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Morusin attenuates LPS-induced inflammation and oxidative stress in lung epithelial cells by inhibiting NLRP3 inflammasome activation

Yan Li[a], Li Zhang[b]*

[a]*Department of Respiratory Medicine, Wuhan Hankou Hospital, Wuhan, Hubei Province, China*
[b]*Department of Orthopedics, Wuhan Hankou Hospital, Wuhan, Hubei Province, China*

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Abstract
Acute lung injury (ALI) is a pulmonary disorder characterized by severe lung tissue damage and inflammation. Morusin (a flavone, a type of flavonoid; $C_{25}H_{24}O_6$), a flavonoid isolated from mulberry root bark, possesses notable anti-inflammatory and antioxidant properties. However, its regulatory effects on ALI progression remain unclear. This study aimed to investigate whether morusin could mitigate the development of ALI. Morusin was shown to enhance cell viability and reduce apoptosis in lipopolysaccharide (LPS)-stimulated A549 cells. Furthermore, morusin alleviated inflammation by decreasing the levels of interleukin-1β (IL-1β), IL-6, and prostaglandin E2 (PGE2) in LPS-challenged A549 cells. In addition, morusin effectively reduced oxidative stress in these cells. Mechanistically, morusin was found to suppress the activation of NLRP3 (nucleotide-binding domain, leucine rich-containing family, pyrin domain containing 3) inflammasome in LPS-stimulated A549 cells. Finally, this study demonstrated for the first time that morusin ameliorates LPS-induced inflammation and oxidative stress in lung epithelial cells by inhibiting both nuclear factor *kappa B* signaling pathway and NLRP3 inflammasome activation. However, this project was limited to cell experiments, and no animal experiments or clinical verifications were conducted. Our findings suggest that morusin could serve as a promising therapeutic agent for the treatment of ALI.

***Corresponding author**: Li Zhang, Department of Orthopedics, Wuhan Hankou Hospital, No. 7 Erqi Side Road, Jiang'an District, Wuhan City, Hubei Province 430012, China. *Email address*: zhangli4082101@163.com

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Introduction

Acute lung injury (ALI) is a serious clinical condition, characterized by extensive inflammation and lung dysfunction, which progresses to acute respiratory distress syndrome (ARDS).[1,2] The pathological features of ALI include disruption of alveolar–capillary barrier, excessive release of chemokines and pro-inflammatory cytokines, and subsequent lung tissue injury.[3] ALI severely affects patients' health and survival while also imposing considerable economic pressure on their families. A549 cells have been usually treated with lipopolysaccharide (LPS) to establish an *in vitro* ALI model.[4–6] Therefore, the development of effective therapeutic strategies to reduce inflammation and oxidative stress is essential for improving ALI outcomes.

Plant-derived compounds have attracted increasing attention as potential therapeutic agents because of their favorable characteristics, including safety, natural origin, and lack of harmful residues.[7] Among them, the root bark of *Morus* species is a rich source of bioactive compounds, such as triterpenoids, flavonoids, and steroids.[8] Traditionally, it has been used in herbal medicine for its anti-inflammatory, immunomodulatory, antioxidant, anti-tumor, and antibacterial properties.[9] Morusin, a flavone, a type of a flavonoid, extracted from mulberry root bark, is shown to possess significant anti-inflammatory and antioxidant activities.[10]

Recent studies have revealed the regulatory effects of morusin ($C_{25}H_{24}O_6$) in various diseases. Morusin is reported to inhibit receptor activator of nuclear factor-κB ligand (RANKL)-induced osteoclastogenesis, thereby preventing osteoporosis.[11] It also enhances the therapeutic effect of temozolomide in glioblastoma by promoting cytoplasmic vacuolization.[12] In addition, morusin suppresses inflammatory signal transduction by inhibiting the phosphorylation of signal transducer and activator of transcription 1 (STAT1) and nuclear factor *kappa B* (NF-κB).[13] Moreover, it alleviates colitis by reducing the production of pro-inflammatory and fibrotic markers[14] and attenuates mycoplasma pneumonia through the inhibition of Wnt/β-catenin and NF-κB signaling pathways to mitigate mycoplasma pneumonia.[15] Despite these findings, the effects of morusin on ALI progression have remained unclear.

Therefore, the present study aimed to investigate the potential role of morusin in ALI. This is the first investigation of morusin's effects on ALI progression. Our results demonstrated that morusin improved LPS-induced inflammation and oxidative stress in lung epithelial cells by inhibiting the NF-κB pathway and restraining the activation of the NLRP3 (nucleotide-binding domain, leucine rich-containing family, pyrin domain containing 3) inflammasome. These findings provide novel evidence supporting the therapeutic potential of morusin in the treatment of ALI.

Materials and Methods

Cell lines and treatment

The human lung adenocarcinoma cell line A549 was obtained from the American Type Culture Collection (ATCC, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, New York, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) in a humidified incubator at 37°C with 5% CO_2.

To establish an *in vitro* ALI model, A549 cells were treated with lipopolysaccharide (LPS; 10 μg/mL) for 24 h. Morusin (S091502; purity: 99.99%) was purchased from Shanghai Selleck Chemicals Co. Ltd. (Shanghai, China) and applied to A549 cells at concentrations of 12.5, 25, 50, and 100 μg/mL.

Cell counting kit-8 (CCK-8) assay

Cell viability was assessed using the cell counting kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan). A549 cells were seeded into 96-well plates at a density of 1000 cells per well. After treatment, 10 μL of CCK-8 solution was added to each well, and the absorbance was measured using a microplate reader (Bio-Rad, CA, USA).

Flow cytometry

Cell apoptosis was analyzed by flow cytometry. After treatment, the A549 cells were resuspended and stained using 5 μL of fluorescein isothiocyanate (FITC)-conjugated Annexin V and 5 μL of propidium iodide (PI) (BD Biosciences, Franklin Lakes, USA) according to the manufacturer's instructions. Apoptotic cells were quantified using a flow cytometer (BD Biosciences).

Enzyme-linked immunosorbent serological assay (ELISA)

The levels of interleukin (IL)-1β, IL-6, and prostaglandin E2 (PGE2) in cell culture supernatants were measured using commercial ELISA kits (IL-1β: ab214025; IL-6: ab178013; PGE2: ab287802; all from Abcam, Shanghai, China) following the manufacturers' protocols.

Detection of reactive oxygen species (ROS) (DCFDA staining)

The intracellular ROS levels were assessed using the 2',7'-dichlorofluorescein diacetate (DCFDA) cellular ROS detection assay kit (E004-1-1; Nanjing Jiancheng Technology Co. Ltd., Nanjing, China). Briefly, A549 cells were incubated with dichlorodihydrofluoresce in diacetate (DCFH-DA) for 20 min at 37°C. After washing with phosphate-buffered saline (PBS), fluorescence was observed under a fluorescence microscope (Olympus, Tokyo, Japan).

Detection of MDA, SOD, and GSH levels

The levels of malondialdehyde (MDA; ab118970), superoxide dismutase (SOD; ab65354), and glutathione (GSH; ab65322) were determined using commercial assay kits (Abcam) according to the manufacturers' instructions.

Western blot analysis

Proteins were extracted from A549 cells using the radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China). The protein concentrations were determined, and equal amounts of protein were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transfer onto polyvinylidene fluoride (PVDF) membranes (Beyotime). The membranes were blocked with non-fat milk and incubated overnight at 4°C with primary antibodies targeting NLRP3 (1:1000; ab263899), apoptosis-associated speck-like protein (ASC; 1:1000; ab309497), caspase-1 (1:1000; ab207802), IL-1β (1:1000; ab283818), phosphorylated p65 (p-p65; 1:1000; ab76302), p65 (1:1000; ab32536), IκBα (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha; 1:1000; ab32518), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:1000; ab8245) (all obtained from Abcam). After washing, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:1000; ab7090) for 2 h at room temperature. The corresponding protein bands were visualized using a chemiluminescence detection kit (Thermo Fisher Scientific, USA).

Statistical analysis

All experiments were performed in triplicate (n = 3). Data are presented as mean ± standard deviation (SD). Statistical analysis was conducted using the GraphPad Prism Software 9 (GraphPad Software, USA). Comparisons among groups were performed using one-way analysis of variance (ANOVA), followed by Tukey's *post hoc* test; $P < 0.05$ was considered statistically significant.

Results

Morusin enhanced cell viability and attenuated apoptosis in LPS-stimulated A549 cells

The molecular structure of morusin is shown in Figure 1A. Treatment with different concentrations of morusin alone did not significantly affect the viability of A549 cells (Figure 1B). However, LPS exposure markedly reduced cell viability, an effect that was reversed by morusin in a dose-dependent manner, particularly at concentrations of 50 and 100 μg/mL (Figure 1C). In addition, LPS treatment significantly increased apoptosis in A549 cells whereas

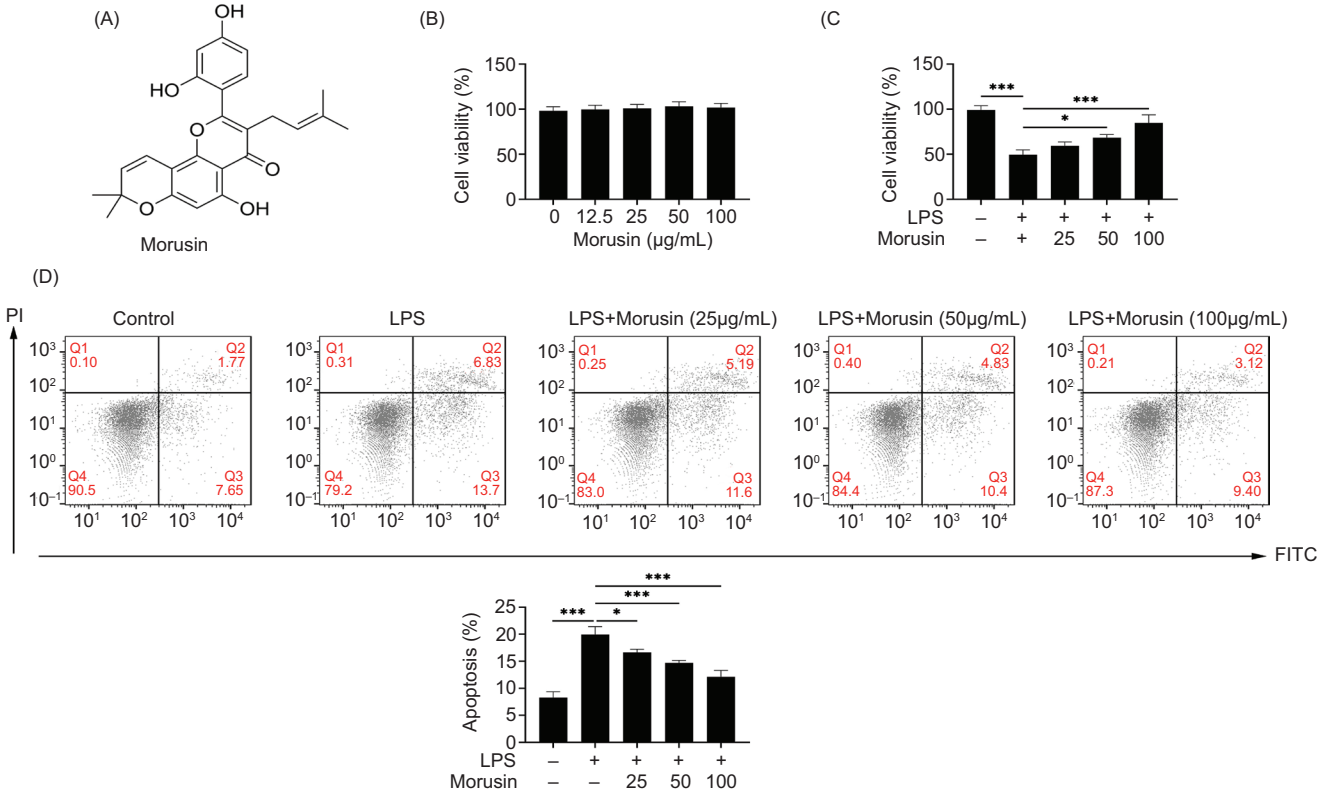

Figure 1 Morusin enhances cell viability and attenuates apoptosis in LPS-stimulated A549 cells. (A) The molecular structure of morusin. (B) Cell viability was assessed by CCK-8 assay in A549 cells treated with 0, 12.5, 25, 50, or 100 μg/mL morusin. (C) Cell viability was assessed by CCK-8 assay in the control, LPS, LPS+25 μg/mL morusin, LPS+50 μg/mL morusin, and LPS+100 μg/mL morusin groups. (D) Cell apoptosis was analyzed by flow cytometry in the control, LPS, LPS+25 μg/mL morusin, LPS+50 μg/mL morusin, and LPS+100 μg/mL morusin groups. *P < 0.05, ***P < 0.001.

morusin administration at 25, 50, and 100 μg/mL notably attenuated this increase (Figure 1D). These findings indicate that morusin enhances cell viability and reduces apoptosis in LPS-stimulated A549 cells.

Morusin alleviated inflammation in LPS-stimulated A549 cells

Following LPS stimulation, the levels of pro-inflammatory cytokines IL-1β, IL-6, and prostaglandin E2 (PGE2) were significantly elevated. However, morusin treatment at 25, 50, and 100 μg/mL effectively reduced the expression of these inflammatory mediators in a dose-dependent manner (Figures 2A-C). These results suggest that morusin can alleviate LPS-induced inflammatory responses in A549 cells.

Morusin mitigated oxidative stress in LPS-stimulated A549 cells

lipopolysaccharide stimulation significantly increased the production of ROS in A549 cells whereas morusin treatment significantly decreased ROS levels in a concentration-dependent manner (Figure 3A). Furthermore, LPS exposure elevated MDA levels while decreasing SOD and GSH levels, and these changes could be effectively reversed by morusin treatment, particularly at higher concentrations (Figure 3B). Overall, these findings demonstrate that morusin mitigates oxidative stress in LPS-stimulated A549 cells.

Morusin inhibited the NF-κB pathway and suppressed NLRP3 inflammasome activation in LPS-stimulated A549 cells

Western blot analysis revealed that LPS stimulation significantly increased the protein expression of p-p65, total p65, and IκBα. Morusin treatment markedly reduced the levels of these proteins in a dose-dependent manner (Figure 4A). Moreover, the protein expression levels of NLRP3, ASC, caspase-1, and IL-1β were upregulated following LPS exposure, but morusin treatment at 25, 50, and 100 μg/mL significantly suppressed their expressions (Figure 4B). Collectively, these results suggest that morusin inhibits the NF-κB signaling pathway and suppresses NLRP3 inflammasome activation in LPS-stimulated A549 cells.

Discussion

In recent years, increasing attention has been directed toward the application of herbal extracts in the treatment of inflammatory diseases. Morusin, a flavonoid derived from mulberry root bark, possesses notable anti-inflammatory and antioxidant properties and is reported as involved in the regulation of various disease processes.[11-15] However, its potential role in the progression of ALI has remained largely unexplored. In the present study, it was demonstrated that morusin enhanced cell viability and reduced apoptosis in LPS-stimulated A549 cells, suggesting a protective effect against LPS-induced cellular injury.

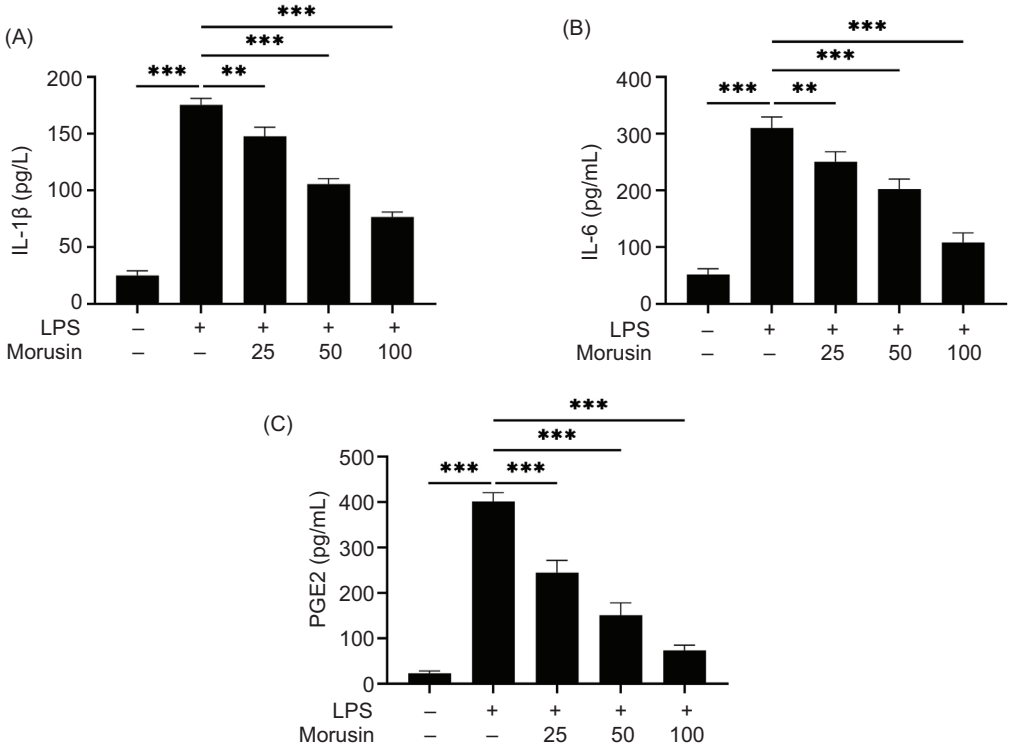

Figure 2 Morusin alleviates inflammation in LPS-stimulated A549 cells. A549 cells were divided into the control, LPS, LPS+25 μg/mL morusin, LPS+50 μg/mL morusin, and LPS+100 μg/mL morusin groups. The levels of (A) IL-1β, (B) IL-6, and (C) PGE2 were measured by ELISA. **P < 0.01, ***P < 0.001.

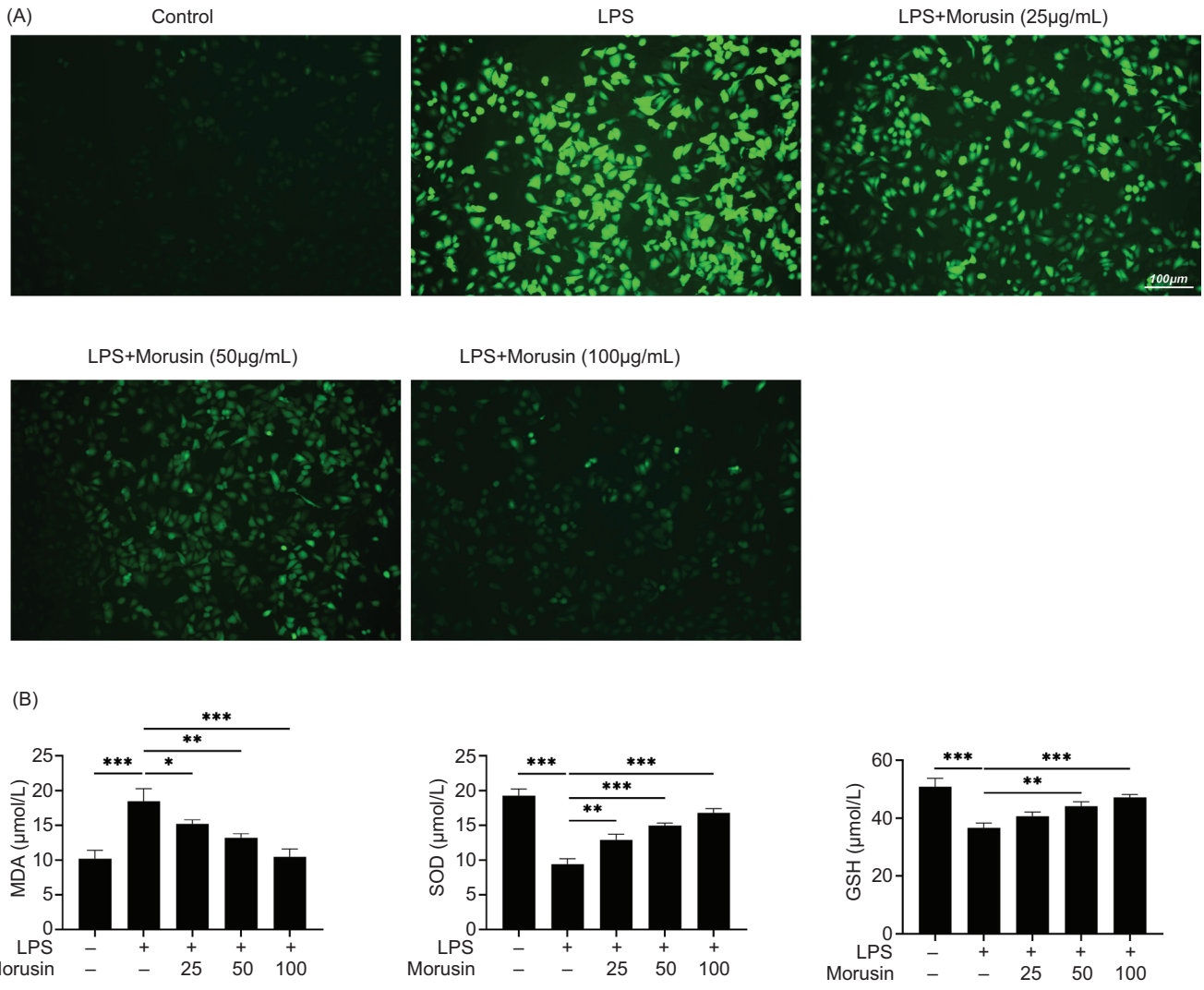

Figure 3 Morusin mitigates oxidative stress in LPS-stimulated A549 cells. A549 cells were divided into the control, LPS, LPS+25 μg/mL morusin, LPS+50 μg/mL morusin, and LPS+100 μg/mL morusin groups. (A) The intracellular ROS level was determined by DCFDA staining. (B) The levels of MDA, SOD, and GSH were measured using commercial assay kits. *P < 0.05, **P < 0.01, ***P < 0.001.

Inflammation is a key pathological feature of ALI, contributing to tissue damage and disease progression. Previous studies have highlighted the importance of targeting inflammatory responses to ameliorate ALI. For instance, forsythiaside A was shown to suppress inflammation and preserve epithelial barrier integrity by modulating the peroxisome proliferator-activated receptor gamma-retinoid X receptor alpha (PPAR-γ/RXR-α) complex.[16] Similarly, trelagliptin was found to reduce LPS-induced inflammation and oxidative stress,[17] while Broncho-Vaxom attenuated inflammatory responses in LPS-induced ALI models.[18] Baicalein also demonstrated anti-inflammatory effects by inhibiting glycolysis through regulation of the hypoxia-inducible factor 1-alpha (HIF-1α) signaling pathway.[19] Consistent with these findings, the present study showed that morusin significantly reduced the production of inflammatory mediators, including IL-1β, IL-6, and PGE2, in LPS-stimulated A549 cells, thereby alleviating the inflammatory response.

Oxidative stress, often resulting from excessive production of reactive ROS, plays a pivotal role in the pathogenesis of ALI. Hyperinflammation in ALI exacerbates oxidative stress, leading to further tissue injury.[20] Several agents are reported to attenuate oxidative stress and improve ALI outcomes. For example, Mucin1 is shown to inhibit oxidative stress and protect against ALI progression.[21] Additionally, lentinus edodes polysaccharides reduce oxidative stress and ameliorate ALI.[22] In addition, fibroblast growth factor 1 (FGF1) and oxaloacetic acid are reported to reduce oxidative stress and mitigate ALI induced by LPS and paraquat, respectively.[23,24] In agreement with these observations, the present study found that morusin significantly decreased ROS levels and modulated oxidative stress markers, indicating that its protective effects against ALI are partly mediated through the suppression of oxidative stress.

The NLRP3 inflammasome is a central component of innate immune response and is implicated in the amplification of inflammation during ALI.[25] Modulating the

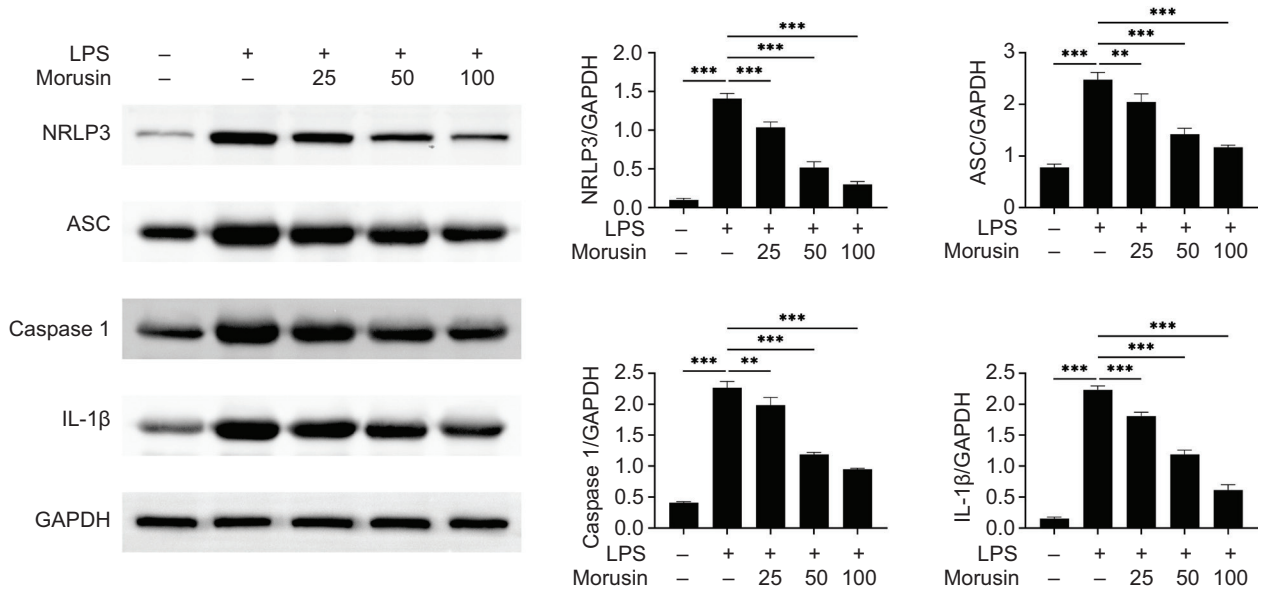

Figure 4 Morusin inhibits the NF-κB pathway and suppresses NLRP3 inflammasome activation in LPS-stimulated A549 cells. A549 cells were divided into the control, LPS, LPS+25 μg/mL morusin, LPS+50 μg/mL morusin, and LPS+100 μg/mL morusin groups. (A) The protein levels of p-p65, p65, and IκBα were analyzed by Western blot analysis. (B) The protein expression levels of NLRP3, ASC, caspase-1, and IL-1β were analyzed by Western blot analysis. *P < 0.05, **P < 0.01, ***P < 0.001.

activation of NLRP3 inflammasome is recognized as a potential therapeutic strategy. Notably, inhibition of the inositol-requiring transmembrane kinase/endoribonuclease 1α-X-box binding protein 1 (IRE1α/XBP1) axis suppresses thioredoxin-interacting protein (TXNIP)/NLRP3 inflammasome activation, thereby attenuating LPS-induced ALI.[26] However, the effects of morusin on NLRP3 inflammasome in ALI was not addressed previously. In this study, it was demonstrated that morusin inhibited the activation of NLRP3 inflammasome in LPS-stimulated A549 cells, providing further insight into its anti-inflammatory mechanism.

Limitations of this study must be acknowledged. The study was limited to *in vitro* experiments, and other phenotypic aspects, such as cell migration and epithelial barrier function, were not assessed. In addition, *in vivo* validation using appropriate animal models was not performed. This project was limited to cell experiments (*in vitro*) and no animal experiments or clinical verifications were conducted, which could limit the practical applicability of the findings.

Conclusion

This is the first study to demonstrate that morusin ameliorates LPS-induced inflammation and oxidative stress in lung epithelial cells by inhibiting the NF-κB signaling pathway and restraining the activation of NLRP3 inflammasome. Higher organization level models, such as human lung organoids, air-liquid interface models, spheroid, organoid, and lung-on-a-chip models, must be investigated in the future.[27,28] Future studies could further elucidate the regulatory functions of morusin in ALI by incorporating animal models and clinical samples to better define its therapeutic potential.

Ethical Approval

This study did not contain any experiments with human participants or animals performed by any of the authors.

Data Availability

The authors declare that all data supporting the findings of this study are available within the paper and any raw data can be obtained from the corresponding author upon request.

Author Contributions

Yan Li and Li Zhang designed the study and carried the same. Both authors supervised data collection, data analysis, data interpretation, manuscript preparation for publication, and reviewed draft of the manuscript. Both authors had read and approved the final manuscript.

Conflict of Interest

The authors had no conflict of interest to disclose.

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