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MicroRNA-147b alleviates inflammation and apoptosis in acute lung injury via inhibition of p38 MAPK signaling pathway

Q. XU, G.-D. HUANG, G.-C. DUAN, H.-J. QIN

Department of Emergency Medicine, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, Shanghai, China

Abstract. – OBJECTIVE: The pathogenesis of acute lung injury (ALI) is complicated, the condition is developing rapidly, and the mortality rate is high. It is a common acute and critical illness in clinic. Here, we aimed to demonstrate the function and molecular mechanism of microRNA-147b (miR-147b) in ALI.

MATERIALS AND METHODS: MiR-147b mimic or miR-147b inhibitor was transfected into A549 cells to upregulate or downregulate miR-147b. The inflammatory response of A549 cells was observed by measuring the levels of inflammatory cytokines (TNF-α, IL-6, IL-1β) and chemokines (CCL2, CCL4) by enzyme-linked immunosorbent assay (ELISA) assay. The detection of apoptosis in A549 cells relies on Cell Counting Kit-8 (CCK-8) assay, caspase-3 activity assay, and flow cytometry. Quantitative Real Time-Polymerase Chain Reaction (RT-PCR) and Western blot were employed to detect the expression of miRNA and protein.

RESULTS: MiR-147b was downregulated in lipopolysaccharide (LPS)-induced ALI rats and LPS-treated A549 cells. Upregulation of miR-147b markedly suppressed LPS-induced inflammation and apoptosis of A549 cells, which was manifested by the reduction of inflammatory cytokines (TNF-α, IL-6, IL-1β) and chemokines (CCL2, CCL4), the reduction of LDH contents, the increase of cell viability, and the decrease of caspase-3 activity and apoptosis rate of A549 cells. The downregulation of miR-147b further induced inflammation and apoptosis of A549 cells caused by LPS, which was alleviated by inhibition of p38 MAPK pathway.

CONCLUSIONS: Taken together, miR-147b was downregulated in ALI, and the overexpression of miR-147b inhibited LPS-induced inflammation and apoptosis in A549 cells *via* inhibition of p38 MAPK signaling pathway.

Key Words:

Acute lung injury, MiR-147b, p38 MAPK, Lipopolysaccharide, Inflammation, Apoptosis.

Introduction

Acute lung injury (ALI) is an acute diffuse inflammatory lung injury caused by various intrapulmonary and extrapulmonary pathogenic factors, such as severe infection, trauma, poisoning, disseminated intravascular coagulation (DIC), etc., which in turn leads to acute respiratory failure, clinically manifested as progressively increased dyspnea, and refractory hypoxemia[1,2]. ALI is characterized by diffuse edema of alveolar epithelial cells, pulmonary interstitium and alveoli, and injury of microvascular endothelial cells, resulting in acute hypoxic respiratory insufficiency, which is a common delayed complication of severe diseases, such as sepsis. The incidence of ALI is about 6.8%, and the mortality rate is as high as 40% to 50%, which seriously threatens the life safety of patients[3].

Sepsis is still the main cause of ALI. The degree of ALI caused by sepsis is closely related to the concentration of lipopolysaccharide (LPS), a specific component of the cell wall of Gram-negative bacteria in the blood. LPS can cause acute diffuse damage to alveolar capillary endothelial cells, alveolar epithelial cells, and lung interstitium. Its essence is the activation and infiltration of various inflammatory cells in the lung and the release of a series of inflammatory mediators, causing lung damage. At the same time, more inflammatory cells are activated and more inflammatory mediators or cytokines are released, further amplifying and strengthening the lung damage signals, forming an inflammatory waterfall effect, and leading to alveolar-capillary membrane damage[4]. The massive release and activation of these inflammatory mediators cause structural damage, dysfunction, apoptosis, and necrosis of cells, that play an important role in the occurrence and development of ALI.

Corresponding Author: Haijun Qin, MM; e-mail: haijunqin@163.com

MicroRNA (miRNA) is a type of endogenous non-protein-encoded single-chain small molecule RNA found in eukaryotes with a length of 20-25 nucleotides[5]. Mature miRNAs are produced by the cleavage and processing of longer primary transcripts by a series of nucleases, and then, assembled into an RNA-induced silencing complex (RISC). MiRNA recognizes the target mRNA through base pairing, and guides the RISC to degrade the target mRNA or inhibit the target mRNA translation according to the degree of complementarity[6]. MiRNA is involved in regulating various cellular processes, such as cell proliferation, differentiation, aging, apoptosis, metabolism, inflammation and immune response, organ formation, and tumor formation[7]. In recent years, some scholars have conducted preliminary discussions on the regulatory role of miRNA during ALI. In various types of ALI animal models, miRNA expression increased, decreased, or remained unchanged. Changing the expression of miRNA may provide a potential new method for the treatment of ALI. Yang et al[8] found that miR-140-5p expression decreased in ALI mice, while the upregulation of miR-140-5p inhibited inflammation in ALI. MiRNA-1246 was found to be involved in the regulation of inflammation and apoptosis in ALI *via* the NF-κB and Wnt/β-catenin pathways[9]. However, there are few studies on the role of miR-147b in ALI.

In this study, we established animal and cell models of ALI using LPS to detect miR-147b expression. Moreover, the role of miR-147b in ALI was studied by upregulation and downregulation of miR-147b. Our results suggested that miR-147b could be a potential therapeutic target for ALI.

Materials and Methods

Rat ALI Model

This study was approved by the Animal Ethics Committee of Shanghai Jiao Tong University Animal Center. 20 male Sprague Dawley (SD) rats (7-8 weeks old, 200-250 g) (Shanghai Experimental Animal Center of Chinese Academy of Sciences, Shanghai, China) were taken and randomly divided into 2 groups, each with 10 rats, namely the control group and the ALI model group. The rats were fasted for 12 hours before the experiment and had free access to water. The ALI model was established by LPS tracheal instillation. LPS (Sigma Aldrich, Darmstadt, Germany) is formulated with sterile saline to a concentration of 5 mg/ml. The rats were intraperitoneally injected with 10% chloral hydrate (0.35 g/kg) for anesthesia, and then, the cervical trachea of the rats was separated, and the ALI model was replicated by instilling LPS from the trachea to the lungs with a microsyringe. The control group was infused with an equal volume of normal saline. After 24 hours, the lung tissues of the two groups of rats were collected and stored in liquid nitrogen.

Cell Culture and Transfection

The complete medium for culturing A549 cells consists of Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA), 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 1% penicillin/streptomycin (Gibco, Rockville, MD, USA). A549 cells were cultured in a constant 37°C cell incubator containing 95% O_2 and 5% CO_2.

According to the protocols, synthetic RNAs (miR-147b mimic, negative control (NC) mimic, miR-147b inhibitor and NC inhibitor) (RiboBio, Guangzhou, China) were transfected into A549 cells using Transfection Kit (RiboBio, Guangzhou, China). After placing the cells in the incubator for 48 hours, A549 cells were treated with LPS for 4 hours, with or without the pretreatment of p38 MAPK inhibitor SB203580 (p38i) for 1 hour.

Quantitative Real-Time Polymerase Chain Reaction (RT-PCR) Analysis

After 50 mg of rat lung tissue was shredded, 1 mL of TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was added and placed in a tissue homogenizer for homogenization treatment. For A549 cells in a 24-well plate, 0.5 mL of TRIzol reagent was added, followed by grinding with a pipette tip. Subsequently, chloroform was added, and the Eppendorf (EP) tube was vigorously shaken for 30 seconds and allowed to stand for 5 minutes. After that, the EP tube was centrifuged at 12,000 g at 4°C for 15 minutes, and the upper aqueous phase was collected, and isopropyl alcohol was added, mixed, and allowed to stand for 10 minutes. After that, the EP tube was centrifuged at 12,000 g at 4°C for 10 minutes. The supernatant was discarded, 75% ethanol was added, and after mixing, it was centrifuged at 12,000 g and 4°C for 5 minutes. Finally, the obtained RNA was dissolved in 20 μL of diethyl pyrocarbonate (DEPC)-treated Water (Beyotime, Shanghai, China). Thermo Nanodrop 2100 spectrophotometer was

used to detect RNA purity and concentration. DEPC-treated Water was used as a blank control, and the RNA concentration of each sample and its OD260/OD280 value were recorded. OD260/OD280 is 1.8-2.0, indicating that the RNA purity is high. To establish a miRNA cDNA library, reverse transcription was performed using All-in-One™ miRNA First-Strand complementary deoxyribose nucleic acid (cDNA) Synthesis Kit (GeneCopoeia, Guangzhou, China) in accordance with the protocols. RT-PCR was performed using All-in-One™ miRNA qPCR Kit (GeneCopoeia, Guangzhou, China). U6 was the internal control of miR-147b. All the primers were listed in Table I.

Detection of Inflammatory Cytokines and Chemokines

The A549 cell supernatant was collected and centrifuged at 200 g for 5 minutes to remove cell debris. The contents of inflammatory cytokines (TNF-α, IL-6, IL-1β) and chemokines (CCL2, CCL4) in the cell supernatant were detected by corresponding ELISA assay kits (Bestbio, Shanghai, China).

Cell Counting Kit-8 (CCK-8) Assay

A549 cells were placed in each well of the 96-well plates. After the cells have undergone the above treatment, CCK-8 solution (10 μL; Beyotime, Shanghai, China) was added to each well and the cells were incubated for other 2 hours in the cell incubator. The absorbance at 450 nm was detected by a spectrophotometer.

Lactate Dehydrogenase (LDH) Contents

The supernatant of A549 cells was collected and LDH contents in the cell supernatant were detected using LDH ELISA kit (DOJINDO, Shanghai, China).

Caspase-3 Activity

Caspase-3 activity was detected using Caspase-3 activity detection kit (Beyotime, Shanghai, China).

Western Blot

Total protein of A549 cells and rat lung tissue was collected using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) and protein concentration was measured using the bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). The proteins were separated by electrophoresis in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). After that, the separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Then, the membranes were incubated with 5% skimmed milk for 2 hours. Subsequently, the membranes were incubated with primary antibodies (Phospho-p38 MAPK, CST, Danvers, MA, USA, Rabbit, 1:1000; p38 MAPK, CST, Danvers, MA, USA, Rabbit, 1:1000; glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Abcam, Cambridge, MA, USA, Rabbit, 1:1000) at 4°C overnight. Then, the membranes were incubated with secondary antibody for 2 hours. Blots were developed using Super enhanced chemiluminescence (ECL) Detection Reagent (Yeasen, Shanghai, China) in Image Lab™ Software.

Flow Cytometry

A549 cells were collected using trypsin (Beyotime, Shanghai, China). Then, we centrifuged the cell suspension at 200 g for 5 minutes, discarded the supernatant, washed the cells with phosphate-buffered saline (PBS), and centrifuged again to obtain cells. The above steps were repeated 3 times. After that, we discarded the supernatant and resuspended the cells with 200 μL of binding buffer, and added 5 μL of Annexin V-FITc (KeyGen, Shanghai, China) and 5 μL of propidium iodide (KeyGen, Shanghai, China) in the dark. Finally, the apoptosis rate was analyzed by flow cytometry.

Statistical Analysis

Statistical analysis was performed using Statistical Product and Service Solutions (SPSS)

Table I. Real time PCR primers.

Gene name	Forward (5′>3′)	Reverse (5′>3′)
miR-147b	CGTGTGCGGAAATGCTT	GTTGTTGGTTGGTTGGTTGT
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT

RT-PCR, quantitative real-time polymerase chain reaction.

22. 0 software (IBM Corp., Armonk, NY, USA). Data were represented as mean ± Standard Deviation (SD). The *t*-test was used for analyzing measurement data. The differences between the two groups were analyzed by using the Student's *t*-test. The comparison between multiple groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ indicated the significant difference.

Results

MiR-147b Was Downregulated in ALI

Through RT-PCR analysis, miR-147b was significantly downregulated in lung tissues of ALI rats (Figure 1A) and in LPS-treated A549 cells (Figure 1B). After transfection with miR-147b mimic, miR-147b was markedly upregulated in A549 cells (Figure 1C).

Up-Regulation of MiR-147b Inhibited LPS-Induced Inflammation and Apoptosis of A549 Cells

After using miR-147b mimic to overexpress miR-147b in A549 cells, the contents of three inflammatory cytokines (TNF-α, IL-6 and IL-1β) and two chemokines (CCL2, CCL4) in the cell supernatants was detected. Compared with the control group, the contents of inflammatory cytokines and chemokines in the LPS group was remarkably increased, while the upregulation of miR-147b reduced their contents significantly (Figure 2A-2E). In addition, the LDH contents in the cell supernatant of the LPS group was also significantly higher than that in the control group, and the overexpression of miR-147b could reverse this effect (Figure 2F). The upregulation of miR-147b can greatly alleviate the damage in the viability of A549 cells caused by LPS (Figure 2G). The treatment of LPS can mediate the apoptosis of A549 cells, which is shown by the increased activity of caspase-3 and the increase of apoptosis rate. The overexpression of miR-147b remarkably inhibited the apoptosis of A549 cells (Figure 2H and 2I).

The p38 MAPK Pathway Was Activated in ALI

Since the P38 MAPK signaling pathway plays a vital role in the inflammatory response, and its inhibitor SB203580 has a strong inhibitory effect on LPS-induced inflammation, we focused on this pathway. Through Western blot, we detected the p38 MAPK pathway in ALI rat model and LPS-treated A549 cells. In ALI, the phosphorylation of p38 was significantly enhanced (Figure 3A and 3B). The overexpression of miR-147b could notably inhibit the phosphorylation level of p38, while miR-147b silencing could enhance the phosphorylation level of p38 (Figure 3C).

MiR-147b Inhibited Inflammation and Apoptosis in ALI is p38 MAPK Pathway Dependent

The downregulation of miR-147b further aggravated LPS-induced inflammation and apoptosis of A549 cells. Compared with the LPS + inhibitor-NC group, the levels of inflammatory cytokines (TNF-α, IL-6, IL-1β) in the LPS + inhibitor group was markedly increased, the release of LDH was greatly increased, and the caspase-3 activity and apoptosis rate were further increased. However, after pretreatment with p38

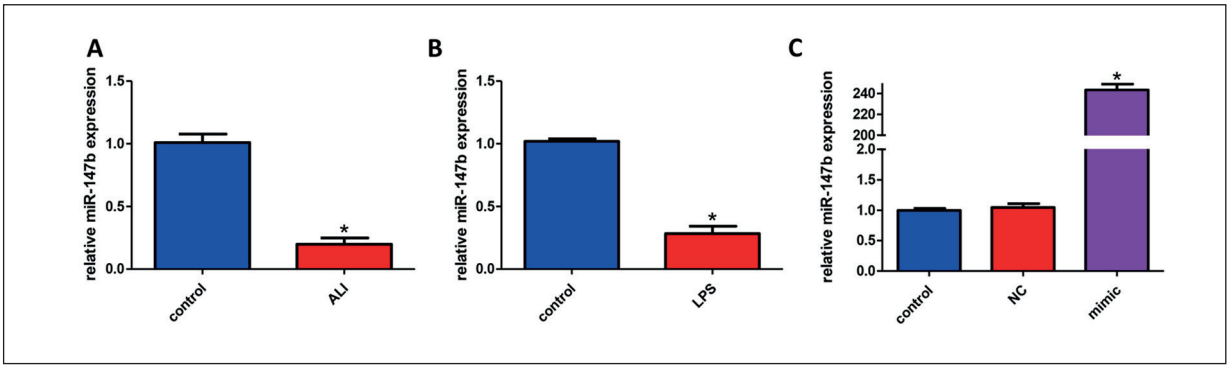

Figure 1. MiR-147b was downregulated in ALI. **A,** MiR-147b expression in ALI rats was detected by RT-PCR ("*" $p<0.05$ *vs.* control, n = 6). **B,** MiR-147b expression in LPS-treated A549 cells was detected by RT-PCR ("*" $p<0.05$ *vs.* control, n = 3). **C,** MiR-147b expression in A549 cells transfected with miR-147b mimic was detected by RT-PCR ("*" $p<0.05$ *vs.* control, n = 3).

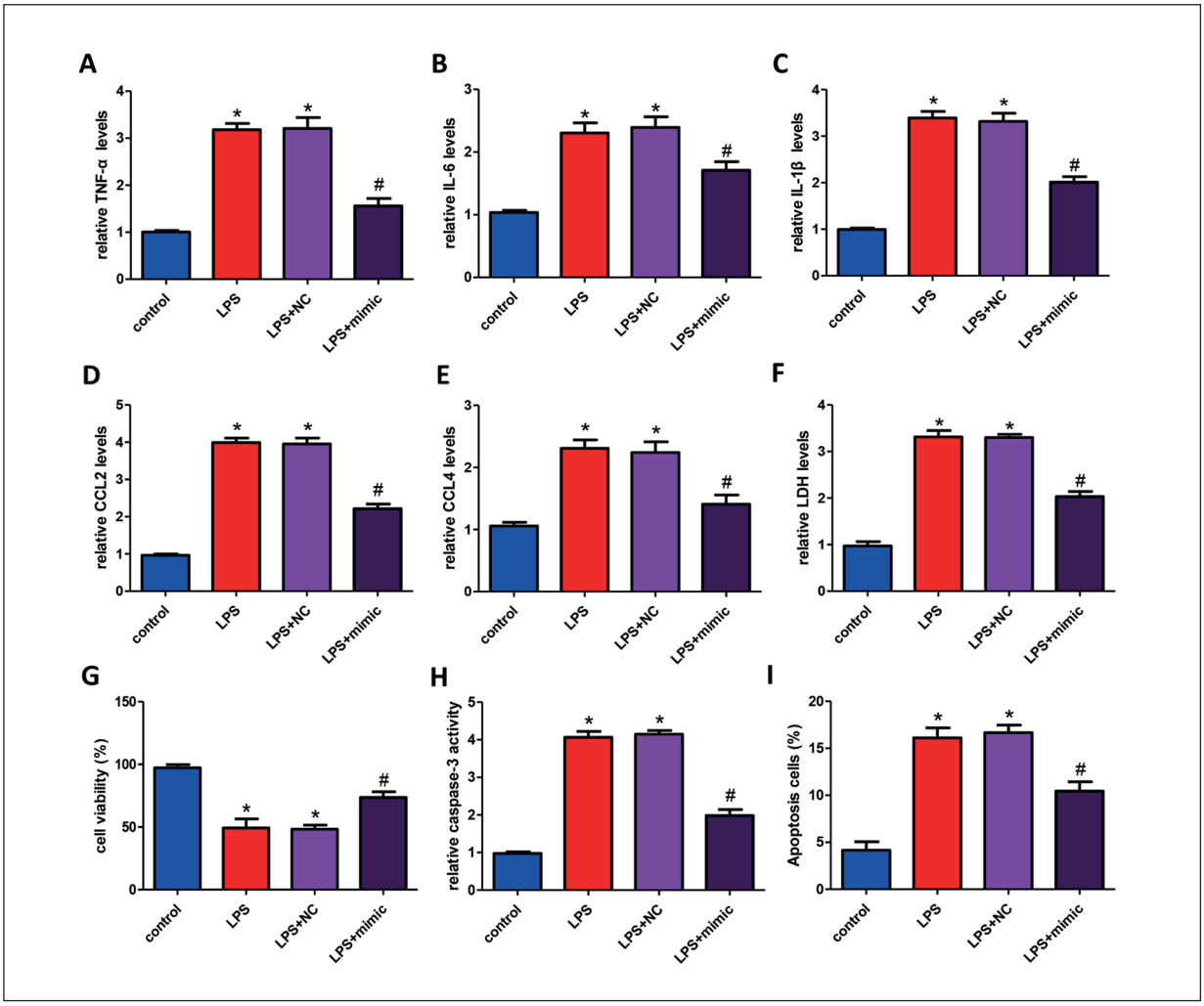

Figure 2. Upregulation of miR-147b inhibited LPS-induced inflammation and apoptosis of A549 cells. The contents of TNF-α **(A)**, IL-6 **(B)**, and IL-1β **(C)** in the cell supernatant were detected using ELISA assay kits ("$_{*}$" $p<0.05$ *vs.* control, "$_{\#}$" $p<0.05$ *vs.* LPS+NC, n=3). The contents of CCL-2 **(D)** and CCL-4 **(E)** in the cell supernatant were detected using ELISA assay kits ("$_{*}$" $p<0.05$ *vs.* control, "$_{\#}$" $p<0.05$ *vs.* LPS+NC, n=3). **F,** The contents of LDH were detected using LDH ELISA kit ("$_{*}$" $p<0.05$ *vs.* control, "$_{\#}$" $p<0.05$ *vs.* LPS+NC, n=3). **G,** The cell viability was detected by CCK-8 assay ("$_{*}$" $p<0.05$ *vs.* control, "$_{\#}$" $p<0.05$ *vs.* LPS+NC, n=3). **H,** The caspase-3 activity of A549 cells was detected ("$_{*}$" $p<0.05$ *vs.* control, "$_{\#}$" $p<0.05$ *vs.* LPS+NC, n=3). **I,** Apoptosis rate was detected by flow cytometry ("$_{*}$" $p<0.05$ *vs.* control, "$_{\#}$" $p<0.05$ *vs.* LPS+NC, n=3).

MAPK inhibitor, the above effects were all observably reversed (Figure 4A-4F).

Discussion

ALI is acute progressive respiratory failure caused by various pathogenic factors inside and outside the lung. The pathogenesis of ALI includes complex inflammatory responses, lung tissue damage, and activation and inhibition of cytokines, chemokines, and apoptosis[10]. Alveolar epithelial cells are not only the target cells of various injury factors, but also active participants in the inflammatory response. Alveolar epithelial cells can be activated by LPS and release inflammatory mediators, which in turn causes structural damage, dysfunction, apoptosis, and necrosis of the cells[11,12]. It plays an important role in the occurrence and development of acute lung injury.

MiRNA is widely involved in the immune regulation mechanism of systemic inflammatory response syndrome and septic shock[13-15]. During the occurrence of systemic inflammatory response, miRNA is involved in regulating the expression of various inflammatory genes and transcription

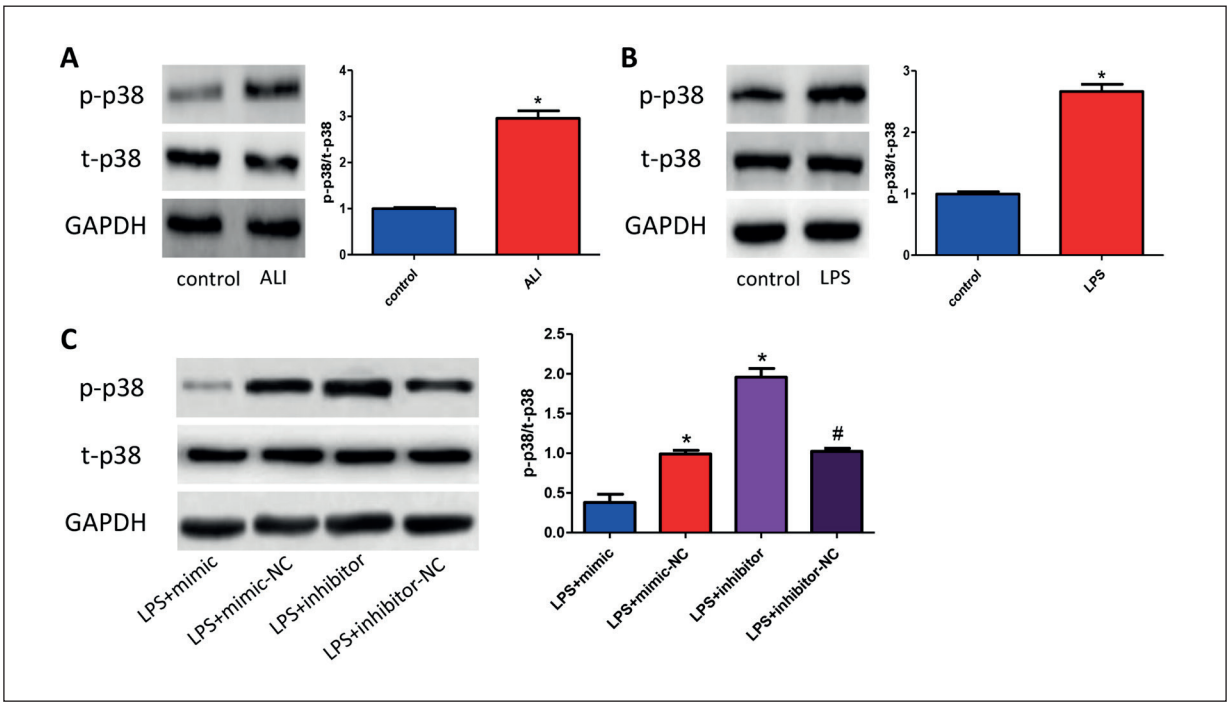

Figure 3. The p38 MAPK pathway was activated in ALI. **A,** The expression of phospho-p38 and total p38 in ALI rats was detected using Western blot ("$_*$" $p<0.05$ *vs.* control, "$_\#$" $p<0.05$ *vs.* LPS+NC, n=6). **B,** and **C,** The expression of phospho-p38 and total p38 in A549 cells was detected using Western blot ("$_*$" $p<0.05$ *vs.* control, "$_\#$" $p<0.05$ *vs.* LPS+NC, n=3).

factors, the proliferation, differentiation and apoptosis of various inflammatory cells, the production and release of various cytokines and inflammatory mediators, and the expression of various inflammatory factor receptors. However, at present, there are few studies on the role of miRNA in the development of ALI caused by sepsis.

In this study, we found that the treatment of A549 cells with LPS could induce inflammatory response and promote apoptosis. In addition, miR-147b was downregulated in LPS-treated A549 cells and ALI rats. The upregulation of miR-147b remarkably reduced the levels of inflammatory cytokines and inhibited apoptosis of A549 cells, while the downregulation of miR-147b further induced inflammation and apoptosis of A549 cells. Moreover, we found that the p38 MAPK pathway was significantly activated in ALI, and the use of p38 MAPK pathway inhibitors could inhibit LPS-induced inflammation and apoptosis of A549 cells.

p38 MAPK is an intracellular serine/threonine protein kinase, an important class of signal transduction enzymes, which regulates gene transcription and translation by transducing extracellular signals into the nucleus[16]. The p38 MAPK pathway plays an important role in the development of ALI. The role of p38 MAPK in the inflammatory response was first discovered after the use of the anti-inflammatory drug SB203580 in mice, which was manifested as inhibition of LPS-induced monocyte production of tumor necrosis factor, sepsis and endotoxin shock[17]. Zheng et al[18] proved that the use of SB239063 to inhibit p38α and β can inhibit the expression of IL-1β, reduce the inflammatory response, and protect the ALI caused by intestinal ischemia reperfusion. Dolinay et al[19] also have shown that inhibiting p38 MAPK can inhibit the activity of Caspase 3/7 to prevent apoptosis in the body. Our research also found that miR-147b inhibits ALI inflammation and apoptosis by inhibiting p38 MAPK pathway. There are some limitations in this present study. First, further research is needed on why up-regulation of miR-147b can inhibit the p38 MAPK signaling pathway. Second, functional experiments in vivo are also very meaningful and necessary. These are worthy of further study.

Conclusions

This paper revealed for the first time that reconstructed dysregulation of miR-147b expres-

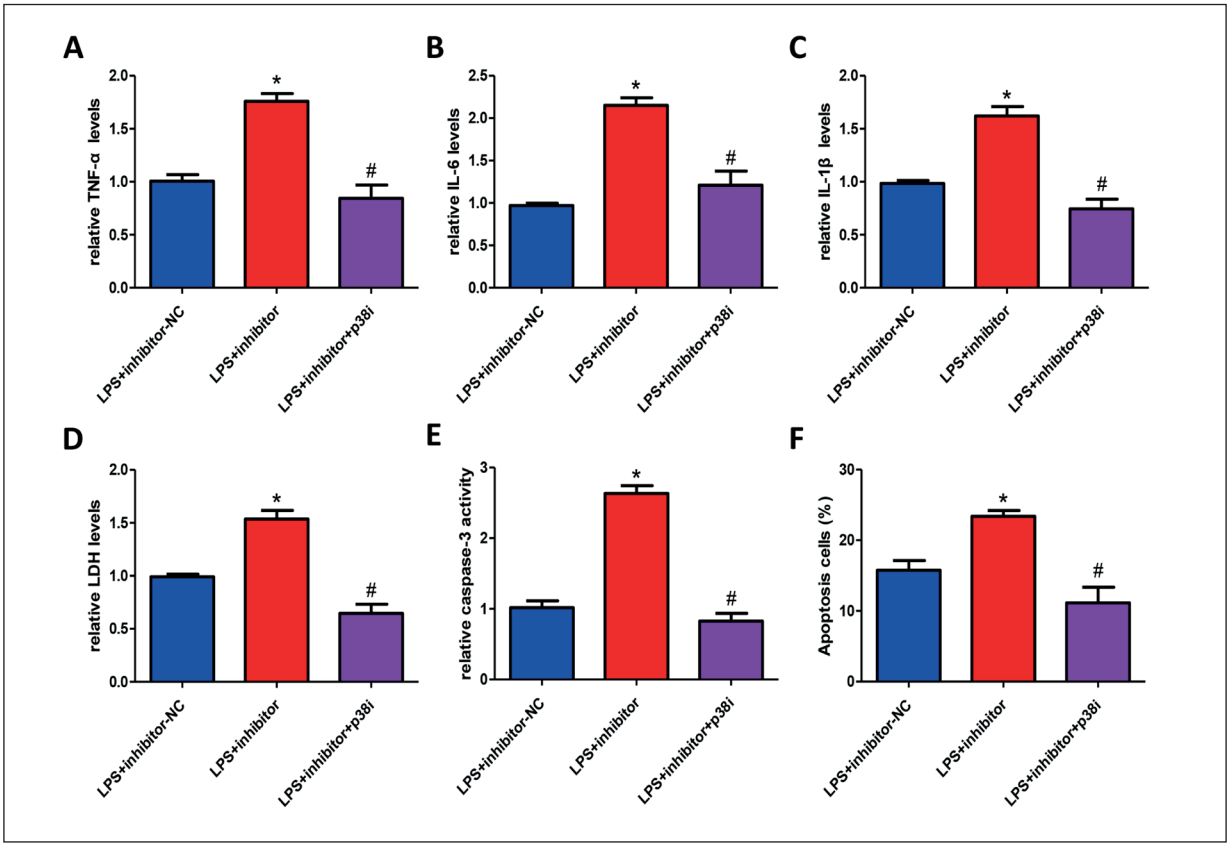

Figure 4. MiR-147b inhibited inflammation and apoptosis in ALI is p38 MAPK pathway dependent. The contents of TNF-α **(A)**, IL-6 **(B)**, and IL-1β **(C)** in the cell supernatant were detected using ELISA assay kits ("$_{*}$" $p<0.05$ *vs.* control, "$_{\#}$" $p<0.05$ *vs.* LPS+NC, n=3). **D,** The contents of LDH were detected using LDH ELISA kit ("$_{*}$" $p<0.05$ *vs.* control, "$_{\#}$" $p<0.05$ *vs.* LPS+NC, n=3). **E,** The caspase-3 activity of A549 cells was detected ("$_{*}$" $p<0.05$ *vs.* control, "$_{\#}$" $p<0.05$ *vs.* LPS+NC, n=3). **F,** Apoptosis rate was detected by flow cytometry ("$_{*}$" $p<0.05$ *vs.* control, "$_{\#}$" $p<0.05$ *vs.* LPS+NC, n=3).

sion induced by LPS can inhibit LPS-induced lung inflammation and apoptosis. Mechanically, LPS treatment induced the activation of the p38 MAPK pathway, which can be inhibited by overexpression of miR-147b. To sum up, miR-147b expression was remarkably decreased in ALI, and upregulation of miR-147b could inhibit LPS-induced inflammation and apoptosis of A549 cells via inhibiting p38 MAPK signaling pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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