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# MiR-24 inhibits inflammatory responses in LPS-induced acute lung injury of neonatal rats through targeting NLRP3

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ABSTRACT

Inflammation plays an important role in the development of acute lung injury (ALI) in preterm infants. Despite the critical role of microRNA in inflammatory response, little is known about its function in ALI. In this study, we investigate the role of MicroRNA-24 (miR-24) in lipopolysaccharide (LPS) induced neonatal rats ALI and its potential mechanism. LPS was used to induce ALI neonatal animal model. miR-24 expression in the lung tissues of LPS-challenged neonatal rats was detected by qPCR. Proinflammatory factors, including tumor necrosis factor-alpha (TNF-α), IL-1β, IL-18 in the bronchoalveolar lavage fluid and lung tissues of LPS-challenged neonatal rats were measured by qRT-PCR and western blot, respectively. The mRNA levels of surfactant protein A (SP-A) and D (SP-D) was measured by qRT-PCR. Direct binding of miR-24 and pyrin domain-containing 3(NLRP3) were determined by dual luciferase assay. The levels of NLRP3, apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain (ASC) and caspase-1 protein expression were detected by immunohistochemistry (IHC) staining and western blot, respectively. Our data indicated that LPS-induced lung injury in neonatal rats and resulted in significant downregulated of miR-24 expression. Overexpression of miR-24 significantly reduced LPS-induced lung damage and decreased the release of proinflammatory cytokine TNF-α, IL-6, IL-1β and SP-A, SP-D expression induced by LPS. In addition, miR-24 inhibited the expression of NLRP3 by directly targeting to the CDS region of NLRP3 mRNA. Furthermore, miR-24 overexpression attenuated lung inflammation and deactivated the NLRP3/caspase-1/IL-1β pathway in LPS-challenged neonatal rats. These data show that miR-24 alleviated inflammatory responses in LPS-induced ALI via targeting NLRP3.

## 1. Introduction

Acute lung injury (ALI) characterized by severe noncardiogenic pulmonary inflammatory response with a high incidence and mortality rate [1]. Newborns are very susceptible to ALI, which is one of the most common causes of neonatal death [2]. Recently, advances has been made in understanding the epidemiology and pathogenesis of ALI. However, clinical studies suggested that pharmacotherapy is not effective for reducing mortality in patient with ALI [3]. Therefore, it is urgently needed to explore the molecular and immunological mechanisms of neonatal ALI.

The NLRP3 (NOD-like receptor family, pyrin domain containing 3) inflammasome is a protein complex consists of the NLR family member NLRP3, the adaptor protein apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain (ASC) and the effector protein caspase-1 [4]. NLRP3 inflammasome orchestrates immune responses to infection and cell stress through activation of caspase-1 and inflammatory cytokines [5]. Activation of NLRP3 plays a key role in a mouse model of ALI [6]. Several studies suggested that inhibition of the NLRP3 inflammasome could be a potential target for treatment of ALI [7–9].

MicroRNAs (miRNAs) are a class of 18–23 nucleotide long noncoding RNAs, which posttranscriptional downregulated and suppressed target gene expression through binding to specific sites of the target mRNA [10]. MiRNAs have important functions in a broad spectrum of biological processes, including adaptive and innate immunity [11]. Previous study has reported that miRNAs are increasingly involved in several lung diseases [12,13]. A role for miRNAs in the lung injury was demonstrated when microRNA such as miR-155, miR-127, miR-146a was shown to inhibit lung inflammation [14–16]. However, the functions of miR-24 in LPS-induced ALI remain unknown and little is known about the repression of inflammatory genes by miR-24 during acute lung injury.

In the present study, we investigated the role of miR-24 in LPS-

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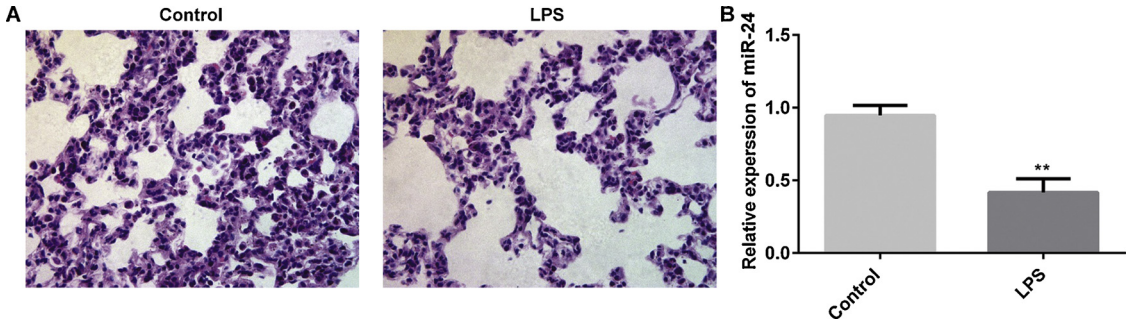


**Fig. 1. miR-24 is downregulated in LPS-challenged ALT neonatal rat.** (A) Hematoxylin and eosin staining for the lung tissues of neonatal rat treated with or without LPS. magnification × 400. (B) qRT-PCR was used to determine miR-24 expression levels in the lung tissues of LPS-induced ALT neonatal rats. Data are represented as mean ± SEM (n = 10). $^{**}P < 0.01$ vs. the control group. LPS, lipopolysaccharide. miR-24, microRNA-24.

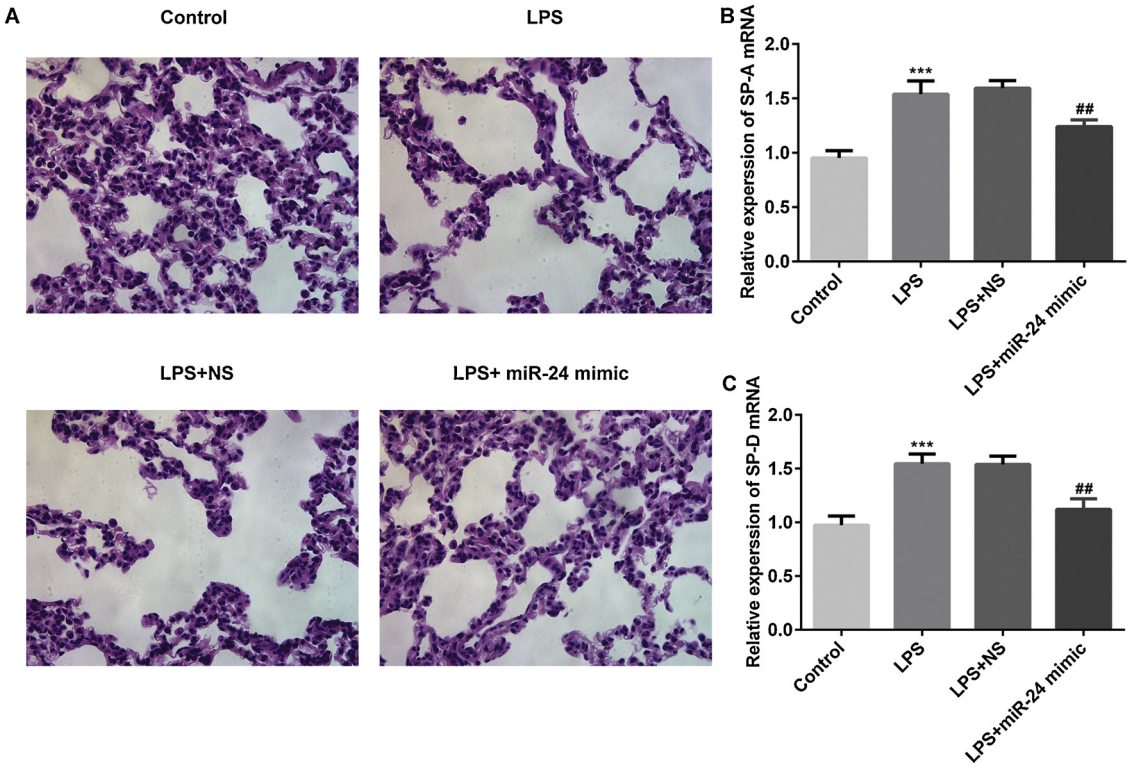


**Fig. 2. Overexpression of miR-24 ameliorates lung injury in LPS-induced ALT neonatal rat.** (A) Hematoxylin and eosin staining for the lung tissues of LPS-induced ALT neonatal rats treated with or without miR-24 mimic. magnification × 400. The levels of SP−A (B) and SP−D (C) in the lung tissues of LPS-induced ALT neonatal rats were detected by qRT-PCR. Data are represented as mean ± SEM (n = 10). $^{***}P < 0.001$ vs. the control group. $^{\#\#}P < 0.01$, vs. the LPS group.

induced lung injury and its potential mechanism.

## 2. Materials and methods

### 2.1. Neonatal rat and preparation of ALI model

Newborn Sprague-Dawley rats (3–8 day old, 8–14 g bodyweight) were purchased from the Zhejiang Center of Laboratory Animals. The animals were housed in a specific pathogen-free condition in groups of five per standard cage on 12 h light/dark cycle. The air temperature was maintained at 22 ± 2°C. Neonatal rat were anesthetized with diethyl ether and then intraperitoneal injected with 3 mg/kg LPS (Sigma-Aldrich, St Louis, MO, USA). Infected animals were divided into three groups (n = 10 per group) randomly and subjected intraperitoneal injections of miR-24 mimic or miR-24 NC at doses of 30 mg/kg body weight for 3 days after LPS exposure. The control group, the rats were intraperitoneally injected with normal saline. The bronchoalveolar lavage fluid (BALF) was collected from neonatal rat according to the previous study [17]. This study was approved by the first hospital of jilin university ethics committee.

### 2.2. HE staining

The lung tissue samples were collected and fixed overnight with 4% paraformaldehyde. After embedded with paraffin, samples were sectioned at 5 μm thickness. Next, hematoxylin and eosin (HE, Sigma) were used to stain. Histopathological viewed using a light microscope (Leica Microsystems, Wetzlar, Germany) equipped with × 400 magnification. Six sections of each lung tissue were randomly selected and scored (n = 10).

### 2.3. Immunohistochemistry (IHC) staining

Lung tissues samples were collected and processed as previously described. The lung sections were then deparaffinized in xylene and dehydrated in graded alcohol (100, 90, 80 and 70%), and performed

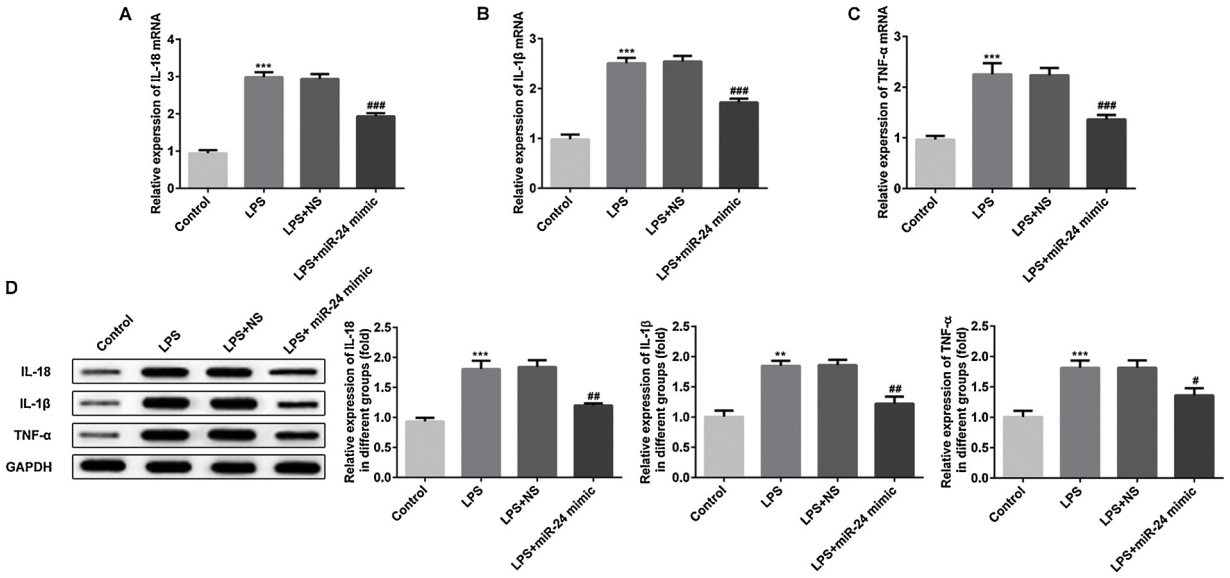


**Fig. 3. Overexpression of miR-24 ameliorates inflammatory response in LPS-induced ALT neonatal rat.** The mRNA levels of IL-18(A), IL-1β (B) and TNF-α (C) in the BALF of LPS-induced ALT neonatal rats were detected by qRT-PCR. (D) The protein levels of IL-18, IL-1β and TNF-α in the lung tissue of LPS-induced ALT neonatal rats were detected by western blot. Data are represented as mean ± SEM (n = 10). $^{***}P < 0.001$ vs. the control group. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. the LPS group. TNF-α, Tumor necrosis factor-alpha, IL-18, Interleukin-18, IL-1β, Interleukin-1β, LPS, lipopolysaccharide. NC, nagetive control. miR-24, microRNA-24.

antigen retrieval. Lung tissues sections were stained with NLRP3 antibody (cat. no. ab214185, Abcam, Cambridge, MA) using a 1:200 dilution overnight at 4℃. Then, HRP-conjugated goat anti-rabbit IgG (cat. no. ab6721 Abcam, Cambridge, MA) was applied and incubated at room temperature for 1 h. Sections were viewed using a Leica DM2000 microscope equipped with ×200 magnification and analyzed using LAS software. NLRP3-positive cells was counted in 6 separate parts of lung tissue per animal (n = 10) and quantitative analyzed using ImageJ (National Institutes of Health, Bethesda, MD, USA).

### 2.4. Western blot analysis

Total protein was exacted using RIPA lysis buffer (Sigma) containing protease and phosphatase inhibitors (Wolsen, xi'an, China). Total protein extracts (20 μg) from lung tissues were separated by 10% SDS polyacrylamide gel electrophoresis and then transferred onto a polyvinylidene difluoride membrane (Millipore, USA). The membrane was blocked with 5% non-fat milk for 1 h at 4℃. Then, the blots were incubated with primary antibodies: IL-18(1:600, cat.no 06-1115, Sigma-Alorich, USA), IL-1β (1:1000, cat.no 12703, Cell Signaling Technology, Inc., Danvers, MA, USA), TNF-α (1: 1000, cat.no 11948, Cell Signaling Technology, Inc., Danvers, MA, USA) NLRP3 (1:1,000, cat.no 13158, Cell Signaling Technology, Inc., Danvers, MA, USA) and ASC (1:1,000, cat.no 67824, Cell Signaling Technology, Inc., Danvers, MA, USA), Caspase-1 (1:1000, cat.no 3866, Cell Signaling Technology, Inc., Danvers, MA, USA) and GAPDH (1:1000, cat.no 5174, Cell Signaling Technology, Inc., Danvers, MA, USA) at 4℃ overnight, followed incubated by horseradish peroxidase-conjugated secondary antibody (1:1000, cat.no 7074, Cell Signaling Technology, Inc., Danvers, MA, USA) for 1 h at room temperature and detected by chemiluminescence detection kit (Millipore, Billerico, MA, USA).

### 2.5. Real-time PCR

Total RNA was isolated from lung tissues or BALF using Trizol (sigma). 5 ug of total RNA was transcribed to cDNA with a reverse transcription kit (Takara). Quantify analysis with real-time PCR assay using the SYBR Green real-time PCR Master Mix regents (Toyobo, Osaka, Japan) on the ABI PRISM 7000 Sequence Detection System (ABI/Perkin Elmer, Foster City, CA) according to the standard protocol. The primers are showed as follows: miR-24-F: 5′-CGGGGTACCCTTTCATAGTCGCCAGTC-3′ miR-24-R: 5′−CCGCTCGAGCACATACCAACAATCCCT-3′; TNF-α-F: 5′-AGCACACAAGTGGCACAACG-3′, TNF-α-R: 5′-CATGCGGTCGTAGTCCATAAT-3′; IL-18-F: 5′- SGCCTGTGTTCGAGGATATGACT-3′; IL-18-R: 5′−CCTTCACAGAGAGGGTCACAG-3′; IL-1β-F: 5′-GCTGCTTCCAAACCTTTGAC-3′, IL-1β-R: 5′-AGCTTCTCCACAGCCACAAT-3′; SP-A-F: 5′-CTTCACCCTCTTCTTGACTGTTG-3′; SP-A-R: 5′-TCTCCCTTGTCTCCACGTCCT-3′; SP-D-F:5′-ACTCATCACAGCCCACAACA-3′; SP-D-R:TCAGAACTCACAGATAACAAG; GAPDH-F: 5′-TGGCCTTCCGTGTTCCTAC-3′, GAPDH-R: 5′-GAGTTGCTGTTGAAGTCGCA-3′; U6-F: 5′-GCTTCGGCAGCACATATACTAAAAT-3′, U6-R: 5′-CGCTTCACGAATTTGCGTGTCAT-3′.

### 2.6. Bioinformatics analysis and miRNA prediction

microRNA databases (http://www.mircode.org/), TargetScan 6.2 (http://www.targetscan.org/index.html) and miRWalk (http://mirwalk.uni-hd.de/) were used to identify miR-24 potential targets.

### 2.7. Luciferase reporter assay

To determine whether NLRP3 is a direct target of miR-24, the CDS region of NLRP3 mRNA containing putative miR-24 binding site was amplified by PCR and cloned into pmiR-Report vector downstream of the luciferase coding gene to generate wile type (WT) NLRP3-CDS. Mutations in the miR-24 predicted target site in the CDS were generated the mutagenic oligonucleotide primers using the QuikChange Multi Site-directed Mutagenesis kit (Stratagene, LaJolla, CA). For luciferase assay, 293 T cells were cultured in 24-well plates, then pmiR NLRP3-CDS or their mutant constructs was transfected into 293 T cells. 48 h post transfection, firefly luciferase activity was normalized using a Renilla luciferase plasmid and measured using dual luciferase assays (Promega).

### 2.8. Statistical analysis

Statistical analysis was carried out with GraphPad Prism 5.0 (GraphPad, San Diego, CA, USA). Data are expressed as the mean ± SEM. Differences between multiple groups were analyzed using one-way ANOVA. Statistical difference was considered at

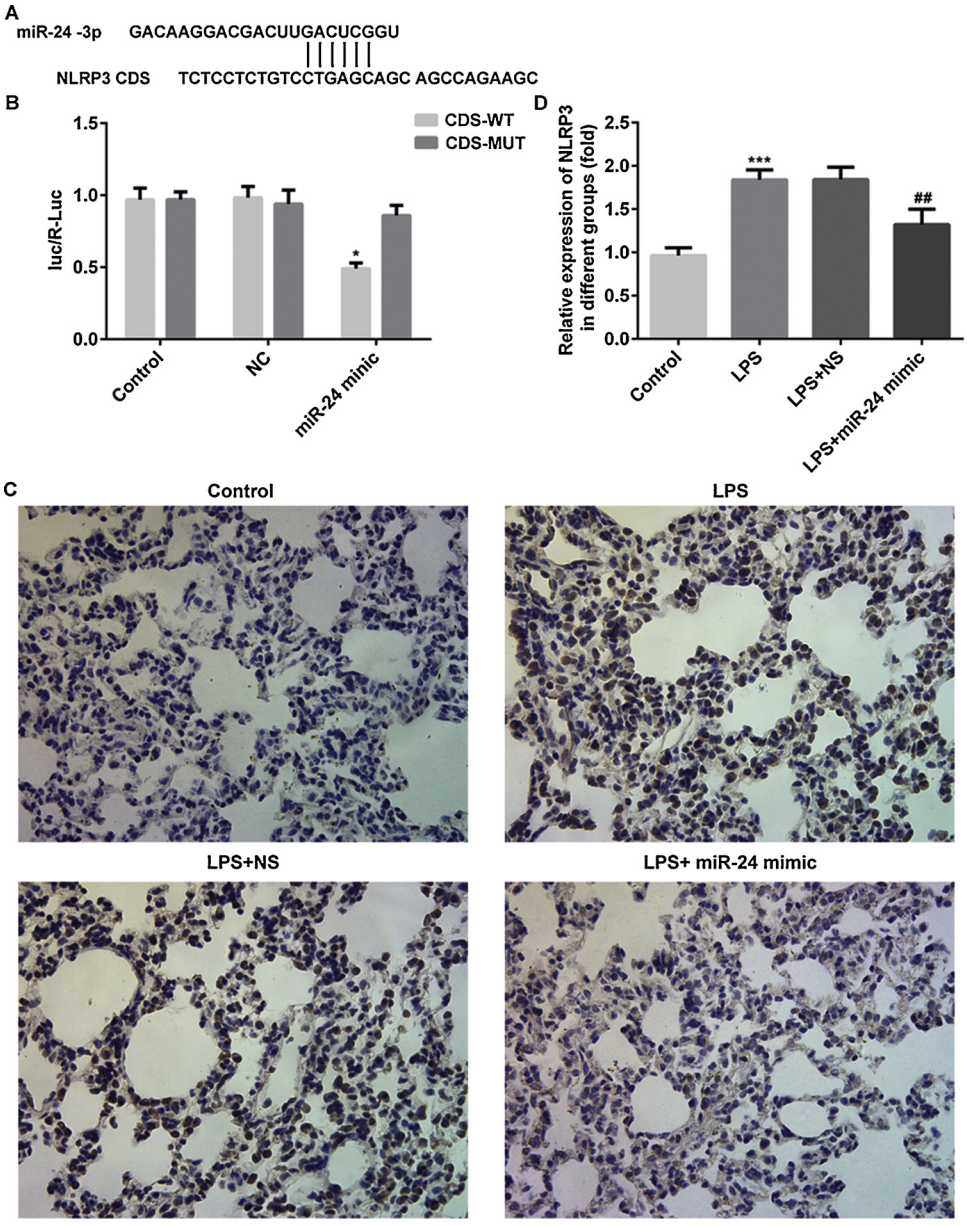


**Fig. 4. NLRP3 is a directly target gene of miR-24.** (A) Bioinformatical predication showed that NLRP3 was a putative target gene of miR-24. (B) The dual luciferase reporter assay was used to detect the luciferase activity in cells co-transfected with NLRP3-CDS WT/MUT and miR-24 mimic/mimic control. (C) Immunohistochemical staining for NLRP3 in the lung tissues of LPS-induced ALT neonatal rat treated with or without miR-24 mimic. magnification × 200. (D) Quantitative analysis of NLRP3 expression. Data are represented as mean ± SEM (n = 10). $^{*}P < 0.05$, $^{***}P < 0.001$ vs. the control group. $^{\#\#}P < 0.01$, vs. the LPS group. NC, nagetive control. NLRP3, NOD-like receptor family, pyrin domain containing 3.

$p < 0.05$.

## 3. Results

### 3.1. miR-24 is downregulated in LPS-challenged neonatal rat

As shown in Fig. 1A, compared with the control group, the lungs of LPS-challenged neonatal rat showed the presence of interstitial edema, cytoplasmic vacuolization, indicating that LPS successfully induced ALF in neonatal rat. We measured miRNA-24 expression by qRT-PCR at 6 h of LPS treatment. As shown in Fig. 1B, compared with the control group, miRNA-24 expression was significantly downregulated in LPS-challenged neonatal rat.

### 3.2. Overexpression of miR-24 ameliorates inflammatory response in LPS-challenged neonatal rat

To confirm the physiological function of miR-24 in LPS-induced ALI in vivo, we intraperitoneal injected miR-24 mimic into neonatal rat after LPS challenged. Subsequently, HE staining was conducted to detect the inflammatory status in lungs. As shown in Fig. 2A, compared with the control group, the lungs of LPS-challenged neonatal rat showed the presence of alveolar structure disorder, interstitial edema, cytoplasmic vacuolization, and the alveolar septum was significantly widened. miR-24 mimic injection significantly improved histological damage induced by LPS. Alveolar structure disorder, interstitial edema, cytoplasmic vacuolization, and the alveolar septum in Neonatal rat were less severe in LPS + miR-24 mimic group than in LPS group. In addition, we analyzed the pulmonary surfactant proteins (SP−A and SP−D) expression in the neonatal rat lung tissue. As shown in Fig. 2B-C, compared with the control group, the levels of SP−A and SP−D were significantly increased after LPS challenge. miR-24 overexpression decreased the levels of SP−A and SP−D induced by LPS.

Furthermore, we investigate the function of miR-24 on the expression of proinflammatory cytokines TNF-α, IL-18 and IL-1β in LPS-challenged neonatal rat. As shown in Fig. 3A-D, compared with the control group, the mRNA levels of IL-18, IL-1β and TNF-α in the BALF (Fig. 3A-C) and the protein levels of these cytokines (Fig. 3D) in the lung tissues of neonatal rat were significantly increased after LPS challenged. Overexpression of miR-24 decreased these cytokines

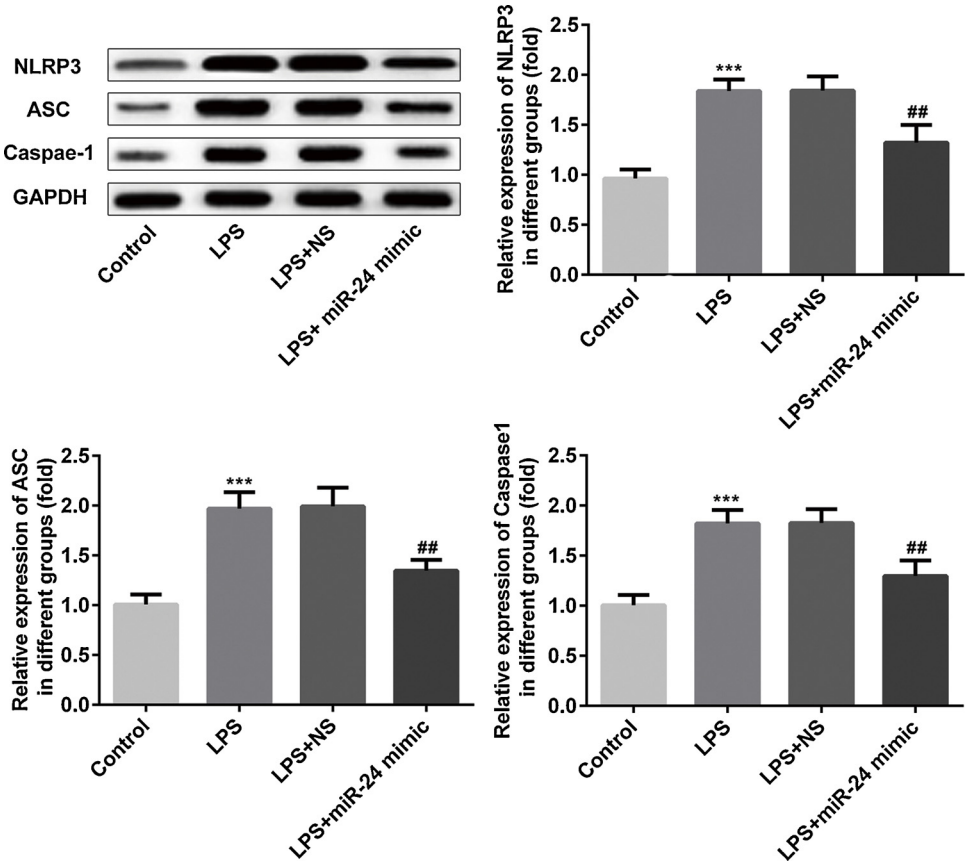


**Fig. 5. MiR-24 overexpression inhibits the activation of NLRP3 inflammasome in LPS-challenged neonatal rat.** The protein expression of NLRP3, ASC and Caspase-1 in the lung tissue of LPS-induced ALT neonatal rats treated with or without miR-24 mimic were detected by western blot. Data are represented as mean ± SEM (n = 10). $^{***}P < 0.001$ vs. the control group. $^{\#\#}P < 0.01$ vs. the LPS group. NC, nagetive control. NLRP3, NOD-like receptor family, pyrin domain containing 3. ASC, apoptosis-associated speck-like protein containing a c-terminal caspase recruitment domain.

expression induced by LPS. These results indicated that miR-24 overexpression decreases the inflammatory response by reducing the production of inflammatory cytokines in LPS-challenged neonatal rat.

### 3.3. MiR-24 directly targets NLRP3

Bioinformatics analysis was performed to predicate the putative targets of miR-24, and found that NLRP3 might be a target gene of miR-24 (Fig. 4A). Luciferase reporter assay showed that miR-24 mimic significantly reduced the luciferase activity of the wild-type NLRP3 CDS reporter gene, whereas has no effected on the mutant reporter of NLRP3 CDS reporter gene, indicating that NLRP3 is a direct target of miR-24 (Fig. 4B). We next identify the regulatory effect of miR-24 on NLRP3 expression in LPS-challenged neonatal rat. IHC staining results showed that compared with control group, the levels of NLRP3 was significantly increased in the lungs of LPS-challenged neonatal rat. miR-24 mimic transfection down-regulated NLRP3 expression in the lungs of LPS-challenged neonatal rat (Fig. 4C-D).

### 3.4. MiR-24 overexpression inhibits the activation of NLRP3 inflammasome in LPS-challenged neonatal rat

We next identify the regulatory effect of miR-24 on NLRP3 inflammasome expression in LPS-challenged neonatal rat. The expression of NLRP3 inflammasome related protein NLRP3, ASC and Caspase-1 in the lungs of LPS-challenged neonatal rat were measured after miR-24 mimic transfection. As shown in Fig. 5, the protein expression of NLRP3, ASC and Caspase-1 were significantly increased in the lung tissues of neonatal rat after LPS challenge, which were attenuated by miR-24 mimic transfection. Our results indicated that miR-24 inhibits the activation of NLRP3 inflammasome.

## 4. Discussion

ALI is characterized by a prominent pulmonary and systemic inflammatory response. The mortality of children with acute lung injury is higher than 60% [18]. Clinicians treating children with ALI have used protocols described as similar to ARDSNet protocols [19]. Pediatric intensivists commonly use different modes of mechanical ventilation (MV). Partial liquid ventilation is considered to be a less harmful form of respiratory support for these children [20]. However, the potential mechanism of neonatal ALI remains unclear. In the present study, our results showed that miR-24 was downregulated in LPS-challenged neonatal rat. NLRP3 was found as a direct target of miR-24. Overexpression of miR-24 ameliorates inflammatory response and inhibits the activation of NLRP3 inflammasome in LPS-challenged neonatal rat. Our study indicated that miR-24 might be a protective factor in LPS-induced lung injury of neonatal rat.

LPS is a critical component of the cell wall of Gram-negative bacteria and is a commonly trigger of ALI by activating inflammatory cascade and prominent etiological factor [1,21]. LPS is widely used to induce animal models of ALI. As expected, LPS was successfully induced ALI neonatal animal model. MicroRNAs (miRNA) have reported play a crucial role in multiple complicated diseases, including ALI. For example, inhibition of miR-203 effectively attenuated LPS-induced interstitial pneumonia and ALI [22]. Recent study revealed that miR-24 was down-regulated in acute respiratory distress syndrome (ARDS), which characterized by pulmonary epithelial injury and extensive inflammation of the pulmonary parenchyma [23]. In the present study, our results showed that miR-24 expression was significantly down-regulated in the lung tissues of LPS-induced neonatal rat.

Bioinformatics analysis predicted that NLRP3 was a putative target mRNA of miR-24. A dual luciferase reporter was confirmed that miR-24 mimic transfection significant decrease the luciferase activity of the wild-type NLRP3 CDS reporter gene. Moreover, miR-24 mimic injection

prevented the LPS-induced increase of NLRP3 expression.

NLRP3 inflammasome is a major intracellular inflammatory pathway, which participate in innate immune response [24]. When NLRP3 inflammasome was activated, the cytokines like IL-1β and IL-18 etc were secreted [25]. Previous study has reported that NLRP3 inflammasome activation results in inflammatory tissue damage in ALI [26]. Inhibition of NLRP3 inflammasome activation could alleviated ALI. For example, miR-223 inhibits the activity of the NLRP3 inflammasome to alleviate acute lung injury [9]. Recently studies has reported that miR-24 limits aortic vascular inflammation [27] and suppress allergic inflammation [28], indicating miR-24 exhibited anti-inflammatory action. In our present study, we found that NLRP3 inflammasome was activated in LPS-induced neonatal rat. Overexpression of miR-24 markedly decreased LPS-induced the proinflammatory cytokines Il-1β, IL-18, and TNF-α expression and compromised LPS-induced activation of NLRP3 inflammasome. These findings indicated that miR-24 alleviated the progression of ALI in neonatal rats by inhibiting NLRP3 inflammasome activation.

In conclusion, this study demonstrated that miR-24 inhibits inflammatory responses via targeting NLRP3. These findings highlights the miR-24 might be a potential prognostic biomarker and therapeutic strategy for ALI in neonatal rats.

## Conflict of interest

The authors declare no conflict of interest.

## Ethics statement

This study was approved by the first hospital of jilin university ethics committee.

## Financial disclosure

None.

## Acknowledgement

None

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