MicroRNA-421 attenuates macrophage-mediated inflammation by inhibiting PDCD4 *in vitro*

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Abstract. The exact pathogenesis of acute lung injury (ALI) has not been fully clarified. Previous studies have demonstrated that ALI is associated with inflammation. Recent studies have demonstrated that microRNA-421 (miR-421) prevents inflammation in cerebellar ischemia reperfusion injury. However, the role of miR-421 in ALI remains unclear. The present study investigated the role of miR-421 in ALI and the mechanism underlying this. ALI was induced *in vitro* by treatment of RAW 264.7 macrophages with lipopolysaccharide (LPS). Cells were then transfected with miR-421 mimic, miR-421 mimic control, programmed cell death 4 (PDCD4) siRNA and PDCD4 siRNA control using Lipofectamine 2000. Cell viability was measured using the Cell Counting kit-8. Reverse transcription-quantitative polymerase chain reaction was used to detect the expression of miR-421 and PDCD4 in RAW 264.7 macrophages. The concentrations of IL-1β and TNF-α were detected by ELISA. Dual-luciferase reporter assays were used to investigate the interaction between miR-421 and PDCD4 mRNA. LPS inhibited cell viability and miR-421 expression. The miR-421 mimic promoted RAW 264.7 cell viability and inhibited the expression of iNOS and COX-2 as well as the release of IL-1β and TNF-α. PDCD4 siRNA inhibited the expression of iNOS and COX-2 expression as well as the release of IL-1β and TNF-α. In addition, miR-421 mimic and PDCD4 siRNA inhibit the expression of p-NF-κB (p65) in RAW 264.7 cells. In conclusion, the present study demonstrated the protective effect of miR-421 on ALI and showed that miR-421 attenuates LPS-induced ALI by inhibiting PDCD4 and NF-κB. These findings provided a theoretical basis for the treatment of ALI.

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Introduction

Acute lung injury (ALI) is a type of severe acute lung inflammation that causes diffuse lung injury induced by various direct or indirect factors (1). The main manifestations of ALI are increased permeability of the alveolar wall and pulmonary capillaries, pulmonary interstitial and alveolar edema, eventually leading to acute respiratory insufficiency, and even acute respiratory distress syndrome (2).

The etiology and mechanism of ALI are complex. During the past half-century, there has been considerable progress in the field of ALI research, although the exact pathogenesis has not been fully clarified. Previous studies have suggested that excessive activation of inflammatory cells, release of excessive inflammatory mediators, mutual activation and the interactions of numerous inflammatory factors and effector cells, combined with uncontrolled inflammatory responses are the main pathophysiological changes (3).

A variety of inflammatory cells are recruited and activated in the lung in response to various direct and indirect harmful stimuli, leading to the release of a large number of pro-inflammatory mediators. This 'inflammatory cascade' of cytokines results in the induction of the inflammatory response (4). Therefore, strategies for the prevention and treatment of ALI should be focused on inhibiting the overexpression and hyperactivation of inflammatory mediators. Regulating the occurrence and development of the inflammatory response has important clinical significance in preventing the occurrence of ALI and decreasing mortality (5).

Although there are numerous strategies for ALI treatment in the clinic, including cytokine therapy, stem cell therapy and hormone therapy, the incidence rate and mortality rate of ALI remains high (6). Comprehensive elucidation of the molecular pathological mechanism of ALI is required to improve the clinical efficacy of these therapeutic strategies.

MicroRNAs (miRNAs) are small (22-25 nucleotide), non-coding RNAs that regulate gene expression by inhibiting translation or promoting the degradation of target mRNAs and regulating a variety of important cell activities by specifically pairing with the 3'-untranslated region (3'-UTR) of the target gene (7). miRNAs are also associated with the occurrence and development of lung diseases, including pneumonia, lung cancer and pulmonary fibrosis (8,9). For example, Shen *et al* (10) demonstrated that miR-200b may attenuate

cellular senescence and inflammatory responses by targeting ZEB2 in pulmonary emphysema. Furthermore, Liang *et al* (11) demonstrated that miR-187 may present a novel therapeutic target in non-small-cell lung cancer based on its ability to regulate cyclins-related protein expression (11). miR-140 was also shown to suppress interstitial lung disease (ILD) development by downregulating osteoglycin (OGN) via the Wnt signaling pathway (12).

Recent studies have demonstrated the involvement of miRNAs in the regulation of the occurrence and development of the immune response that leads to ALI. For example, Huang *et al* (13) demonstrated that downregulation of miR-27b enhanced the expression of Nrf2 and HO-1, inhibited NF-κB signaling pathway, and protected against LPS-induced ALI in mice. In addition, Ju *et al* (14) reported that miR-27a attenuates LPS-induced ALI in mice by blocking activation of the TLR4/MyD88/NF-κB axis. Another study indicated that miR-326 activates the NF-κB signaling pathway by targeting the *BCL2A1* gene, leading to an enhanced inflammatory response and the lung injury associated with septic shock in ALI induced in mice (15).

Recent studies have demonstrated that miR-421 regulates inflammatory responses. Zheng *et al* (16) reported that miR-421 inhibited p65 mRNA translation by targeting YTHDF1 to prevent inflammation in cerebral ischemia reperfusion injury. In addition, Jiang *et al* (17) demonstrated that miR-421 promotes the inflammatory response of fibroblast-like synoviocytes in rheumatoid arthritis by downregulating the expression of SPRY1. Furthermore, inhibition of miR-421 promotes the development of bronchopulmonary dysplasia by upregulating Fgf10 (18). However, the effect of miR-421 on ALI is not clear. In this study, we used the lipopolysaccharide-induced model of ALI to investigate the effect of miR-421 on ALI *in vitro* and the underlying mechanism.

Materials and methods

RAW 264.7 macrophages cells culture. RAW 264.7 macrophage cells were purchased from the American Type Culture Collection (ATCC). RAW 264.7 cells were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% (volume/volume) fetal bovine serum (FBS; HyClone), 100 U penicillin and 100 μg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C under 5% CO_2 in a humidified incubator. For the LPS-induced model of ALI, RAW 264.7 cells were plated into 12-well plates ($1x10^6$ cells/well) and incubated for 24 h before treatment with 0, 50, 100 or 200 ng/ml LPS (Beijing Solarbio Science & Technology Co., Ltd.) for a further 24 h, as previously described (19).

Transfection. miR-421 mimic, miR-421 mimic control, programmed cell death 4 (PDCD4) siRNA and PDCD4 siRNA control were designed and synthesized by GenePharma (Shanghai GenePharma Co., Ltd.). RAW 264.7 cells in the logarithmic growth phase were seeded into 6-well plates and incubated for 12 h at 37˚C under 5% CO_2 in a humidified incubator prior to transfection with miR-421 mimic (5'-AUCAAC AGACAUUAAUUGGGCGC-3', 20 μM), miR-421 mimic control (5'-UUCUCCGAACGUGUCACGUTT-3', 20 μM), PDCD4 siRNA (5'-GAGGCUAUGAGAGAAUUUATT-3', 20 μM) and PDCD4 siRNA control (5'-UAGCCUAGU CCAAAGCAGCAT-3', 20 μM) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C for 48 h. After 48 h, RNA and protein were extracted for further experiments.

Cell Counting kit-8 (CCK-8). Cell viability was measured using the CCK-8 (Dojindo Molecular Technologies, Inc.) method. In brief, RAW 264.7 macrophages were seeded into 96-well plates ($5x10^3$ cells/well) and incubated for 12 h at 37˚C under 5% CO_2 in a humidified incubator. Subsequently, 10 μl CCK-8 reagent was added to each well and the RAW 264.7 cells were incubated for 1 h. The optical density in each well was measured at 450 nm using an iMark Microplate Absorbance Reader (Bio-Rad Laboratories, Inc.).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RAW 264.7 cells were harvested and total RNA was extracted with RNAiso (Takara Bio, Inc.) according to manufacturer's protocols. The concentration and purity of the isolated RNA was determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc.). RNA was reverse-transcribed into cDNA using PrimeScript™ RT reagent kit (Takara Bio, Inc.) and Mir-X miRNA RT-qPCR TB Green® Kit (Takara Bio, Inc.). RT was performed at 37˚C for 15 min, then 85˚C for 5 sec. qPCR was performed on an ABI Prism 7500 (Thermo Fisher Scientific, Inc.) using TB Green® Premix Ex Taq™ II (Tli RNaseH Plus; Takara Bio, Inc.) according to manufacturer's protocols. The thermocycling conditions were as follows: Initial denaturation at 95˚C for 30 sec; 40 cycles of 95˚C for 5 sec and 60˚C for 31 sec. The sequences of the primers as follows: miR-21 forward, 5'-TAGCTTATCAGACTGATGTTGA-3' and reverse, 5'-AAC GCTTCACGAATTTGCGT-3'; U6 forward, 5'-CTCGCT TCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATT TGCGT-3'; PDCD4 forward, 5'-AGGCCGAGGTGGGCG GATCACTTGA-3' and reverse, 5'-GCCACCATGCCTGGC TACT-3'; and GAPDH forward, 5'-CCTCTGACTTCAACA GCGACAC-3' and reverse, 5'-TGGTCCAGGGGTCTTACTC C-3'. The relative expression level was then calculated using $2^{-\Delta\Delta Cq}$ method (20).

Western blotting. Total proteins were extracted from RAW 264.7 cells using a RIPA kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocols. Nuclear proteins were extracted using the Nuclear and Cytoplasmic Protein Extraction kit (Beyotime Institute of Biotechnology). The protein concentration was measured with BCA kits (Beyotime Institute of Biotechnology) according to the manufacturer's protocols. The proteins were separated by 10% SDS-PAGE for 1.5 h. Next, the protein was transfected into PVDF (Millipore) membrane (40 μg/lane). The PVDF membrane was blocked with 5% skimmed milk at room temperature for 1 h before incubation overnight at 4˚C with primary antibodies for the detection of p-NF-κB (p65) (cat. no. ab76302; dilution, 1:1,000; Abcam), NF-κB (p65; cat. no. ab76311; dilution, 1:1,000; Abcam) and β-actin (cat. no. ab8227; dilution, 1:2,000; Abcam). Membranes were then incubated with horseradish peroxidase (HRP)-conjugated

goat anti-rabbit secondary antibodies (cat. no. ab6721; 1:5,000; Abcam) for 1 h at room temperature. Immunoreactive bands were visualized using the ECL Plus Western Blotting Substrate (Pierce; Thermo Fisher Scientific, Inc.) kit and the FluorChem FC3 imaging system (ProteinSimple).

ELISA. RAW 264.7 cells in the logarithmic growth phase were seeded into 6-well plates and cultured at 37˚C under 5% CO_2 in a humidified incubator for 24 h. The culture supernatant was collected and the concentrations of IL-1β (cat. no. MLB00C) and TNF-α (cat. no. MTA00B) were detected using ELISA kits (R&D Systems, Inc.) according to the manufacturer's protocols. The optical density at 450 nm was measured using an iMark Microplate Absorbance Reader (Bio-Rad Laboratories, Inc.).

Dual-luciferase reporter assay. StarBase 3.0 (starbase.sysu.edu.cn/) was used to predict that miR-421 binds to the 3'-UTR of PDCD4 mRNA. PDCD4 reporter plasmids (pmirGLO) with the wild-type miR-421 binding site (pmirGLO-PDCD4-WT) and a mutant-type miR-421 binding site (pmirGLO-PDCD4-MUT) in the 3'-UTR of PDCD4 mRNA were synthesized by Shanghai GenePharma Co., Ltd. using a site-directed mutagenesis kit (cat. no. 200518; Agilent Technologies Inc.). Next, pmirGLO-PDCD4-WT and pmirGLO-PDCD4-MUT were co-transfected with synthesized reporter vectors (0.8 μg) and miR-421 mimic (0.8 μg) or miR-421 mimic control (0.8 μg) using Lipofectamine 2000 reagent at 37˚C for 48 h. *Renilla* luciferase activity was measured using a Dual-Luciferase Reporter Assay system (Promega Corporation), according to the manufacturer's protocol.

Statistical analysis. SPSS 19.0 (IBM Corp.) was used for statistical analysis, and the measurement data are expressed as the mean ± standard deviation. Differences between two groups were compared using t-tests. Differences between multiple groups were compared using one-way analysis of variance, followed by Tukey's post-hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

LPS inhibits RAW 264.7 macrophage cell viability and miR-421 expression. RAW 264.7 macrophages were treated with different concentrations of LPS for 24 h. At 100 ng/ml, LPS significantly inhibited the viability of RAW 264.7 cells. Furthermore, the inhibitory effect of LPS at 200 ng/ml was greater than that at 100 ng/ml (Fig. 1A). Therefore, LPS at 100 ng/ml was selected as the suboptimal inhibitory dose and LPS was added at 100 ng/ml in the subsequent experiments. Furthermore, RT-qPCR demonstrated that LPS (100 ng/ml) significantly inhibited miR-421 expression in RAW 264.7 cells (Fig. 1B).

miR-421 upregulation inhibits the release of inflammatory factors by RAW 264.7 macrophages. The miR-421 mimic significantly increased miR-421 expression in RAW 264.7 macrophages (Fig. 2A). LPS (100 ng/ml) treatment inhibited RAW 264.7 cell viability. In addition, the miR-421 mimic reversed the inhibitory effect of LPS (100 ng/ml) on RAW 264.7 cell viability (Fig. 2B). The miR-421 mimic inhibited the LPS-induced expression of COX-2 and iNOS in RAW 264.7 cells (Fig. 2C and D). LPS (100 ng/ml) treatment promoted the release of IL-1β and TNF-α by RAW 264.7 cells and this effect was inhibited by the miR-421 mimic (Fig. 2E).

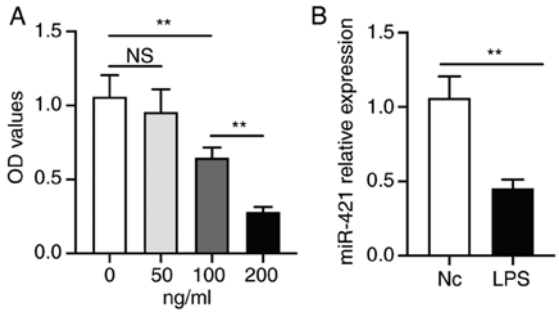

Figure 1. LPS inhibits RAW 264.7 macrophage cell viability and miR-421 expression. (A) Inhibitory effect of LPS (0, 50, 100, 200 ng/ml) on the viability of RAW 264.7 macrophages (24 h). (B) Inhibitory effect of LPS (100 ng/ml) on the miR-421 expression level of RAW 264.7 macrophages. **P<0.01. LPS, lipopolysaccharide; miR, microRNA; NS, no significance; NC, negative control; OD, optical density.

miR-421 directly inhibits PDCD4 expression and PDCD4 downregulation inhibits RAW 264.7 cell production of inflammatory factors. Using StarBase 3.0, it was predicted that miR-421 binds to a specific site in the 3'-UTR of PDCD4 mRNA (Fig. 3A). Dual-luciferase reporter assay confirmed that miR-421 directly targeted the 3'-UTR of PDCD4 mRNA to inhibit its expression (Fig. 3B). Further studies on the function of the *PDCD4* gene demonstrated that PDCD4 siRNA blocked the inhibitory effect of LPS on RAW 264.7 cell viability (Fig. 4A and B). PDCD4 siRNA also inhibited LPS-induced expression of COX-2 and iNOS in RAW 264.7 cells (Fig. 4C and D). In addition, PDCD4 siRNA inhibited the LPS-induced increase of IL-1β and TNF-α production by RAW 264.7 cells (Fig. 4E).

miR-421 mimic and PDCD4 siRNA inhibit p-NF-κB (p65) expression in RAW 264.7 cells. Western blot analysis demonstrated that the miR-421 mimic inhibited p-NF-κB (p65) expression in RAW 264.7 cells (Fig. 5A). Furthermore, PDCD4 siRNA also inhibited p-NF-κB (p65) expression in RAW 264.7 cells (Fig. 5B).

Discussion

LPS stimulates monocytes and macrophages to release pro-inflammatory factors that mediate inflammatory reactions. Excessive inflammatory reactions may cause injury to tissues such as the lung (21). LPS may also damage pulmonary vascular endothelial cells, increase vascular permeability, cause pulmonary edema and lead to progressive disorder of gas exchange. These changes are consistent with the histopathological and physiological changes of ALI. Therefore, LPS is generally considered as an ideal stimulant to induce ALI (21).

Tang *et al* demonstrated that increased iNOS expression serves a key role in the pathogenesis of LPS-induced

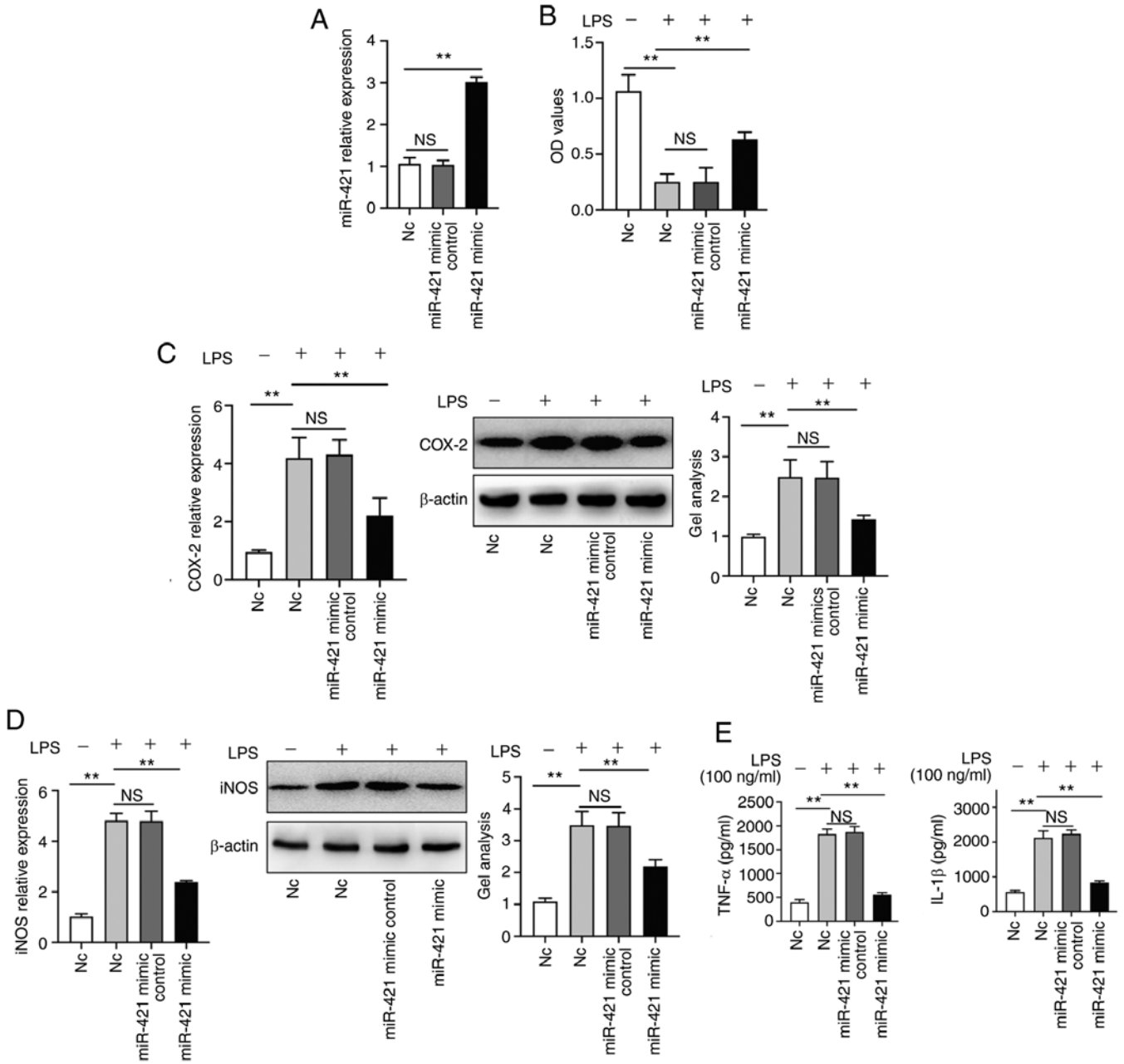

Figure 2. miR-421 upregulation inhibits the release of inflammatory factors by RAW 264.7 macrophages. (A) miR-421 mimic significantly increased the expression of miR-421. (B) miR-421 mimic reversed the inhibitory effect of LPS (100 ng/ml) on RAW 264.7 macrophage cell viability. (C) miR-421 mimic inhibited LPS-induced COX-2 expression by RAW 264.7 cells. (D) miR-421 mimic inhibited LPS-induced iNOS expression by RAW 264.7 cells. (E) miR-421 mimic inhibited the LPS (100 ng/ml)-induced increase in IL-1β and TNF-α expression by RAW 264.7 cells. **P<0.01. miR, microRNA; NS, no significance; LPS, lipopolysaccharide; NC, negative control; OD, optical density.

ALI (22). LPS stimulates iNOS expression in a variety of cell types found in the lung, including macrophages, neutrophils and endothelial cells (23). The present study demonstrated that miR-421 alleviated the increase in iNOS expression induced in RAW 264.7 macrophages by treatment with LPS. Therefore, we hypothesized that the protective effect of miR-421 on ALI may be associated with its inhibitory effect on iNOS.

Cyclooxygenase (COX), which converts arachidonic acid into prostaglandins, is considered to serve an important role in the occurrence and development of numerous inflammatory diseases, including ALI (24). As an inducible COX subtype, COX-2 may be induced by cytokines, growth factors, viruses, LPS and other inflammatory stimuli associated with ALI (25). In the present study, COX-2 expression was significantly increased in RAW 264.7 cells following LPS stimulation, while miR-421 attenuated this change, suggesting that the protective effect of miR-421 on ALI is associated with its inhibitory effect on COX-2.

Inflammatory mediators, particularly cytokines, serve key roles in the development and prognosis of ALI (26). In the early stage of ALI, numerous inflammatory factors, including TNF-α and IL-1β, serve a decisive role in the initiation of the early inflammatory response, and may maintain the continuous development and expansion of inflammation through a variety of mechanisms (26). While inflammatory

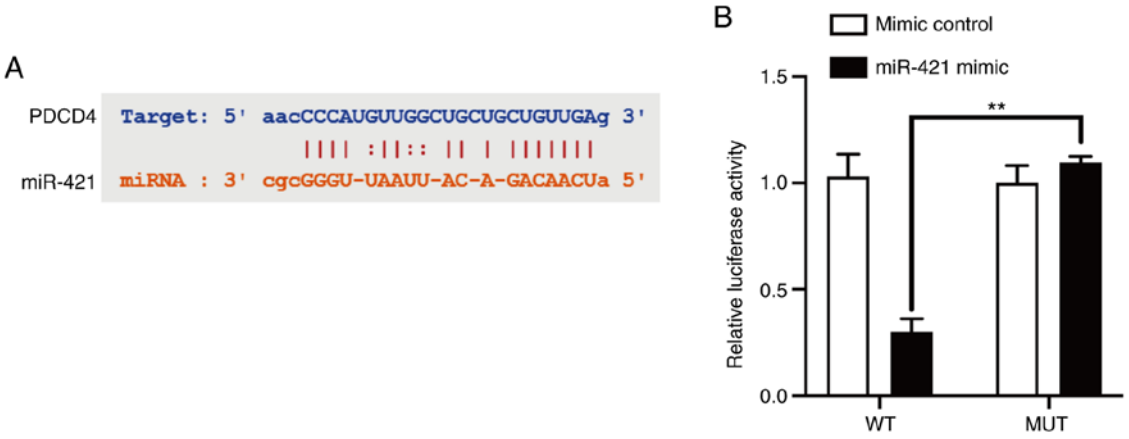

Figure 3. miR-421 directly inhibits PDCD4 expression (A) StarBase 3.0 online prediction results. (B) Dual-luciferase reporter assay demonstrated that miR-421 directly targets the 3'-UTR of PDCD4 mRNA. **P<0.01. NS, no significance; miR, microRNA; WT, wild-type; MUT, mutant.

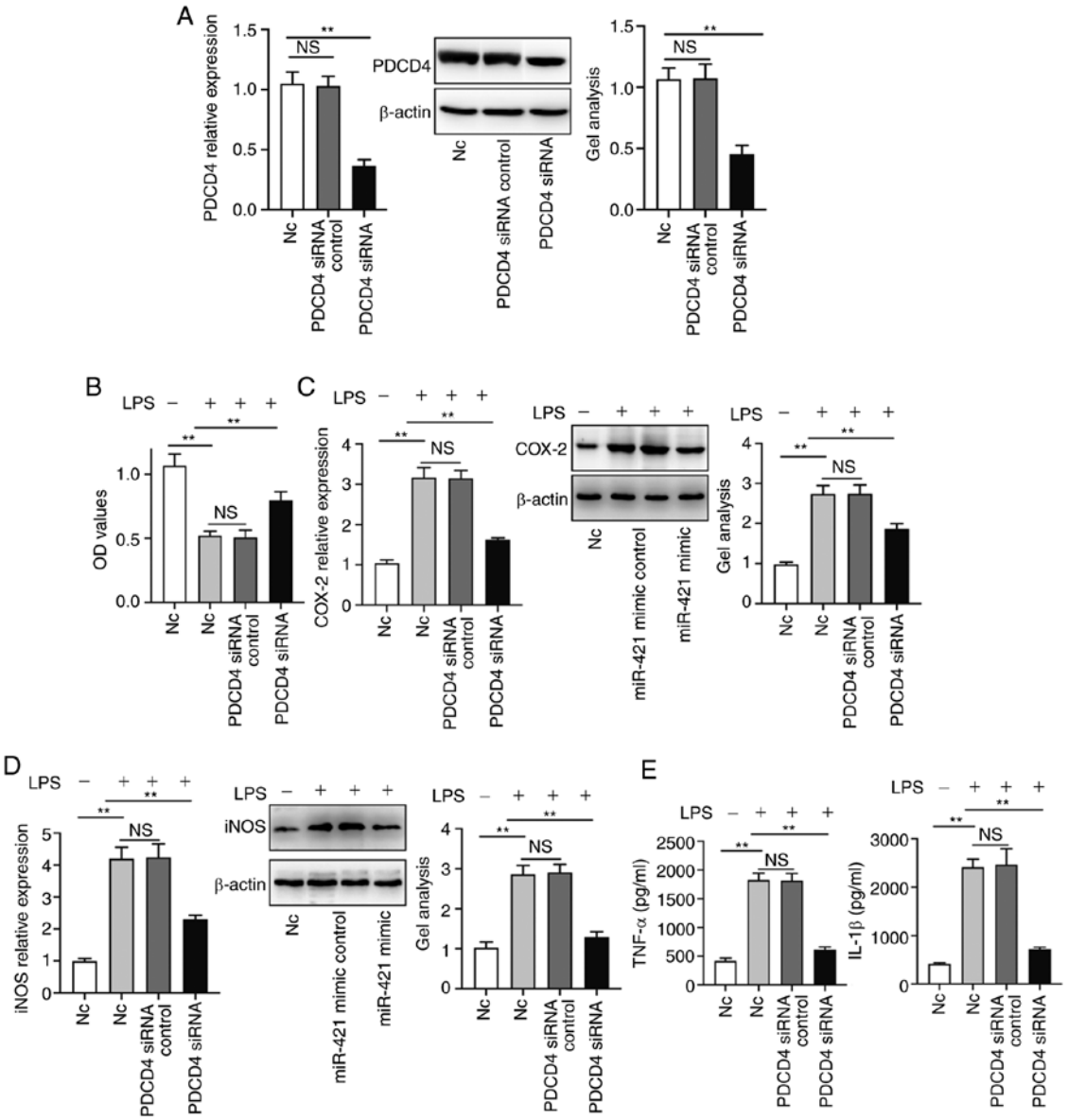

Figure 4. PDCD4 downregulation inhibits RAW 264.7 cell production of inflammatory factors. (A) PDCD4 siRNA inhibited PDCD4 mRNA expression. (B) PDCD4 siRNA reversed the inhibitory effect of LPS (100 ng/ml) on the viability of RAW 264.7 macrophage cells. (C) PDCD4 siRNA inhibited LPS-induced COX-2 expression by RAW 264.7 macrophage cells. (D) PDCD4 siRNA inhibited LPS-induced iNOS expression by RAW 264.7 macrophage cells induced by LPS. (E) PDCD4 siRNA reversed the LPS (100 ng/ml)-induced increase in IL-1β and TNF-α expression by RAW 264.7 macrophage cells. **P<0.01. LPS, lipopolysaccharide; NS, no significance; NC, negative control.

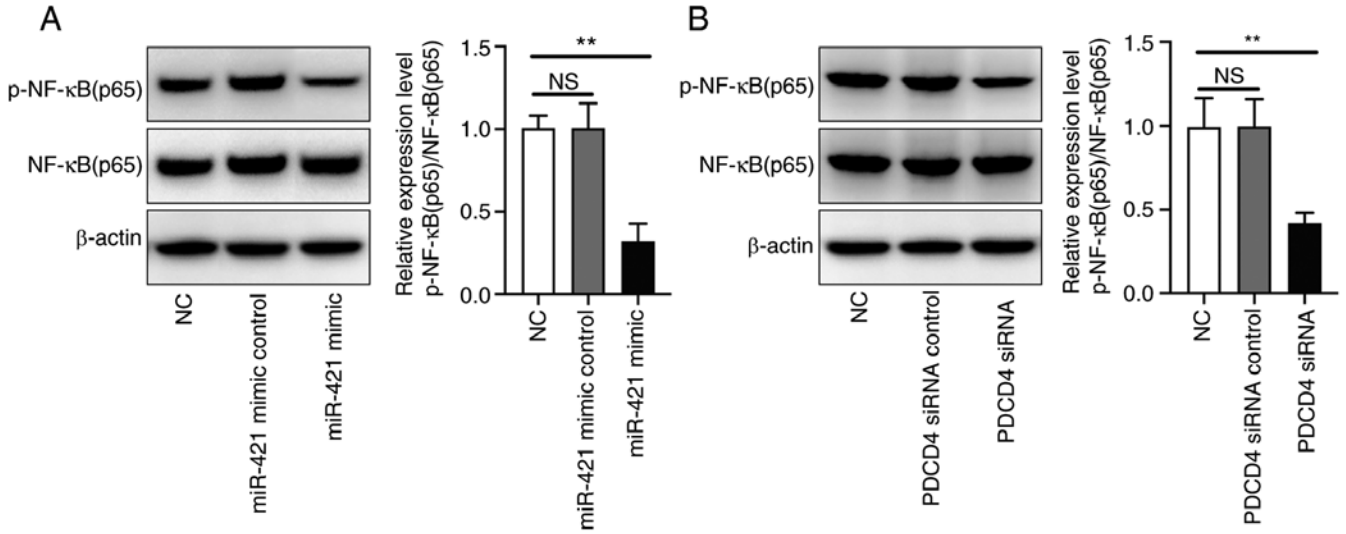

Figure 5. miR-421 mimic and PDCD4 siRNA inhibit p-NF-κB (p65) expression in RAW 264.7 cells. (A) miR-421 mimic inhibited the expression of p-NF-κB (p65). (B) PDCD4 siRNA inhibited the expression of p-NF-κB (p65). **P<0.01. miR, microRNA; NS, no significance; NC, negative control.

factors may remove harmful microorganisms, excessive production of inflammatory factors will cause damage to lung tissue (27).

High levels of TNF-α may also cause severe systemic inflammatory reactions, shock, vascular leakage and pulmonary edema (28,29). In the present study, cells were stimulated with 100 ng/ml LPS for 24 h, and it was found that LPS (100 ng/ml) treatment for 24 h induced a significant increase in the production of TNF-α in RAW 264.7 cells and this effect was significantly inhibited by the miR-421 mimic.

IL-1β may also activate neutrophils, which are considered to serve a very important initiating role in the initiation of the inflammatory cascade (30,31). In the present study, it was demonstrated that LPS (100 ng/ml) treatment for 24 h induced a significant increase in the production of IL-1 β in RAW 264.7 cells and this effect was significantly inhibited by the miR-421 mimic.

Using StarBase 3.0, it was predicted that miR-421 directly targets a site in the 3'-UTR of PDCD4 mRNA. As reported previously, Yang *et al* (32) reported that miR-421 promotes the proliferation and invasion of non-small cell lung cancer cells through targeting PDCD4. This was also confirmed in the present study using dual-luciferase reporter assays. Furthermore, Western blot analysis demonstrated that the miR-421 mimic inhibited PDCD4 in RAW 264.7 cells. Li *et al* (33) demonstrated that PDCD4-overexpression significantly attenuated the anti-apoptotic effect of MSC-Exo in lung cells.

In ALI, NF-κB activation enhances the transcription of numerous pro-inflammatory factors, including adhesion molecules, cytokines and chemokines, and induces the expression of enzymes, including COX-2 and iNOS (34). It was demonstrated that PDCD4 siRNA inhibited the phosphorylation of NF-κB, indicating that NF-κB serves a role in the protective effect of miR-421 on ALI.

The results of the present study demonstrated the protective effect of miR-421 on ALI. Furthermore, it was demonstrated that miR-421 may attenuate LPS-induced ALI by inhibiting PDCD4 and NF-κB. These results provided a theoretical basis for the development of strategies for the prevention and treatment of ALI.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

LW designed the experiments. HL, JS, LG, LF and DC performed the experiments. HL collected and analyzed the data. All authors confirmed the authenticity of all the raw data. HL and LW wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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