JNK-IN-8 treatment alleviates lipopolysaccharide-induced acute lung injury via suppression of inflammation and oxidative stress regulated by JNK/NF-κB signaling

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Abstract. JNK serves critical roles in numerous types of inflammation- and oxidative stress-induced disease, including acute lung injury (ALI). JNK-IN-8 is the first irreversible JNK inhibitor that has been described. However, whether JNK-IN-8 can prevent lipopolysaccharide (LPS)-induced ALI by inhibiting JNK activation and its downstream signaling is poorly understood. The objective of the present study was to investigate the specific therapeutic effects of JNK-IN-8 on LPS-induced ALI and the molecular mechanisms involved. JNK-IN-8 attenuated myeloperoxidase activity, malondialdehyde and superoxide dismutase content and the lung wet/dry ratio, and improved the survival rate following lethal injection of LPS. Additionally, JNK-IN-8 decreased bronchoalveolar lavage fluid protein levels, lactate dehydrogenase activity, neutrophil infiltration and the number of macrophages (as demonstrated by flow cytometry), as well as the production of TNF-α, IL-6 and IL-1β (as evaluated via ELISA). In addition, reverse transcription-quantitative PCR and ELISA showed that JNK-IN-8 attenuated LPS-induced inflammatory cytokine production and oxidative stress in primary murine peritoneal macrophages and RAW264.7 cells *in vitro*. Furthermore, the present study demonstrated that the JNK/NF-κB signaling pathway was involved in the therapeutic effect of JNK-IN-8 against LPS-induced injury both *in vivo* and *in vitro*. In conclusion, these findings indicated that JNK-IN-8 had a therapeutic effect on LPS-induced ALI in mice. The mechanism may be associated with inhibition of the JNK/NF-κB signaling pathway. JNK-IN-8 may be a potential therapeutic agent for the treatment of ALI.

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Introduction

Acute lung injury (ALI) is a disease with high incidence and mortality rates and can cause acute respiratory distress syndrome (ARDS), which seriously threatens human health (1-3). A prospective epidemiological study in 1999-2000 estimated an annual incidence of ALI and ARDS of 190,000 adult patients in the United States of America (1). As a critical disease, ALI has attracted increasing attention in clinical practice. The primary manifestations of ALI are alveolar capillary system damage, increased pulmonary vascular permeability, overactivation of macrophages and neutrophils, excessive inflammatory response and increased reactive oxygen species (ROS) production, which eventually lead to respiratory function damage (4). Previous evidence has revealed that inflammation and oxidative stress are associated with ALI (5).

The mechanisms of inflammation and oxidative stress work in different ways. MAPKs serve as typical inflammation-associated signals and comprise ERK-1/2, p38 and JNK (6). JNK is primarily involved in inflammation and oxidative stress (6). JNK mediates activation of NF-κB signaling, the main upstream regulator of proinflammatory cytokines in ALI (7). Activated NF-κB/p65 translocates to the nucleus to regulate a number of inflammatory cytokines (8,9) and promote oxidative stress (10). Previous studies have demonstrated that, following paraquat poisoning, JNK is activated in the lung, and the JNK inhibitor SP600125 can alleviate ALI caused by paraquat (11,12). Another JNK inhibitor, JNK-IN-8 (13), has an inhibitory effect on the regulation of NF-κB signaling activation by JNK, and can inhibit inflammation and improve neurological function in ischemic brain injury (14). These studies (6-14) suggest that JNK inhibitors serve a major role in the molecular pathology of numerous types of disease. Nevertheless, to the best of our knowledge, the exact role of JNK-IN-8 in inflammation and oxidative stress associated with ALI is not clear.

JNK-IN-8, as an effective and specific JNK inhibitor, has a notable inhibitory effect on the JNK/NF-κB signaling pathway (14,15) which is associated with inflammation and oxidative stress in ALI. Therefore, its impacts may extend to ALI. However, to the best of our knowledge, the

in vitro and *in vivo* effects of JNK-IN-8 on lipopolysaccharide (LPS)-induced ALI have not been reported. Therefore, the aim of the present study was to investigate the protective effects of JNK-IN-8 in LPS-induced ALI, and the association between its molecular mechanism and inflammation and oxidative stress.

Materials and methods

Reagents and antibodies. JNK-IN-8 was purchased from MedChemExpress. Mouse TNF-α (cat. no. CSB-E04741m), IL-6 (cat. no. CSB-E04639m) and IL-1β (cat. no. CSB-E08054m) ELISA kits were purchased from Cusabio Technology LLC. Alexa Fluor® 488 anti-mouse Ly-6G (cat. no. 127625) and 594 anti-mouse F4/80 antibody (cat. no. 123140) were purchased from BioLegend, Inc. Specific primary antibodies against NF-κB p65 (cat. no. 8242), phosphorylated (p)-p65 (Ser536; cat. no. 3303), JNK (cat. no. 9252), p-JNK (Thr183/Tyr185; cat. no. 4668) and β-actin (cat. no. 3700) were purchased from Cell Signaling Technology, Inc.

Animals. A total of 75 animals (age, 8 weeks) received humane care in 2019, according to the Guide for the Care and Use of Laboratory Animals (16). Male specific pathogen-free grade C57BL/6 mice (weight, 18-22 g) were provided by Shanghai SLAC Laboratory Animal Co., Ltd., and were housed (5 mice/individually vented cage) using a 12-h light/dark cycle under optimal temperature (20-26˚C) and humidity (40-70%). Mice were provided with free access to food and water. Prior to the experiment, the mice were habituated to the environment for ≥1 weeks. The specific criteria of humane endpoints were selected according to the Guide for the Care and Use of Laboratory Animals: Eighth Edition (17). The humane endpoints included dying (indicated by weak response to leg clamping, very faint heartbeat, irregular breathing, depression and hypothermia), loss of appetite (>24 h) and weakness (unable to feed, drink or stand). Euthanasia was performed according to the American Veterinary Medical Association Guidelines for the Euthanasia of Animals: 2013 Edition (18). The displacement rate used for CO_2 inhalation euthanasia was 20% to avoid distress. Standard evidence of death included dilated pupils and absence of heartbeat, as well as failure to respond to a toe pinch or touch of the eye. Cervical dislocation was performed following euthanasia to ensure death. All animal experiments were approved by the Ethics Committee of Zhejiang University (approval no. ZJU20170913; Hangzhou, China).

Establishment of the ALI mouse model. The ALI mouse model was established by intratracheal administration of LPS (20 μg/50 μl PBS). A total of 24 mice were divided into four groups at random (n=6/group): Sham operation (equivalent PBS via intraperitoneal injection), JNK-IN-8, LPS and LPS + JNK-IN-8 group. Mice in the JNK-IN-8 and LPS + JNK-IN-8 groups received JNK-IN-8 (10 mg/kg) via intraperitoneal injection. After 1 h, mice in the LPS + JNK-IN-8 group also received LPS treatment. LPS (0.4 mg/kg; Sigma-Aldrich; Merck KGaA) was injected into the mice in the LPS and LPS + JNK-IN-8 groups via the trachea following anesthetization with sevoflurane (induction, 5%; maintenance, 2.5%), as previously described (18). The physical condition of animals was monitored every 2 h and sacrificed at 6 h after LPS injection by CO_2 inhalation. Subsequently, bronchoalveolar lavage fluid (BALF) and lung tissues were collected and stored at -80˚C until use. In the ALI model, no mice died during the experiment. In the survival experiment, 48 mice were divided into four groups at random (n=12/group): Sham operation (equivalent PBS via intraperitoneal injection), JNK-IN-8, LPS and LPS + JNK-IN-8 group. Mice in the JNK-IN-8 and LPS + JNK-IN-8 groups received JNK-IN-8 (10 mg/kg) via intraperitoneal injection. After 1 h, mice in the LPS and LPS + JNK-IN-8 groups were given a lethal dose of LPS (20 mg/kg). Animals were monitored every 6 h and the survival rate was recorded every 12 h for 72 h, as previously described (19). In the survival experiment, 15 mice died due to LPS-induced ALI and two were euthanized upon reaching a humane endpoint of dying. All surviving mice were euthanized by CO_2 inhalation followed by cervical dislocation after the experiment.

Inflammatory cell count in BALF. C57BL/6 mice used for the collection of BALF were euthanized by CO_2 inhalation, after which an intermediate incision was made to open the chest to expose the trachea, as previously described (20). The alveoli were washed twice with 0.5 ml PBS during endotracheal intubation to collect 0.8 ml BALF. The numbers of inflammatory cells (specifically, total living cells, $ly6g^+$ neutrophils and $F4/80^+$ macrophages) were counted using flow cytometry (BD Biosciences). Cells in BALF (500 μl) were stained with ly6g for neutrophils and F4/80 for macrophages, as previously described (21,22). Subsequently, flow cytometry was used to count the cells. The $ly6g^+$ and $F4/80^-$ cells were considered to be 'neutrophils'. The $ly6g^-$ and $F4/80^+$ cells were considered to be 'macrophages'. The $ly6g^-$ and $F4/80^-$ cells were considered to be other cells. The sum of the three types of cells was the number of 'total living cells'. Gates were artificially set according to the cell community location. Finally, the data were analyzed using FlowJo software (version 10; Tree Star, Inc.).

Wet/dry (W/D) weight ratio of lung tissue in the ALI model. Following euthanasia, the right lung tissue was collected and the wet weight was recorded. Subsequently, the lung tissue was covered with foil and placed in an incubator at 80˚C until the weight remained stable to obtain the dry weight. Finally, the W/D ratio of the lung tissues was calculated to evaluate the degree of pulmonary edema.

Histology evaluation. At 6 h after LPS injection, the thoraces of the mice in the ALI model were opened under deep anesthesia, and the mice were perfused with 50 ml 0.01 M PBS (pH, 7.4) followed by 4% paraformaldehyde in 0.1 M PBS (pH, 7.4) through the ascending aorta. Subsequently, the left upper lung lobe was placed in 4% paraformaldehyde, dehydrated with ethanol and embedded in paraffin. Lung tissues were cut into slices (thickness, 4-5 μm) and then subjected to hematoxylin and eosin and periodic acid Schiff staining. Pathological changes were observed under an optical light microscope (magnification, x200) and two experienced pathologists blindly scored the changes, as previously described (23): 0, normal tissue; 1, minimal inflammatory change; 2, no obvious damage

to the lung architecture; 3, thickening of the alveolar septae; 4, formation of nodules or areas of pneumonitis that distorted the normal architecture; and 5, total obliteration of the field.

ELISA. Expression levels of cytokines, including IL-1β, IL-6 and TNF-α, in BALF, lung tissue and cell supernatant were detected using ELISA kits according to the manufacturer's protocols.

Cell culture. Mouse macrophage (RAW264.7) cells were purchased from the American Type Culture Collection. RAW264.7 cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) with 5% CO_2 and 37˚C. Injecting thioglycolate into the abdominal cavity of mice creates a sterile inflammatory environment in which macrophages accumulate and can be obtained by lavage of the abdominal cavity (8). A total of three 8-week-old male C57BL/6 mice were injected intraperitoneally with 3 ml 3% thioglycolate (Sigma-Aldrich; Merck KGaA), as previously described (24). After 3 days, the mice were sacrificed by CO_2 inhalation followed by cervical dislocation. The abdomen was soaked with 75% alcohol for 5 min at room temperature, then a small incision along the midline was made with sterile scissors. Subsequently, 10 ml cold Dulbecco's PBS was injected into the peritoneal cavity. The lavages were collected by centrifugation at 1,500 x g for 10 min at room temperature and resuspended in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.). Cells were then incubated at 37˚C for 6 h and washed with PBS to remove the non-adherent cells. The remaining adherent cells were used as the peritoneal macrophages in subsequent experiments. In order to confirm these isolated cells to be macrophages, LPS-induced M1 polarization experiments were performed. For macrophages, including TNF-α, IL-6 and IL-1β, these M1 polarization indexes increase following LPS stimulation (3). In order to investigate the effect of JNK-IN-8 on the inflammatory response of macrophages, cells were pretreated with JNK-IN-8 at 5% CO_2 and 37˚C for 1 h, followed by treatment with LPS (100 ng/ml) at 5% CO_2 and 37˚C for 6 h.

Cell viability. Following treatment, cell viability was assessed using a Cell Counting Kit-8 (CCK-8) cell proliferation/cytotoxicity assay kit (Beyotime Institute of Biotechnology). Primary macrophages and RAW 264.7 cells were seeded into 96-well plates at a density of $2x10^3$ cells/well at 5% CO_2 and 37˚C for 24 h. Cells were then treated with increasing concentrations of JNK-IN-8 (0.00, 0.39, 0.78, 1.56, 3.13, 6.25, 12.50, 25.00, 50.00 and 100.00 μM) at 5% CO_2 and 37˚C for 24 h. At the end of the experimental period, cells were incubated with CCK-8 reagent and absorbance at a wavelength of 450 nm was measured using an ELX800 absorbance microplate reader. The experiments were performed in triplicate.

Calculation of biochemical parameters. Lung samples were homogenized and dissolved in extraction buffer from commercially available assay kits (Beijing Solarbio Science & Technology Co., Ltd.) to measure lactate dehydrogenase (LDH; cat. no. BC0685), malondialdehyde (MDA; cat. no. BC0025), glutathione (GSH; cat. no. BC1175), superoxide dismutase (SOD; cat. no. BC0170) and myeloperoxidase (MPO; cat. no. BC0073) levels, according to the manufacturer's protocols.

Reverse transcription-quantitative (RT-q)PCR assay. Total RNA from cells/tissue was extracted using the RNeasy Mini kit (Qiagen). After evaluating the quality of the RNA based on the A260/A280 ratio, cDNA was synthesized using 1 mg RNA from each sample, 2 ml 5X PrimeScript RT Master Mix (Takara Bio, Inc.), and 4 ml RNase-free distilled water in a total volume of 10 ml. RT-qPCR was performed using an ABI Prism 7500 system (Applied Biosystems; Thermo Fisher Scientific, Inc.) with SYBR Green QPCR Master Mix (Takara Bio, Inc.). The total volume (20 μl) of each PCR reaction consisted of 10 μl SYBR-Green QPCR Master Mix, 6 μl ddH2O, 2 μl cDNA and 10 μM each of forward and reverse primers. RT-qPCR was performed using the following thermocycling conditions: 95˚C for 10 min, followed by 40 cycles of 95˚C for 10 sec, 60˚C for 20 sec and 72˚C for 20 sec, and a final extension at 72˚C for 1 min. GAPDH served as the internal control. The murine primer sequences were as follows: GAPDH forward, 5'-ACCCAGAAGACTGTGGATGG-3' and reverse, 5'-CACATTGGGGGTAGGAACAC-3'; TNF-α forward, 5'-CGGGCAGGTCTACTTTGGAG-3' and reverse, 5'-ACCCTGAGCCATAATCCCCT-3'; IL-6 forward, 5'-AACGATGATGCACTTGCAGA-3' and reverse, 5'-TGTGACTCCAGCTTATCTCTTGG-3'; and IL-1β forward, 5'-TGCCACCTTTTGACAGTGATG-3' and reverse, 5'-CAAAGGTTTGGAAGCAGCCC-3'. The experiments were performed in triplicate.

Western blot analysis. Nucleoprotein and total protein were extracted from lung tissue or cells using RIPA reagent (Beijing Solarbio Science & Technology Co., Ltd.). A BCA protein assay kit (Thermo Fisher Scientific, Inc.) was used to determine the protein concentration of samples from lysed lung tissues or cells. Each well of a 10% SDS-PAGE gel was loaded with an equal volume of protein, and the proteins were separated by SDS-PAGE, after which they were transferred to a PVDF membrane. After incubation with 5% milk at room temperature for 1 h, the membrane was incubated with primary antibodies (all 1:1,000; all Cell Signaling Technology, Inc.), including anti-NF-κB p65 (cat. no. 8242), anti-p-NF-κB p65 at Ser536 (cat. no. 3033), anti-β-actin (cat. no. 3700) and anti-Lamin B (cat. no. 12255), at 4˚C overnight. Then, the membrane was incubated with corresponding secondary antibodies (1:2,000; cat. no. 6990; Cell Signaling Technology, Inc.) at room temperature for 1 h. The protein bands were visualized using an ECL western blot kit (Bio-Rad Laboratories, Inc.) and visualized on a ChemiDoc XRS System (Bio-Rad Laboratories, Inc.). Protein expression was calculated by measuring the optical density using Image Lab software (version 3.0, Bio-Rad Laboratories, Inc.). Target protein expression levels were normalized to those of β-actin.

Statistical analysis. Data are presented as the mean ± SD of three independent repeats. SPSS 16.0 software (SPSS, Inc.) was used for statistical analysis. Figures were created using Prism 5.0 software (GraphPad Software, Inc.). Survival data were analyzed with Kaplan-Meier plots and log-rank tests. Differences among the experimental groups were compared

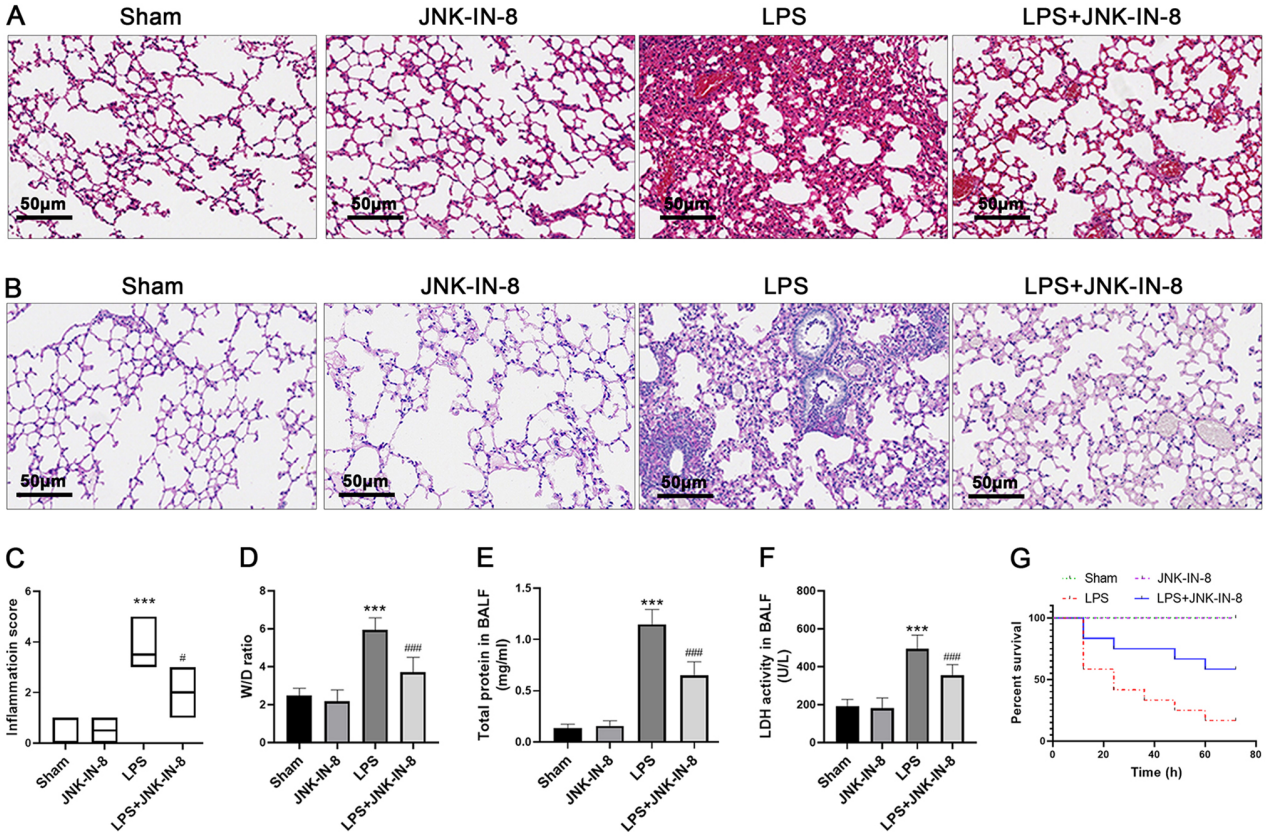

Figure 1. JNK-IN-8 alleviates LPS-induced ALI in mice. Mice with LPS-induced ALI were pretreated with JNK-IN-8. (A) Hematoxylin and eosin and (B) Periodic acid Schiff staining, and (C) lung inflammation score were detected to evaluate the histopathological changes of the lung (n=6). Scale bar, 50 μm. (D) W/D ratio of lung tissue was measured. (E) Total protein and (F) LDH activity in BALF were measured (n=6). (G) Survival of mice given a lethal dose of LPS was monitored every 12 for 72 h (n=12). Data are presented as the mean ± SD. The inflammation score was analyzed using the Kruskal-Wallis test followed Dunn's post hoc test. ***P<0.001 vs. Sham; $^{\#}$P<0.05 and $^{\#\#\#}$P<0.001 vs. LPS. LPS, lipopolysaccharide; ALI, acute lung injury; W/D, wet/dry; LDH, lactate dehydrogenase; BALF, bronchoalveolar lavage fluid.

by one-way ANOVA and then analyzed by Tukey's multiple comparison test. The inflammation score was analyzed using a Kruskal-Wallis test followed by Dunn's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

JNK-IN-8 decreases lung injury induced by LPS in mice. Compared with the Sham and JNK-IN-8 groups, LPS caused marked pathological changes by increasing the accumulation of inflammatory cell damage in alveolar tissue (Fig. 1A and B). JNK-IN-8 treatment alleviated severe histopathological changes caused by LPS, including inflammatory cell infiltration, tissue destruction and glycogen deposition. In accordance with this finding, the lung injury score of the LPS + JNK-IN-8 treatment group was significantly lower than that of the LPS group (Fig. 1C). The W/D ratio of lung tissue and total protein levels in BALF (which represent the severity of pulmonary edema) were significantly higher in the LPS group than in the Sham and JNK-IN-8 groups, whereas pretreatment with JNK-IN-8 significantly decreased the W/D ratio and total protein levels (Fig. 1D and E). Furthermore, JNK-IN-8 treatment decreased LDH activity in the BALF of LPS-challenged mice (Fig. 1F). Pretreatment with JNK-IN-8 improved the survival rate within 72 h of lethal injection of LPS (Fig. 1G). These findings suggest that JNK-IN-8 alleviated pathological damage of the lungs induced by LPS.

JNK-IN-8 decreases the inflammatory response and oxidative stress in the lungs of mice treated with LPS. Subsequently, the role of JNK-IN-8 in the inflammatory response and oxidative stress in the lungs of mice challenged with LPS was determined. The mRNA expression levels of TNF-α, IL-6 and IL-1β in lung tissue were significantly decreased by JNK-IN-8 pretreatment (Fig. 2A), and this also decreased the serum levels of TNF-α, IL-6 and IL-1β (Fig. 2B). In addition, JNK-IN-8 treatment effectively inhibited the increase in MPO activity and MDA generation caused by LPS (Fig. 2C). It also significantly decreased LPS-induced SOD depletion (Fig. 2C), suggesting that JNK-IN-8 treatment decreased the inflammatory response and oxidative stress in mice with LPS-induced ALI.

Pretreatment with JNK-IN-8 significantly decreased the levels of TNF-α, IL-6 and IL-1β in BALF compared with those in the LPS group (Fig. 3A). Compared with the Sham group, the numbers of total cells, neutrophils and macrophages in BALF were increased following LPS stimulation, and this change was inhibited by pretreatment with JNK-IN-8 (Fig. 3B and C).

JNK-IN-8 decreases LPS-induced inflammatory cytokine production and oxidative stress in primary murine peritoneal macrophages and RAW264.7 cells in vitro. Before assessing

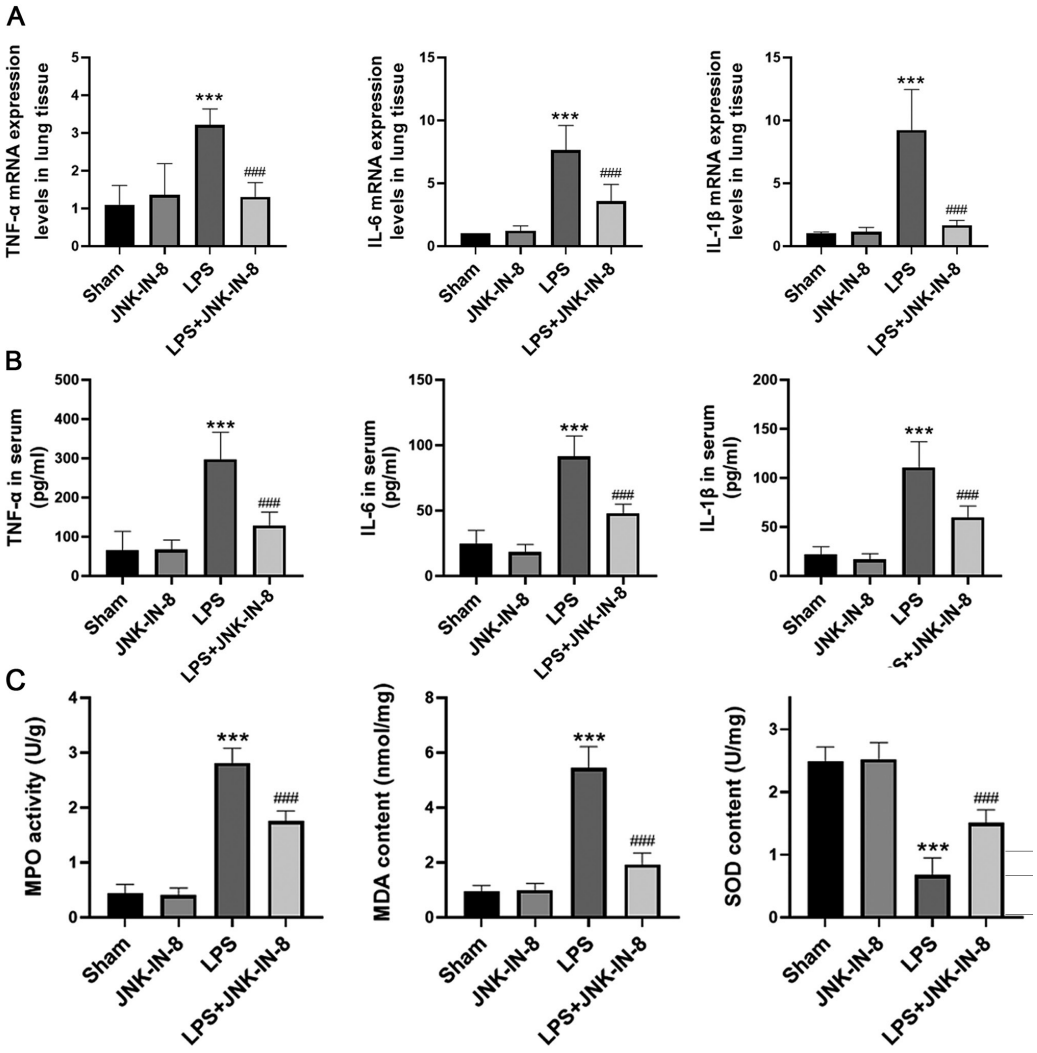

Figure 2. JNK-IN-8 decreases inflammatory responses and oxidative stress in ALI mice. Mice with LPS-induced ALI were pretreated with JNK-IN-8. TNF-α, IL-6 and IL-1β (A) mRNA levels in lung tissue samples, and (B) protein levels in serum were determined by reverse transcription-quantitative PCR and ELISA, respectively. (C) MPO activity, SOD and MDA content, in lung tissue samples were examined. Data are presented as the mean ± SD (n=6). ***P<0.001 vs. Sham; ###P<0.001 vs. LPS. ALI, acute lung injury; LPS, lipopolysaccharide; MPO, myeloperoxidase; SOD, superoxide dismutase; MDA, malondialdehyde.

the effects of JNK-IN-8 on primary murine peritoneal macrophages, the cytotoxic effect of JNK-IN-8 on cells was assessed using a CCK-8 assay (Fig. 4A). No significant cytotoxic effects of JNK-IN-8 were observed at concentrations of ≤12.50 μM in primary macrophages (Fig. 4A). Based on these data, a maximal concentration of 10 μM was selected to analyze the effects of JNK-IN-8 in primary macrophages.

In order to investigate the anti-inflammatory effect of JNK-IN-8, primary macrophages were pretreated with JNK-IN-8 for 1 h and then stimulated with LPS (100 ng/ml) for 6 h. mRNA expression levels and secretion of TNF-α, IL-6 and IL-1β in the LPS group were significantly increased compared with those in the Sham group, but these were decreased in the LPS + JNK-IN-8 group compared with the LPS group (Fig. 4B and C). Subsequently, the role of JNK-IN-8 in oxidative stress was investigated. JNK-IN-8 pretreatment significantly decreased MDA content and inhibited the LPS-induced decrease in SOD activity (Fig. 4D) in primary macrophages.

The effects of JNK-IN-8 on the macrophage cell line RAW264.7 were assessed. In the CCK-8 assay, no cytotoxic effects of JNK-IN-8 were observed at concentrations ≤12.50 μM in RAW264.7 cells (Fig. 5A). RAW264.7 cells were cultured and treated with JNK-IN-8 *in vitro*. The trend was the same as that of primary macrophages. RAW264.7 cells were pretreated with JNK-IN-8 for 1 h and then stimulated with LPS (100 ng/ml) for 6 h. The gene expression levels and secretion of TNF-α, IL-6 and IL-1β were decreased by JNK-IN-8 pretreatment compared with those in the LPS group (Fig. 5B and C). JNK-IN-8 administration significantly decreased the MDA content and inhibited the LPS-induced decrease in SOD activity (Fig. 5D).

JNK-IN-8 regulates JNK and NF-κB activation to affect inflammation and oxidative stress in LPS-treated cells. In order to investigate the mechanism by which JNK-IN-8 inhibits inflammation, its effects on the JNK/NF-κB signaling pathway were analyzed. A significant increase was observed in the protein levels of p-JNK, as well as p-NF-κB p65, in LPS-stimulated lung tissues, and JNK-IN-8 treatment significantly inhibited this increase (Fig. 6A and B). JNK-IN-8 also decreased the phosphorylation of JNK and NF-κB p65, which was stimulated by LPS in a dose- and time-dependent manner in primary macrophages *in vivo* (Fig. 6C-F). Further

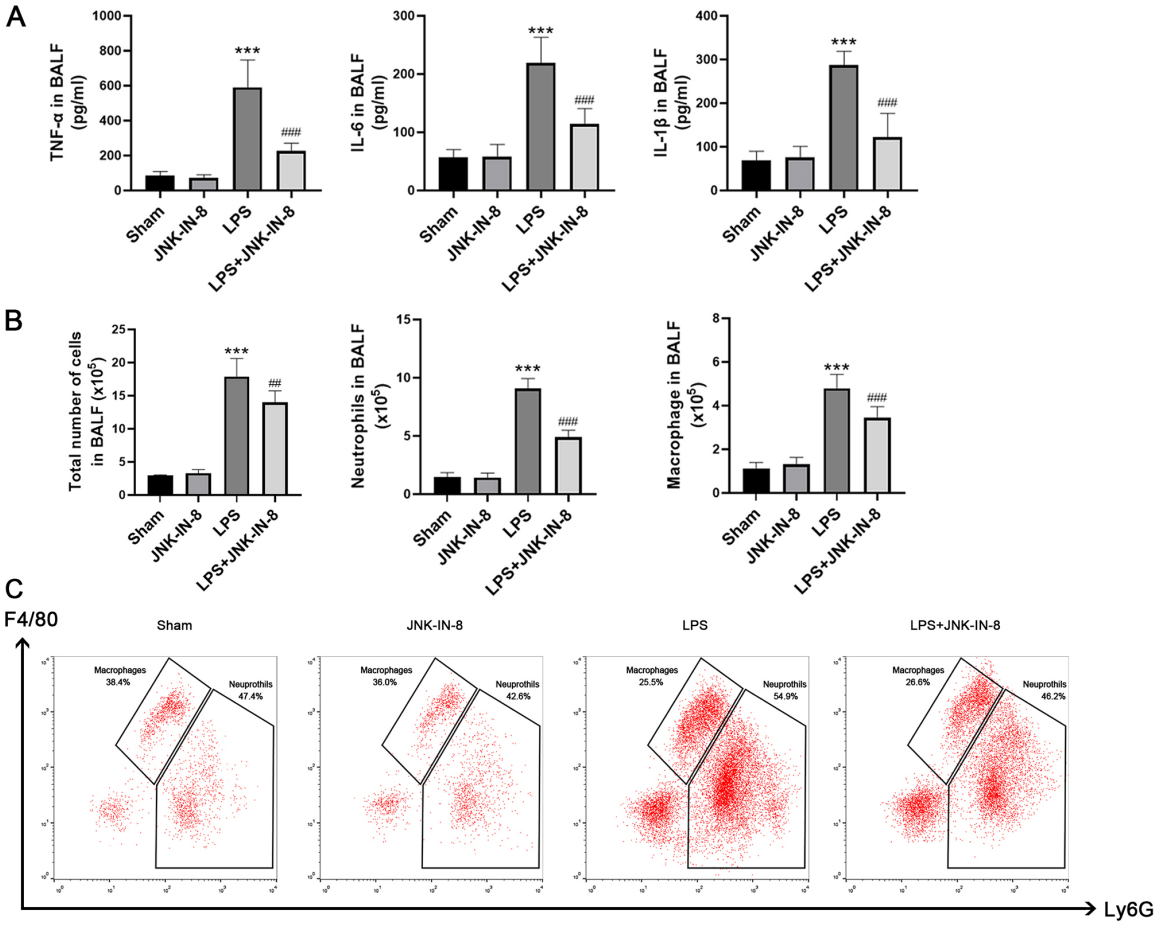

Figure 3. JNK-IN-8 decreases the inflammatory response in BALF. Mice with LPS-induced acute lung injury were pretreated with JNK-IN-8. (A) TNF-α, IL-6 and IL-1β protein levels (assessed via ELISA) and (B) total cells, neutrophils and macrophages in BALF were determined. (C) Representative images of flow cytometry dot plots. Data are presented as the mean ± SD (n=6). ***P<0.001 vs. Sham; $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. LPS. BALF, bronchoalveolar lavage fluid; LPS, lipopolysaccharide.

experiments revealed that JNK-IN-8 treatment exhibited a significant inhibitory effect on the nuclear translocation of p65 induced by LPS (Fig. 6G and H). In conclusion, these data provided evidence that JNK-IN-8 decreased LPS-induced injury via the JNK/NF-κB signaling pathway.

Discussion

Excessive inflammation and oxidative stress are key factors that regulate the development of ALI. The expression levels of proinflammatory regulators can be enhanced by oxidative stress (5). Furthermore, inflammatory cells induce the overproduction of ROS in a similar manner, thus forming a cycle to promote the occurrence and development of ALI (25-27). JNK-IN-8 was used in the present study to demonstrate that inhibition of JNK may be a potential therapy for the treatment of ALI. The present *in vivo* experiments revealed that inhibition of JNK by JNK-IN-8 attenuated ALI in an LPS-induced mouse model. Furthermore, *in vitro* experiments demonstrated that JNK-IN-8 inhibited secretion of inflammatory factors and oxidative stress in LPS-stimulated mouse peritoneal macrophages, as well as RAW264.7 cells, by inhibiting JNK/NF-κB signaling. Therefore, it was concluded that JNK-IN-8 inhibited the activation of JNK, as well as NK-κB, both *in vivo* and *in vitro*. This indicated that JNK contributed to the regulation of inflammation and oxidative stress. Furthermore, this could offer a novel strategy for treating ALI.

LPS is a well-known agent for inducing ALI (28). LPS stimulates macrophage activation and inflammatory cell infiltration via the JNK and NF-κB signaling pathways, causing uncontrolled release of inflammatory cytokines and oxidative stress (29-30). JNK signaling has been reported to be associated with inflammation and oxidative stress. As a result, JNK is a promising candidate for novel therapeutic targets of inflammation and oxidative stress (6). In inflammatory diseases, a number of JNK-associated synthetic inhibitors, such as the micromolecules SP600125 (31) and CC-930 (32), have been reported. Shen *et al* (12) reported that SP600125 treatment suppresses the JNK/AP-1 signaling pathway to decrease oxidative stress and the inflammatory response, thus decreasing the apoptosis of A549 cells. Notably, different JNK inhibitors exhibit numerous physiological characteristics due to the different types of JNKs (11-14). Therefore, further identification of selective JNK inhibitors is a key goal.

JNK-IN-8, the first irreversible JNK inhibitor to be described, suppresses IL-1β-stimulated c-Jun phosphorylation in IL-1R cells (13). A recent study revealed that JNK-IN-8 inhibits neuroinflammation in ischemic brain injury by inhibiting JNK/NF-κB (14). In the present study, JNK-IN-8 also effectively inhibited JNK/NF-κB signaling to suppress IL-1β,

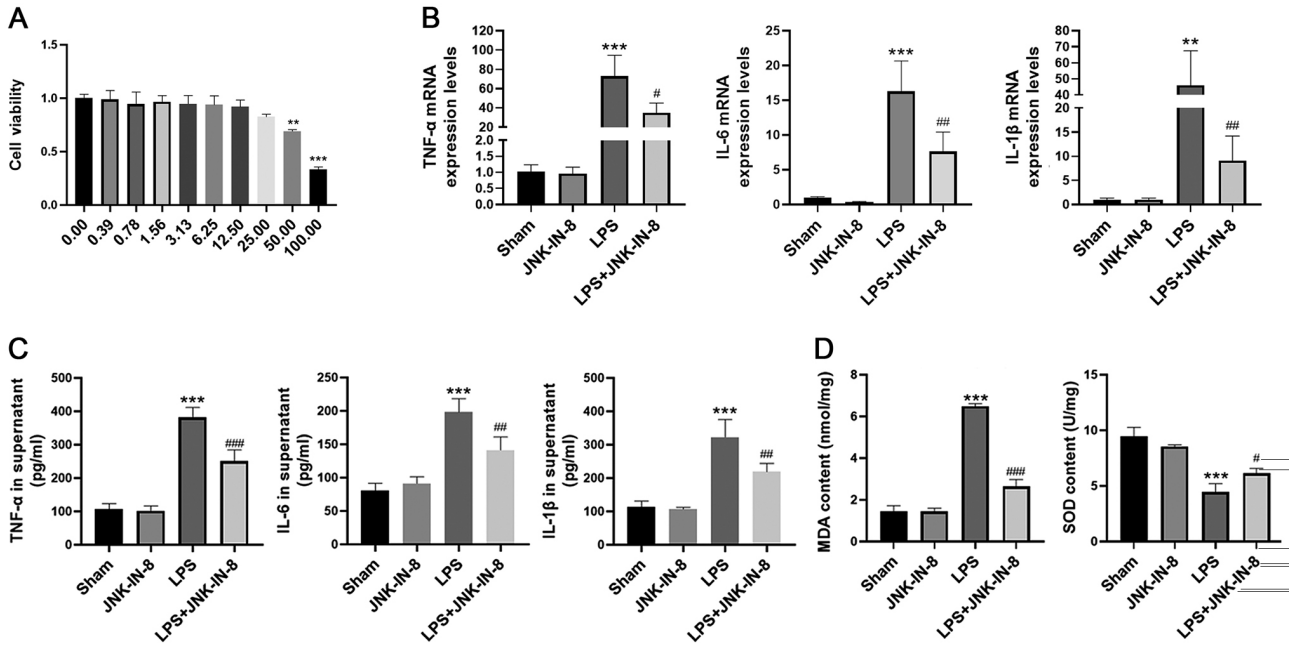

Figure 4. JNK-IN-8 attenuates LPS-induced inflammatory cytokine production and oxidative stress in primary murine peritoneal macrophages *in vitro*. (A) Cytotoxic effects of JNK-IN-8 on primary macrophages were assessed using a Cell Counting Kit-8 cell viability assay at 24 h. TNF-α, IL-6 and IL-1β (B) mRNA levels in primary macrophages and (C) protein expression levels in the supernatant of primary macrophages were determined via reverse transcription-quantitative PCR and ELISA, respectively. (D) MDA activity and MPO content of primary macrophages were determined. Data are presented as the mean ± SD (n=3). **P<0.01 and ***P<0.001 vs. Sham or 0.00; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. LPS. LPS, lipopolysaccharide; MDA, malondialdehyde; MPO, myeloperoxidase; SOD, superoxide dismutase.

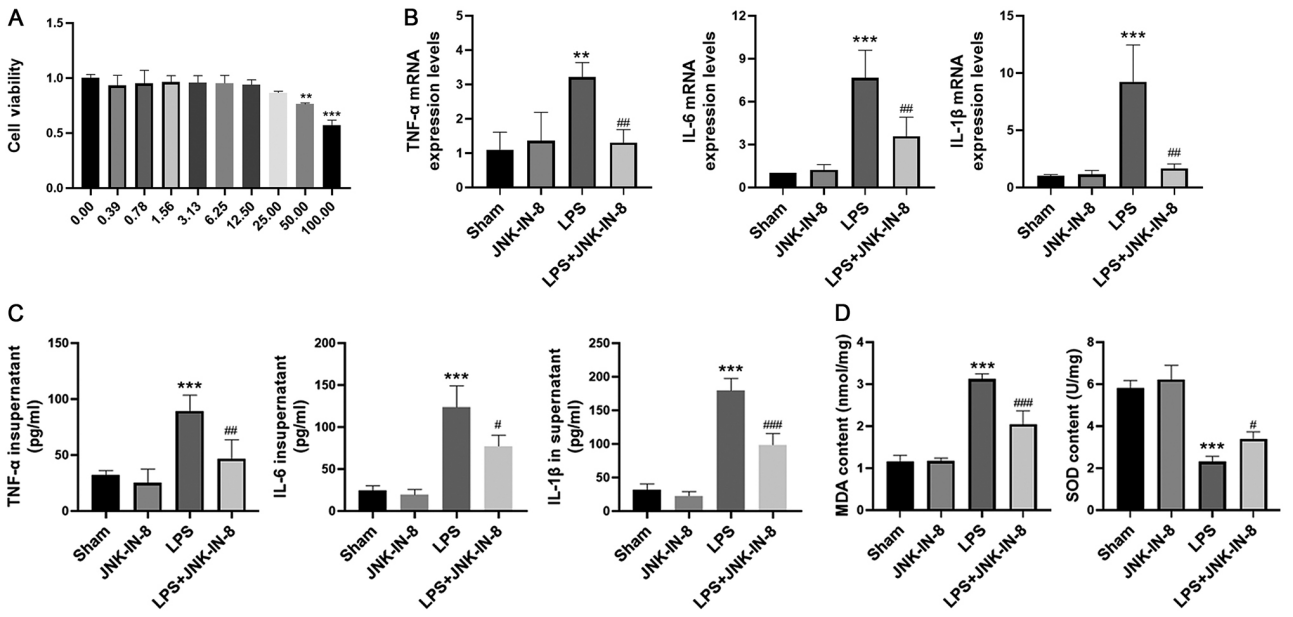

Figure 5. JNK-IN-8 attenuates LPS-induced inflammatory cytokine production and oxidative stress in RAW264.7 cells *in vitro*. (A) Cytotoxic effects of JNK-IN-8 on RAW264.7 cells were assessed using a Cell Counting Kit-8 cell viability assay at 24 h. TNF-α, IL-6 and IL-1β (B) mRNA levels in RAW264.7 cells and (C) protein expression levels in the supernatant of RAW264.7 cells were determined by reverse transcription-quantitative PCR and ELISA, respectively. (D) MDA and SOD activity of RAW264.7 cells were determined. Data are presented as the mean ± SD (n=3). **P<0.01 and ***P<0.001 vs. Sham or 0.00; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. LPS. LPS, lipopolysaccharide; MDA, malondialdehyde; SOD, superoxide dismutase.

TNF-α and IL-6 expression levels and release in primary peritoneal macrophages following LPS treatment. These data demonstrated that the JNK/NF-κB signaling pathway serves an important role in inflammatory disease. In addition, the inhibition of JNK-mediated NF-κB activation by JNK-IN-8 may be a potential target for treatment of inflammatory diseases.

Excessive oxidative stress is involved in the pathogenesis of ALI (29). When lung tissue is damaged in ALI, levels of MDA, a marker of lipid peroxidation, increase, whereas those of SOD, an antioxidant enzyme, decrease (26). The present results demonstrated that LPS increased the MDA content and decreased the activity of SOD in lung tissues and primary peritoneal

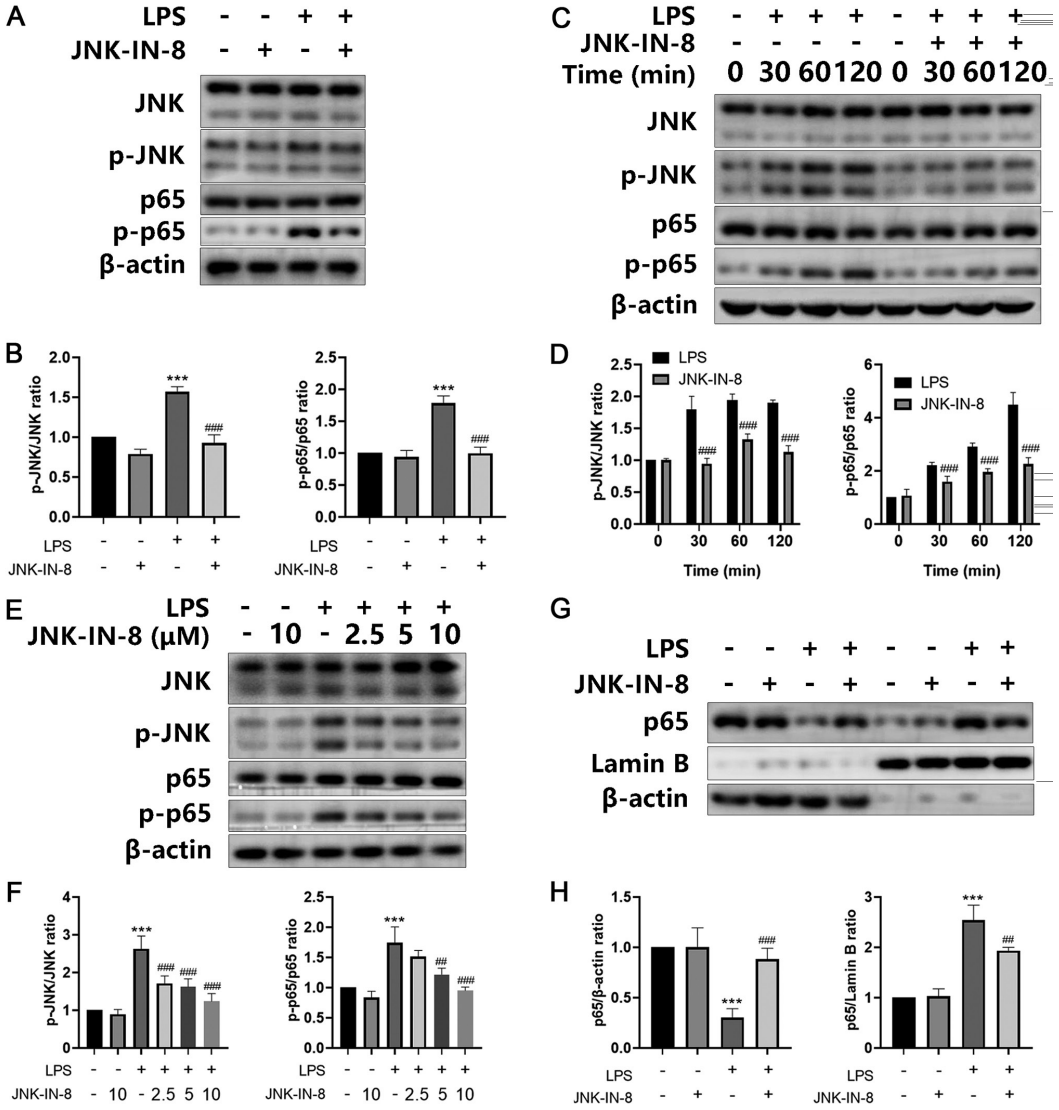

Figure 6. JNK-IN-8 suppresses the JNK/NF-κB signaling pathway in LPS-induced acute lung injury *in vivo* and *in vitro*. (A) Primary macrophages stimulated by LPS and JNK-IN-8 (10 mM) for 2 h were extracted and subjected to western blot analysis. The protein expression levels of JNK, p-JNK, p65 and p-p65 were assessed and (B) analyzed by densitometry. (C) Primary macrophages, stimulated with LPS in the presence or absence of JNK-IN-8 (10 mM) for 0, 30, 60 or 120 min, were extracted and subjected to western blot analysis. The protein expression levels of JNK, p-JNK, p65 and p-p65 were assessed and (D) analyzed by densitometry. (E) Protein expression levels of JNK, p-JNK, p65 and p-p65 in lung tissue were assessed and (F) analyzed by densitometry. β-actin was used as the internal loading control. (G) Primary macrophages were stimulated with LPS in the presence or absence of JNK-IN-8 (10 mM) for 2 h. Cytoplasmic and nuclear NF-κB p65 protein expression levels were determined and (H) analyzed by densitometry. β-actin and Lamin B were used as internal loading controls. Data are presented as the mean ± SD (n=3). ***P<0.001 vs. Sham; $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. LPS. LPS, lipopolysaccharide; p-, phosphorylated.

macrophages. JNK-IN-8 inhibited the increase in MDA content and decreased the LPS-induced SOD activity *in vivo* and *in vitro*, indicating that JNK-IN-8 alleviated the oxidative stress induced by LPS and protected against lung damage.

In the present study, JNK-IN-8 contributed to the regulation of inflammation and oxidative stress following ALI. JNK-IN-8 treatment decreased LPS-induced inflammation and oxidative stress activation both *in vivo* and *in vitro*. TNF-α, IL-1β and IL-6 were upregulated following LPS stimulation, whereas treatment with JNK-IN-8 decreased LPS-induced upregulation of TNF-α, IL-1β and IL-6, suggesting that JNK-IN-8 treatment contributed to the decrease in inflammation. Compared with the control group, JNK-IN-8 inhibited JNK phosphorylation. This finding indicated that LPS-induced JNK activation was prevented by JNK-IN-8. In addition, JNK-IN-8 decreased NF-κB signaling activation. In summary, the present study demonstrated that targeting JNK-IN-8 is a potent prospective therapy for the treatment of ALI.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JD designed and performed the experiments, analyzed the data and wrote the manuscript. GW designed the experiments. GW, HL, NL and JX interpreted the data. JX conceptualized and supervised the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were approved by the Ethics Committee of Zhejiang University (approval no. ZJU20170913).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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