Protective effects of lipid mediators, obtained from docosahexaenoic acid via soybean lipoxygenase, on lipopolysaccharide-induced acute lung injury through the NF-κB and Nrf2/HO-1 signaling pathways

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Received February 26, 2025; Accepted May 30, 2025

DOI: 10.3892/mmr.2025.13598

Abstract. Acute lung injury (ALI), marked by acute and chronic inflammation, causes damage to alveolar epithelial and capillary endothelial cells. The present study investigated lipid mediators (LM) effects on lipopolysaccharide (LPS)-induced RAW264.7 cells and ALI mice. LM, comprising 17S-monohydroxy docosahexaenoic acid (DHA), resolvin D5 and protectin DX (in a 3:47:50 ratio), were derived from DHA via soybean lipoxygenase and demonstrated anti-inflammatory properties. *In vitro* experiments revealed that LM decreased nitric oxide (NO) and prostaglandin E2 (PGE2) levels caused by LPS via downregulating inducible nitric oxide synthase and cyclooxygenase-2. Additionally, LM inhibited the inflammation by suppressing NF-κB signaling. The results also indicated that LM reduced oxidative stress by lowering reactive oxygen species and malondialdehyde (MDA) levels while enhancing glutathione (GSH) content and superoxide dismutase (SOD) activities, probably through activation of nuclear factor erythroid 2-related factor 2 (Nrf2)/heme oxygenase-1 (HO-1) signaling pathway. Moreover, the benefits of LM on inflammation and oxidative stress were reversed when pretreated with ML385, an Nrf2 inhibitor. *In vivo* studies revealed that LM reduced the lung wet/dry ratio, increased GSH, catalase and SOD activities, along with lowered myeloperoxidase and MDA levels. In addition, LM reduced inflammatory cytokine levels in serum and bronchoalveolar lavage fluid. Mechanistically, LM inhibited NF-κB signaling and activated Nrf2/HO-1 signaling pathways.

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Key words: acute lung injury, oxidative stress, inflammation, nuclear factor erythroid 2-related factor 2/heme oxygenase-1, NF-κB

Introduction

Acute lung injury (ALI) is a pulmonary inflammation syndrome marked by enhanced alveolar-capillary barrier permeability and alveolar epithelium damage, potentially progressing to severe respiratory distress syndrome (1,2). Research indicates that both inflammation and oxidative stress exert critical roles in ALI pathogenesis (3,4), making these processes attractive therapeutic targets (5-7).

Lipopolysaccharide (LPS) triggers the recruitment of inflammatory cells, especially neutrophils, to lung tissue, driving inflammation and oxidative stress (8-11). Thus, suppressing both processes offer a significant therapeutic potential for ALI.

In a resting state, NF-κB subunits are sequestered and inactivated through their association with inhibitor of κB (IκB). Following LPS exposure, IκB becomes phosphorylated and is subsequently degraded at a fast rate, allowing NF-κB to move into the nucleus. This nuclear activation of NF-κB promotes the upregulation and release of inflammatory mediators (12,13). In ALI, excessive neutrophils and macrophages generate reactive oxygen species (ROS), which aggravate both inflammation and oxidative stress (14,15). This process results in elevated levels of malondialdehyde (MDA), reduced glutathione (GSH) levels and decreased activity of superoxide dismutase (SOD) and catalase (CAT), ultimately impairing the antioxidant defense system and contributing to lung tissue damage (16). Moreover, nitric oxide (NO) and prostaglandin E2 (PGE2) contribute to the progression of inflammation and oxidative stress (17). Oxidative stress further amplifies inflammation, perpetuating the development of ALI (18). Nuclear factor erythroid 2-related factor 2 (Nrf2) is crucial in mitigating diseases such as ALI and asthma (19). Upon

activation by inducers, Nrf2 dissociates from the combination, translocating to the nucleus, thus, triggering the expression of antioxidant genes, thereby reducing oxidative stress (20,21). Therefore, suppressing NF-κB activation while enhancing Nrf2 signaling may alleviate ALI.

Inflammation is a key contributor to the pathogenesis of numerous chronic diseases, including liver disorders, cardiovascular conditions and cancer. Natural compounds with anti-inflammatory properties have gathered increasing attention due to their potential for therapeutic development with minimal side effects. Lipid mediators (LM), specifically 17S-monohydroxy docosahexaenoic acid (DHA), resolvin D5 and protectin DX (3:47:50 ratio), derived from DHA via soybean lipoxygenase were depicted in Fig. S1. Our previous studies showed that LM exhibit anti-inflammatory properties (22,23). However, the effects of LM against ALI remain poorly understood. The present study explores the anti-inflammatory and antioxidant properties of LM in LPS-stimulated RAW264.7 cells and mice.

Materials and methods

Quantification of lipid mediators. Normal-phase high-performance liquid chromatography (NP-HPLC) of lipid mediators was performed using a SUPELCOSIL LC-DIOL column (SUPELCO, 25x3 mm, 5 μm). The mobile phase consisted of heptane/2-propanol/acetic acid (95:5:0.1, v/v/v) at a flow rate of 0.5 ml/min, with the column temperature maintained at 10˚C. Lipid mediators were detected by monitoring UV absorbance at 237, 242, and 270 nm. Concentrations were determined by correlating peak areas with standard curves of known concentrations.

Cell culture and viability assay. RAW264.7 cells (KCLB-40071, mycoplasma negative, Korea Cell Line Bank) were retrieved and sub-cultured 3-5 times to achieve a stable and favorable condition. All experiments used cells within 10 passages. Cells were treated with LM from 1 to 100 μg/ml for 3 h, subsequently incubated with lipopolysaccharides (LPS) at 1 μg/ml (from *Escherichia coli* O111:B4; cat. no. L2630, MilliporeSigma) for 24 h. MTT assay kit (cat. no. ab211091; Abcam) was used to detect the cell viability (23). Briefly, the existing media was replaced with a mixture of 50 μl serum-free media and 50 μl of MTT Reagent and the plate was incubated at 37˚C for 3 h. Subsequently, 150 μl of MTT Solvent was added to each well, followed by an incubation for 15 min in the dark. Finally, the absorbance at 590 nm was measured by microplate spectrophotometer (BioTeK; Agilent Technologies, Inc.).

Intracellular ROS measurement. Cells were pre-treated with LM (0.5, 1 and 2 μg/ml) for 3 h (23), followed by stimulation with LPS for 24 h. After that, cells were exposed to DCFH-DA solution for 30 min. ROS was assessed via fluorescence microscopy (Leica Microsystems GmbH).

Inflammatory mediator assay. Cells were pre-incubated with or without 5 μM ML385 (MedChemExpress) for 2 h, followed by LM treatment for 3 h. Afterward, cells were exposed to LPS for 24 h. The concentrations of pro-inflammatory cytokines in the supernatant were quantified using ELISA kits (all Abcam) for IL-6; cat. no. ab222503), tumor necrosis factor-α (TNF-α; cat. no. ab208348), and IL-1β (cat. no. ab100704), following the manufacturer's instructions. PGE2 was calculated by ELISA kit (cat. no. MOEB2492; Assay Genie). NO levels were measured with the Griess Reagent System (cat. no. G2930; Promega Corporation).

Animal experiments. The animal experiment was approved by the Institutional Animal Care and Use Committee of the Korea Research Institute of Bioscience and Biotechnology as well as the Institutional Animal Ethics Committee (approval no. KRIBB-AEC-23233). A total of 18 female BALB/c mice (6 weeks old; Orient Bio) were maintained under standard conditions (22±2˚C; 65±5% humidity; 12-h light/dark cycle). ALI was induced via intranasal LPS administration (2). Mice were assigned to three groups (n=6/group): Normal control (NC), treated with saline, LPS (5 mg/kg) and LPS (5 mg/kg) + LM (10 μg/kg/daily). The dosage was chosen based on a previous study (23). LM was administered orally for 7 days (based on the preliminary data). One h after the final treatment, LPS was administered intranasally to induce lung injury. Bronchoalveolar lavage fluid (BALF) was collected 12 h later using 1 ml of phosphate-buffered saline (PBS) under anesthesia (2,14). Mice were initially placed in an induction chamber with 3% isoflurane and once unconscious, they were transferred to a facemask to maintain anesthesia with 1.5% isoflurane during the procedure. All animals were sacrificed under 8% isoflurane anesthesia followed by cervical dislocation, in accordance with ethical guidelines. Additionally, mice showing signs of distress, such as weight loss exceeding 20% of baseline, labored breathing, or cyanosis, were designated for humane sacrifice using the same method. However, no such symptoms were observed in any of the animals throughout the study. BALF was centrifuged at 4˚C and 15,000 x g for 10 min. The supernatant was collected for cytokine detection via ELISA, including IL-6 (cat. no. ab222503), TNF-α (cat. no. ab208348) and IL-1β (cat. no. ab100704) (Abcam), and the cell pellets were resuspended for cell counting.

Lung tissue was collected for the measurement of wet/dry (W/D) ratio according to the previous study (20). Hematoxylin and eosin (H&E) staining of lung tissue was processed and graded as 0-4 according to the severity of damage as follows: 0=no injury; 1=mild injury (25% of the field); 2=moderate injury (50% of the field); 3=severe (75% of the field); and 4=most severe (90% of the field) (2,24).

Oxidative stress assay. The right lung was excised, homogenized and prepared in extraction buffer. Levels of myeloperoxidase (MPO) in lung tissue was assessed via ELISA kit (cat. no. RAB0374; MilliporeSigma). MDA (cat. no. ab118970; Abcam), GSH (cat. no. ab239727; Abcam), CAT (cat. no. ab83464; Abcam) and SOD (cat. no. ab65354; Abcam) in lung tissue or cells were assessed via colorimetric assay kit (Abcam).

Western blotting. The isolation and separation of protein was conducted as the previous study (25). Proteins were extracted from cells using RIPA buffer (Bio Solution Co., Ltd.), then

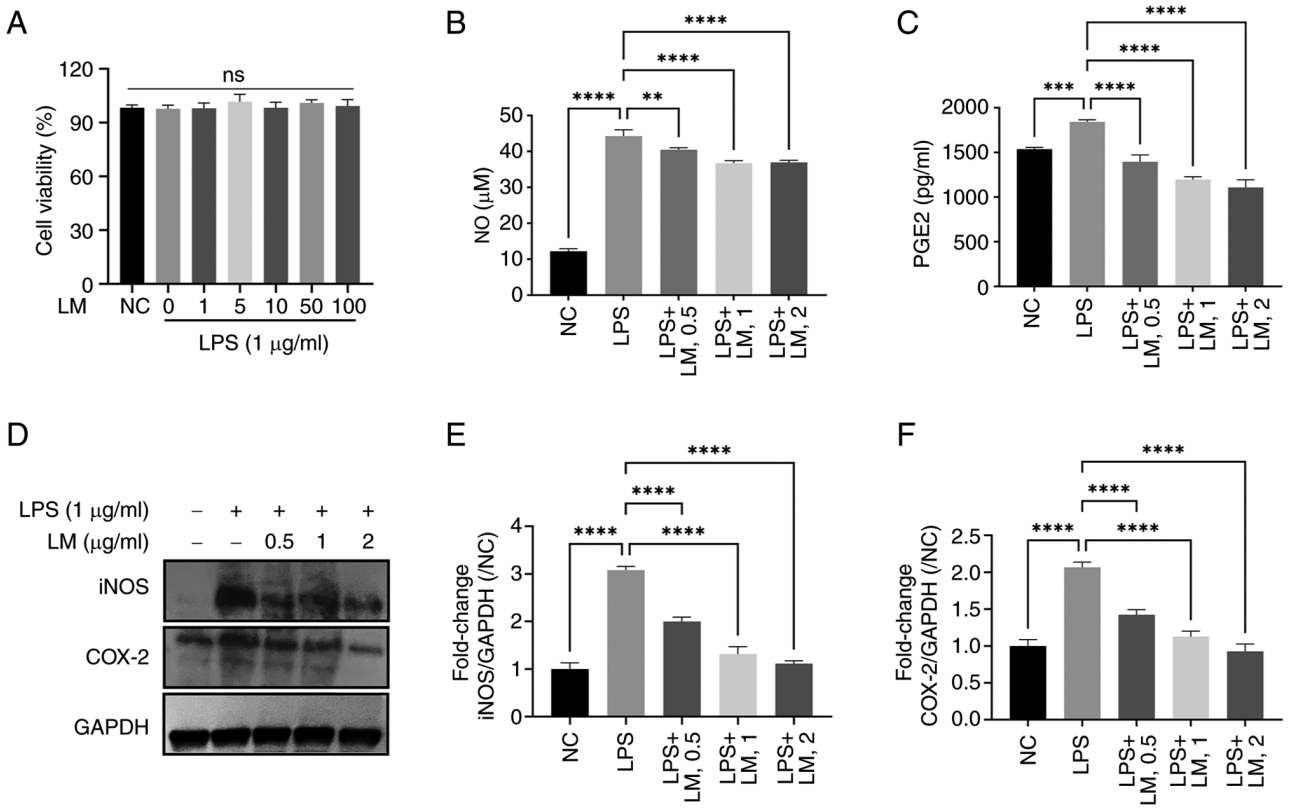

Figure 1. LM mediates the production of NO and PGE2 *in vitro*. (A) RAW264.7 cells were treated with LM for 3 h, followed by stimulation with LPS at 1 μg/ml for 24 h. MTT assay was performed to detect the cell viability. The production of (B) NO and (C) PGE2 were quantified. (D-F) Western blot analysis was conducted to assess the expression of iNOS and COX-2. Data are presented as mean ± SD and was determined using Tukey's test. **P<0.01, ***P<0.001, ****P<0.0001. LM, lipid mediators; NO, nitric oxide; PGE2, prostaglandin E2; LPS, lipopolysaccharide; iNOS, inducible nitric oxide synthase; COX-2, cyclo-oxygenase-2.

kept on ice for 30 min. After centrifugation at 15,000 x g for 10 min at 4˚C, protein concentration was measured using a BCA kit (Thermo Fisher Scientific, Inc.). Samples were mixed with loading buffer (Beijing Solarbio Science & Technology Co., Ltd.) and heated at 100˚C for 10 min. Equal amounts of protein (~25 μg) were separated by 10% SDS-PAGE, transferred to PVDF membranes (MilliporeSigma) and blocked with 5% BCA (Thermo Fisher Scientific, Inc.) at room temperature for 40 min. Membranes were incubated with rabbit monoclonal antibodies against inducible nitric oxide synthase (iNOS; cat. no. ab178945; 1:1,000), cyclooxygenase-2 (COX-2; cat. no. ab179800; 1:1,000), Nrf2 (cat. no. ab92946; 1:1,000), heme oxygenase-1 (HO-1; cat. no. ab52947; 1:2,000), p65 (cat. no. ab16502; 1:1,000), pp65 (cat. no. ab76302; 1:1,000) and GAPDH (cat. no. ab181602; 1:20,000; all Abcam) overnight at 4˚C, then with HRP-conjugated goat anti-rabbit secondary antibody (cat. no. ab205718; Abcam; 1:20,000) for 2 h at room temperature. After washing, signals were detected using ECL substrate (Thermo Fisher Scientific, Inc.) and visualized on film (Thermo Fisher Scientific, Inc.). Band intensity was analyzed using ImageJ (1.48v; National Institutes of Health).

Statistical analysis. The data are expressed as means ± standard deviations (SDs). Statistical analysis was conducted using one-way ANOVA followed by Tukey's post hoc test for parametric data, or Kruskal-Wallis followed by Dunn's post hoc test for non-parametric analysis (only for inflammation score) using GraphPad Prism 9.5.1 (Dotmatics). P<0.05 was considered to indicate a statistically significant difference.

Results

LM mediates NO and PGE2 expression in vitro. MTT assay confirmed that LM combined with LPS (1 μg/ml) was non-cytotoxic (Fig. 1A). First, the effect of LM alone on RAW264.7 cells was examined. It was observed that LM alone showed no significant effect on NO or IL-6 levels (Fig. S2). To evaluate the anti-inflammatory properties of LM, its ability to suppress LPS-induced proinflammatory mediators was analyzed. LPS stimulation significantly elevated NO to 44.25±1.76 μM (P<0.0001 vs. NC) and increased PGE2 to 1,842.67±23.67 pg/ml (P<0.001 vs. NC; Fig. 1B and C). However, LM at 0.5, 1 and 2 μg/ml markedly reduced these mediators. Similarly, LPS significantly enhanced the expression of iNOS and COX-2 (P<0.0001 vs. NC, respectively; Fig. 1D to F), whereas, LM markedly suppressed the expression of both proteins.

LM inhibits inflammatory cytokines via NF-κB signaling pathway in vitro. LPS significantly elevated IL-6 (4,470.72±125.18 pg/ml; P<0.0001), TNF-α (2,865.04±57.34 pg/ml; P<0.0001) and IL-1β (1,870.72±165.18 pg/ml; P<0.0001) when compared with normal conditions, while, LM treatment notably reduced these

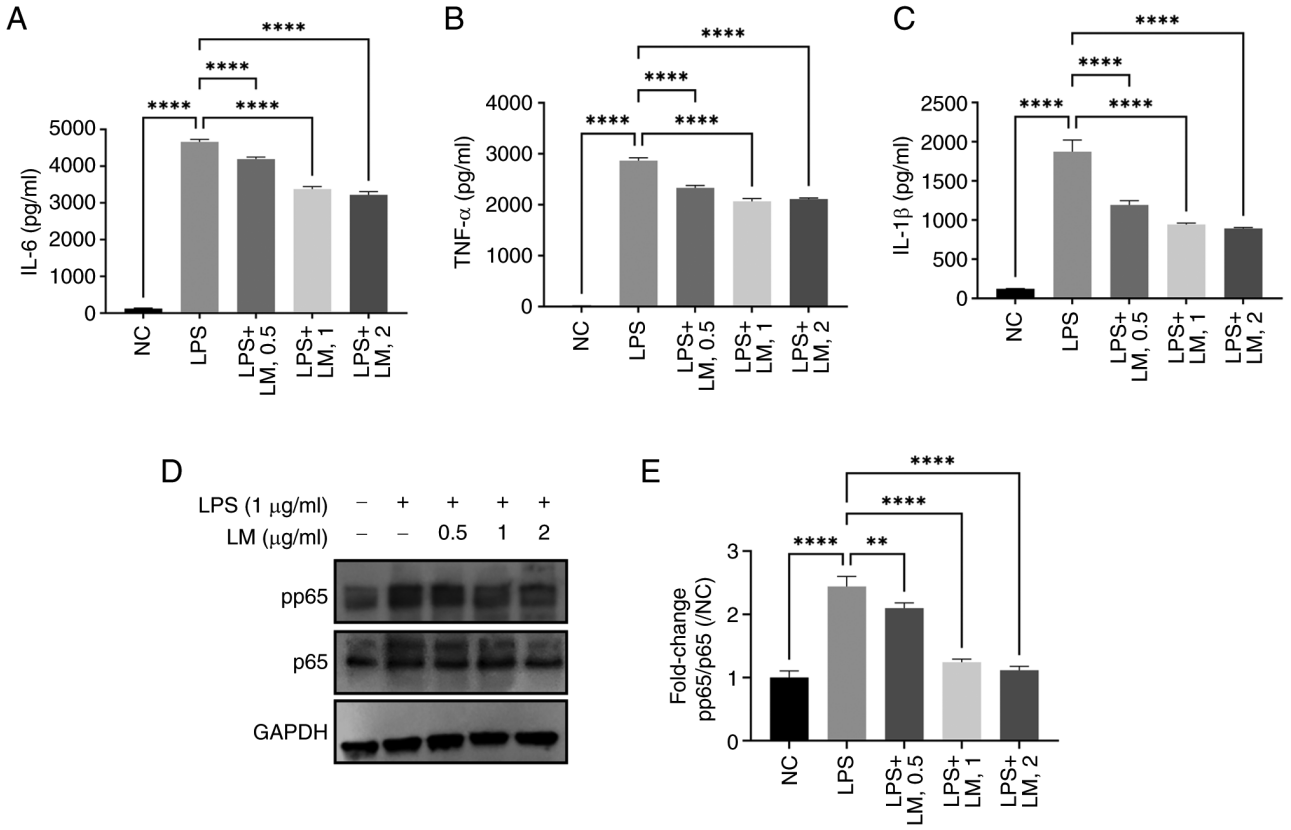

Figure 2. LM inhibits inflammatory cytokines via NF-κB signaling pathway *in vitro*. RAW264.7 cells were pre-incubated with LM for a period of 3 h, followed by LPS at 1 μg/ml for 24 h. (A) IL-6, (B) TNF-α and (C) IL-1β were measured via ELISA. (D and E) Western blot analysis was conducted to evaluate the expression levels of pp65. Data are presented as mean ± SD and was determined using Tukey's test. **P<0.01, ****P<0.0001. LM, lipid mediators; IL, interleukin; TNF-α, tumor necrosis factor-α; ELISA, enzyme-linked immunosorbent assay; NC, normal control.

cytokines (Fig. 2A-C). Western blot analysis revealed that LPS stimulation significantly increased NF-κB phosphorylation (pp65; P<0.0001 vs. NC), while LM pretreatment markedly mitigated phosphorylated p65 (Fig. 2D and E).

LM suppresses oxidative stress in vitro. To assess the antioxidant potential of LM, ROS accumulation, MDA content, GSH levels, CAT and SOD activities were measured. LPS exposure significantly promoted ROS accumulation (P<0.0001 vs. NC group), as shown by fluorescence imaging, but this was markedly reduced by LM pretreatment at different concentrations (Fig. 3A). LPS also decreased GSH level and SOD activities while increasing MDA content (P<0.0001 vs. NC group, respectively; Fig. 3B-D). However, LM pretreatment reversed these effects, elevating GSH and enhancing SOD activities while reducing MDA level. In addition, Nrf2 and HO-1 levels were highly enhanced by LM in comparison to LPS stimulation (Fig. 3E-G).

LM suppresses the oxidative stress and inflammation via activation of Nrf2. In order to explore the role of Nrf2 signaling in mediating LM's effects on oxidative stress and inflammation, the present study administered an inhibitor of Nrf2, ML385 (5 μM) for 2 h before LM treatment, followed by LPS incubation. Nrf2 expression was assessed using western blot analysis. As shown in Fig. 4A and B, LM treatment led to a significant increase in Nrf2 level (P<0.0001 vs. LPS group). However, activation of Nrf2 was downregulated in pretreatment with ML385 and LM group compared with LM alone treatment (P<0.01). Furthermore, LM treatment significantly reduced IL-6 and TNF-α (P<0.0001 vs. LPS group, respectively) whereas these cytokines were increased in LM + ML385 treatment compared with LM treatment (P<0.01, P<0.05, respectively; Fig. 4C and D). The inhibition of both cytokines caused by LM was offset by pretreatment with ML385.

LM inhibits the lung injury in ALI mice. Histological analysis of lung tissue revealed significant pathological changes in the LPS-induced ALI group (P<0.001 vs. NC group), including thickened alveolar septa, alveolar wall edema and inflammatory cell infiltration, which were significantly reduced with LM pretreatment (P<0.05 vs. ALI group; Fig. 5A and B). The lung W/D ratio further indicated that LM significantly reduced edema severity in comparison to LPS group (P<0.0001; Fig. 5C).

MPO, a key marker of neutrophil infiltration into lung tissue (2,7), was significantly elevated to 3 times in the ALI mice when compared with normal levels (P<0.0001; Fig. 5D). LM administration significantly reduced MPO levels by ~30% to 0.68±0.08 U/g (P<0.01 vs. ALI group).

LM reduces the inflammatory cytokines in BALF and serum. LPS exposure significantly raised the levels of TNF-α, IL-6

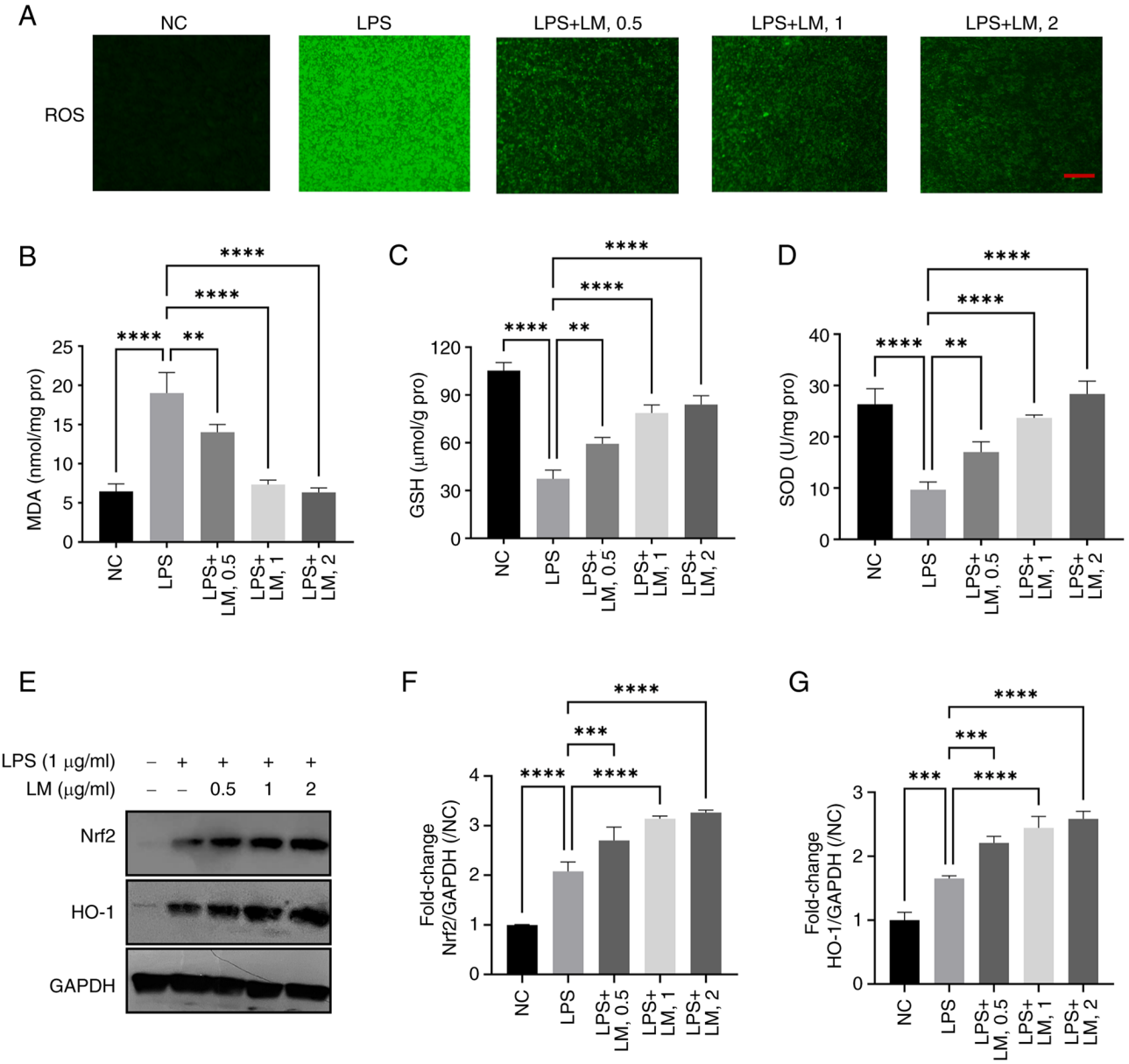

Figure 3. LM suppresses the oxidative stress *in vitro*. (A) RAW264.7 cells were pretreated with LM for 3 h, followed by a 24-h exposure to LPS. Cells were then stained with DCFH-DA to detect ROS. Fluorescence intensity was visualized using a fluorescence microscope, scale bar, 100 μm. Levels of (B) MDA, (C) GSH and (D) SOD in the cells were measured. (E) Western blot analysis was performed to evaluate the expression of Nrf2 and HO-1. Relative expression of (F) Nrf2 and (G) HO-1 was quantified using ImageJ software. Data are presented as mean ± SD and was determined using Tukey's test. **P<0.01, ***P<0.001, ****P<0.0001. LM, lipid mediators; LPS, lipopolysaccharide; ROS, reactive oxygen species; MDA, malondialdehyde; GSH, glutathione; SOD, superoxide dismutase; Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase-1; NC, normal control.

and IL-1β in BALF (P<0.0001 vs. NC group, each), whereas LM pretreatment effectively moderated these cytokine elevations, reducing them by >50% compared with the ALI group (P<0.001, P<0.0001, P<0.001; Fig. 6A-C). Similarly, serum TNF-α rose to 151.33±11.19 pg/ml, IL-6 increased to 248.74±19.19 pg/ml and IL-1β elevated to 54.64±2.08 pg/ml (P<0.0001 vs. NC group, respectively; Fig. 6D-F). LM pretreatment significantly reduced these levels to 90.33±6.17, 132.68±5.39 and 35.14±3.21 pg/ml (P<0.001, P<0.0001, P<0.001 vs. ALI group; Fig. 6D-F).

LM alleviates the oxidative stress in ALI mice. To assess the potential of LM in mitigating LPS-induced oxidative stress, key antioxidant levels were evaluated as shown in Fig. 7. LPS exposure significantly increased MDA level (~3-fold; P<0.0001 vs. NC group), while reducing SOD activity by almost a half (P<0.001 each vs. NC group), CAT and GSH levels to nearly one-third (P<0.0001 each vs. NC group). Pretreatment with LM substantially reduced MDA level to 4.01±0.21 nmol/g tissue (P<0.001 vs. ALI group) and restored SOD and CAT activity to 5.13±0.42 U/mg protein and 9.67±0.95 U/mg protein (P<0.01 vs. ALI group, respectively), as well as enhanced GSH level to 4.08±0.13 μmol/g tissue (P<0.001 vs. ALI group).

LM alters NF-κB and Nrf2/HO-1 signaling pathways in ALI mice. The NF-κB pathway was analyzed by western blotting to determine the effect of LM. LPS significantly increased the phosphorylation of pp65 (P<0.001 vs. NC group), promoting

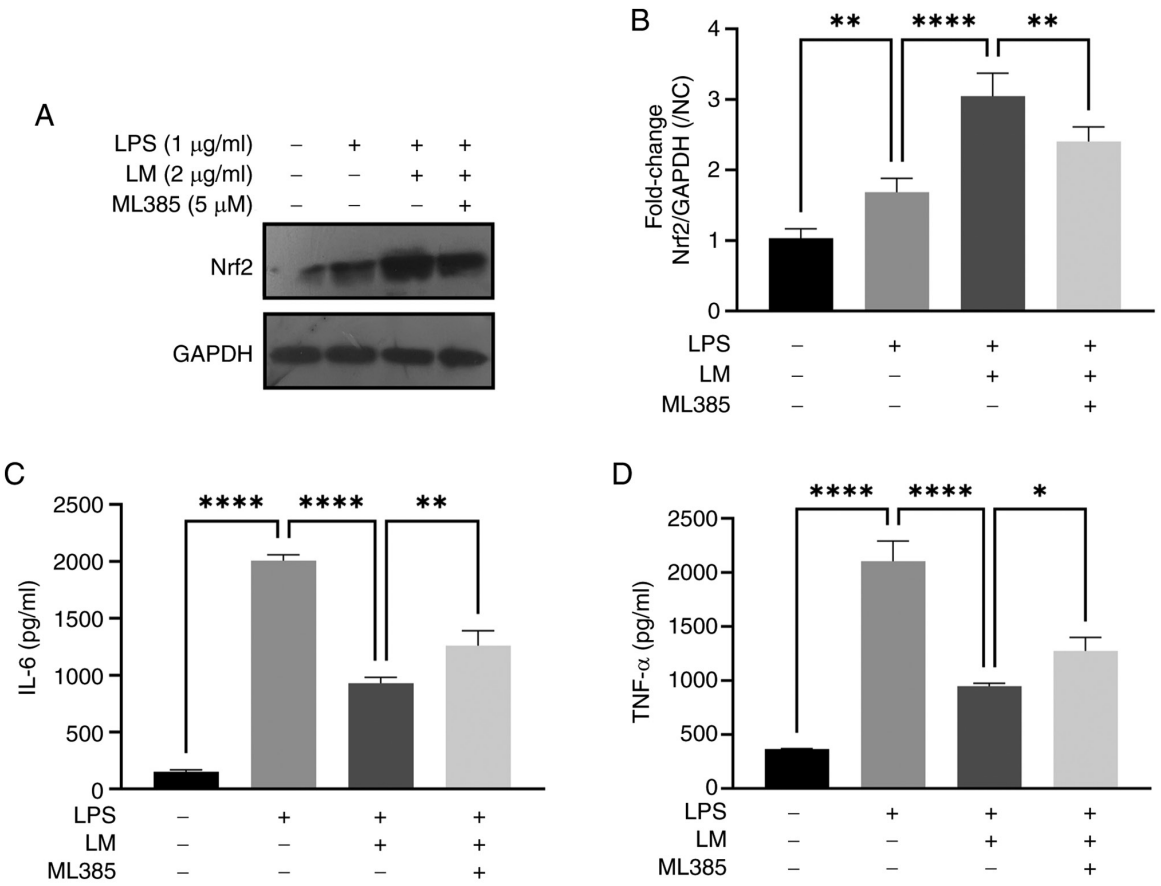

Figure 4. LM suppresses the inflammation via Nrf2 signaling pathway *in vitro*. RAW264.7 cells were pretreated with ML385 at 5 μM for 2 h and then LM (2 μg/ml) for further 3 h, followed by stimulated with LPS for 24 h. (A) and (B) Nrf2 expression levels were assessed by western blotting. The levels of (C) IL-6 and (D) TNF-α were measured using ELISA kits. Data are presented as mean ± SD and determined using Tukey's test. *P<0.05, **P<0.01, ****P<0.0001. LM, lipid mediators; Nrf2, nuclear factor erythroid 2-related factor 2; LPS, lipopolysaccharide; IL, interleukin; TNF-α, tumor necrosis factor-α; ELISA, enzyme-linked immunosorbent assay.

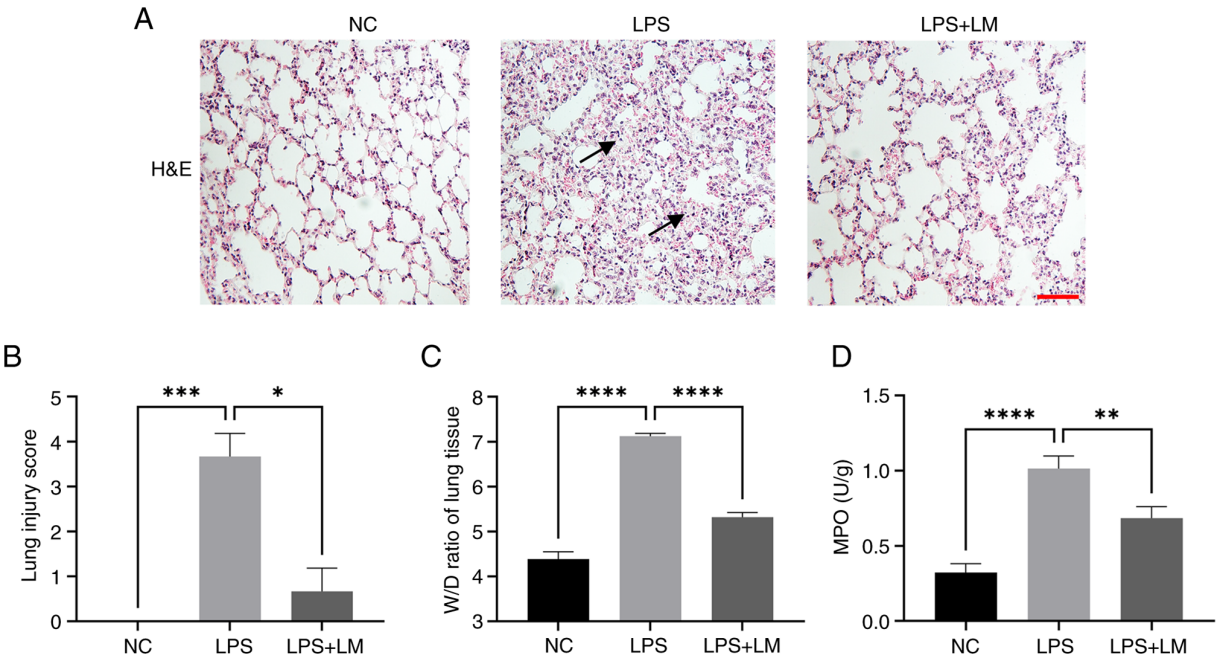

Figure 5. LM inhibits the pathological alterations in LPS-induced ALI mice. (A) H&E-stained images of lung tissue injury. Scale bar, 100 μm. (B) Histological scoring of lung injury; black arrows indicate hemorrhage and destruction of alveolar structure. Data are presented as mean ± SD and was determined using Kruskal-Wallis followed by Dunn's post hoc test. *P<0.05, ***P<0.001. (C) The W/D ratio was measured in the LPS-induced ALI model. (D) MPO from lung was determined. Data are presented as mean ± SD and was assessed using Tukey's test. **P<0.01, ****P<0.0001. LM, lipid mediators; LPS, lipopolysaccharide; ALI, acute lung injury; H&E, hematoxylin and eosin; W/D, wet-to-dry; MPO, myeloperoxidase.

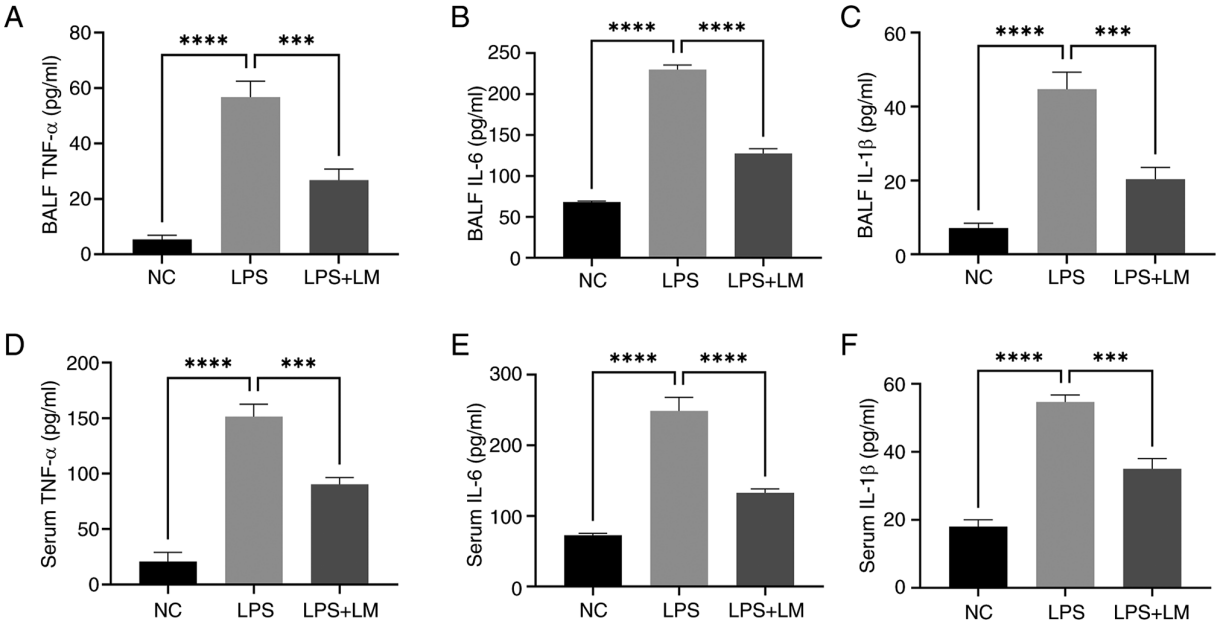

Figure 6. LM reduces the inflammatory cytokines in BALF and serum in LPS-induced ALI mice. (A) TNF-α, (B) IL-6 and (C) IL-1β in BALF obtained from LPS-induced ALI mice, was measured by ELISA kits. (D) TNF-α, (E) IL-6 and (F) IL-1β levels in serum of LPS-induced ALI mice, was detected by ELISA kits. Data are presented as mean ± SD and determined using Tukey's test. ***P<0.001, ****P<0.0001. LM, lipid mediators; BALF, bronchoalveolar lavage fluid; LPS, lipopolysaccharide; ALI, acute lung injury; TNF-α, tumor necrosis factor-α; IL, interleukin; ELISA, enzyme-linked immunosorbent assay.

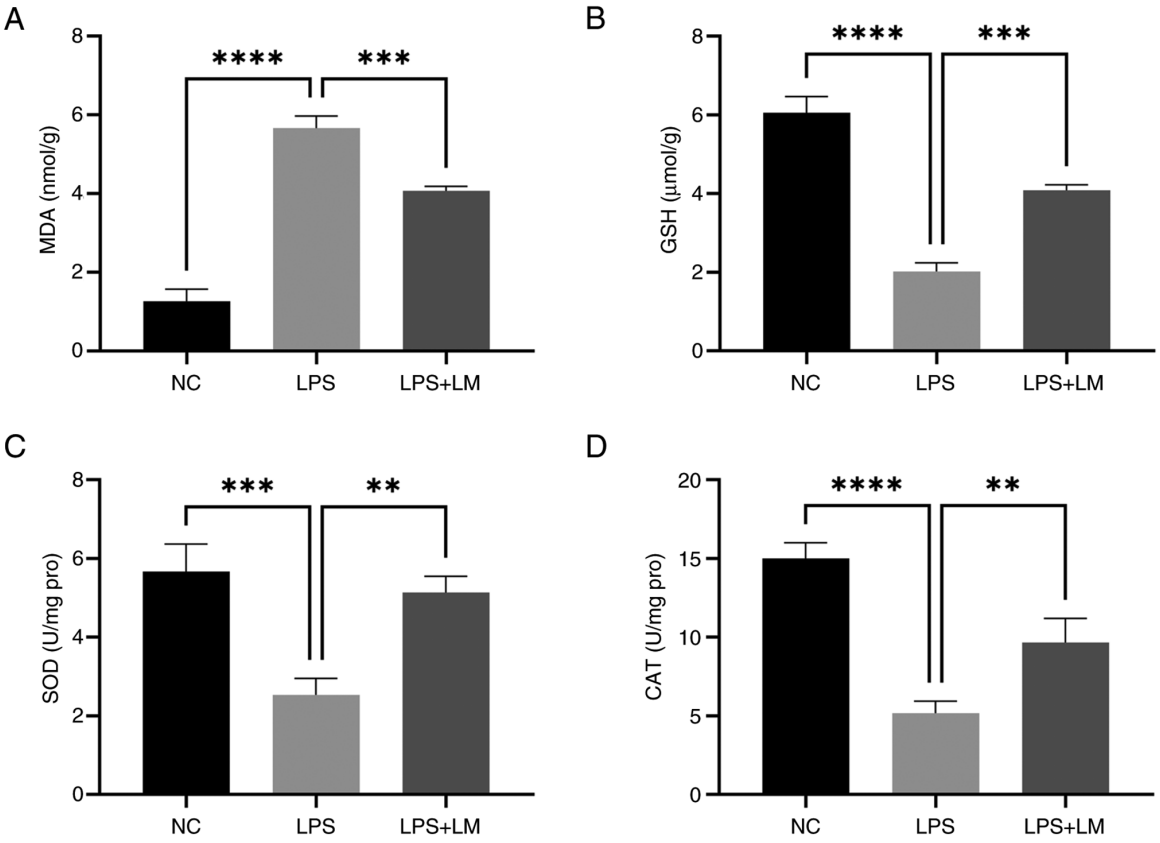

Figure 7. LM alters oxidative stress in LPS-induced ALI mice. The level of (A) MDA, (B) GSH, (C) SOD and (D) CAT were determined in lung homogenates from LPS-induced ALI mice. Data are presented as mean ± SD and determined using Tukey's test. **P<0.01, ***P<0.001, ****P<0.0001. LM, lipid mediators; LPS, lipopolysaccharide; ALI, acute lung injury; MDA, malondialdehyde; GSH, glutathione; SOD, superoxide dismutase; CAT, catalase; NC, normal control.

inflammation in ALI mice whereas LM pretreatment significantly reduced pp65 expression (P<0.001 vs. ALI group), effectively suppressing NF-κB activation (Fig. 8A and B). The role of LM in modulating the Nrf2/HO-1 pathway was

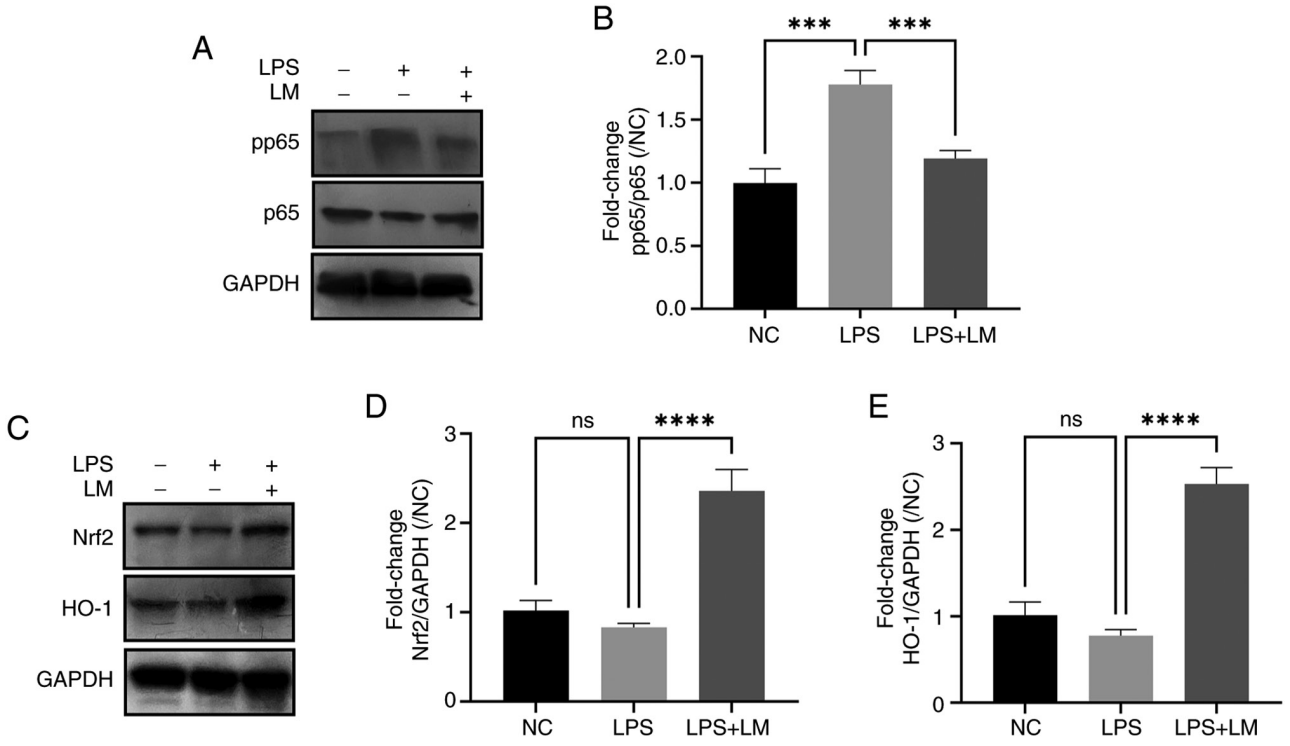

Figure 8. LM modulates NF-κB and Nrf2/HO-1 signaling in LPS-induced ALI mice. (A) Western blotting was conducted to determine p65 and pp65. (B) Quantification of these proteins via densitometric analysis with GAPDH as the internal control. (C) The expression of Nrf2 and HO-1 were analyzed by western blotting. (D and E) Quantification of Nrf2 and HO-1 expression via densitometric analysis. Data are presented as mean ± SD and determined using Tukey's test. ***P<0.001, ****P<0.0001. LM, lipid mediators; HO-1, heme oxygenase-1; LPS, lipopolysaccharide; ALI, acute lung injury; NC, normal control.

investigated. LM pretreatment significantly upregulated Nrf2 expression compared with the NC and LPS groups (P<0.001, respectively; Fig. 8C and D). Additionally, LM obviously enhanced HO-1 expression (P<0.0001 vs. NC or ALI group, respectively; Fig. 8C and E), a downstream target of Nrf2. These results indicated that LM activated the Nrf2/HO-1 pathway in LPS-induced ALI mice.

Discussion

ALI is a severe inflammatory condition characterized by damage to epithelial and endothelial cells, along with disruption of the alveolar-capillary barrier, leading to lung tissue edema and collapse (13,26). This barrier disruption triggers activation of alveolar macrophages and neutrophils in response to exudate in the alveolar spaces, promoting the release of proinflammatory cytokines and oxidants that exacerbate epithelial and endothelial cell damage (19). Consequently, ALI manifests as a complex airway disease involving amplified oxidative stress and inflammatory responses (27,28). Specialized pro-resolving lipid mediators (SPMs), derived from omega-3 polyunsaturated fatty acids, play a key role in regulating inflammatory diseases such as arthritis, ALI and asthma (29,30). SPMs exert effects through various G protein-coupled receptors, which was evidenced by receptor loss impairing inflammation resolution (31,32). While downstream signaling events triggered by SPMs receptor activation remain partially ununderstood, pathways such as NF-κB and Nrf2 signaling have been implicated (33,34). LM has demonstrated anti-inflammatory effects in models of dermatitis and arthritis (23,35). The objective of the present study was to investigate the potential of LM in ameliorating lung tissue injury.

The intratracheal or intranasal administration of bacterial products such as LPS is a widely used model for studying ALI (19). The present study observed severe diffuse lung damage characterized by infiltration of inflammatory cells, thickening of the alveolar walls and enlargement of the spaces between tissues. The increased lung W/D ratio further indicated lung edema, a hallmark of ALI (36,37). LPS exposure markedly elevated the W/D ratio and triggered extensive neutrophil migration into lung tissue, leading to increased MPO levels and severe inflammation (7,33). Consistent with previous findings, the results confirmed that LPS markedly raised MPO levels in lung tissue. Notably, LM pretreatment effectively mitigated histological damage and reduced MPO activity, demonstrating its potential to alleviate LPS-induced lung injury.

LPS triggers excessive release of proinflammatory mediators, which are critical in acute and chronic inflammatory disease pathogenesis (38-41). The present study demonstrated that LM pretreatment effectively reduced TNF-α, IL-6 and IL-1β secretion in LPS-stimulated RAW264.7 cells and ALI mice. Additionally, NO and PGE2, key inflammatory markers regulated by iNOS and COX-2, separately (4,25), were markedly suppressed by LM, along with reduced iNOS and COX-2 expression. Following LPS challenge, NF-κB pathway is activated, contributing to inflammatory diseases (42,43). Thus, targeting NF-κB is critical for anti-inflammatory strategies. Our previous studies demonstrated that LM inhibits NF-κB

activation in chronic inflammatory conditions (22,23,35). In parallel, the results in present study suggested that LM pretreatment effectively suppressed NF-κB pathway in LPS induced cells and mice.

ROS, generated by inflammatory cells or enzymatic systems in alveolar epithelial and capillary endothelial cells, is a key contributor to ALI pathogenesis (44,45). Evidence shows that antioxidants can mitigate ALI severity, highlighting oxidative stress as a pivotal factor (46-48). Endogenous antioxidants such as SOD, CAT and GSH are markedly depleted during oxidative stress (7,14). Consistent with this, LPS exposure increased ROS production and MDA level while reducing CAT, SOD activities and GSH level. LM pretreatment, however, reduced ROS production and MDA content while restoring antioxidants levels. Nrf2 is a critical regulator of ROS-induced oxidative stress (49,50). Upon ROS signaling, Nrf2 is activated and promotes the expression of antioxidant genes, including HO-1 (51-53). Resolvin D1 attenuated oxidative stress via Nrf2/HO-1, but this effect was abolished by an inhibitor of Nrf2, ML385, and an inhibitor of HO-1, ZnPP (54). Similarly, the protective effects on the ischemia/reperfusion-induced lung injury of maresin 1 could be diminished by Znpp-IX and Brusatol (an Nrf2 antagonist) (55). These investigations suggested the Nrf-2/HO-1 signaling pathway contributed to mitigation of oxidative stress by SPMs. The present study also found that LM markedly activated Nrf2/HO-1 axis in RAW264.7 cells and ALI mice. Recent evidence suggests that the Nrf2/HO-1 pathway plays a critical role in cellular antioxidant and anti-inflammatory defense, while NF-κB is a central mediator of pro-inflammatory responses (56,57). Given the reported crosstalk between these two pathways, it was hypothesized that LM exerts its anti-inflammatory effects through modulation of the Nrf2 and NF-κB signaling pathways. The present study aimed to investigate the anti-inflammatory properties of LM and its potential regulation of these key molecular pathways. The findings indicated that LM exerted anti-inflammatory effects, which appear to be at least partially mediated through the Nrf2 pathway, as evidenced by the diminished effect observed upon Nrf2 inhibition with ML385. These results support the role of Nrf2 activation in the protective mechanism of LM, potentially via HO-1 induction.

In summary, LM exhibits protective properties against lung tissue damage in ALI by inhibiting the NF-κB signaling pathway and activating the Nrf2/HO-1 signaling pathway. However, there are some limitations to the present study. To begin with, additional studies are needed to evaluate the standalone effects of LM in animal experiments. Moreover, while LPS induced ALI is widely used for studying inflammatory lung conditions, it was recognized that future studies should explore other ALI models (such as bacterial or viral infection-induced ALI) to enhance the generalizability of the present findings. Furthermore, although the findings strongly supported the involvement of Nrf2 in the anti-inflammation of LM, it is acknowledged that further mechanistic studies, such as dual-pathway inhibition or genetic interventions, would be beneficial to confirm direct regulatory interactions. In addition, while the present study did not explore the synergistic or distinct effects of individual LM components, future research could focus on component-specific studies using purified LM derivatives to further elucidate their individual roles.

Acknowledgements

Not applicable.

Funding

The present study was funded by the Microbial Biotechnology Research Center, Korea Research Institute of Bioscience and Biotechnology from Ministry of Science and ICT (grant no. KGM1222511) and the Bio and Medical Technology Development Program of the National Research Foundation of the Korea government (RS-2024-00396026).

Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

YS was responsible for conceptualizing and designing the study, conducting experiments, analyzing data and writing as well as revising the manuscript. HSC contributed to data analysis and provided critical feedback during revisions, ultimately helping finalize the manuscript. SKK and YH participated in performing experiments and analyzing data. SCC and JHS conducted literature reviews, supplied necessary materials and assisted with data analysis. YSJ played a key role in analyzing data and refining the intellectual content through critical revisions. JHC contributed to data analysis and helped draft and finalize the manuscript. JWS was involved in conceptualizing the study and contributed markedly to drafting and finalizing the manuscript. YS and JWS both confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was reviewed and approved by the Institutional Animal Care and Use Committee of the Korea Research Institute of Bioscience and Biotechnology as well as the Institutional Animal Ethics Committee (KRIBB-AEC-23233).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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