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Original Research Article

MiR-574-5p alleviates sepsis-induced acute lung injury by regulating TRAF6/NF-κB pathway

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Abstract

Purpose: *To investigate the protective effect of miR-574-5p pretreatment against acute lung injury (ALI) induced by sepsis.*
Methods: *A male C57BL/6 mouse model of sepsis-induced ALI was established by cecal ligation and puncture (CLP) and treated with miR-574-5p agomir (intravenous injection, 80 mg/kg per day, 3 days). After that, blood and lung samples were obtained for histopathological observation. Myeloperoxidase (MPO) activity, inflammatory cell infiltration, and cytokine expression were analyzed. The target gene of miR-574-5p was predicted using TargetScan prediction, and verified by luciferase assay and western blot.*
Results: *In sepsis-induced ALI mice model, downregulation of miR-574-5p was observed. Pretreatment of miR-574-5p significantly alleviated ALI by suppressing histological damage, and reducing MPO activity and inflammatory cell infiltration, as well as decreasing cytokine expression. The underlying mechanism was that miR-574-5p targeted TNF receptor associated factor 6 (TRAF6) and suppressed the downstream NF-κB pathway. Moreover, TRAF6 overexpression reversed the effects of miR-574-5p on ALI.*
Conclusion: *MiR-574-5p pretreatment suppresses inflammatory responses, thus reducing lung injury induced by sepsis in mice, partly via the regulation of TRAF6 and NF-κB pathway. Therefore, this approach can potentially be used for the clinical management of ALI in humans*

Keywords: *Sepsis, Acute lung injury, MiR-574-5p, TRAF6, NF-κB pathway*

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INTRODUCTION

Sepsis refers to the systemic inflammatory response caused by some bioactive chemicals, such as infectious pathogen-produced lipopolysaccharide (LPS) [1]. When the inflammatory response intensifies, a large number of toxins, inflammatory mediators and metabolites are produced, leading to sepsis as defined by acute respiratory distress syndrome, cardiovascular dysfunction, or even multi-organ dysfunction [2]. Because the lungs and respiratory tract are directly contact with inhaled microorganisms and particles, pulmonary infection is the most common cause of sepsis [3].

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However, little studies considering the molecular mechanism of sepsis-induced ALI.
MicroRNAs (miRNA) regulate the post-transcriptional expression level of target genes by binding to its 3'-untranslation region (UTR), which have abilities to regulate sepsis-induced organ damage. For example, miR-146a inhibits the expression of IRAK1and TNF receptor associated factor 6 (TRAF6) by targeting erb-b2 receptor tyrosine kinase 4 (ErbB4), thereby reducing the myocardial dysfunction caused by sepsis [4]. In severe sepsis and sepsis-induced cases of ALI, plasma miR-155 and miR-146a are serve as novel biomarkers [5]. In particular, in the serum from patients with type 2 diabetes, studies have shown that miR-574-5p was decreased and is associated with miR-146a due to its anti-inflammatory effects [6]. In addition, serum miR-574-5p expression in sepsis survivors was different from that in non-survivors, and it could be used as a prognostic factor for patients with sepsis [7]. However, its role in septic-induced ALI is unknown.

Here, the role of miR-574-5p in a mouse model of sepsis were investigated. Further analysis verified the targeting relationship between miR-574-5p and TRAF6. This study indicated thatmiR-574-5p could be a new approach for the treatment of ALI induced by sepsis.

EXPERIMENTAL

Mouse model

Male C57BL/6 mice (6 - 8 weeks, 16 - 18 g) were obtained from Animal Research Center of Nanjing University (Nanjing, China). The animal study was performed with the approval (approval no. wydw2017-0159) of the institutional ethical committee, and followed the guidelines of the Care and Use of Laboratory Animals published by the National Institutes of Health [8]. To develop sepsis-induced ALI, the cecal ligation and puncture (CLP) model was employed, in which the release of fecal material into the peritoneal cavity induces sepsis. For anesthesia, mice were intraperitoneally injected with pentobarbital and supine fixed on an operating table. The abdominal cavity was opened layer by layer. The cecum was carefully separated and pulled out, and the mesangial membrane was then separated. After that, the distal cecum with silk thread was ligated, and the procedures were as follows: ran a needle through the cecum 5 mm below the ligation line, squeezed out some intestinal contents, and then placed a rubber drainage strip to prevent pinhole closure. The cecum was carefully reinserted, and the abdominal incision was sutured layer by layer.

Twenty-four mice were divided into 6 mice/group, randomly: Sham (anesthesia only) + negative control (NC) agomir, Sham + miR-574-5p agomir, CLP + NC agomir and CLP + miR-574-5p agomir. The Sham group only received anesthesia without cecal ligation. Mice in NC agomir or miR-574-5p agomir (GenePharma, Shanghai, China) group were intravenously injected (80 mg/kg/day, 3days). 24 h after the last administration of NC agomir or miR-574-5p agomir, CLP model was established. Six hours later, mice were sacrificed, and blood and lung samples were obtained for further analysis.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total lung tissue RNA was extracted and then used for reverse transcription. The cDNAs were obtained and then amplified with the ExTaq Kit (Takara) to determine miRNA levels. The U6 small nuclear RNA (snRNA) was used to normalize miR-574-5p expression. The primers were as follows: U6 (forward): 5'- AACGCT TCACGAATTTGCGT-3', U6 (reverse): 5'-CTCG CTTCGGCAGCACA-3'; miR-574-5p (forward): 5'-ACACTCCAGCTGGGTGTGTGAGTGTGTGT-3', miR-574-5p (reverse): 5'-CTCAA CTGGTGTC GTGGAGTCGGCAATTCAGTTGAGCACACTCA -3'.

Histopathology analysis of lung tissue

Lung specimens were fixed, embedded, sectioned, dehydrated, and stained with hematoxylin and eosin (H&E).A grading score was assigned (0 to 10) as described previously [9]. Ten images were randomly selected from each slide and scored by two independent pathologists.

Isolation of bronchoalveolar lavage fluid (BALF)

The isolation of BALF was collected as previously described [10]. The lungs collected from animals were lavaged three times using sterile phosphate buffer saline (PBS, 500 µl) through a tracheal cannula. The fluid samples were further centrifugated (1000 ×*g*, 10 min). The resultant supernatant aliquots were isolated and saved at 80°C.

Myeloperoxidase (MPO) activity

An MPO Colorimetric Activity Assay Kit (Sigma-Aldrich, St. Louis, MO, USA) was used. The supernatant was incubated with a KPO_4 buffer (50 mM). The activity of MPO was obtained by

analyzing the alterations of absorbance at 460 nm, which were expressed as U/g protein (U represents a unit of enzyme activity).

Determination of cell population

The cell pellet at the bottom of BALF was suspended in PBS solution. The number of neutrophils and macrophages were obtained by light microscope with Wright-Giemsa staining.

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of TNF-α, IL-1β and IL-6 in the BALF supernatant were measured using commercial ELISA kits (R&D Systems, Minneapolis, MN, USA).

Cell culture and cell transfection

Mouse microvascular pulmonary endothelial cells (MPVECs) and human embryonic kidney 293 (HEK293) cells were from ATCC cell lines (Manassas, VA, USA) and cultured in DMEM (Dulbecco Modified Eagle Medium; 5 % CO_2; 37 °C). For *in vitro* sepsis challenge simulation, the MPVECs were incubated with lipopolysaccharide (LPS) solution (2 μg/mL) for 4 h. Cell transfections, including miR-574-5p mimics, NC mimics, TRAF6 expressing vector (TRAF6) and control vector (Vector), were accomplished by Lipofectamine 2000 (Thermo Fisher Scientific).

Luciferase reporter assay

First, DNA fragments of the TRAF6 3'-untranslated region (UTR) that contain miR-574-5p binding sites were amplified and cloned into a vector (pGL3; Promega, Madison, WI). Another vector with mutated miR-574-5p putative binding sites was used as a control. After transfection, the luciferase activity was finally measurement.

Protein extraction and western blot

Western blotting was described in previous studies [11,12]. Total protein was extracted, separated and transferred onto PVDF, which were blocked and incubated with primary antibodies: anti-TRAF6, p-IKKβ, IKKβ, p-p65, and p65 (Cell Signaling Technology, Danvers, MA, USA).

Secondary antibodies (goat anti-rabbit IgG or goat anti-mouse IgG) conjugated with horseradish peroxidase were used to incubate the membranes. β-Actin expression was used as control. Proteins were visualized a nd analyzed by Image J software (https://imagej.net/Citing).

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Statistical differences between two groups and more than two groups were analyzed using Student's *t*-test and one-way ANOVA, respectively; $p < 0.05$ was considered statistically significant.

RESULTS

MiR-574-5p attenuated mouse sepsis-induced ALI

The qRT-PCR results showed that, in lung tissues from mice in the CLP + NC agomir group, the miR-574-5p level was lower than those in the Sham + NC agomir group (Figure 1 A). In addition, administration of the miR-574-5p agomir resulted in a significant upregulation of miR-574-5p in the lungs (Sham + miR-574-5p agomir and CLP + miR-574-5p agomir). The histological analysis showed that, compared to control mice (sham + NC agomir), sepsis stimulation led to the significant histological damage, including the markedly congested lung tissues and collapsed alveoli in CLP model mice (Figure 1 B). Histological score analysis showed that, in CLP + miR-574-5p agomir group, the lung injury score was significantly reduced as compared to that in CLP + NC agomir group. These findings indicated that miR-574-5p reduced ALI induced by sepsis.

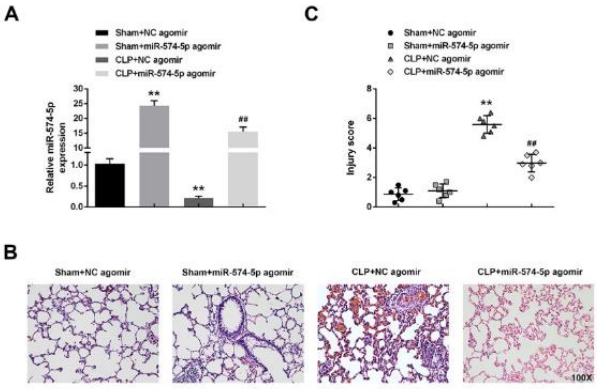

Figure 1: Effect of miR-574-5p mimics on sepsis-induced mouse ALI. (A) Relative miR-574-5p level in lung tissues from mice by qRT-PCR. (B) H&E staining of lung sections from mice. Magnification, 20 × 100. (C) Grading scores were assigned based on severity of lung injury and shown as scatter diagram; **/##$p < 0.01$

MiR-574-5p inhibited inflammatory cell infiltration and cytokine expression

As compared to the control group, the MPO activity and the number of neutrophil and macrophage were increased in the CLP + NC agomir group, but miR-574-5p pretreatment significantly reversed it (Figure 2 A). Furthermore, the dramatic upregulations in the levels of TNFα, IL-1β and IL-6 were observed in the CLP + NC agomir group as compared to the control group (Figure 2 B). However, the sepsis-induced upregulation of proinflammatory cytokines was dramatically reduced by pretreatment with miR-574-5p.

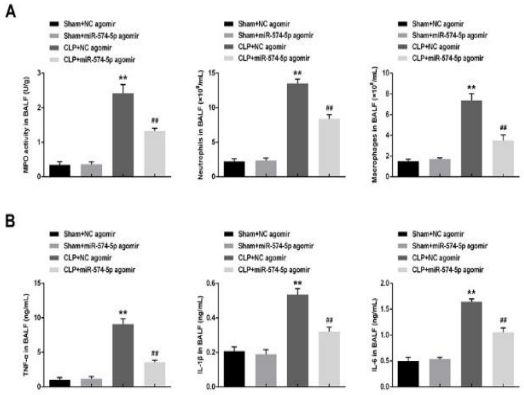

Figure 2: Effect of miR-574-5p on sepsis-induced inflammatory cell infiltration and cytokine production. (A) Myeloperoxidase (MPO) activity and neutrophil and macrophage counts in BALF from mice. (B) Cytokine concentrations in BALF from mice were determined using ELISA; **##p < 0.01

MiR-574-5p targeted TRAF6

The molecular mechanism was investigated. TargetScan predicted that TRAF6 was a potential downstream target of miR-574-5p (Figure 3 A). The luciferase reporter experiment demonstrated that miR-574-5p mimics reduced the luciferase activity of TRAF6 WT-3'-UTR reporter plasmid as compared to that of MUT-TRAF6 3'-UTR reporter plasmid (Figure 3 B). The protein expression of TRAF6 in MPVECs was downregulated by miR-574-5p mimics (Fig. 3 C). As compared to the control group, pulmonary TRAF6 protein level was higher in the CLP + NC agomir group. In addition, as compared to that in the CLP + NC agomir group, TRAF6 expression was markedly induced in the CLP + miR-574-5p agomir group (Figure 3 D). These data indicate that miR-574-5p targeted TRAF6.

TRAF6 upregulation eliminated the protective effect of miR-574-5p

The role of TRAF6 in mouse ALI induced by sepsis was explored. LPS treatment caused the decreased miR-574-5p expression and increased TRAF6 expression in MPVECs (Figures 4 A and B). In addition, the decreased expressions of TNFα, IL-1β and IL-6 after LPS stimulation in miR-574-5p treated mice were reversed by TRAF6 overexpression (Figure 4 C). Furthermore, miR-574-5p treatment downregulated the phosphorylation of IKKβ and p-65 after LPS stimulation, which were reversed by TRAF6 overexpression (Figure 4 D).

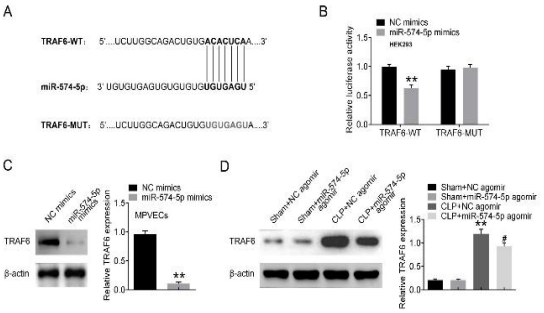

Figure 3: MiR-574-5p targeted TRAF6. (A) for the interaction sites between miR-574-5pand 3'-UTR of TRAF6 were marked in bold. (B) Relative luciferase activity of the TRAF6 3'-UTR reporter plasmids was measured in HEK293 cells after transfecting miR-574-5p mimics or NC mimics. (C) TRAF6 protein levels in MPVECs was determined by western blotting. (D) Pulmonary TRAF6 protein levels in mice was determined using western blotting; #p < 0.05, **p < 0.01

DISCUSSION

Severe sepsis is commonly caused by severe infection and after surgery and leads to life-threatening multi-organ dysfunction including ALI [1]. ALI is characterized by edema of pulmonary interstitium and damage of alveolar, and accompanied by the inflammatory cell infiltration and alterations in the expression of proinflammatory factors which may cause high mortality and morbidity in ALI patients [13]. MiRNAs are known to participate in the pathogenesis of ALI. For example, miR-145 has been shown to target TGFBR2, suppress downstream effector SMAD3 and ameliorate sepsis-induced lung injury [14]. MiR-155 has been reported to be significantly upregulated in septic lung injury, and its downregulation alleviated inflammation by targeting sirtuin1 (SIRT1) in mouse and cell models [15].

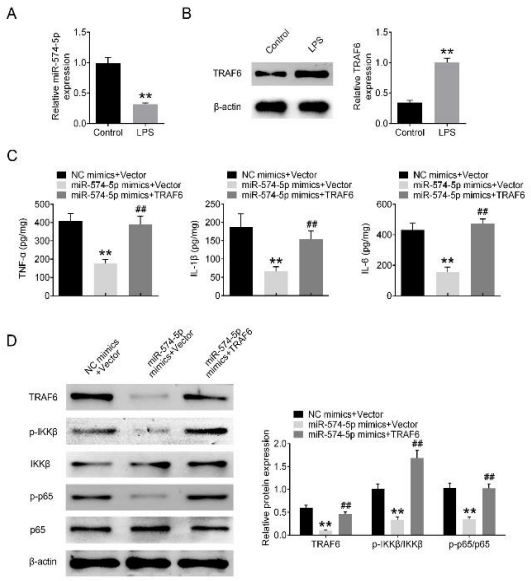

Figure 4: Involvement of TRAF6 in the protective effect of miR-574-5p on MPVECs. (A) Relative miR-574-5p levels and (B) protein expression level of TRAF6 in MPVECs with LPS treatment was determined using qRT-PCR and western blotting. (C) Cytokine concentration in MPVECs from mice was determined using ELISA. (D) Protein expression levels were determined by western blotting. **##$p < 0.01$

In addition, some other miRNAs have been reported to be promoters or suppressors of ALI, including miR-218 that inhibits RUNX family transcription factor 2 (RUNX2) [16], miR-326 that mediates the NF-κB signaling pathway [17], and others. Here, miR-574-5p expression was decreased in the lung tissues from sepsis model mice, and its overexpression reduced the level of inflammatory factors and the severity of lung injury and in lung endothelial cells by targeting TRAF6. Therefore, it is worth considering whether these miRNAs can play a synergistic role in alleviating ALI.

The importance of miR-574-5p has been demonstrated in various tumors. Upregulation of miR-574-5p could promote the development of thyroid cancer , through regulation of the Wnt/β-catenin pathway by targeting Quaking proteins [18]. MiR-574-5p acts as an regulator of CUGBP1to stimulate human lung tumor growth [19]. Although high miR-574-5p levels are related to the death of patients with sepsis, its function in sepsis-induced ALI remains unclear [7]. Herein, this is the first evidence demonstrating a novel protective function of miR-574-5p by negatively regulating TRAF6 and reduced inflammation in sepsis-induced ALI.

TRAF6 act as a transducer for the IL-1R/TLR signaling and TNF receptor superfamily through ubiquitination and activating downstream pathways including the NF-κB pathway, which causes alterations in the release of proinflammatory cytokines [20]. Initially, TRAF6 was investigated in the pathogenesis of acute pancreatitis because TRAF6 downregulation is related to the inflammatory responses in the pancreas and lung [21]. Genetic variation in TRAF6 was later found to be closely related to the susceptibility to sepsis-induced ALI [22].

So far, research has discovered several TRAF6 regulators. Tabersonine, an indole alkaloid mainly isolated from *Catharanthus roseus*, and isoalantolactone [23], a sesquiterpene lactone extracted from roots of *Inula helenium* L. [24], were found to inhibit TRAF6 ubiquitination and alleviate ALI. Regarding miRNA regulation, miR-146a-5p could regulate the expression of TRAF6 in neuropathic pain and other diseases [25]. However, the potential miRNA that may regulate TRAF6 in sepsis-induced ALI needs to be further explored. Here, it was observed that miR-574-5p could directly target TRAF6 and inhibit the NF-κB pathway. The joint use of various methods to regulate TRAF6 and downstream signals to treat acute lung damage requires future research.

CONCLUSION

The expression of miR-574-5p is downregulated in ALI mice induced by sepsis. Administration of miR-574-5p suppresses inflammatory responses and reduces sepsis-induced lung injury. Therefore, the regulatory effects of miR-574-5p on NF-κB pathway might be the mechanism underlying its protective effect against sepsis-induced lung injury. Thus, this approach may be suitable for the clinical management of sepsis-induced ALI.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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