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# MicroRNA-17 contributes to the suppression of the inflammatory response in lipopolysaccharide-induced acute lung injury in mice via targeting the toll-like receptor 4/nuclear factor-κB pathway

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**Abstract.** Acute lung injury (ALI) is a common lung disease with a high mortality rate, which is characterized by an excessive uncontrolled inflammatory response. MicroRNA (miR)-17 has previously emerged as a novel regulatory molecule of inflammatory response in various complex diseases; however, the anti-inflammatory action and associated molecular mechanisms of miR-17 in ALI have not been fully elucidated. The aim of the present study was to investigate the role of miR-17 in the inflammatory response in ALI and to elucidate the potential underlying mechanism. Using a lipopolysaccharide (LPS)-induced ALI mouse model, it was observed that miR-17 was significantly downregulated in lung tissues compared with the control group. In this model, ectopic expression of miR-17 attenuated lung pathological damage, reduced lung wet/dry ratio and lung permeability, and increased survival rate in ALI mice. In addition, agomiR-17 injection significantly suppressed LPS-induced inflammation, as evidenced by a reduction in the activity of myeloperoxidase and the production of interleukin (IL)-6, IL-1β and tumor necrosis factor-α in lung tissues. Of note, toll-like receptor (TLR) 4, an upstream regulator of the nuclear factor (NF)-κB inflammatory signaling pathway, was directly targeted by miR-17, and its translation was suppressed by miR-17 *in vitro* and *in vivo*. Using an LPS-induced RAW264.1 macrophage injury model, it was observed that miR-17 overexpression suppressed the pro-inflammatory effect of LPS, while these inhibitory effects were markedly abrogated by TLR4 overexpression. In addition, TLR4 knockdown by si-TLR4 mimicked the effects of miR-17 overexpression on LPS-induced cytokine secretion in the *in vitro* model. Further experiments revealed that miR-17 significantly reduced the expression of key proteins in the NF-κB pathway, including IKKβ, p-IκBα and nuclear p-p65, and suppressed the NF-κB activity in ALI mice. Collectively, these results indicated that miR-17 protected mice against LPS-induced lung injury via inhibiting inflammation by targeting the TLR4/NF-κB pathway; therefore, miR-17 may serve as a potential therapeutic target for ALI.

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*Key words:* acute lung injury, microRNA-17, inflammatory response, toll-like receptor 4, nuclear factor-κB pathway

## Introduction

Acute lung injury (ALI) is a severe clinical condition with a high mortality rate among critically ill patients (1,2). Although there have been significant advancements in the therapeutic strategies of ALI, the morbidity and mortality rates remains high (3,4). In recent years, it has become widely accepted that inflammation is an important pathological characteristic of ALI, and contributes to the initiation and development of ALI (5). Therefore, inhibition of the inflammatory response may be key to the improvement of ALI patient outcomes.

It is well known that toll-like receptors (TLRs) play an important role in the inflammatory process triggered by lipopolysaccharide (LPS) (6,7). After TLR4 recognizes LPS, a series of cascades, including myeloid differentiation factor 88 (MyD88), are initiated, followed by activation of the nuclear factor (NF)-κB pathway and the secretion of pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6, ultimately resulting in ALI (8). Once the TLR4 pathway is inhibited, the inflammatory response can be alleviated, thereby attenuating ALI. Extensive studies have demonstrated, using an LPS induced *in vivo* ALI model, that inhibition of the TLR4 pathway is beneficial in ALI (9,10). For example, Zhang *et al* reported that inhibition of the TLR4/NF-κB signaling pathway improved the oxidative stress and inflammatory response in the lung tissues of ALI rats (11). Therefore, suppression of the activation of the TLR4/NF-κB pathway may alleviate inflammation-induced ALI.

MicroRNAs (miRNAs) are a family of short non-coding RNAs (with a mean size of 22 nucleotides), which suppress target gene expression through either translation repression or RNA degradation (12). Accumulating evidence has demonstrated that miRNAs potentially contribute to the development of ALI via regulation of target genes (13-15). For example,

Yang *et al* observed that miR-140-5p inhibited LPS-induced inflammatory response in ALI via blocking the TLR4 pathway (16). Ling *et al* demonstrated that miR-494 inhibition improved lung injury through suppressing the inflammatory response in ALI rats (17). miR-17, a member of the miR-17-92 cluster, has been found to play an important role in ameliorating inflammatory response, particularly pulmonary inflammation (18,19). More importantly, a recent study has identified decreased expression of miR-17 in ALI mice, and miR-17 negatively regulates lung FOXA1 expression, which plays an important role in ALI by promoting the apoptosis of alveolar type II epithelial cells *in vitro* and *in vivo* (20). However, the function of miR-17 in inflammatory response in ALI has yet to be fully elucidated.

In the present study, an *in vivo* mice model of ALI and an *in vitro* LPS-induced RAW264.7 cell injury model were established to investigate the role and underlying mechanism of action of miR-17 in the regulation of inflammation in ALI. The aim was to determine whether miR-17 may hold promise as a novel treatment target for the prevention and treatment of ALI.

## Materials and methods

*Ethics statement*. The protocol of the present study was approved by the Ethics Committee of the Affiliated Hospital of Inner Mongolia University for Nationalities (permit no. 2018-0139). The mice were treated humanely, and all measures were undertaken to minimize animal suffering. The mice were monitored every 12 h over a period of 1 week for health and behavior. A humane endpoint was used in our experiments according to previous report (21). The specific signs used to determine the endpoint included: i) Loss of >25% body weight compared with the starting weight; ii) decreased food or water intake; iii) decreased mobility/activity, lethargy, rough hair coat. Sacrifice was performed by intraperitoneal injection of sodium pentobarbital (50 mg/kg) followed by cervical dislocation, and death was confirmed when no spontaneous breathing for 2-3 min and no blinking reflex were observed (22). No animals died before meeting these endpoints. All mice (n=60) were euthanized as mentioned above.

*Animals*. A total of 60 male BALB/c mice (6-8 weeks old, weighing 18-22 g) were obtained from the Shanghai SLAC Laboratory Animal Co. Ltd. BALB/c mice were housed under standard conditions (12-h light-dark cycle, 25-27˚C, ~40% humidity) with free access to food and water throughout the duration of the experiments. A total of 20 mice were randomly divided into four groups (n=5/group) as follows: i) Control, ii) LPS, iii) LPS + agomir-17 and iv) LPS + agomir-negative control (NC) groups. LPS group mice were injected through the tail vein with 2 mg/kg LPS. The control group received the same volume of normal saline. Mice in the agomir-17 or agomir NC groups were injected intravenously with agomir-17 or agomir NC (8 mg/kg), respectively (20), 24 h prior to LPS induction. All mice were anesthetized with pentobarbital sodium (50 mg/kg, intraperitoneal injection), and euthanized by cervical dislocation at 24 h after LPS treatment (except those included in the survival experiment), then the lung tissues and bronchoalveolar lavage fluid (BALF) were collected for subsequent analysis.

For the survival rate analysis, 40 mice were divided into four groups (n=10/group) as mentioned above. The survival rate from 0 to 7 days was observed and calculated using the Kaplan-Meier method. Agomir-17/NC were designed and synthesized by RiboBio. The sequences were as follows: Agomir-17: 5'-CAAAGUGCUUACAGUGCAGGUAG-3'; and agomir NC: 5'-GUCCUGAGAAGGCUAGCAUAGAU-3'.

*Reverse transcription-quantitative PCR (RT-qPCR) analysis*. Total RNA was isolated from lung tissues or cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). RT of miR-17 was performed using the miScript II RT kit (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C for 30 min. miR-17 expression was measured using the Exiqon SYBR Green Master Mix (Exiqon; Qiagen, Inc.) on a Light Cycler instrument (Bio-Rad Laboratories, Inc.). The thermocycling conditions were as follows: A hot start step at 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 sec and 60˚C for 1 min. The primers for RT-qPCR analysis were as follows: miR-17 forward, 5'-AGGCCCAAAGTGCTGTTCGT-3' and reverse, 5'-GTGCAGGGTCCGAGGT-3'; U6 forward, 5'-TGCGGG TGCTCGCTTCGCAGC-3' and reverse, 5'-CCAGTGCAG GGTCCGAGGT-3'. The miRNA relative expressions were analyzed using the $2^{-\Delta\Delta Cq}$ method (23).

*Lung histology*. After a 24-h LPS challenge, the mice were sacrificed and the lung tissues were harvested and fixed in 10% formalin for 24 h at 4˚C, embedded in paraffin, and cut into 4-$\mu$m sections. The sections were stained using hematoxylin for 5 min and rinsed with distilled water, followed by color separation with alcohol hydrochloric acid at room temperature. Subsequently, the samples were stained with eosin for 2 min, dehydrated, cleared, dried and mounted at room temperature. Histological changes were observed and photographed under a light microscope (E-800M, Nikon Corporation) at a magnification of x200 and 400.

*Evaluation of lung permeability*. The Evans Blue (EB) dye extravasation method was used to assess pulmonary permeability as previously described (24). Briefly, EB dye (20 mg/kg, Sigma-Aldrich; Merck KGaA) at a concentration of 0.5% (5 mg/ml) in normal saline was injected into the mice of each group through the tail vein. After 2 h, the mice were sacrificed and then the dye was extracted by incubation in formamide for 24 h at 60˚C. The light absorbance at 620 nm was measured, and the dye concentration in lung homogenate was calculated against a standard curve and was expressed as $\mu$g of Evans blue dye per g of lung tissue.

*Lung wet/dry (W/D) ratio*. The W/D ratio was used to assess pulmonary edema. After a 24-h LPS challenge, the right lung of the mice was harvested and immediately weighted, then dried in an incubator at 80˚C for 60 h.

*Measurement of IL-6, IL-1$\beta$ and TNF-$\alpha$*. The supernatant from the BALF was collected by centrifugation at 12,000 x g for 10 min at 4˚C. For cultured cells, the supernatant was carefully collected by centrifugation 12,000 x g for 10 min

at 4˚C. The concentrations of IL-6, IL-1β and TNF-α were analyzed by using IL-6 (cat. no. p1330), IL-1β (cat. no. p1305) and TNF-α (cat. no. pt518) ELISA kits, respectively, according to the instructions of the manufacturer (Beyotime Institute of Biotechnology).

*Measurement of myeloperoxidase (MPO) activity*. Lung tissue homogenate was subjected to MPO assay using a commercial kit (cat. no. ab105136, Abcam) and the absorbance at 460 nm was detected using a microplate reader (Bio-Tek Instruments, Inc.).

*Cell culture and transfection*. RAW264.7 cells were obtained from Sciencell Research Laboratories and maintained in DMEM supplemented with 10% FBS (Sigma-Aldrich; Merck KGaA), 1% penicillin and streptomycin (Sigma-Aldrich; Merck KGaA) at 37˚C and 5% $CO_2$ in an incubator.

After RAW264.7 cells in 6-well plates had grown to ~80% confluence, 20 nM miR-17 mimics, 20 nM miR-17 inhibitor, 2 μg pcDNA-TLR4 or 30 nM si-TLR4 were transfected into the cells at 37˚C for 24 h using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). miR-17 inhibitor, miR-17 mimics and the corresponding control vectors were purchased from RiboBio Co., Ltd. The TLR4 overexpressing vector pcDNA-TLR4 and empty vector pcDNA were constructed by Qiagen, Inc. In addition, TLR4 siRNA (si-TLR4) and corresponding negative control siRNA (si-Scramble) were purchased from RiboBio Co., Ltd. The sequences were as follows: miR-17-5p mimic: 5'-CAAAGUGCUUACAGUGCA GGUAG-3'; mimics NC: 5'-GUCCUGAGAAGGCUAGCA UAGAU-3'; miR-17-5p inhibitor: 5'-CUACCUGCACUGUAA GCACUUUG-3', inhibitor NC 5'-CUAUGCUAGCCUUCUC AGGACUU-3'.

*Cell proliferation*. The effect of LPS (2 μg/ml) on RAW264.7 cells was measured by using the MTT assay. At the end of the transfection, 20 μl MTT solution (Sigma-Aldrich; Merck KGaA) was added into each well ($2x10^5$/well), and RAW264.7 cells were cultured for another 2 h. Then, the absorbance at 450 nm was detected by a microplate reader (Bio-Tek Instruments, Inc.).

*Luciferase assay*. miRNA target prediction tools, including PicTar version 2007 (https://pictar.mdc-berlin.de/) and TargetScan Release 7.0 (http://targetscan.org/) were used to search for the putative targets of miR-17. The dual luciferase reporter assay was performed as described previously (25). RAW264.7 cells were treated with miR-17 mimics, miR-17 inhibitor the luciferase reporter plasmids (wt-TLR4-UTR-pGL3 or mt-TLR4-UTR-pGL3), and pRL-TK-Renilla control plasmid (Promega Corporation) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). At 48 h post-transfection, luciferase activity was detected using the Dual Luciferase Reporter kit (Beyotime Institute of Biotechnology).

*Western blot analysis*. Protein was extracted from the mouse lung tissues using the Nuclear and Cytoplasmic Protein Extraction kit (Beyotime Institute of Biotechnology). The protein concentration was measured using a protein analysis kit (Bio-Rad Laboratories, Inc.). Extracted protein samples (40 μg) were separated by 12% SDS-PAGE (w/v) and transferred onto a PVDF membrane (EMD Millipore). The membrane was blocked with 5% skimmed milk for 2 h at room temperature, followed by incubation with primary antibodies against TLR4 (cat. no. 14358, 1:2,000 dilution), nuclear p-p65 (Ser-468, cat. no. 3039, 1:1,000 dilution), p-IκB-α (Ser-32, cat. no. 2859, 1:1,000 dilution), IκB-α (cat. no. 4814, 1:1,000 dilution), Histone H3 (cat. no. 9728, 1:1,000 dilution) and β-actin (cat. no. 3700, 1:1,000) (all from Cell Signaling Technology, Inc.) at 4˚C overnight, followed by HRP-conjugated goat anti-rabbit IgG (1:10,000; cat. no. 205718; Abcam). β-actin and Histone H3 served as internal controls. The protein bands were developed using an ECL kit (GE Healthcare) and blot bands were quantified with ImageJ version 1.46 (Rawak Software, Inc.).

*Statistical analysis*. GraphPad Prism 5.0 (GraphPad Software, Inc.) was used to perform the statistical analyses. When only two groups were compared, Student's t-test was used. One-way analysis of variance followed by Tukey's post hoc test was applied to compare differences between multiple groups. Survival analysis was performed using the Kaplan-Meier method. All data are presented as the mean ± standard deviation. $P<0.05$ was considered to indicate statistically significant differences.

## Results

*miR-17 is downregulated in lung tissues of ALI mice*. First, an LPS-induced ALI mouse model was established, which is widely used to simulate the pathological conditions of severe lung injury *in vivo* (26). Following LPS treatment, the pathological changes of lung tissues, lung W/D ratio (an indicator of lung edema), pulmonary capillary permeability, MPO activity and the inflammatory response were evaluated. As shown in Fig. 1A, the H&E staining results revealed that LPS treatment caused obvious pathological changes, including inflammatory cell infiltration and widespread increased alveolar wall thickness, compared with the control group (n=5/group). The W/D ratio of mice in the LPS group (n=5/group) was markedly higher compared with that in the control group (Fig. 1B). The pulmonary capillary permeability was measured by the EB dye method (n=5/group) and the results demonstrated that LPS treatment led to a significant increase in EB dye extravasation compared with the control group (Fig. 1C). The MPO activity, determined by a commercial assay (n=5/group), was markedly upregulated following LPS stimulation compared with that in the control group (Fig. 1D). Furthermore, the expression of IL-1β, IL-6 and TNF-α was measured by ELISA (n=5/group). As shown in Fig. 1E, the levels of these cytokines were obviously increased in the LPS group compared with those in the control group (Fig. 1E). All these data indicated that the LPS-induced ALI mouse model was successfully constructed.

To investigate the potential involvement of miR-17 in ALI, the expression level of miR-17 was measured in lung tissues from ALI mice (n=10/group). It was observed that miR-17 was markedly downregulated in the lung tissues from ALI mice (Fig. 1F), suggesting that miR-17 may play an important role in the pathogenesis of ALI.

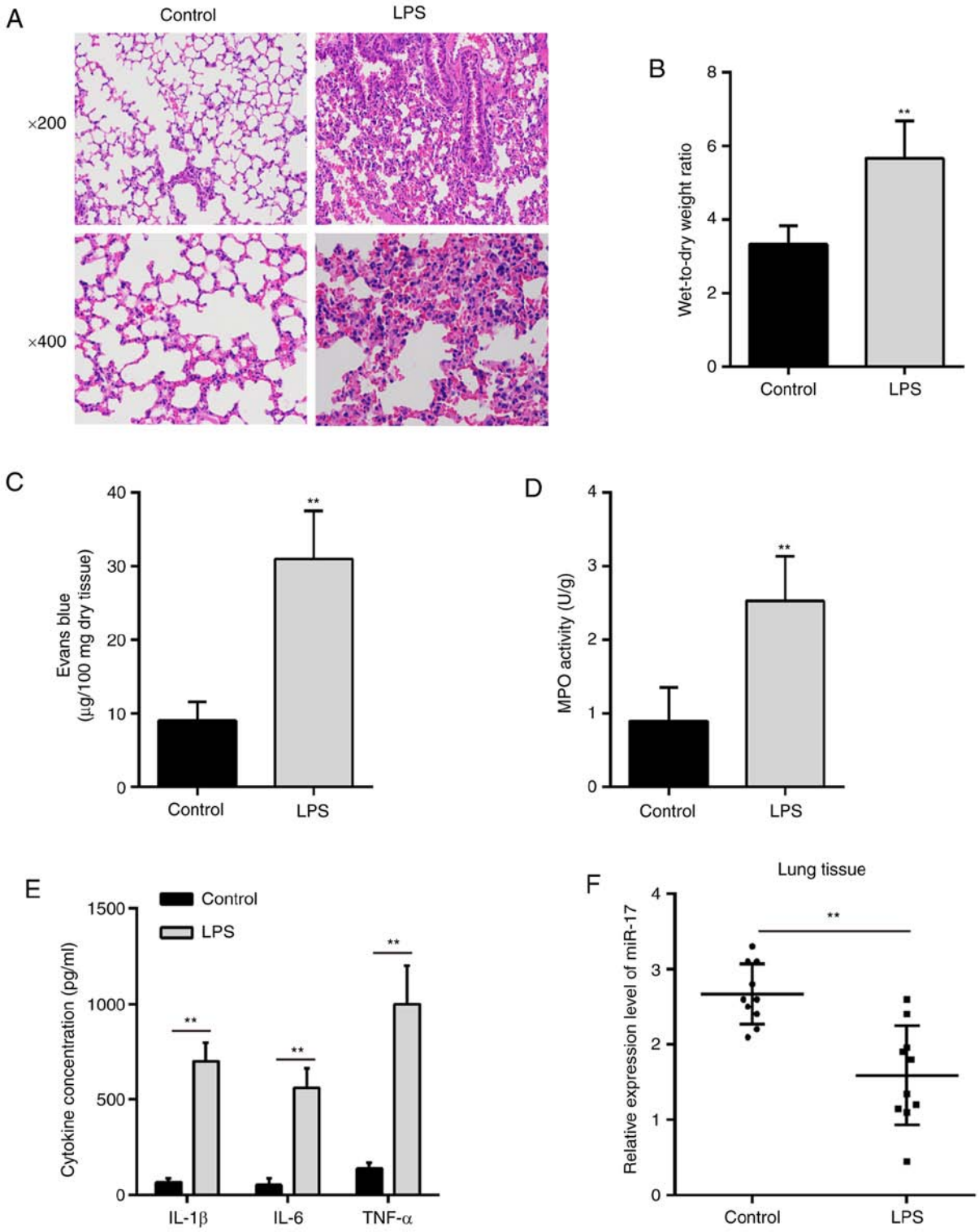


Figure 1. miR-17 was downregulated in mice with LPS-induced ALI. The mice were intratracheally administered LPS for 24 h. Subsequently, the mice were sacrificed and lung tissues were collected for analysis (n=5 mice per group). (A) Histopathological analysis of lung tissue was performed following hematoxylin and eosin staining. Original magnification, x200 and x400. (B) Pulmonary edema was evaluated using the lung W/D ratio. (C) Lung permeability was assessed using the Evans Blue dye extravasation method. (D) Infiltration of neutrophils into the lung tissues was assessed by MPO activity. (E) The levels of the cytokines IL-1β, IL-6 and TNF-α were detected by ELISA. (F) The expression of miR-17 was detected in the lung tissues of LPS-treated mice by quantitative PCR analysis (n=10 mice per group). Data are expressed as mean ± standard deviation of three independent experiments. $^{**}$P<0.01 vs. control group. LPS, lipopolysaccharide; ALI, acute lung injury; W/D ratio, wet/dry ratio; MPO, myeloperoxidase; IL, interleukin; TNF, tumor necrosis factor.

*Overexpression of miR-17 improves LPS-induced ALI in vivo.* To further examine the therapeutic effect of miR-17 in LPS-induced ALI, agomiR-17 was intravenously administered to the mice, followed by LPS treatment. The miRNA transfection efficiency was first evaluated using RT-qPCR. The results demonstrated that the miR-17 expression in lung tissues from ALI mice was increased following agomiR-17 injection (n=5/group; Fig. 2A). Subsequently, the pathological changes,

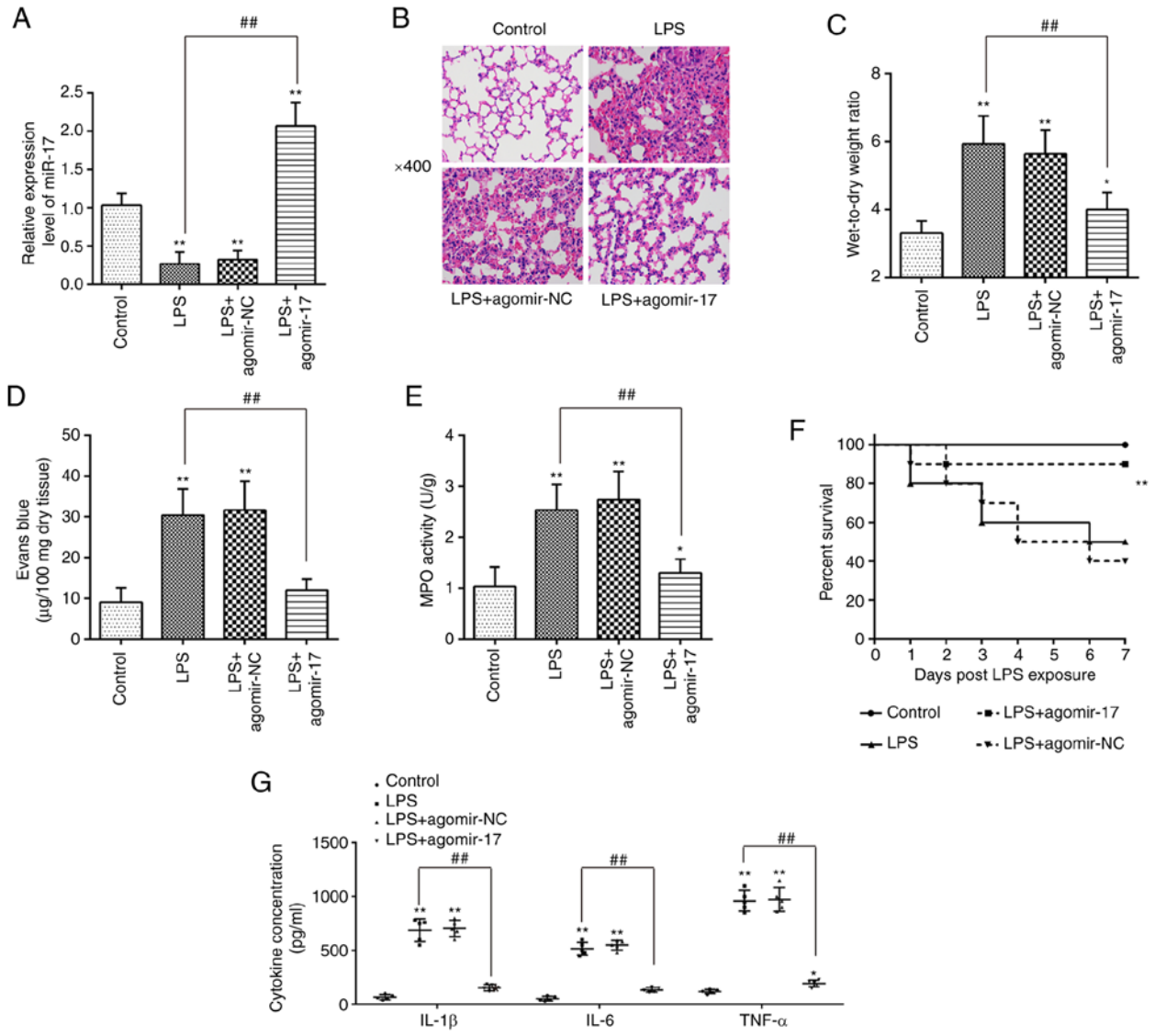


Figure 2. Overexpression of miR-17 improved LPS-induced ALI *in vivo*. The mice were injected intravenously with agomir-17 or agomiR-NC 24 h prior to LPS treatment. The mice were sacrificed after LPS administration for 24 h, and then the lung tissues and BALF were collected for analysis (n=5 mice per group). (A) The miR-17 expression level was measured by RT-qPCR analysis. (B) Histopathological analysis of lung tissue was performed following with hematoxylin and eosin staining. Original magnification, x400. (C) Pulmonary edema was evaluated using the lung W/D ratio. (D) Lung permeability was assessed using the Evans Blue dye extravasation method. (E) Infiltration of neutrophils into the lung tissues was assessed by MPO activity. (F) Kaplan-Meier survival curves of mice in all treatment groups (n=10 mice per group). (G) The levels of the cytokines IL-1β, IL-6 and TNF-α were detected by ELISA. Data are expressed as mean ± standard deviation of three independent experiments. $^{*}$P<0.05, $^{**}$P<0.01 vs. control group. $^{##}$P<0.01 vs. LPS group. LPS, lipopolysaccharide; ALI, acute lung injury; MPO, myeloperoxidase; RT-qPCR, reverse transcription-quantitative PCR; W/D ratio, wet/dry ratio; BALF, bronchoalveolar lavage fluid; IL, interleukin; TNF, tumor necrosis factor.

pulmonary edema, pulmonary capillary permeability and MPO activity were determined with the use of H&E staining, EB extravasation, W/D ratio and MPO assays, respectively (n=5/group). It was observed that LPS treatment was associated with a higher extent of lung injury compared with the control group, while agomir-17 alleviated the severity and distribution of lung lesions caused by LPS (Fig. 2B). As shown in Fig. 2C and D, agomir-17 treatment markedly reduced W/D ratio and EB extravasation in ALI mice, suggesting that miR-17 effectively improved the pulmonary edema and pulmonary capillary permeability. It was also observed that the increased MPO activity induced by LPS was obviously inhibited by agomir-17 (Fig. 2E). In addition, the Kaplan-Meier method revealed that 50% of mice in the LPS group died within 7 days, whereas the 7-day survival rate after agomiR-17 injection was markedly higher compared with the LPS group (n=10/group; Fig. 2F). More importantly, the protein levels of cytokines, including IL-1β, IL-6 and TNF-α, induced by LPS were significantly decreased following agomir-17 injection (n=5/group; Fig. 2G). Collectively, these data indicate that miR-17 upregulation may improve LPS-induced ALI.

*TLR4 is a direct target of miR-17.* To investigate the potential molecular mechanism of miR-17-regulated inflammatory events in ALI, TargetScan and PicTar analyses were conducted to predict the target genes of miR-17. As shown in Fig. 3A and B, TLR4 was identified as a potential target gene of miR-17, with the target site located in the 3'-UTR of TLR4 mRNA. It is well known that TLR-4 is a common receptor of LPS, and its downstream signaling effector, NF-κB, is closely

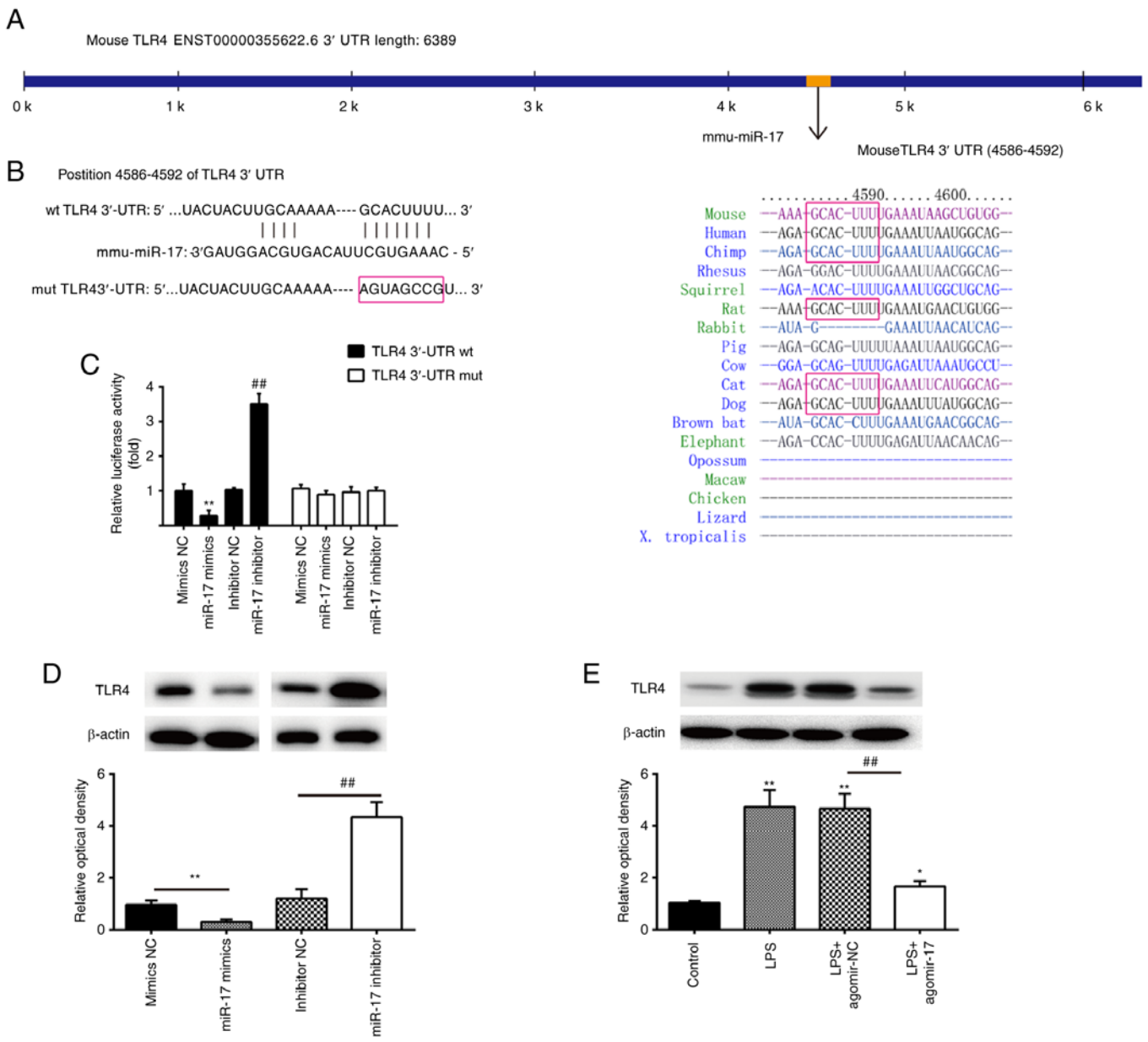


Figure 3. TLR4 is a direct target of miR-17. (A and B) Putative binding site of miR-17 and TLR4. (C) Luciferase activity was detected by a dual luciferase assay in RAW264.7 cells co-transfected with firefly luciferase constructs containing the TLR4 wt or mut 3'-UTRs and miR-17 mimics, mimics NC, miR-17 inhibitor or inhibitor NC, as indicated (n=3). (D) The expression of the TLR4 protein was detected by western blotting following transfection with miR-17 mimics and miR-17 inhibitor. Data are expressed as mean ± standard deviation of three independent experiments, $^{**}$P<0.01 vs. mimics NC, $^{\#\#}$P<0.01 vs. inhibitor NC. (E) The expression of the TLR4 protein was measured by western blotting in lung tissues from agomir-17- and agomir-NC-injected ALI mice. β-actin was used as the internal control. Data are expressed as mean ± standard deviation of three independent experiments (n=5 mice per group). $^{*}$P<0.05, $^{**}$P<0.01 vs. mimics NC, $^{\#\#}$P<0.01 vs. inhibitor NC. ALI, acute lung injury; TLR, toll-like receptor; wt, wild-type; mut, mutant; UTR, untranslated region.

associated with inflammatory response in ALI (14,27-30). Thus, TLR4 was selected for the subsequent investigation. The potential targeting interaction between miR-17 and TLR-4 was verified by dual luciferase reporter assays. The results demonstrated that the miR-17 mimics significantly inhibited the luciferase activity of the TLR4-3'UTR wt, and that miR-17 inhibitor transfection resulted in a significant increase in luciferase activity; however, no changes were observed in the cells following co-transfection of TLR4 3'-UTR-mut with either miR-17 mimics or inhibitor (Fig. 3C). miR-17 was then introduced into RAW264.1 cells and the expression of TLR4 was analyzed by western blotting. It was observed that miR-17 overexpression suppressed the protein expression of TLR4, while miR-17 inhibition promoted TLR4 expression in RAW264.1 macrophages (Fig. 3D). Of note, the protein expression of TLR4 was also found to be decreased in lung tissues of ALI mice after agomiR-17 injection (n=5/group; Fig. 3E). These results indicate that TLR4 may be a functional target of miR-17 against lung inflammatory response in ALI mice.

*miR-17 is downregulated in LPS-treated RAW264.1 macrophages*. In the physiopathology of ALI, macrophages play an important role in the regulation of inflammation (31,32). To further confirm whether miR-17 is involved in LPS-mediated immune response, RAW264.1 macrophages were treated with

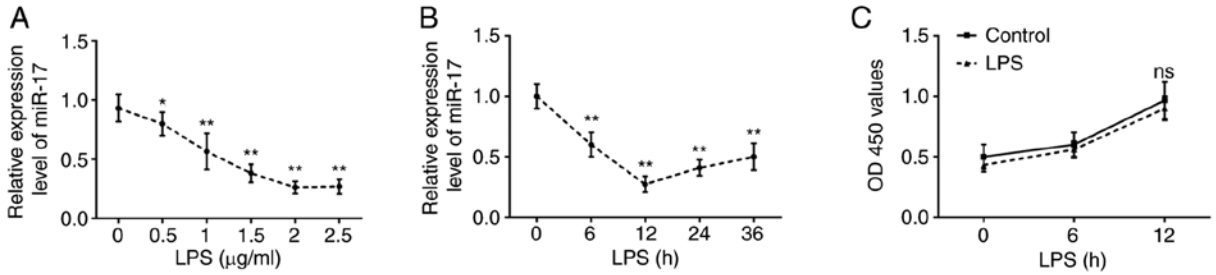


Figure 4. miR-17 was downregulated in LPS-stimulated RAW264.7 macrophages. (A) RAW264.7 cells were stimulated with different concentrations of LPS for 12 h, and the miR-17 expression level was measured by RT-qPCR analysis. (B) RAW264.7 cells were stimulated with 2 mg/ml LPS for different times, and then the miR-17 expression level was measured by RT-qPCR analysis. (C) The viability of RAW264.7 cells after treatment with LPS (2 mg/ml) was assessed using a Cell Counting Kit-8 assay. Data are expressed as mean ± standard deviation of three independent experiments. $^{*}$P<0.05, $^{**}$P<0.01 vs. control group (LPS untreated). LPS, lipopolysaccharide; RT-qPCR, reverse transcription-quantitative PCR.

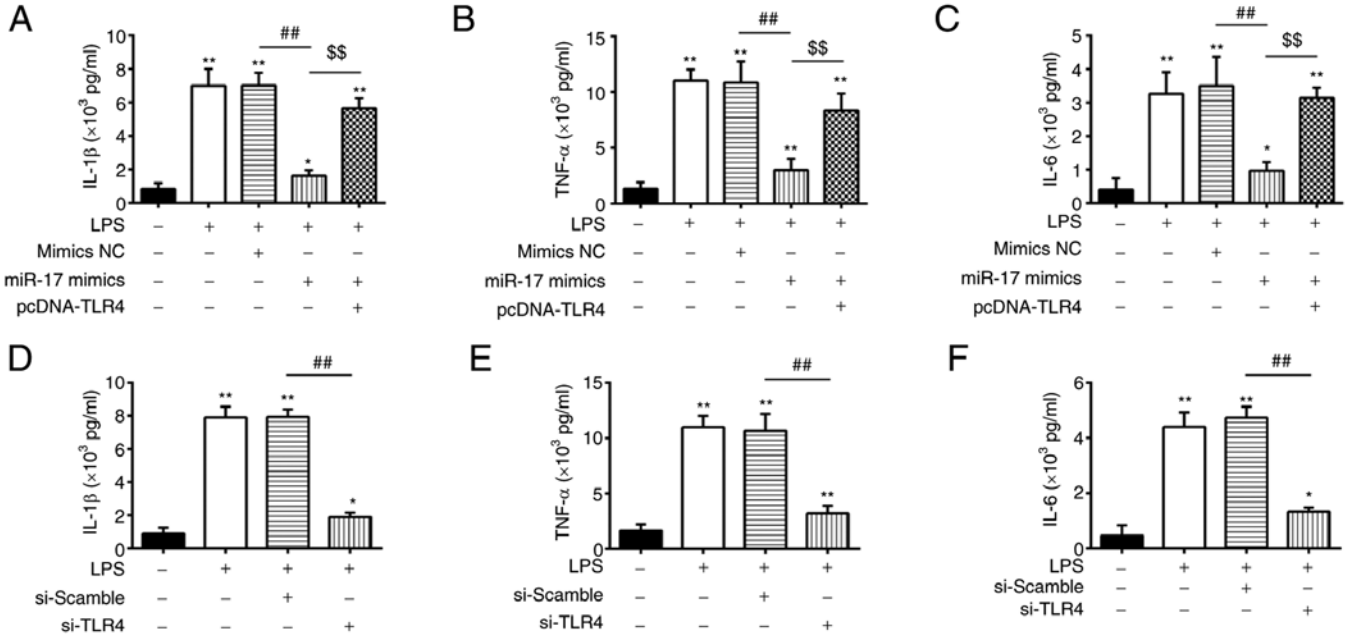


Figure 5. miR-17 regulates the inflammatory response in LPS-treated RAW264.7 cells through targeting TLR4. (A-C) IL-1β, IL-6 and TNF-α levels were measured using ELISA in RAW264.7 cells co-transfected with pcDNA-TLR4 and miR-17 mimics, in addition to LPS treatment. (D-F) IL-1β, IL-6 and TNF-α levels were measured using ELISA in RAW264.7 cells transfected with si-TLR4, in addition to LPS treatment. Data are expressed as mean ± standard deviation of three independent experiments. $^{*}$P<0.05, $^{**}$P<0.01 vs. control group. $^{\#\#}$P<0.01 vs. LPS + mimics NC group or LPS + si-scramble, $^{\$\$}$P<0.01 vs. LPS + miR-17 mimics group. LPS, lipopolysaccharide; TLR, toll-like receptor; IL, interleukin; TNF, tumor necrosis factor.

LPS at different concentrations (0-2.5 μg/ml) for different times (0-36 h), as previously described (33). After LPS treatment of RAW264.1 cells, the expression level of miR-17 decreased in a dose-dependent manner (Fig. 4A). Furthermore, it was also observed that miR-17 expression time-dependently decreased, while the expression of miR-17 reached a nadir at 12 h after LPS treatment (Fig. 4B). Additionally, the MTT assay was used to assess the effect of LPS (2 μg/ml) on the viability of RAW264.7 cells, and the results demonstrated that cell viability was not affected by LPS at any of the different time points (Fig. 4C). Based on the data mentioned above, treatment with 2 μg/ml LPS for 12 h was selected as the optimal conditions for subsequent experiments.

*Overexpression of miR-17 improves the inflammatory response by targeting TLR4 in LPS-treated RAW264.7 cells.* As mentioned above, TLR4 was found to be a direct target of miR-17 in RAW264.7 cells; therefore, it was further investigated whether miR-17 regulates the inflammatory response in ALI through targeting TLR4 by co-transfecting miR-17 mimics and pcDNA-TLR4 into RAW264.7 cells, followed by treatment with LPS. Consistently with the results of the inflammatory cytokine generation *in vivo*, LPS treatment resulted in significant increases in the levels of IL-1β, IL-6 and TNF-α, whereas these effects caused by LPS were attenuated by overexpression of miR-17. Interestingly, TLR4 overexpression reversed the inhibitory effects of miR-17 overexpression on the expression of these cytokines in this cell model (Fig. 5A-C). In addition, RAW264.7 cells were transfected with si-TLR4 to determine the role of TLR4 in the regulation of inflammatory response. As shown in Fig. 5D-F, the production of these cytokines induced by LPS was markedly reduced by TLR4 knockdown, which was similar to the effect of miR-17 mimics on LPS-treated RAW264.7 cells. These findings demonstrated

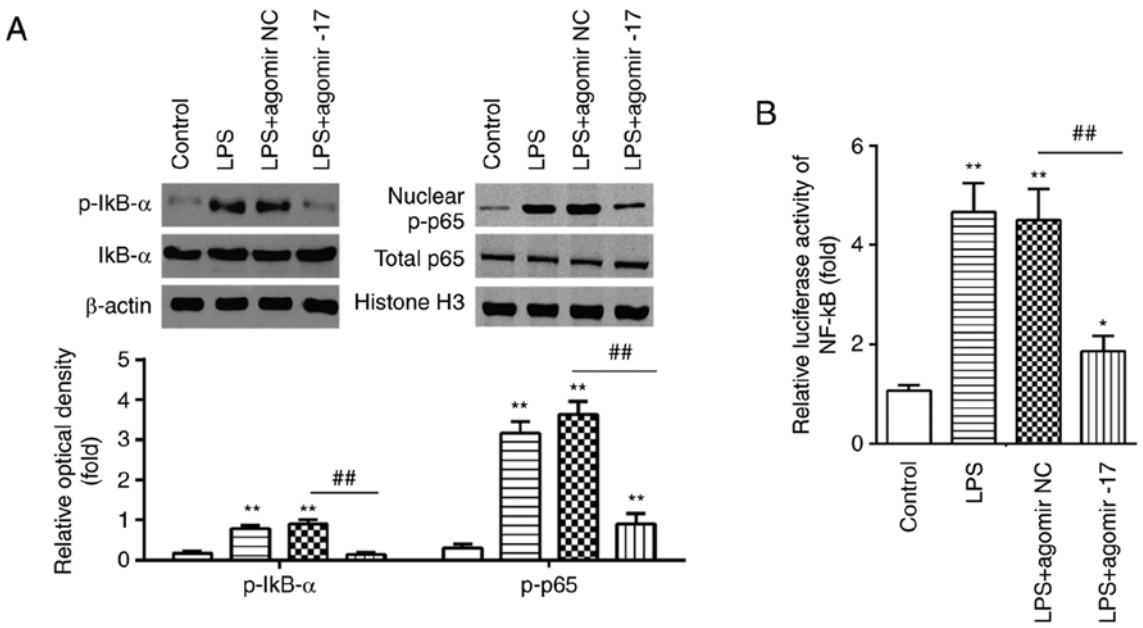


Figure 6. Overexpression of miR-17 inhibits the NF-κB pathway in ALI mice. The mice were injected intravenously with agomiR-17 or agomiR-NC for 24 h, followed by exposure to LPS. Subsequently, the mice were sacrificed and lung tissues were harvested for analysis. (A) The levels of nuclear p-p65, IκB-α and p-IκB-α were measured by western blot analysis. The bands were semi-quantitatively analyzed using ImageJ software. β-actin protein was used as the internal control of the cytoplasmic proteins; Histone H3 protein was used as the internal control of nuclear proteins. The histogram presents the changes in the relative ratio of the phosphorylated protein levels to the total expression level (phospho/total protein ratio). (B) NF-κB activity was assessed using the NF-κB activity assay. Data are expressed as mean ± standard deviation of three independent experiments (n=5 mice per group). $^{*}$P<0.05, $^{**}$P<0.01, vs. control group; $^{\#\#}$P<0.01, vs. LPS + agomiR-17 group. ALI, acute lung injury; NF-κB, nuclear factor-κB.

that miR-17 improves the inflammatory response by targeting TLR4 in an ALI cell model.

*Overexpression of miR-17 blocks the activation of the NF-κB signaling pathway*. According to previous research, TLR4 is a common receptor of LPS, and its downstream signaling effector, the NF-κB pathway, plays a crucial role in the inflammatory response in ALI (34,35). Therefore, to further investigate the possible mechanisms underlying the role of miR-17 in the inflammatory response, we focused on the phosphorylation/activation of the NF-κB signaling pathway in ALI mice using western blot assay (n=5/group). The data demonstrated that LPS treatment significantly upregulated the levels of p-IκBα and nuclear p-p65 compared with the control group. However, compared with the LPS group, the levels of these proteins were markedly downregulated in the LPS + agomiR-17 group (Fig. 6A). In addition, it was also observed that LPS led to a significant increase in NF-κB activity, whereas agomiR-17 markedly reduced the increased NF-κB activity caused by LPS (Fig. 6B). These results demonstrated that miR-17 exerts its potent inhibitory effects against LPS-induced lung injury via the NF-κB signaling pathway.

## Discussion

In the present study, miR-17 was significantly downregulated in both LPS-induced ALI mice and LPS-treated RAW264.7 cells. Further analysis revealed that miR-17 improved LPS-induced lung injury through inhibition of the inflammatory response through blocking the activation of the TLR4/NF-κB pathway. These results suggest that miR-17 may be of value as a potential therapeutic approach to ALI.

Accumulating evidence has demonstrated that miRNAs participate in the regulation of the inflammatory response in several types of diseases (36,37). A number of miRNAs, such as miR-9, miR-147 and miR-132, have been reported to exert an inhibitory effect on inflammatory response (38-40). Rao *et al* demonstrated that miR-17 attenuated staphylococcal enterotoxin B-induced lung injury via suppressing the pulmonary inflammatory response (41). Wang and Zhang found that miR-17 overexpression mediated the protective effects of resveratrol against LPS-induced cell injury through reducing the production of pro-inflammatory cytokines in the human keratinocyte cell line HaCaT (18). However, the mechanism underlying the association between miR-17 and LPS-induced ALI remains elusive. Therefore, it is necessary to further investigate whether miR-17 affects the inflammatory response in ALI. The present study demonstrated that the expression of miR-17 was low in the lung tissues of ALI mice, whereas agomiR-17 injection reduced tissue damage and lung edema, and inhibited the LPS-induced inflammatory response in an LPS-induced ALI mouse model. Furthermore, the results of the present study demonstrated that agomiR-17 markedly improved the survival rate of mice with LPS-induced ALI. Using an *in vitro* RAW264.7 macrophage model, downregulation of miR-17 in RAW264.7 cells was observed after LPS stimulation. Consistently with the results *in vivo*, this demonstrated that miR-17 can attenuate LPS-induced inflammatory response. Collectively, these data suggest that upregulation of miR-17 may improve LPS-induced ALI by inhibition of the inflammatory response.

TLR4 is an essential LPS signaling receptor, which is known to play an important role in inflammatory response in animal or cell models of ALI (42,43). For example, Hu *et al*

reported that the inhibition of the TLR4 signaling pathway suppressed the production of inflammatory cytokines, thereby improving ALI in rats (28). He *et al* demonstrated that the LPS/TLR4 axis can activate the NF-κB signaling pathway, resulting in inflammatory cell infiltration (44). A previous study also reported that miR-27a alleviated LPS-induced ALI in mice via inhibiting inflammation through modulating the TLR4/MyD88/NF-κB pathway (45). Interestingly, the expression levels of TLR4 were previously reported to be regulated by miR-17 in an LPS-induced sepsis mouse model (46); however, it is ambiguous whether miR-17 serves as a protective factor in LPS-induced ALI through regulation of TLR4. In the present study, TLR4 was identified as a target of miR-17. Furthermore, TLR4 overexpression abrogated the inhibitory effects of miR-17 on inflammatory response in LPS-induced RAW 264.7 cells. By contrast, silencing TLR4 by siTLR4 had a similar effect to that of miR-17 on LPS-treated RAW 264.7 cells. Collectively, these findings indicate that the miR-17/TLR4 axis may serve as a novel therapeutic target for LPS-induced ALI.

Activation of canonical NF-κB plays important roles in LPS-induced ALI (47,48). In ALI, NF-κB is constitutively activated and is involved in promoting the inflammatory response, which suggests that inhibiting the activity of NF-κB may constitute a promising therapeutic approach to ALI (49). According to previous research, TLR4 is a recognized inducer of the NF-κB signaling pathway, and it has been demonstrated that inhibition of TLR-4/NF-κB signaling may improve LPS-induced ALI in a mouse model (50,51). Therefore, the mechanism through which miR-17 affects the TLR-4/NF-κB signaling pathway requires further investigation. In the present study, it was observed that the overexpression of miR-17 significantly suppressed the LPS-induced activation of NF-κB *in vivo*. These results indicate that miR-17 may alleviate the LPS-induced inflammatory response by inhibiting the TLR4/NF-κB signaling pathway.

In conclusion, the present study demonstrated that miR-17 can alleviate LPS-induced ALI in mice through inhibiting the inflammatory response via the TLR4/NF-κB signaling pathway. Therefore, the miR-17/TLR4 axis may be a candidate target for the treatment of patients with ALI.

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**Availability of data and materials**

All data generated and/or analyzed during the present study are included in this published article.

**Authors' contributions**

SF conceptualized the study design, obtained the experimental materials, performed the experiments, analyzed the data and wrote the paper. The author has read and approved the final version of the manuscript.

**Ethics approval and consent to participate**

All individuals provided informed consent for the use of human specimens for clinical research. The present study was approved by the Affiliated Hospital of Inner Mongolia University for the Nationalities Ethics Committees.

**Patient consent for publication**

Not applicable.

**Competing interests**

The author declares that they have no competing interests.

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