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Original Article

MicroRNA-182-5p inhibits inflammation in LPS-treated RAW264.7 cells by mediating the TLR4/NF-κB signaling pathway

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Abstract: Excessive inflammation is predominantly involved in the pathogenesis of acute lung injury (ALI). Recently, microRNAs (miRNAs) have been shown to act as an important regulator of inflammation. Hence, we investigated the levels of miR-182-5p and its functional role about regulating inflammation in LPS-induced ALI mice and RAW264.7 cells. Here, LPS induced-ALI model was established in male mice. By qRT-PCR, we found that the expression of miR-182-5p was significantly downregulated in lung tissue and bronchoalveolar lavage fluid (BALF), as well as decreased in LPS-treated RAW264.7 cells. In vitro, overexpression of miR-182-5p suppressed the inflammatory response, as evidenced by reduction of pro-inflammatory cytokines including interleukin (IL)-6, IL-1β, and tumor necrosis factor-α (TNF-α). Further experiments revealed that toll-like receptor 4 (TLR4) was the target of miR-182-5p, which was confirmed by luciferase activity assay. Moreover, RAW264.7 cells transfected with pcDNA-TLR4 plasmid abrogated the protective effects of miR-182-5p against LPS-induced inflammatory response. Additionally, we observed that overexpression of miR-182-5p blocked LPS-induced activation of the NF-κB signaling pathway by targeting TLR4. Collectively, the findings of our study indicate that miR-182-5p inhibits the inflammatory response by modulating TLR4/NF-κB signaling pathway in LPS-treated RAW264.7 cells.

Keywords: Acute lung injury, MicroRNA-182-5p, inflammatory response, TLR4/NF-κB signaling pathway

Introduction

Acute lung injury (ALI), a life-threatening syndrome, is considered a severe inflammatory process in lungs and causes a high mortality rate worldwide [1]. Despite recent improvements in therapies and early diagnostic, the mortality of ALI remains up to 40% [2-5]. Uncontrolled inflammatory response is a hallmark features of ALI [6]. Therefore, how to prevent the lung tissue from excessive inflammatory response is an effective treatment strategy for ALI patients.

MicroRNAs (miRNAs) are a family of short, small, noncoding RNAs (~21-23 nucleotides), which modulate post-transcriptional regulation of target genes by binding to the 3'-untranslated regions of messenger RNAs (mRNAs), resulting in translational repression or mRNA degradation [7, 8]. Recently, several miRNAs have been proven to play important roles in inflammatory diseases including ALI, such as miR-122 [9], miR-146a/b [10], miR-181a [11], miR-326 [12] and miR-17 [13]. miRNA-182-5p (miR-182-5p), one member of the miR-183 family, was showed to be involved in developmental and oncogenic processes, such as cancer invasion [14], neuronal development, and maturation [15]. Also, miR-182-5p was reported to inhibit the induction of several pro-inflammatory cytokines in liver ischemia-reperfusion injury through inactivation of TLR4 signaling pathway [16]. However, the role of miR-182-5p in ALI has not yet been investigated. In a prior study, Park et al. found that miR-182-5p was significantly upregulated in rat model of LPS-induced ALI after bone marrow-derived mesenchymal stem cells (BM-MSCs) treatment [17]. Thus, we inferred that miR-182-5p might play a key role in the repression of ALI inflammatory response.

In the present study, we established the LPS-induced ALI mice model and investigated the

miR-182-5p level in lung tissues and BALF. We found that miR-182-5p is downregulated in ALI lung tissues, and demonstrated that overexpression of miR-182-5p attenuated LPS-induced inflammatory response in RAW264.7 cells via the TLR4/NF-κB signaling pathway. These findings may be useful for the development of possible clinical approaches for ALI treatment.

Materials and methods

Animals

Female BALB/c mice (16-20 g) purchased from Shanghai SLAC laboratory Animal Co., Ltd. (Shanghai, China) were kept in a temperature controlled room with 12 h dark/light cycles, and without restriction to water or food. Among the mice, 12 mice were randomly selected as the normal group, and the remaining 12 mice were used to establish the mouse models of ALI. In order to minimize the suffering of animals, all procedures involving animals in this study conformed to and were approved by the Animal Ethics Committee of Fudan University.

Experimental protocol and ALI model

The models were established using 12 BALB/c mice as previously reported [18, 19]. Briefly, female BALB/c mice (n = 12/group) were anaesthetized and challenged with intratracheal instillation of LPS (5 mg/kg, Escherichia coli 055:B5; Sigma). 12 mice (without LPS instillation) were injected with the same volume of PBS to serve as controls. Twenty-four hours after injection of LPS, mice were humanely killed by overdose of anesthesia. Right lung was lavaged using 0.9% saline and bronchoalveolar lavage fluid (BALF) was collected and centrifuged for 15 min at 4°C using a cooling centrifuge (4000 rpm). The BALF supernatants were stored at -80°C until further analysis. A small piece of the left lung was dissected and washed with ice-cold saline then then fixed in 10% neutral buffered formalin for 24 h and submitted for histopathological assessment.

Hematoxylin-eosin (HE) staining

The left lower lung from each mouse was fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 5 m thick sections. Then sections were stained with H&E. Subsequently, lung injury score was measured and calculated by a blinded pathologist as previously described [20].

Reverse transcription polymerase chain reaction (RT-qPCR)

For miR-182-5p analysis, RNA was extracted from lung tissues using the miRNeasy mini kit (Qiagen, West Sussex, UK) according to the manufacturer's instructions. Then the RNA was reversely transcribed to cDNA with a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). To quantify TLR4 mRNA, approximately 1 mg of total RNA was reverse-transcribed into cDNAs using the Oligo dT primer (TaKaRa). qPCR was performed using TaqMan miRNAs Quantitation Kit (Applied Biosystems, Foster City, CA, USA) on A fluorescence quantitative PCR instrument (ABI 7500, Applied Biosystems Inc., Foster City, CA, USA). U6 and GAPDH were used as references for miRNAs and mRNAs, respectively. We used the following primers: miR-182-5p, forward 5'-TTTTAGAGGTTTCGTTAATTTTTTC-3'; reverse, 5'-CTCGATTCGAACCTAAACTCG-3'; TLR4, forward 5'-AGTTGATCTACCAAGCCTTGAGT-3' and reverse 5'-GCTGGTTGTCCCAAAATCACTTT-3'; U6 forward: 5'-GCTTCGGCAGCACATATACTAAAAT-3', reverse: 5'CGCTTCACGAATTTGCGTGTCAT-3'. GAPDH, forward 5'-CTGGGCTACACTGAGCACC-3' and reverse 5'-AAGTGGTCGTTGAGGGCAATG-3'. All reactions were performed in triplicate. The relative expression was calculated using the $2^{-\Delta\Delta}$Ct method.

Cell culture

RAW264.7 cells were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and grown in DMEM/F12 (Abcam, Cambridge, MA) media supplemented with 10% fetal bovine serum (FBS, Gibco, Rockville, MD) and 1% pen-strep at 37°C in a humidified atmosphere with 5% CO_2.

Cell transfection

The miR-182-5p mimics, mimics negative control (mimics NC), miR-182-5p inhibitor, and inhibitor NC were bought from GenePharma (Shanghai, China). TLR4 expression vector was constructed by inserting the overall sequence of TLR4 into the pcDNA 3.1 vector (Invitrogen).

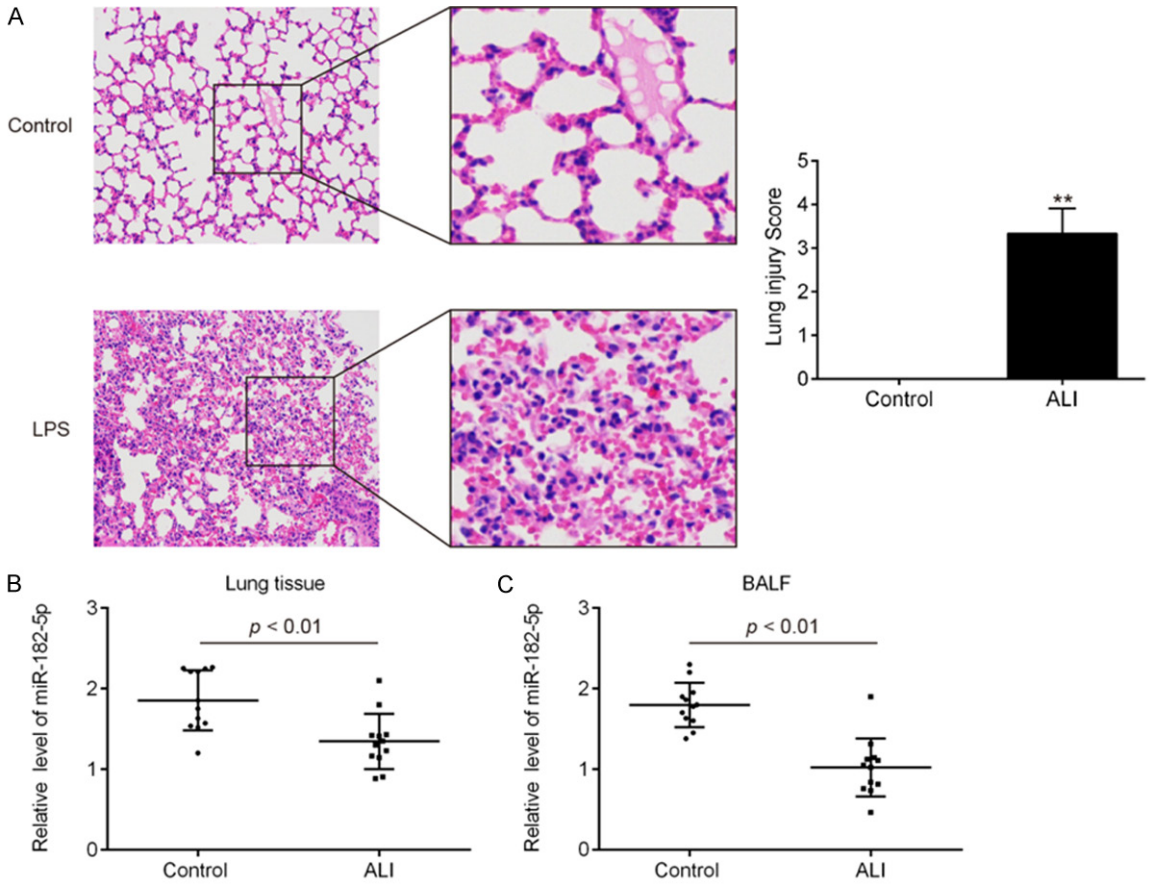

Figure 1. miR-182-5p was downregulated in LPS-induced mice model. Female BALB/c mice were anaesthetized and challenged with intratracheal instillation of LPS (5 mg/kg). Twenty-four hours after injection of LPS, mice were humanely killed by overdose of anesthesia, then lung tissues and bronchoalveolar lavage fluid (BALF) were harvested for subsequent experiments. (A) The histological changes of lung tissues in LPS-induced ALI mice were evaluated using the H&E staining and lung injury score system (n = 12 mice per group). Data were presented as the mean ± SD of three induvial experiments. $^{**}P < 0.01$ vs control group. (B) The miR-182-5p expression was detected using the qRT-PCR analysis in lung tissues and (C) BALF, respectively (n = 12 mice per group). Data are presented as the mean ± SD of three individual experiments. $P < 0.01$ vs control group.

Cellular transfection was performed by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the protocol.

Cell viability

RAW264.7 cells were seeded in 96-well plate at 5000 cells/well and cultured at 37°C (5% CO_2) for 48 h. After different concentrations of LPS (0.1, 1, 2 and 10 μg/ml) treatment for 24 h, cell viability was assessed by Cell Counting Kit-8 (Beyotime, Shanghai, China). Briefly, 10 μL CCK-8 solution was added in the culture media per well and cells were then incubated for another 2 h at 37°C. The absorbance of wells was measured using a Vmax microplate spectrophotometer (Molecular Devices, Sunnyvale, CA) at 450 nm.

Enzyme-linked immunosorbent assay (ELISA)

The levels of pro-inflammatory cytokine including tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) and interleukin-1β (IL-1β) were assessed with murine cytokine-specific Quantikine ELISA kits (R&D Systems).

Dual luciferase activity assay

The fragments from TLR4 3'UTR containing the predicted miR-182-5p binding site were amplified and cloned into the pGL3 vector (Promega, Madison, WI, USA) to form TLR4-wild-type (WT). Mutants at the putative binding site of TLR4-WT were constructed by using Directed Mutagenesis System (Invitrogen) and was referred to TLR4-Mut. Then the vectors were

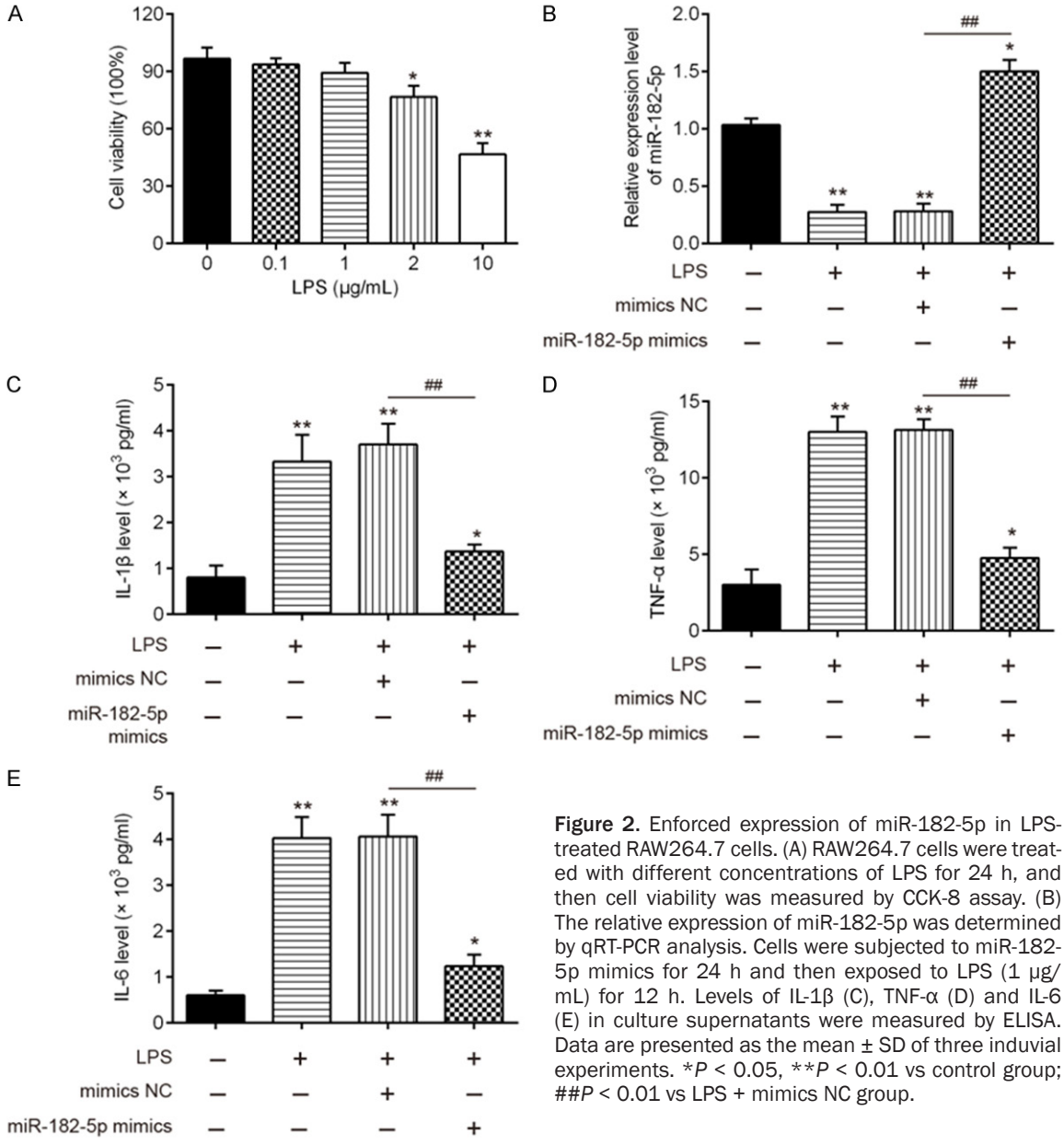

Figure 2. Enforced expression of miR-182-5p in LPS-treated RAW264.7 cells. (A) RAW264.7 cells were treated with different concentrations of LPS for 24 h, and then cell viability was measured by CCK-8 assay. (B) The relative expression of miR-182-5p was determined by qRT-PCR analysis. Cells were subjected to miR-182-5p mimics for 24 h and then exposed to LPS (1 μg/mL) for 12 h. Levels of IL-1β (C), TNF-α (D) and IL-6 (E) in culture supernatants were measured by ELISA. Data are presented as the mean ± SD of three induvial experiments. *$P < 0.05$, **$P < 0.01$ vs control group; ##$P < 0.01$ vs LPS + mimics NC group.

respectively transfected into RAW264.7 cells with miR-182-5p mimics, mimics NC, miR-182-5p inhibitor and inhibitor NC. The luciferase activity was determined by Dual Luciferase Reporter Assay System (Promega).

Western blot

Total proteins were extracted using RIA lysis buffer (Beyotime Biotechnology, Shanghai, China) supplemented with protease inhibitors (Roche, Guangzhou, China). Nuclear proteins were extracted from the treated cells using Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Biotechnology, China). Following BCA protein quantitative assay, the proteins (40 μg) were separated by 8% SDS gel and transferred onto PVDF membranes. After blocking with 5% nonfat milk, the membranes were blotted with primary antibodies for each protein, including nuclear p-65, IκB-α, p-IκB-α and β-actin. Subsequently, the membranes were probed with HRP-conjugated secondary antibodies. Finally, the immunostained bands were detected using an ECL kit (Amersham Biosciences, Buckinghamshire, UK). The results were expressed as a ratio to β-actin protein. The intensity of the bands of interest was analyzed by ImageJ software (Rawak Software, Inc. Munich, Germany).

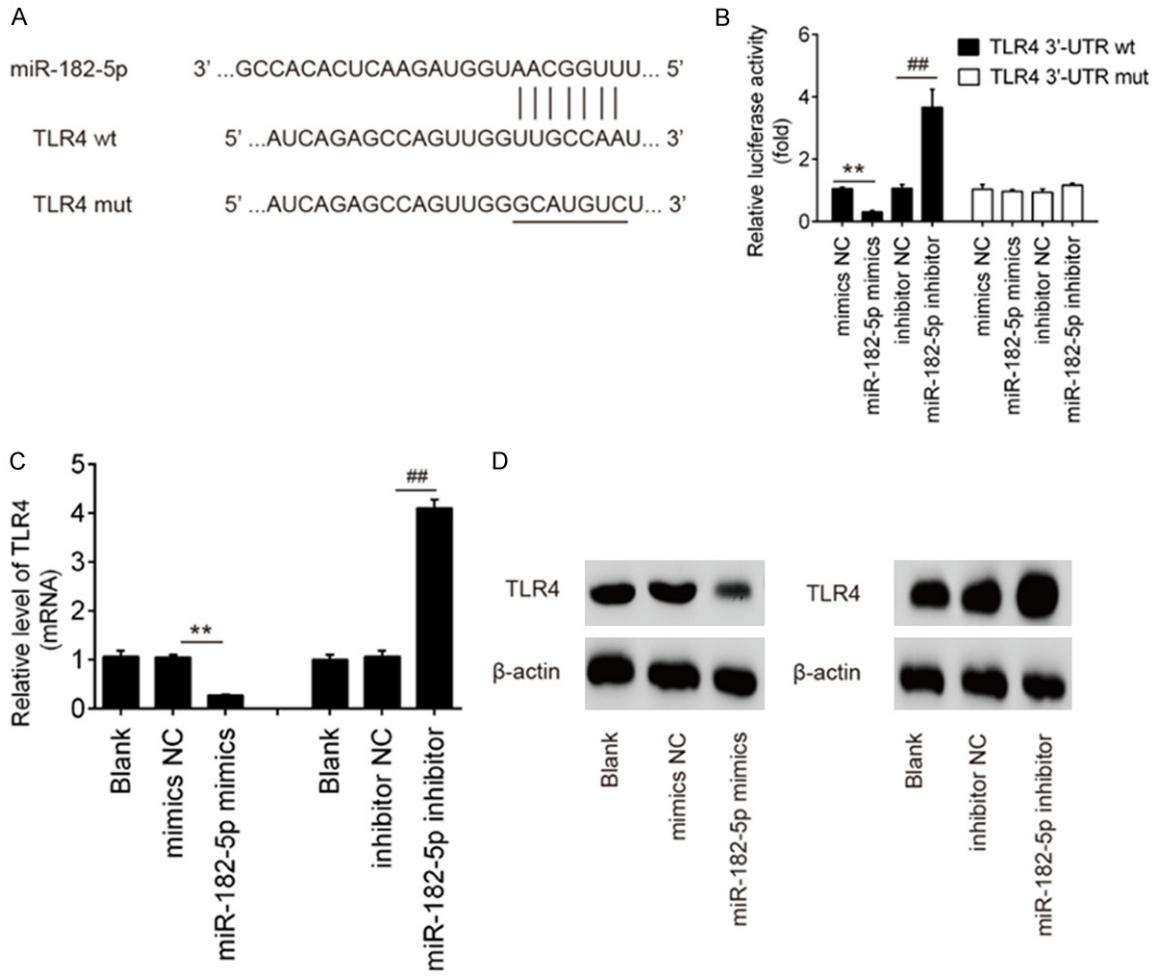

Figure 3. TLR4 is a target of miR-182-5p. A. Schematic of the miR-182-5p putative binding site in human TLR4 3'-UTR. B. RAW264.7 cells were co-transfected with reporter gene containing WT or MUT TLR4 3'UTR along with miR-182-5p mimics, miR-182-5p inhibitor or miR-NC, and the luciferase activity was determined. C, D. TLR4 mRNA and protein expressions in RAW264.7 cells transfected with miR-182-5p mimics or miR-182-5p inhibitor were measured by qRT-PCR and western blot analysis. Data are presented as the mean ± SD of three individual experiments. $^{**}P < 0.01$ vs mimics NC group; $^{\#\#}P < 0.01$ vs inhibitor NC group.

Statistical analyses

Statistical analyses were performed with SPSS 13.0 software (SPSS, Chicago, IL, USA). Data are expressed as the mean ± standard deviation of 3 independent experiments. One-way analysis of variance (ANOVA) or two-tailed Student's t-test was used for comparisons between groups. $P < 0.05$ was considered a significant difference.

Results

miR-182-5p was downregulated in LPS induced mice model

As we stated, LPS was widely used for the construction of an in vivo animal model, mimicking the inflammatory process of ALI. To confirm whether the LPS-induced ALI model was successfully constructed, H&E staining was used to visualize the morphology alteration of lung tissues. As shown in **Figure 1A**, the lung tissues of the control group exhibited normal alveolar structure. However, severe pathological damage, such as inflammatory cell infiltration, lung edema, and obvious alveolar wall thickening was observed in the lung tissues of the LPS group. Correspondingly, the lung injury score in the LPS group was significantly increased compared with that in control group. To determine if miR-182-5p was involved in LPS induced ALI, we examined the expression of miR-182-5p in lung samples. We found that the expression level of miR-182-5p in lung tis-

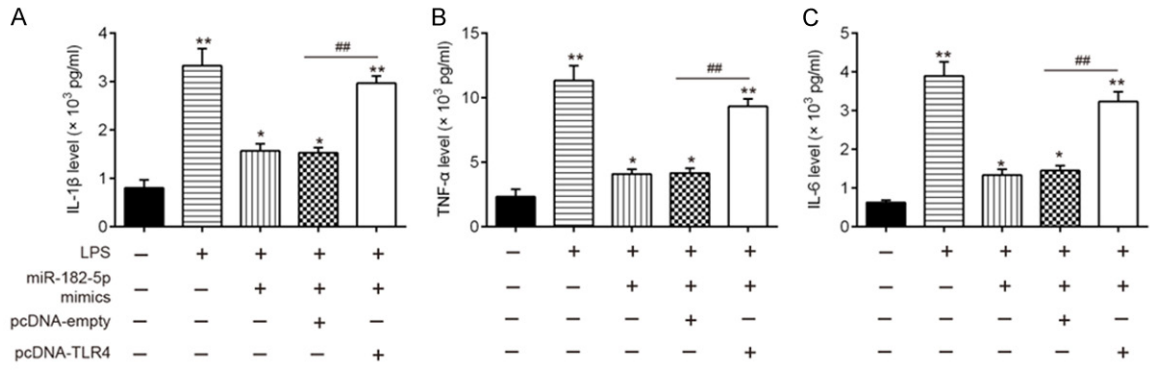

Figure 4. TLR4 overexpression abrogated the anti-inflammatory ability of miR-182-5p in RAW264.7 cells upon LPS treatment. RAW264.7 cells were co-transfected with miR-182-5p mimics and pcDNA-TLR4 plasmids for 24 h, and then treated with LPS for 12 h, followed by the assessment of pro-inflammatory cytokines. Levels of IL-1β (A), TNF-α (B) and IL-6 (C) in culture supernatants were measured by ELISA. Data are presented as the mean ± SD of three individual experiments. **P* < 0.05, ***P* < 0.01 vs control group; ##*P* < 0.01 vs LPS + mimics NC+ pcDNA-empty group.

sues was significantly decreased compared with the control groups (**Figure 1B**). Similarly, we revealed that the expression of miR-182-5p in BAL fluid was also down-regulated in LPS-induced ALI mice (**Figure 1C**). Collectively, these data indicate that down-regulation of miR-182-5p might contribute to the development of ALI.

Enforced expression of miR-182-5p inhibits inflammatory cytokines in LPS-treated RAW264.7 cells

In order to explore the effect of miR-182-5p on LPS-induced inflammatory injury, we first treated RAW264.7 cells with different concentrations of LPS to detect cell viability. As shown in **Figure 2A**, LPS had no inhibitory effect on RAW264.7 cell viability at the concentration of 0.1 and 1 μg/ml, while LPS at concentrations of 2 and 10 μg/ml significantly reduced cell viability. Hence, 1 μg/ml of LPS was selected for subsequent experiments. Then, miR-182-5p mimics was transfected into RAW264.7 cells prior 24 h to LPS stimulation, and we found that the expression of miR-182-5p was decreased in LPS-treated RAW264.7 cells, whereas increased after miR-182-5p mimics transfection (**Figure 2B**). Next, we examined the effect of miR-182-5p overexpression on the concentrations of IL-1β, TNF-α and IL-6 using ELISA, and found that LPS significantly increased the concentrations of these pro-inflammatory cytokines compared to the control cells, and these promoting effects could be reversed by overexpression of miR-182-5p (**Figure 2C-E**). This suggests that the overexpression of miR-182-5p reduces the LPS-induced inflammatory response in RAW264.7 cells.

TLR4 is a target of miR-182-5p

To investigate the potential molecular mechanism by which miR-182-5p functions in the suppression of the ALI inflammatory response, the potential targets of miR-182-5p was predicted using miRNA.org and TargetScan. Among the predicted targets of miR-182-5p, we hypothesized toll-like receptor 4 (TLR4) to be a functionally relevant target for LPS induced ALI due to its importance in mediating inflammatory response [21] and its role in progression of LPS-induced ALI [22]. As suggested in **Figure 3A**, the complementary sequence of miR-182-5p was found in the 3'-UTR of TLR4 mRNA. To confirm the predicted results, we constructed the dual-luciferase reporter system containing the 3'-UTR of TLR4 along with the putative miR-182-5p binding sites. Luciferase reporter gene assay showed that overexpression of miR-182-5p decreased relative luciferase activity of RAW264.7 cells in the presence of the wild-type 3'-UTR, whereas knockdown of miR-182-5p increased the relative luciferase activity (**Figure 3B**). However, we observed that the luciferase activity did not change significantly when the targeted sequence of TLR4 was mutated in the miR-182-5p-binding site. Moreover, the mRNA and protein expression of TLR4 was significantly down-regulated in miR-182-5p mimics group compared with the mimic NC group, while markedly up-regulated in miR-

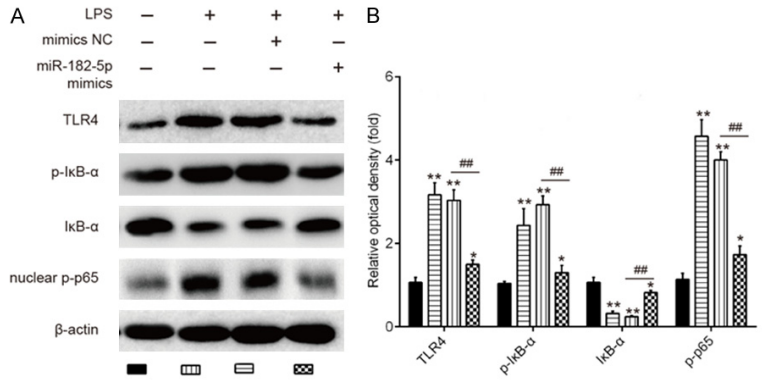

Figure 5. miR-182-5p suppressed LPS activated NF-κB signaling pathway in RAW264.7 cells. Cells were subjected to miR-182-5p mimics for 24 h and then exposed to LPS (1 μg/mL) for 5 h, then cells were harvested for Western Blot. A. The protein expressions of TLR4, p-p65, p-IκB-α and IκB-α were detected by western Blot; B. Protein bands were analyzed semi-quantitatively using Image J software, normalized to β-actin density. Data are presented as the mean ± SD of three individual experiments. **P* < 0.05, ***P* < 0.01 vs control group; ##*P* < 0.01 vs LPS + mimics NC group.

182-5p inhibitor group compared with inhibitor NC group (**Figure 3C** and **3D**). These findings indicated that TLR4 might be a functional target of miR-182-5p.

TLR4 overexpression abrogated the anti-inflammatory ability of miR-182-5p in RAW264.7 cells upon LPS treatment

Given that miR-182-5p directly targeted the 3'-UTR of TLR4 mRNA, and TLR4 can activate inflammatory response, we hypothesized that TLR4 may play an important role in the observed anti-inflammatory ability of miR-182-5p overexpression on ALI. To test this hypothesis, RAW264.7 cells were co-transfected with miR-182-5p mimics and pcDNA-TLR4 plasmids for 24 h, and then treated with LPS for 12 h, followed by the assessment of pro-inflammatory cytokines (IL-1β, TNF-α and IL-6). We found that miR-182-5p mimics significantly decreased the concentrations of these pro-inflammatory cytokines compared to the LPS group, while this inhibitory effect was reversed by overexpression of TLR4 (**Figure 4A-C**). These findings suggest that miR-182-5p inhibited LPS induced inflammatory response through targeting TLR4.

miR-182-5p suppressed LPS activated NF-κB signaling pathway in RAW264.7 cells

Previous studies have demonstrated that activation of the TLR4/NF-κB signaling pathway contributes to the development of LPS-induced ALI, and its inhibition may serve as potential treatment for ALI [22, 23]. Based on the above results that TLR4 is a direct target of miR-182-5p, we speculated that miR-182-5p may modulate the NF-κB signaling pathway via targeting TLR4. We therefore tested the effect of miR-182-5p on LPS-induced the expressions of TLR4, IκB-α, p-IκB-α, and nuclear p-p65 in RAW264.7 cells. As shown in **Figure 5A**, **5B**, LPS stimulation increased the expression of TLR4, p-IκB-α and nuclear p-p65 and reduced the expression of IκB-α in LPS and LPS + mimics NC groups, suggesting that LPS activated the NF-κB signaling pathway. However, overexpression of miR-182-5p suppressed the LPS-induced activation of TLR4 and the subsequent cascade of the NF-κB signaling components including p-IκB-α and p-p65 expression, when compared with LPS + mimics NC group. These data indicated that overexpression of miR-182-5p suppressed LPS-activated NF-κB pathways through modulation of TLR4.

Discussion

In the present study, we present evidence that miR-182-5p was significantly downregulated in a mouse model and cell model of ALI. Overexpression of miR-182-5p inhibited LPS induced inflammatory response through suppression of pro-inflammatory cytokines in RAW264.7 cells. More importantly, we further elucidated the possible underlying mechanisms that miR-182-5p exerts its anti-inflammatory ability through inactivation of TLR4/NF-κB pathways. These results suggest that miR-182-5p is a potential therapeutic target for ALI, which may be valuable for the prognosis of patients with ALI.

A large number of miRNAs are expressed in the lung and are involved in respiratory diseases, including ALI [11, 24]. These widely influence the signaling networks leading to pathologic responses after ALI [25]. In this study, we

showed that miR-182-5p was downregulated in lung tissues and BALF from the ALI mice. Evidence has suggested that miR-182-5p plays a pivotal role in inflammatory response in many organs [16, 26]. For instance, miR-182-5p was the most highly expressed miRNA in alcoholic hepatitis (AH), and overexpression of miR-182-5p led to an upregulation of inflammatory mediators in biliary cells [27]. Another study showed that miR-182 knockdown with antagomirs resulted in significantly lower histological scores of inflammation and tissue destruction in a murine model of arthritis [28]. Therefore, it is reasonable to speculate that miR-182-5p might be involved in the progression of inflammatory response in ALI. As expected, overexpression of miR-182-5p led to the reduction of pro-inflammatory cytokines (IL-1β, TNF-α and IL-6) in LPS treated RAW264.7 cells. These data indicated that miR-182-5p may have a protective effect in ALI by inhibiting the inflammatory response.

TLR4, a transmembrane protein, is one of the members of the toll-like receptor family, which is reported to play an essential role in the inflammatory response in ALI [29-31]. For example, YumikoImai et al. showed that loss of TLR4 expression protected mice from H5N1 virus-induced ALI [31]. Ben et al. found that TLR4 mutant markedly attenuated ALI as determined by improved lung injury and decreased expressions of TNF-α, IL-6, monocyte chemoattractant protein-1 (MCP-1), and macrophage inflammatory protein-2 (MIP-2) [32]. Tang et al. found that TLR-4 deletion or inhibition decreases the severity of acute lung injury through suppression of inflammatory responses [33]. In this study, TLR4 was identified as a target of miR-182-5p in RAW264.7 cells and negatively regulated by miR-182-5p. Moreover, overexpression of TLR4 could significantly abrogate the inhibitory effects of miR-182-5p on inflammatory response in LPS-treated RAW264.7 cells. Thus, we confirm that miR-182-5p could protect RAW264.7 cells from LPS-induced inflammation through targeting TLR4.

It has been reported that activation of the TLR4/NF-κB pathway is pivotal to the pathogenesis in various pulmonary diseases, such as ALI [22, 23, 34, 35]. Previous research corroborated high expression of NF-κB and TLR4 in lung tissue after LPS challenge, and blockade of this pathway attenuated acute inflammatory lung injury [36]. Given this, we further explored whether the TLR4/NF-κB pathway is involved in miR-182-5p-mediated inhibition in inflammatory response. As expected, our results showed that the activation of TLR4/NF-κB pathway induced by LPS in RAW264.7 cells was abrogated by miR-182-5p overexpression. All data indicate that miR-182-5p may attenuate the LPS-mediated inflammatory response by abrogating the TLR4/NF-κB pathway.

In conclusion, the present study substantiated that overexpression of miR-182-5p has a protective effect on LPS-induced inflammatory response through modulation of the TLR4/NF-κB signaling pathway. Despite the lack of clinical data and animal experiments, our study could advance our knowledge into understanding the molecular mechanisms underlying ALI. To further verify the role of miR-182-5p in ALI, more investigations are still required in the future.

Acknowledgements

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Disclosure of conflict of interest

None.

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