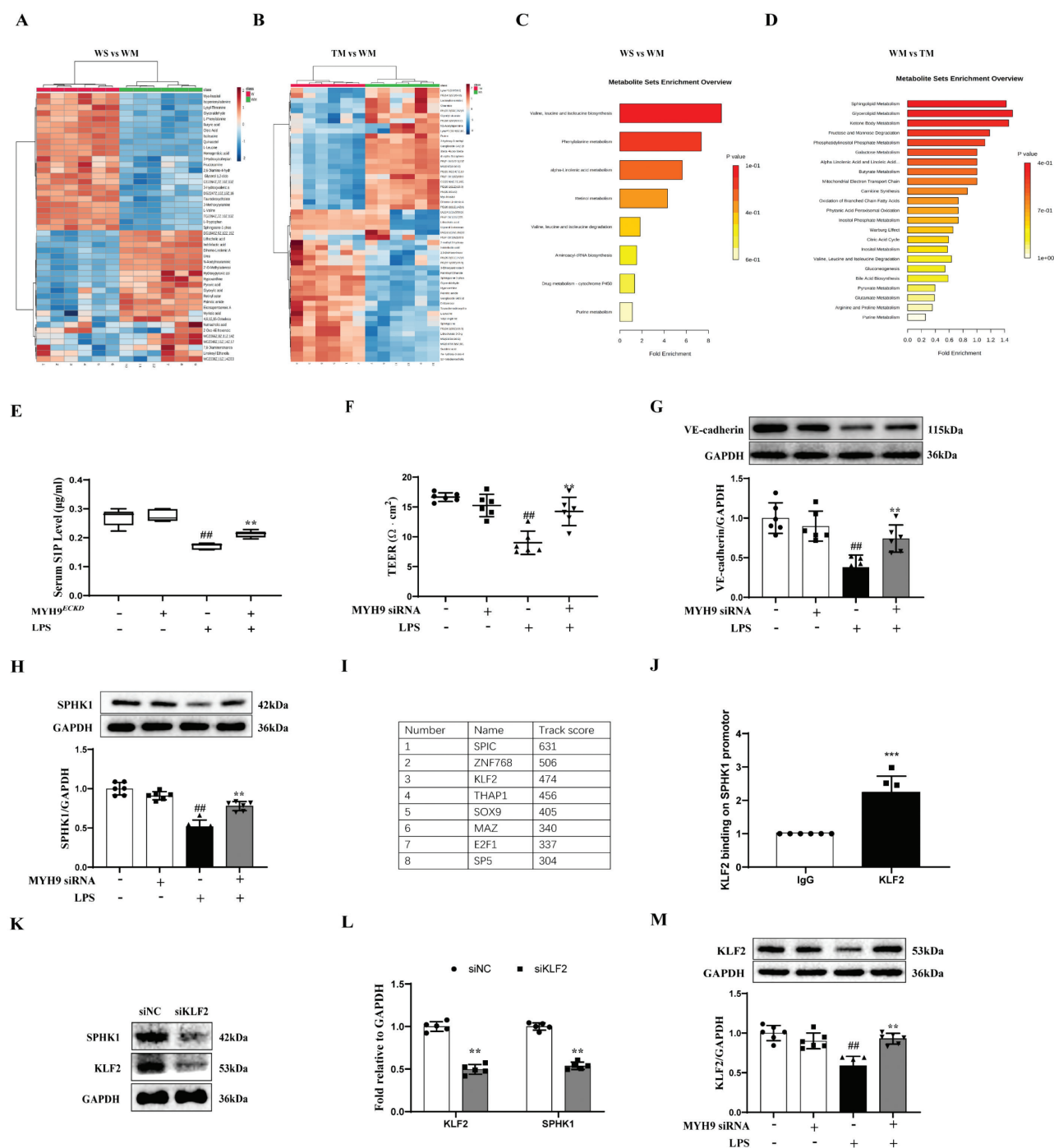


Figure 1 | NMMHC IIA knockdown ameliorated LPS-induced reduction of S1P release from endothelial cells and inhibition of the KLF2/SPHK1 signaling pathway.

Heatmap of the differential endogenous metabolites among the wild-sham (WS), wild-model (WM), knockdown-sham (TS), and knockdown-model (TM) groups based on the HPLC-Q-TOF/MS system for serum analysis. **A**. Heatmap of the differential endogenous metabolites between the WS and WM groups in serum. **B**. Heatmap of the differential endogenous metabolites between the WM and TM groups in serum. **C**. The overview of functional enrichment with the differential metabolites between the WS and WM groups in serum. **D**. The overview of functional enrichment with differential metabolites between WS and WM groups in serum. **E**. Serum S1P in mice were measured by HPLC-QQQ/MS (n = 6). **F**. Trans-endothelial electrical resistance (TEER) assays of HUVECs was performed (n = 6). **G**. The expression of VE-cadherin in HUVECs was measured by western blot (n = 6). **H**. The expression of SPHK1 in HUVECs was measured by western blot (n = 6). **I**. Transcription factor prediction of SPHK1 was scored by the JASPAR database. **J**. The binding ability of KLF2 to the SPHK1 promoter was determined by ChIP (n = 6).

K-M. The expression of SPHK1 and KLF2 in HUVECs was evaluated by western blot and the quantification as shown (n = 6). Data are shown as the mean ± SD. ##P < 0.01 compared to the sham group; **P < 0.01, ***P < 0.001 compared to the model group.

Therefore, S1P exhibited the strongest association with the pulmonary endothelial barrier. The mechanism by which NMMHC IIA regulates serum S1P levels was subsequently investigated.

The results of targeted metabolomics for S1P by high-performance liquid chromatography coupled with triple quadrupole tandem mass spectrometry (HPLC-QQQ-MS/MS) showed that the serum S1P levels were significantly reduced in LPS-induced ALI and markedly restored following endothelial-specific monoallelic knockout of NMMHC IIA (**Figure 1E**). The underlying mechanism by which NMMHC IIA regulates S1P levels *in vitro* was subsequently investigated. MYH9 siRNA-mediated knockdown of NMMHC IIA in HUVECs achieved approximately 50% reduction in protein levels (**Figure S1B**). This intervention significantly ameliorated LPS-induced endothelial barrier dysfunction, as evidenced by improved trans-endothelial resistance and preservation of vascular endothelial cadherin (VE-cadherin) in HUVECs (**Figure 1F, G**).

Notably, sphingosine kinase 1 (SPHK1), the key enzyme responsible for converting sphingosine (SPH) into S1P [20], was markedly downregulated following LPS stimulation but the expression was restored upon MYH9 knockdown (**Figure 1H**), suggesting that NMMHC IIA modulates S1P biosynthesis via SPHK1 in this model.

The JASPAR database was used to predict the possible transcription factors that regulate SPHK1 and reveal the molecular mechanism by which NMMHC IIA regulates SPHK1. The analysis identified Spi-C transcription factor (SPIC), zinc finger protein 678 (ZNF678), and Krüppel-like factor 2 (KLF2) as the transcription factors with the highest affinity scores (**Figure 1I**). Among the transcription factors, KLF2 had the strongest connection to endothelial function. KLF2 has a key role in a variety of diseases, such as atherosclerosis, coronavirus disease 2019 (COVID-19), liver sinusoidal dysfunction [21–23]. However, the effects on SPHK1 remain unknown.

Chromatin immunoprecipitation (ChIP) assays were performed to determine whether KLF2 regulates SPHK1 expression. KLF2 directly binds to the promoter region of SPHK1 (**Figure 1J**). In addition, we used siRNA to knock down KLF2 expression in HUVECs. KLF2 knockdown markedly reduced SPHK1 expression under physiologic conditions (**Figure 1K, L**).

These results indicated that KLF2 regulates the expression of SPHK1. Next, whether NMMHC IIA regulated KLF2 expression was determined. Western blotting showed that the LPS-induced decrease in KLF2 expression in HUVECs was reversed by MYH9 knockdown (**Figure 1M**).

3.2 NMMHC IIA inhibited the KLF2/SPHK1 pathway by promoting FOXO1 nuclear transport in LPS-induced impairment of barrier function in HUVECs

Although reported in different cellular contexts (i.e., NMMHC IIA–FOXO1 interaction promoting FOXO1 nuclear translocation in nasopharyngeal carcinoma and FOXO1-mediated transcriptional repression of KLF2 in stressed endothelial cells [22, 24–26]), these findings suggested a potential NMMHC IIA–FOXO1–KLF2 axis in endothelial inflammation.

To determine whether NMMHC IIA regulates the KLF2/SPHK1 pathway by modulating FOXO1 in LPS-induced HUVECs, FOXO1 siRNA was designed to confirm the effect on the endothelial barrier and KLF2 expression. siRNA-mediated knockdown of FOXO1 significantly ameliorated the LPS-induced reduction in trans-endothelial resistance, EB-albumin leakage, and VE-cadherin degradation in HUVECs (**Figure 2A–C**). Western blot analysis further showed that FOXO1 knockdown reversed the LPS-induced suppression of KLF2 and SPHK1 expression (**Figure 2D–F**).

Whether NMMHC IIA contributes to this regulatory cascade was next determined by directly engaging with FOXO1 in endothelial cells. To this end, the dynamic localization of NMMHC IIA and FOXO1 in HUVECs was determined at various time points (0, 1, 2, 4, and 6 h) after LPS stimulation using immunofluorescence co-localization. Indeed, co-localization of NMMHC IIA and cytoplasmic FOXO1 was observed within the first 1 h of LPS exposure. Over time, both proteins progressively migrated toward the perinuclear region. The cytoplasmic FOXO1 dissociated from NMMHC IIA and entered the nucleus after 2 h of LPS stimulation (**Figure 2G**). To confirm that this co-localization reflects a direct molecular interaction, co-immunoprecipitation (Co-IP) assays were performed. These results confirmed that FOXO1 specifically binds to NMMHC IIA (**Figure S2**). Consistent with this model, western blotting revealed that siRNA-mediated knockdown of MYH9 attenuated the LPS-induced increased phosphorylation of FOXO1 and restored nuclear expression in HUVECs (**Figure 2H, I**).

3.3 NMMHC IIA overexpression exacerbated LPS-induced endothelial barrier dysfunction via the FOXO1/KLF2/SPHK1 pathway in HUVECs

An MYH9 plasmid was constructed to promote the overexpression of NMMHC IIA to perform reverse verification of the above-mentioned signaling pathway. First, NMMHC IIA overexpression was confirmed to promote the LPS-induced reduction in trans-endothelial resistance, EB-albumin leakage, and degradation of

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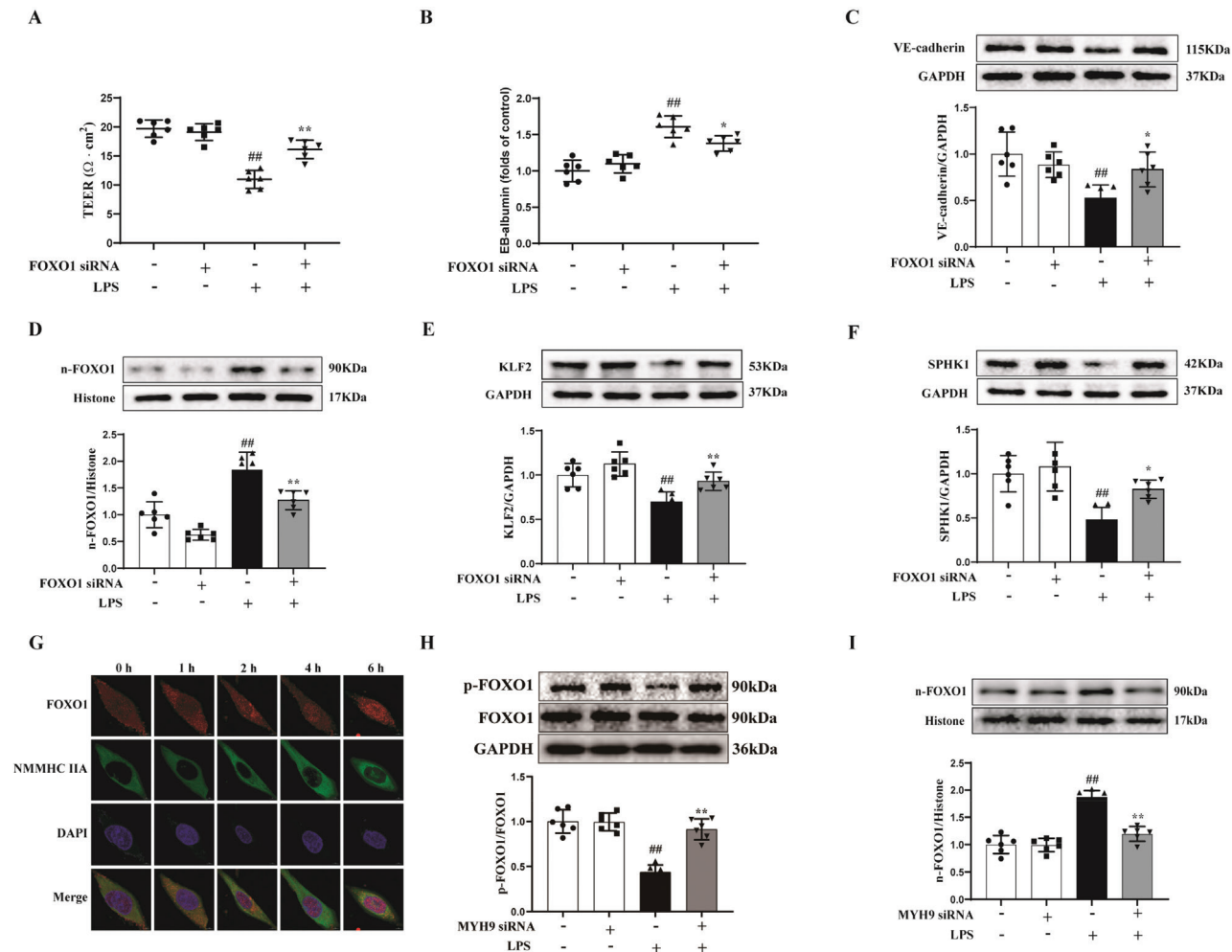


Figure 2 | NMMHC IIA regulated the KLF2/SPHK1 signaling pathway by influencing the nuclear translocation of FOXO1.

A-B. TEER and EBA leakage assays were used to determine the effect of FOXO1 knockdown on LPS-induced hyperpermeability ($n = 6$). **C-F.** The expression of VE-cadherin, n-FOXO1, KLF2, and SPHK1 in HUVECs was measured by western blot ($n = 6$). **G.** Immunofluorescence staining for FOXO1 (red), NMMHC IIA (green), and nuclei (blue) was performed on HUVECs. Scale bar: 5 μm . The co-location of NMMHC IIA and FOXO1 in HUVECs was observed at 0, 1, 2, 4, and 6 h after LPS stimulation. **H-I.** The FOXO1 phosphorylation level and n-FOXO1 expression in HUVECs was measured by western blot 6 h after LPS stimulation ($n = 6$). Data are shown as the mean $\pm$ SD. $\#\#P < 0.01$ compared to the control group; $**P < 0.01$ compared to the model group.

VE-cadherin in HUVECs (Figure S3A-C). Moreover, western blotting revealed that NMMHC IIA overexpression further promoted LPS-induced intranuclear expression of FOXO1 and inhibited KLF2 and SPHK1 expression in HUVECs (Figure S3D-F).

3.4 NMMHC IIA affected pulmonary endothelial barrier dysfunction in LPS-induced ALI by regulating the FOXO1/KLF2/SPHK1 pathway in mice

MYH9^{ECKD} and *MYH9^{iECK1}* mice were then used to confirm whether NMMHC IIA mediates LPS-induced pulmonary endothelial barrier dysfunction by regulating the FOXO1/KLF2/SPHK1 pathway *in vivo*. The

knockdown efficiency in *MYH9^{ECKD}* mice was approximately 50% and the overexpression efficiency in *MYH9^{iECK1}* mice was approximately 40% (Figure S1).

H&E staining of lung tissue sections showed collapse of alveolar structures, marked inflammatory cell infiltration, and thickening of the lung bronchial epithelium after tracheal drip injection of LPS. Endothelial-specific monoallelic knockout of NMMHC IIA ameliorated LPS-induced pathologic lung damage, whereas overexpression aggravated lung injury (Figures 3A-C and 4A). In contrast, EB-albumin leakage and western blotting revealed that NMMHC IIA overexpression exacerbated LPS-induced albumin leakage and VE-cadherin degradation (Figure 4B-D). Moreover, NMMHC IIA endothelial-specific monoallelic knockout ameliorated the

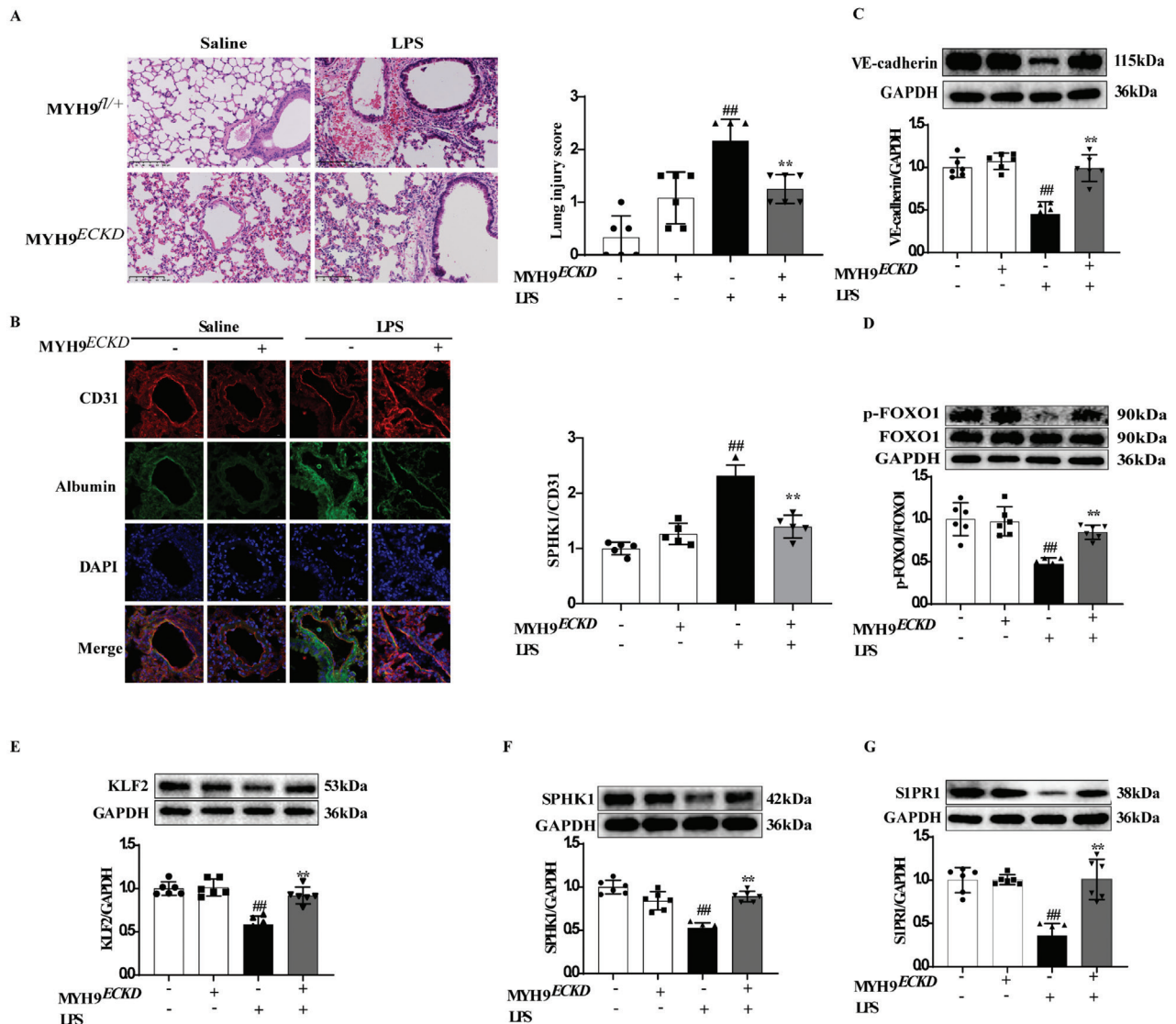


Figure 3 | Endothelium-specific NMMHC IIA monoallelic deletion ameliorated LPS-induced lung injury.

A. Histopathologic change in the lung were evaluated via H&E staining and pathologic injury scores were calculated (n = 6). **B.** Immunofluorescence for extravascular albumin (green) and CD31 (red) in lung frozen sections; nuclei were stained with DAPI (blue [n = 4]). Scale bar = 50 μ m. **C-G.** The FOXO1 phosphorylation level and VE-cadherin, KLF2, SPHK1, and S1PR1 expression in lung tissues of mice was determined by western blot (n = 6). Data are shown as the mean $\pm$ SD. ##P < 0.01 compared to the sham group; **P < 0.01 compared to the model group.

LPS-induced reduction in FOXO1 phosphorylation levels and KLF2, SPHK1, and S1PR1 expression in lung tissues, whereas overexpression had the opposite effects (Figures 3D-G and 4E-H).

3.5 DT-13 directly bound to NMMHC IIA N-terminal and head domains

A previous study indicated that DT-13 inhibits TNF- α -induced inflammation via NMMHC IIA [14]. In the current study whether DT-13 enhanced the thermal stability of NMMHC IIA was evaluated using a thermal shift assay. HUVEC lysates were heated to denature and precipitate

NMMHC IIA and plot the CETSA curve; western blotting was used to detect the remaining soluble NMMHC IIA. CETSA curves revealed that DT-13 significantly improved the thermal stability of NMMHC IIA compared to the control group (Figure 5A). Moreover, ITDRF_{CETSA} confirmed that the thermal stabilization effect of DT-13 on NMMHC IIA was dose-dependent (Figure 5B). In addition, molecular docking analysis demonstrated the interaction between DT-13 and NMMHC IIA (Figure 5H).

In addition, MST analysis was performed to assess the interaction between DT-13 and full-length NMMHC IIA *in vitro*, yielding an equilibrium dissociation constant (K_d) value of 141.52 nM. To further identify the specific

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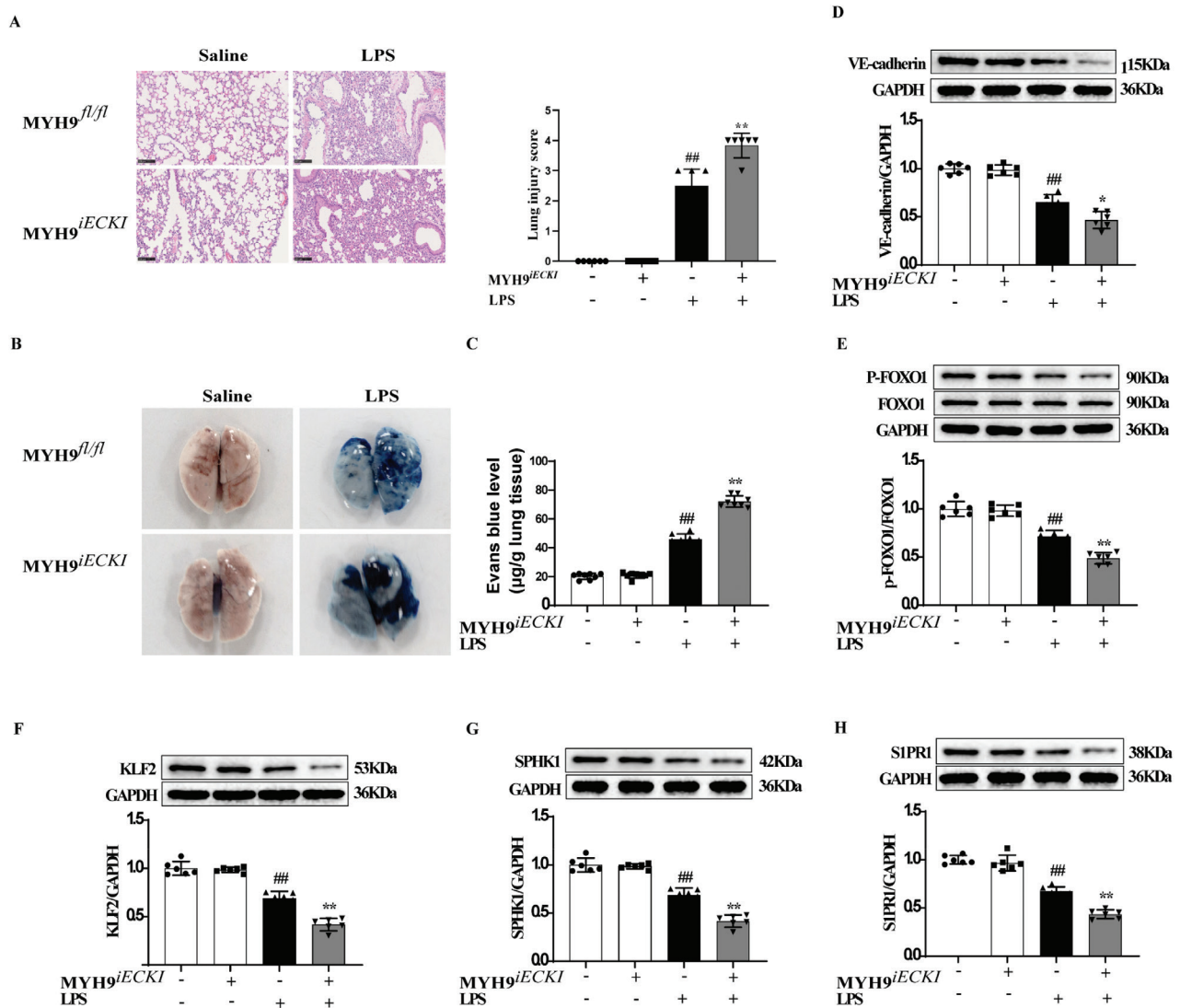


Figure 4 | Endothelium-specific NMMHC IIA overexpression aggravated LPS-induced lung injury.

A. Histopathologic changes in the lung were evaluated via H&E staining and pathologic injury scores were calculated ($n = 6$). **B, C.** Mice were injected intravenously with EBA 4 h after LPS administration. The dye was extracted from lung tissues and quantified after 2 h ($n = 6$). **D-H.** The FOXO1 phosphorylation level and VE-cadherin, KLF2, SPHK1, and S1PR1 expression in lung tissue of mice was determined by western blot ($n = 6$). Data are shown as the mean $\pm$ SD. $##P < 0.01$ compared to the sham group; $*P < 0.05$ and $**P < 0.01$ compared to the model group.

domain of NMMHC IIA targeted by DT-13, the functional regions were mapped, dividing NMMHC IIA into the following four domains: the N-terminal (amino acids 29-69); head (amino acids 83-764); IQ motif (amino acids 775-835); and tail (amino acids 842-1921). MST assays revealed that DT-13 binds to the N-terminal and head domains of NMMHC IIA (Figure 5C-G). These results confirmed that DT-13 directly binds to NMMHC IIA.

3.6 DT-13 inhibited LPS-induced activation of the NMMHC IIA/FOXO1/KLF2/SPHK1 pathway *in vivo*

An ALI mouse model was prepared by intratracheal instillation of LPS to determine the effects of DT-13 *in*

vivo. DT-13 (0.3, 1, and 3 mg·kg⁻¹) was administered orally for 1 h before intratracheal LPS (5 mg·kg⁻¹) injection and lung tissues and BALF were collected 6 h after the LPS challenge. H&E staining revealed that lung injury was significantly attenuated in DT-13-treated mice compared to the model group. Lung injury scores and lung coefficient measurements indicated that DT-13 protected against pulmonary lesions in a dose-dependent manner (Figure 6A). Likewise, the lung index, W:D ratio and EB-albumin leakage assays showed that DT-13 significantly reduced pulmonary edema and albumin leakage in a dose-dependent manner (Figure 6B, C). Furthermore, DT-13 significantly decreased the number of WBC and NEUs in a dose-dependent

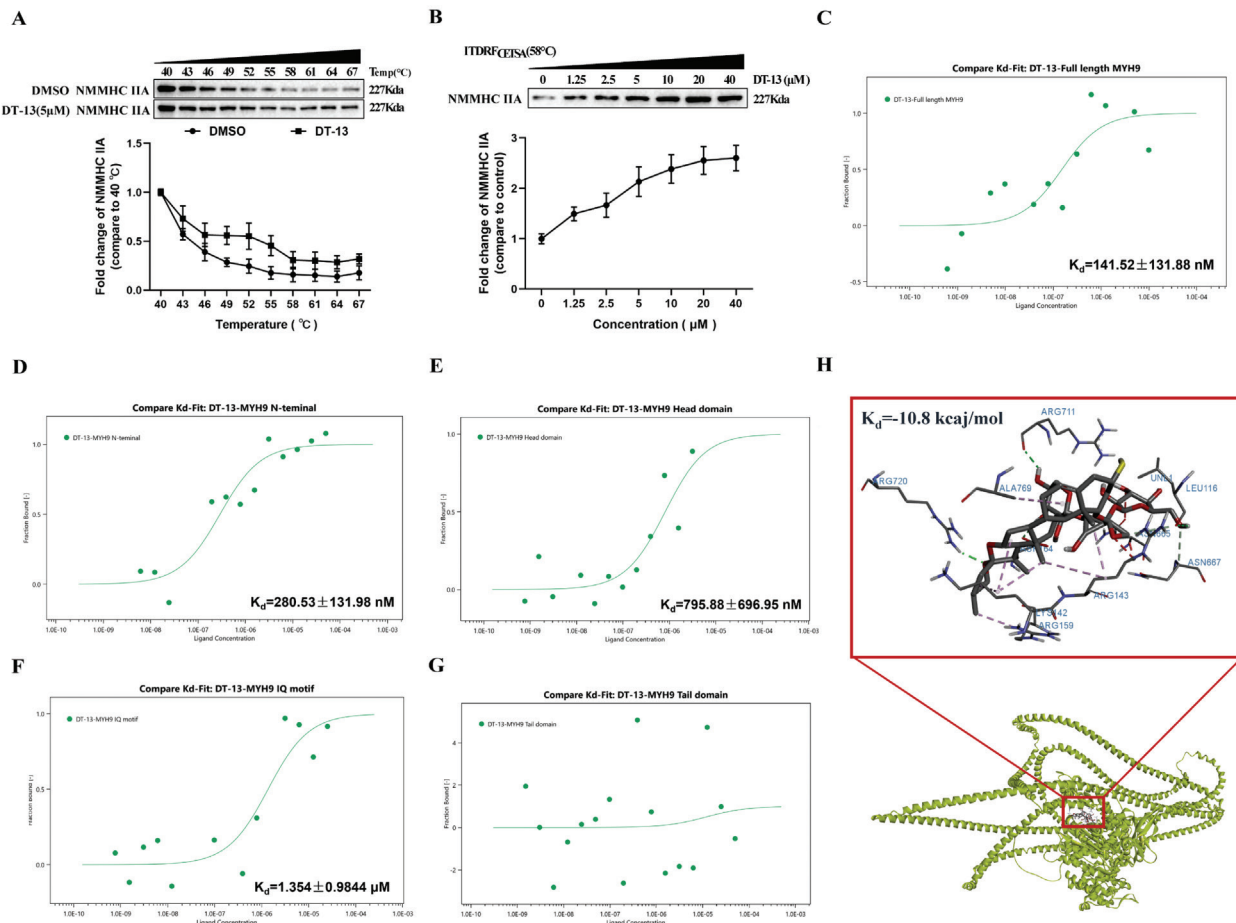


Figure 5 | DT-13 directly bound to the N-terminal and head domain of NMMHC IIA.

A, B. CETSA and ITDRF_{CETSA} were used to evaluate binding between DT-13 and NMMHC IIA at the thermodynamic level. **C.** A microscale thermophoresis (MST) assay was performed to detect the binding kinetics of DT-13 on full-length NMMHC IIA. **D-G.** NMMHC IIA is divided into four major domains according to the analysis of Myh9 cDNA sequence: N-terminal (aa. 29-69); head domain (aa. 83-764); IQ motif (aa. 775-835); and tail domain (aa. 842-1921). HEK293T cells were transfected with expression plasmids for GFP-tagged NMMHC IIA domains and the lysate was collected as an assay buffer for MST assays. **D, E.** DT-13 bound with N-terminal and head domains of NMMHC IIA, **F, G.** There was no binding between DT-13 and the IQ motif and tail domains. **H.** 3D model description of the active binding site, where DT-13 interacts with NMMHC IIA.

manner (Figure 6D, E). The molecular mechanism by which DT-13 ameliorated LPS-induced ALI was investigated. Immunofluorescence results revealed that DT-13 (3 mg·kg⁻¹) significantly reduced LPS-induced NMMHC IIA expression and restored the pFOXO1:FOXO1 ratio, as well as KLF2 and SPHK1 expression in the pulmonary vascular endothelium (Figures 6F-I and S4). Collectively, these findings showed that DT-13 significantly improved LPS-induced ALI by regulating the NMMHC IIA/FOXO1/KLF2/SPHK1 pathway.

3.7 DT-13 inhibited LPS-induced activation of the NMMHC IIA/FOXO1/KLF2/SPHK1 pathway *in vitro*

The molecular mechanism by which DT-13 ameliorated LPS-induced endothelial barrier disruption was verified. TEER and EB-albumin leakage results demonstrated that DT-13 (0.01, 0.1, and 1 μM) improved the LPS-induced

endothelial barrier disruption in a concentration-dependent manner (Figure S5A, B). Western blot and immunofluorescence results revealed that DT-13 (1 μM) significantly ameliorated LPS-induced upregulation of NMMHC IIA and nFOXO1, as well as downregulation of pFOXO1/FOXO1, KLF2, SPHK1, and VE-cadherin in HUVECs (Figure S5C-H). These findings were further supported by multiplexed error-robust fluorescence *in situ* hybridization (MELC) results, which were consistent with previous observations (Figure S6).

3.8 NMMHC IIA knockdown abolished the protective effect of DT-13 against LPS-induced lung injury

Subsequent experiments using *MYH9^{EC}* mice were performed to further demonstrate the critical role of NMMHC IIA in DT-13-mediated protection against

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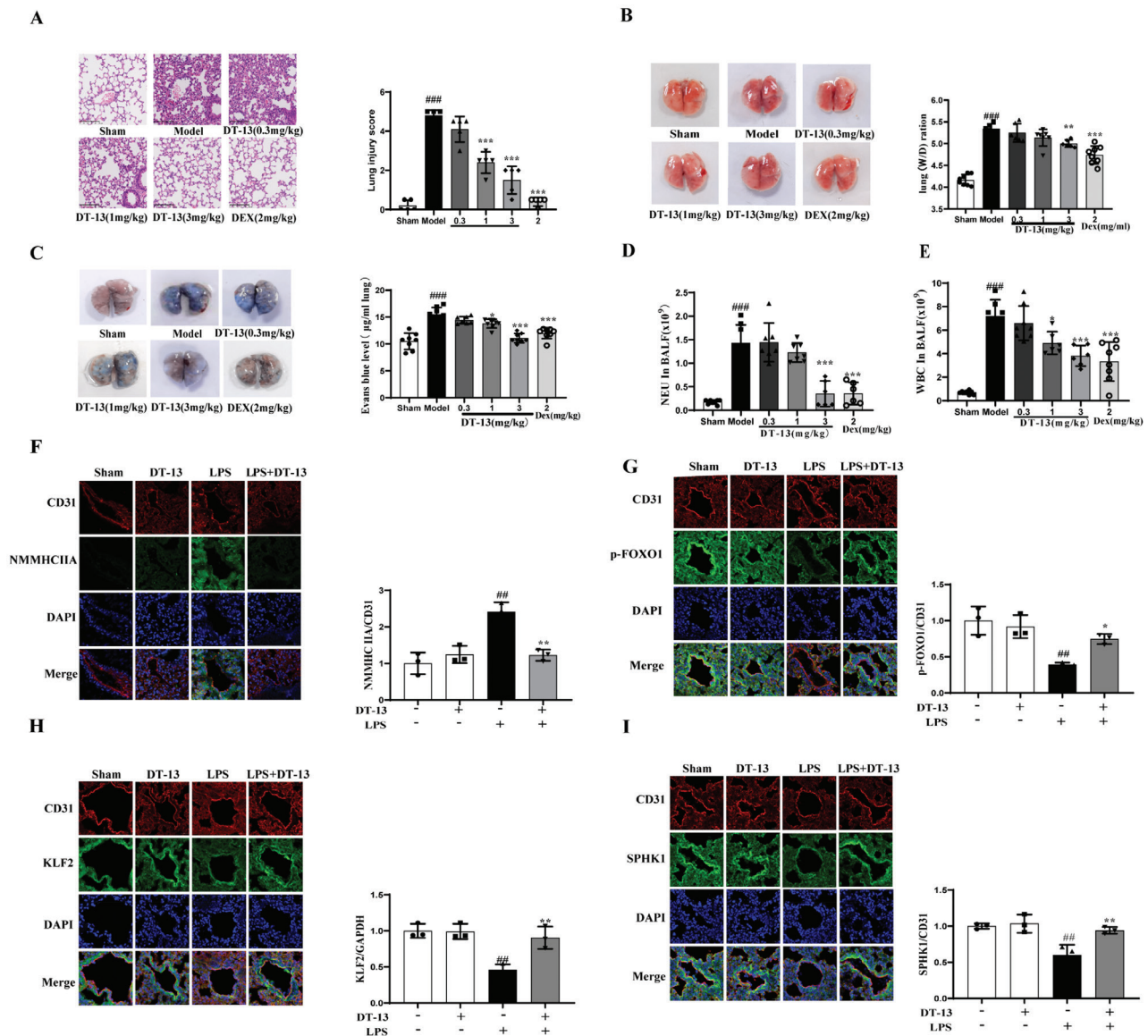


Figure 6 | DT-13 ameliorated LPS-induced pulmonary pathologic damage and lung vascular hyperpermeability in mice.

The mice in the ALI group were challenged with LPS (5 mg·kg⁻¹) by intratracheal instillation for 6 h after inhalation anesthesia with isoflurane and DT-13 and DEX were administered orally 1 h before LPS treatment. **A**. Histopathologic changes in the lung tissue were measured by H&E staining (scale bar = 100 μ m) and lung pathologic injury scores were calculated (n = 5). **B**. The gross morphology of the lung in mice was recorded and the lung wet:dry ratio was measured (n = 6). **C**. Lung gross morphology of mice was recorded and the extravasation of EB-albumin dye into lung parenchyma was determined 2 h after EB-albumin injection (n = 6). **D**, **E**. Mice were sacrificed and lungs were lavaged with PBS after exposure to LPS for 6 h. The number of white blood cells (WBC) and neutrophils (NEU) in bronchoalveolar lavage fluid (BALF) was determined (n = 6). **F-I**. Immunofluorescence staining for NMMHC IIA, p-FOXO1, KLF2, SPHK1 (green), and CD31 (red) was performed on frozen lung sections and the nuclei were stained with DAPI (blue). Scale bar = 20 μ m. The fluorescence intensity of NMMHC IIA, p-FOXO1, KLF2, and SPHK1 in lung endothelium was quantified by Image J (n = 3). Data are shown as the mean $\pm$ SD. $^{##}P < 0.01$, $^{###}P < 0.001$ compared to the sham group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to the model group.

LPS-induced lung endothelial barrier injury. H&E staining and W:D ratio analysis showed that endothelial-specific monoallelic knockout of NMMHC IIA abolished the protective effects of DT-13 on LPS-induced lung pathologic injury and edema (Figure 7A, B). Similarly, immunofluorescence and western blotting revealed

that DT-13 failed to reduce LPS-induced albumin leakage and VE-cadherin degradation in lung vasculature of *MYH9^{ECKD}* mice (Figure 7C, D). Consistently, DT-13 also lost inhibitory effects on LPS-induced reductions in pFOXO1/FOXO1, KLF2, SPHK1, and S1PR1 expression in lung tissues from *MYH9^{ECKD}* mice (Figure 7E-I).

3.9 NMMHC IIA knockdown abolished the protective effect of DT-13 on LPS-induced barrier dysfunction *in vitro*

Finally, the role of NMMHC IIA knockdown was validated in DT-13-mediated protection against LPS-induced endothelial barrier injury *in vitro*. TEER and western blotting demonstrated that NMMHC IIA knockdown eliminated the protective effects of DT-13 on the LPS-induced reduction in trans-endothelial resistance and VE-cadherin degradation in HUVECs (Figure S7A, B). Moreover, NMMHC IIA knockdown prevented DT-13 from ameliorating LPS-induced increases in NMMHC IIA and nFOXO1 expression, as well as reductions in pFOXO1/FOXO1, KLF2, SPHK1, and S1PR1 levels (Figure S7C-H).

4. DISCUSSION

Increasing evidence indicates that pulmonary endothelial cells have a critical regulatory role in the ALI inflammatory response [27]. Moreover, disruption of the pulmonary vascular endothelial barrier is recognized as a key pathologic feature of ALI and preserving endothelial barrier integrity is considered a promising therapeutic strategy [28-30]. The present study demonstrated that inhibition of NMMHC IIA protected the pulmonary endothelial barrier in LPS-induced ALI by regulating the serum S1P levels via the FOXO1/KLF2/SPHK1 pathway during ALI progression and confirmed that DT-13 could attenuate LPS-induced pulmonary endothelial barrier dysfunction by targeting NMMHC IIA to inhibit the FOXO1/KLF2/SPHK1 pathway.

Numerous diseases are characterized by alterations in the expression or levels of various genes, proteins, and metabolites, which offer novel targets for therapeutic intervention. In the current study an attempt was made to identify metabolites in serum affected by endothelial-specific monoallelic knockout of NMMHC IIA and elucidate the therapeutic mechanisms through analysis of high-throughput metabolomics data and multistep validations. Metabolomic analysis revealed that serum levels of glyceraldehyde, L-leucine, sphingosine-1-phosphate, and dihomo-linolenic acid were significantly altered in LPS-induced ALI and these changes were reversed by endothelial-specific monoallelic knockout of NMMHC IIA. In addition, the sphingolipid metabolic pathway was highly correlated with the level of NMMHC IIA expression in LPS-induced ALI (Figure 1). S1P is a simple, biologically active sphingolipid metabolite that has an essential role in endothelial barrier maintenance, anti-inflammatory response, antioxidant activity, and angiogenesis by acting on the receptor S1PR1-3 of endothelial cells to activate a series of signaling cascade responses compared to other metabolites [31]. Moreover, extensive literature suggests that S1P in blood could act as a biomarker for vascular diseases (such as vasculopathy in Alzheimer's disease, vasculitis, and sepsis-related endothelial

barrier damage) and is involved in the regulation of endothelial barrier function [32, 33]. Notably, clinical studies have demonstrated that pneumonia is a major predisposing factor for ALI/ARDS and blood S1P levels can act as a prognostic indicator. Specifically, a negative correlation has been observed between pneumonia severity and serum S1P concentrations with more severe pneumonia cases exhibiting lower S1P levels [34, 35]. The current study delved into the potential involvement of NMMHC IIA in LPS-induced pulmonary endothelial barrier dysfunction, elucidating the potential role in mediating the decrease in blood S1P levels (Figure 1C).

The enzyme, SPHK1, primarily catalyzes the intracellular synthesis of S1P [36]. Studies have revealed that downregulation of SPHK1 impedes the restoration of endothelial barrier function, while overexpression accelerates barrier recovery [37]. The results herein showed that inhibition of NMMHC IIA reversed the LPS-induced reduction in SPHK1 expression in pulmonary endothelial cells (Figure 1H). An integrative analysis of the SPHK1 promoter region was performed and several candidate transcription factors were identified, including SPIC, ZNF678, and KLF2, to explore the transcriptional regulation of SPHK1 (Figure 1I). Among the transcription factors, KLF2 is a well-established guardian of endothelial homeostasis and a critical regulator in ALI [37-39], while the roles of SPIC and ZNF678 in vascular biology remain largely uncharacterized. Given the strong functional link between KLF2 and barrier integrity, KLF2 was the key downstream effector in this context. ChIP assays confirmed that KLF2 directly bound to the SPHK1 promoter, establishing KLF2 as a positive transcriptional regulator of SPHK1. Importantly, inhibition of NMMHC IIA restored KLF2 expression that was suppressed by LPS in lung endothelial cells (Figure 1J-M).

These findings prompted an investigation involving the upstream mechanism responsible for LPS-induced KLF2 suppression, and by extension, how NMMHC IIA exerts a protective effect. As shown previously, high glucose stimulation of HUVECs induces FOXO1 dephosphorylation and subsequent translocation into the nucleus, leading to transcriptional activation. Activated FOXO1 directly binds to multiple conserved sites within the promoter region of the *KLF2* gene, thereby repressing transcriptional activity [40]. Notably, the findings herein demonstrated that this inhibitory regulation extends beyond metabolic stress to inflammatory conditions; specifically, in LPS-challenged pulmonary endothelial cells, FOXO1 suppressed KLF2 expression. This model was further supported by loss-of-function experiments. siRNA-mediated knockdown of FOXO1 ameliorated LPS-induced endothelial barrier disruption and restored the expression of KLF2 and SPHK1 (Figure 2D-F).

Intriguingly, emerging evidence suggested that FOXO1 activity might be modulated by direct protein-protein interactions. FOXO1 directly interacts with NMMHC IIA in nasopharyngeal carcinoma cells.

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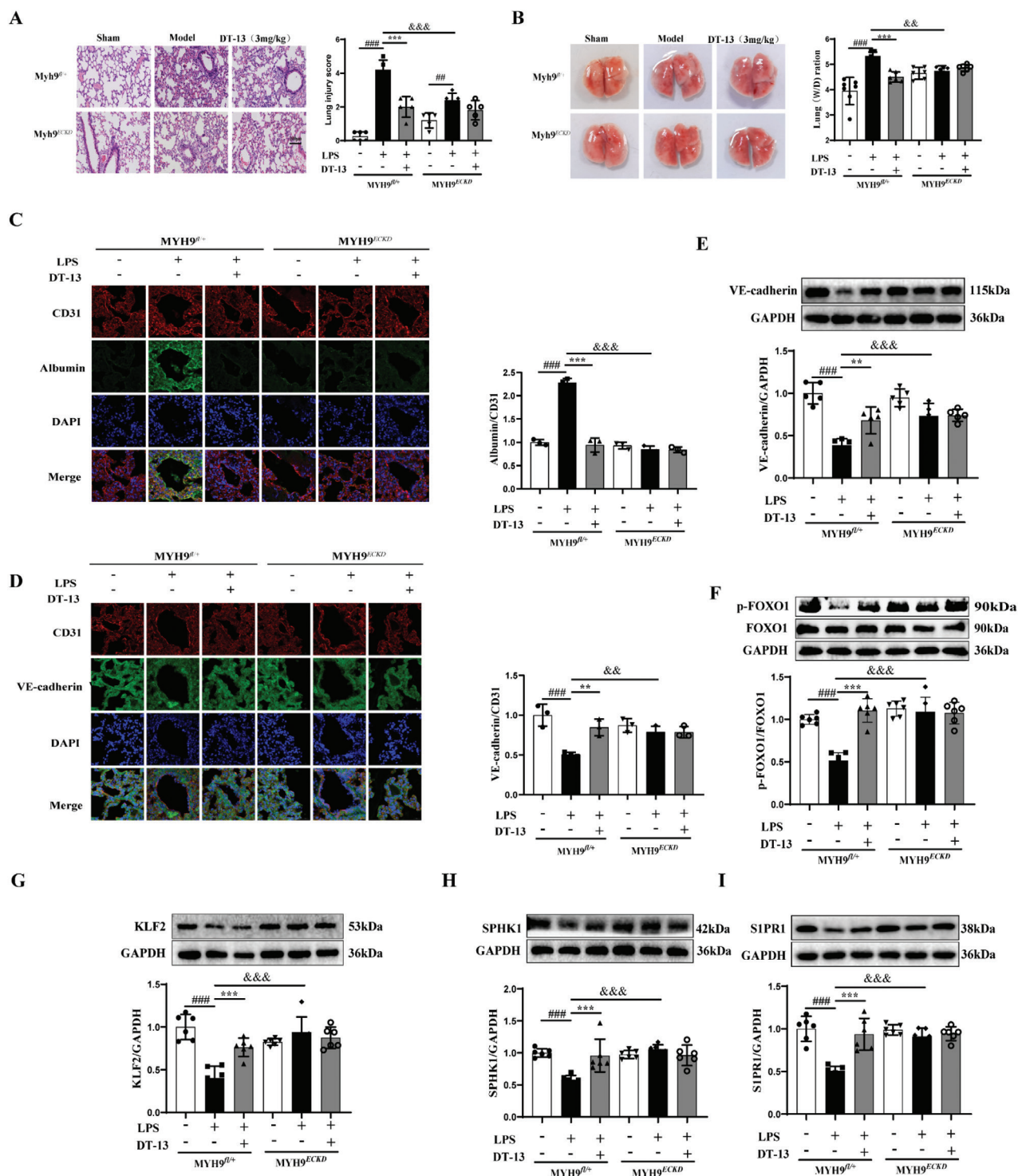


Figure 7 | Endothelium-specific NMMHC IIA monoallelic deletion eliminated the protective effect of DT-13 against LPS-induced injury of the pulmonary vascular endothelial barrier in mice.

A. Lung histopathologic changes in mice were measured by H&E staining (scale bar = 100 μ m) and pathologic injury scores were calculated (n = 5). **B.** The lung morphology of mice was recorded and the lung wet:dry ratio was determined (n = 6). **C, D.** Immunofluorescence staining for albumin, VE-cadherin (green), and CD31 (red) was performed on frozen lung sections and the nuclei were stained with DAPI (blue). Scale bar = 20 μ m. The fluorescence intensity of albumin and VE-cadherin in lung endothelium was quantified by Image J (n = 3). **E.** VE-cadherin expression in HUVECs was measured by western blot (n = 5). **F-I.** S1PR1, KLF2, and SPHK1 expression and the p-FOXO1:FOXO1 ratio in HUVECs was

measured by western blot (n = 6). Data are shown as the mean $\pm$ SD. ####P < 0.001 compared to the sham group; **P < 0.01, ***P < 0.001 compared to the model group; &&P < 0.01, &&&P < 0.001 compared to the WT model group.

Co-immunoprecipitation (Co-IP) and GST pull-down assays confirmed that FOXO1 specifically binds to NMMHC IIA, indicating the formation of a stable protein complex. Furthermore, immunofluorescence co-localization analysis revealed that FOXO1 and NMMHC IIA predominantly co-localize in the cytoplasm. Collectively, these findings demonstrated that NMMHC IIA serves as a functional binding partner of FOXO1 and may modulate stability, subcellular localization, or transcriptional activity through direct physical interaction [41]. Given this physical and functional link between NMMHC IIA and FOXO1, the dynamic subcellular localization and interaction of NMMHC IIA and FOXO1 were examined following LPS stimulation using immunofluorescence co-localization at multiple time points (0, 1, 2, 4, and 6 h). In agreement with prior reports [13] and the validation herein (Figure S2), NMMHC IIA rapidly dissociated from TLR4 after LPS challenge. This release allowed NMMHC IIA to associate with FOXO1 and translocate as a complex to the perinuclear region. During this process, NMMHC IIA appeared to promote dephosphorylation of FOXO1, which facilitated the dissociation of FOXO1 from the NMMHC IIA-FOXO1 complex and enabled subsequent nuclear import. FOXO1 accumulated robustly in the nucleus 2 h after LPS stimulation, where FOXO1 engaged in the transcriptional regulation of downstream targets, including KLF2 and SPHK1 (Figure 2G-I). FOXO1 dissociation from NMMHC IIA and subsequent nuclear import might involve multiple non-exclusive mechanisms that need further confirmation. NMMHC IIA-FOXO1 dissociation might arise from JNK-mediated FOXO1 phosphorylation [42], RhoA/ROCK- or MLCK-driven cytoskeletal remodeling [43], or dephosphorylation-enhanced nuclear import of FOXO1 via NLS in LPS-stimulated pulmonary endothelial cells [44]. Conversely, overexpression of NMMHC IIA via MYH9 plasmid enhanced FOXO1 nuclear translocation, further suppressed KLF2/SPHK1 signaling, and worsened endothelial hyperpermeability (Figure S3). Endothelial-specific monoallelic NMMHC IIA knockout (*MYH9^{ECKD}*) and induced endothelial-specific overexpression (*MYH9^{IECKI}*) mice (~50% knockdown, ~40% overexpression, Figure S1A) were used to validate these findings *in vivo*. The efficiency of knockdown or overexpression has been reported in a previous study [18]. *MYH9^{ECKD}* mice exhibited protected barrier function against LPS-induced ALI, whereas *MYH9^{IECKI}* mice had worse injuries (Figures 3A-C and 4A-D). Mechanistically, *MYH9^{ECKD}* promoted FOXO1 dephosphorylation and KLF2/SPHK1 activation, while *MYH9^{IECKI}* showed the opposite effects (Figures 3D-G and 4E-H). These results clarified how NMMHC IIA regulates S1P release and endothelial barrier integrity, providing insights for therapeutic development.

Liriope muscari, as a substitute for *Ophiopogon japonicus*, has been used to treat lung diseases for a long time in China. DT-13 is the main active ingredient of this herb. Previous studies have shown that DT-13 improves TNF- α -induced vascular endothelial cell hyperpermeability by modulating signaling pathways through NMMHC IIA and Src/PI3K/Akt signaling pathways [16]. Although the original study proposed a potential interaction between DT-13 and NMMHC IIA based solely on *in silico* molecular docking simulations [16], experimental validation was lacking. To address this gap, CETSA and ITDRF_{CETSA} were used, which demonstrated robust and reproducible binding between DT-13 and NMMHC IIA in cells. Further characterization using MST revealed that DT-13 specifically targets functional domains of NMMHC IIA, including the N-terminal region, head domain, and IQ motif (Figure 5), suggesting a mechanism for the regulatory effects. Given that Ruscogenin, the sapogenin of DT-13, could inhibit LPS-induced dissociation of NMMHC IIA from TLR4 to improve the pulmonary endothelial barrier [13], DT-13 was investigated and the same inhibitory effect was confirmed (Figures 6A-E and S8) and further demonstrated that DT-13 exerted a protective effect on LPS-induced ALI by FOXO1/KLF2/SPHK1 pathway (Figures 6F-I and S5). To establish the functional relevance of this interaction, NMMHC IIA knockdown ameliorated LPS-induced pulmonary endothelial barrier dysfunction using MYH9 siRNA *in vitro* and *MYH9^{ECKD}* mice *in vivo*, while simultaneously abolishing the protective effects of DT-13 and reversing regulation of the FOXO1/KLF2/SPHK1 pathway. These results demonstrated that DT-13 exerts endothelial barrier protection primarily by inhibiting NMMHC IIA signaling (Figures 7 and S6), providing new mechanistic insights for ALI treatment.

In conclusion, disruption of pulmonary endothelial barrier homeostasis, leading to protein-rich edema, is a hallmark of ALI/ARDS. The current study identified NMMHC IIA as a key mediator of LPS-induced injury via regulation of the FOXO1/KLF2/SPHK1 pathway and demonstrated that DT-13 (a direct NMMHC IIA inhibitor) protects endothelial barrier function by targeting this pathway (Figure 8). Notably, because S1P is a known endothelial barrier regulator, the serum levels of which correlate with pneumonia severity, the ability to modulate S1P-related pathways may broaden the therapeutic applications of DT-13 to other vascular disorders. The study limitations include unverified NMMHC IIA/FOXO1 intracellular motility, partial pathway validation, and the pending assessment of DT-13 druggability. Nevertheless, these findings established DT-13 as a promising candidate for ALI treatment, while elucidating the role of NMMHC IIA in endothelial pathophysiology.

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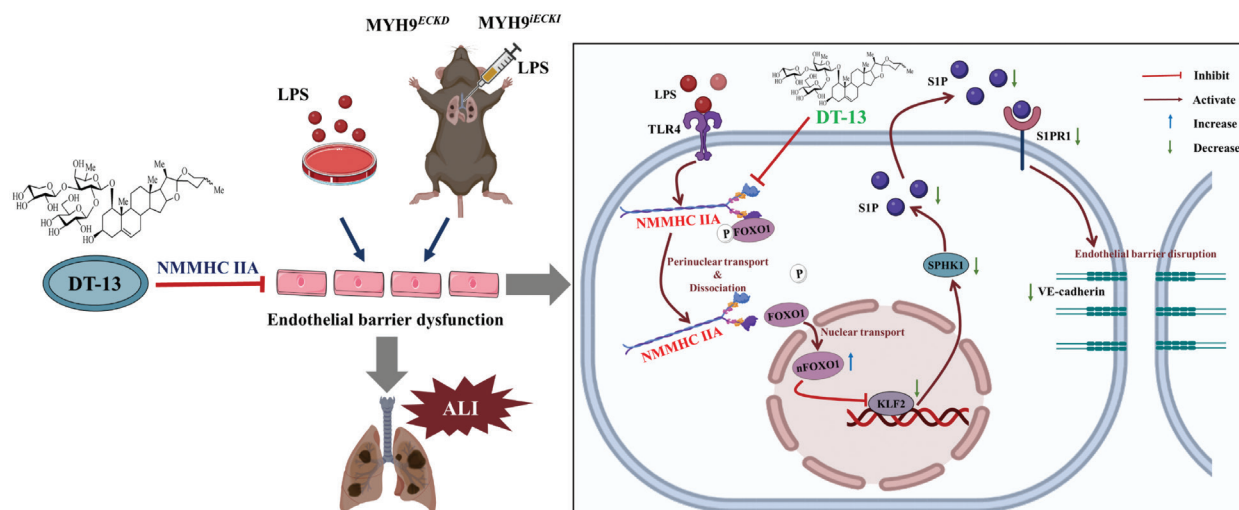


Figure 8 | Schematic diagram showing the mechanism by which DT-13 ameliorated LPS-induced acute lung injury by targeting NMMHC IIA.

LPS stimulation induced the dissociation of NMMHC IIA from TLR4 in lung endothelial cells, then promoted the interaction between NMMHC IIA and FOXO1, leading to suppression of FOXO1 phosphorylation and enhanced trafficking of the NMMHC IIA–FOXO1 complex to the perinuclear region. Subsequent dissociation of the complex enabled FOXO1 nuclear translocation, where FOXO1 repressed KLF2 transcription. This downregulation of KLF2 led to reduced SPHK1 expression and diminished S1P production, ultimately compromising endothelial barrier integrity. DT-13 ameliorated LPS-induced acute lung injury by preserving barrier function through targeting NMMHC IIA to modulate the FOXO1/KLF2/SPHK1 signaling pathway. This schematic diagram was created by Biorender.

ABBREVIATIONS

ALI, acute lung injury; ARDS, acute respiratory distress syndrome; BALF, bronchoalveolar lavage fluid; ChIP, chromatin immunoprecipitation; Co-IP, co-immunoprecipitation; DAPI, 4', 6-diamidino-2-phenylindole; DEX, dexamethasone; EB, Evans blue; FOXO1, Forkhead box O1; H&E, hematoxylin-eosin; HUVECs, human umbilical vein endothelial cells; KLF2, Krüppel-like factor 2; LPS, lipopolysaccharide; MLECs, murine lung endothelial cells; MTT, methyl thiazolyl tetrazolium; NEU, neutrophil; NMMHC IIA, non-muscle myosin heavy chain IIA; S1P, sphingosine 1-phosphate; S1PR1, S1P receptor 1; SPHK1, sphingosine kinase 1; TEER, trans-endothelial electrical resistance; TNFR2, tumor necrosis factor receptor 2; TNF- α , tumor necrosis factor alpha; WBC, white blood cell.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

All animal experimental procedures were carried out according to the National Institutes of Health guidelines, and the protocols were approved by the Animal Ethics Committee of China Pharmaceutical University (certificate no. 2021-10-022).

AUTHOR CONTRIBUTIONS

Junping Kou and Yuanyuan Zhang contributed to the literature search and study design. Jiahui Tang, Jiazhi Zhang, Ziqian Pan,

Huifen Ma, Mengxue Xiao, Jianhao Zhou, and Ling Zhang performed the experiments. Jiahui Tang, Jiazhi Zhang, and Ziqian Pan contributed to data collection and analysis. Jiahui Tang and Jiazhi Zhang participated in the drafting of the article. Junping Kou, Yuanyuan Zhang, Shuaishuai Gong, Fa Li, and Boyang Yu revised the manuscript.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

SUPPLEMENTARY MATERIAL

Supplementary data for this article can be found online at: https://amm-journal.org/wp-content/uploads/2026/03/amm20250077_Suppl.pdf.

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