MicroRNA-21 inhibits lipopolysaccharide-induced acute lung injury by targeting nuclear factor-κB

WEI-DONG ZHU, JIA XU, MAO ZHANG, TIE-MING ZHU, YUN-HUA ZHANG and KE SUN

1Emergency Department; 2Personnel Section, Traditional Chinese Medical Hospital of Zhuji, Zhuji, Zhejiang 311800; 3Emergency Department, The Second Affiliated Hospital of Zhejiang University School of Medicine, Hangzhou, Zhejiang 310052; 4Hepatobiliary Surgery Department; 5Intensive Care Unit; 6Orthopedics Department, Traditional Chinese Medical Hospital of Zhuji, Zhuji, Zhejiang 311800, P.R. China

Abstract. Acute lung injury (ALI) is a frequent, but severe complication following sepsis in patients with critical illness. The present study aimed to investigate the potential role of microRNA-21 (miR-21) in the regulation of inflammation in the ALI induced by lipopolysaccharide (LPS) in vitro and in vivo. The levels of inflammatory cytokines, tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β and IL-10, and the level of miR-21 expression were measured in the lungs of LPS-induced ALI rats and NR8383 alveolar macrophages (AMs). To confirm the regulatory effect of miR-21 in the inflammatory reactions of ALI, NR8383 cells were transfected with a mimic of miR-21 or an anti-miR-21 inhibitor, and the subsequent changes of the miR-21 level and the levels of inflammatory cytokines were detected. The underlying molecular mechanism was also investigated. LPS-induced ALI in rats resulted in significant overexpression of pro-inflammatory cytokines, TNF-α, IL-6 and IL-1β, and miR-21, but reduced the expression of the anti-inflammatory cytokine IL-10. LPS treatment also led to a higher expression level of miR-21 and increased secretion of pro-inflammatory cytokines in NR8383 cells in a time-dependent manner. Manipulation with the miR-21 mimic significantly suppressed the LPS-mediated induction of TNF-α, IL-6 and IL-1β in NR8383 cells, while that induction was upregulated when miR-21 expression was silenced via transfection with the anti-miR-21 inhibitor. Further mechanism experiments revealed that miR-21 regulates LPS-induced inflammation responses via the Toll-like receptor 4 and nuclear factor-κB (NF-κB) signaling pathway. miR-21 negatively regulates inflammatory responses in LPS-induced ALI by targeting the NF-κB signaling pathway, providing further insight into the molecular mechanism of ALI progression.

Introduction

Acute lung injury (ALI) is defined as a clinical syndrome of severe pulmonary inflammation, which is caused by indirect or direct lung injury with alveolar-capillary barrier disruption as well as gas exchange dysfunction (1). ALI is characterized by neutrophilic infiltration, uncontrolled inflammatory process and oxidative stress (2,3). Although various new therapies have improved the survival of patients, the morbidity and mortality rates are still very high, (4,5) which has encouraged researchers to further study the pathophysiological mechanisms of ALI to develop more effective therapeutic regimens.

Until now, although many studies have tried to demonstrate the underlying pathophysiological process, the molecular mechanism of ALI has not been well understood (6). However, it is known that alveolar macrophages (AMs), which serve as the first line of defense in the lung and play a major role in the process of ALI, are activated via a mechanism involving Toll-like receptors (TLRs) to reduce damage to the lungs (7). In addition, previous studies have indicated that inflammation and oxidative stress are closely associated with the pathogenesis of ALI (8). Nuclear factor-κB (NF-κB), a central mediator in inflammation, strictly controls the production of various important pro-inflammatory cytokines secreted by activated AMs in the early stage of ALI, including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-6 (9). Therefore, the suppression of the activation of AMs by targeting NF-κB could alleviate inflammation-induced ALI.

MicroRNAs (miRNAs), endogenous non-coding small RNAs selectively expressed in immunocytes, have promising potential in the regulation of inflammation. Previous studies have revealed that miRNAs, such as miR-155, miR-21 and miR-146a, could modulate the biological behaviors of immunocytes in terms of activation, proliferation and differentiation during inflammatory responses (5,10,11). Of these behaviors, modulations targeting the NF-κB pathway have drawn increasing attention. It has been reported that several miRNAs participate in the ALI process (7). Among them, miR-21 is known to have vital functions in immunological reactions.
A study of miRNA expression during ALI induced by aerosolized LPS in an animal model demonstrated that miR-21 performs a pivotal function in the inflammatory response (8). Another study showed that miR-21 can negatively regulate LPS-induced inflammatory responses (12). These studies indicated that miR-21 may play a vital role in the pathological process. However, the clear mechanism connecting miR-21 and LPS-induced ALI is still not well-understood. Therefore, it is necessary to further investigate the underlying mechanism by which miR-21 regulates ALI. miR-21 has also been shown to be an inhibitor of the production of pro-inflammatory cytokines in a manner that is associated with the NF-κB signaling pathway (12-15). Thus, we hypothesized that miR-21 might also participate in immune modulation in ALI through the NF-κB pathway.

To test our hypothesis, we used an in vitro AM model and an in vivo lipopolysaccharide (LPS)-induced ALI model to evaluate the role of miR-21 (referred to as miR-21-5p hereafter) in the regulation of inflammation in ALI. A miR-21 mimic and an inhibitor were also used to investigate the potential mechanism of miR-21-5p in ALI.

Materials and methods

Animal model. Sprague Dawley rats (approximately 8 weeks old) purchased from the Traditional Chinese Medical Hospital of Zhuji (Zhuji, China) were used through the study for the in vitro assays. All animal experiments were approved by the Animal Committee of the Traditional Chinese Medical Hospital of Zhuji. The ALI model was established in the rats according to the methods in previous reports (6). Briefly, rats were intraperitoneally anesthetized with pentobarbital sodium (50 mg/kg). Then, 7.5 mg/kg LPS was intratracheally instilled. Animals in the control group were subjected to the same procedure, but only PBS was instilled. Rats were sacrificed 8 h later. Unilateral lungs were processed to measure the levels of the cytokines TNF-α, IL-6, IL-1β and IL-10 in the cell culture supernatants and the bronchoalveolar lavage fluid (BALF) were detected by ELISA kits according to the instruction manuals (MultiSciences Co., Ltd). The absorbance at 450 nm was measured to determine the concentrations.

RNA expression levels. Quantitative real-time PCR was performed to measure the TNF-α mRNA and miR-21 expression levels in cells and in the lungs of rats with ALI in different groups. Total RNA was extracted from lungs and NR8383 cells with TRIzol (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) according to the instructions. RNA expression levels were evaluated by the ratio of 260/280. Total RNA was reverse-transcribed to complementary DNA with the oligo (dT) primer. The data were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the 2^ΔΔCq method (16). The primer sequences for miR-21 were 5'‑TGGCGCTAGC TTATCAGACTGAT-3' (sense) and 5'-CCAGTGCGAGGT CGAGGTATT-3' (antisense). The primer sequences for TNF-α were 5'-TCTCAAAACTCGAGTGCAAG-3' (sense) and 5'-AGTTGTGTGTGCTCTTGAGATT-3' (antisense). The primer sequences for GAPDH were 5'-TGGCGCTAGCT TCAGACTGAT-3' (sense) and 5'-CGCTGGAATACATTG GAACATGT-3' (antisense). U6 snRNA was used as an internal control to quantify and normalize the expression of miR-21. The primer sequences for U6 snRNA were 5'-GCTCGAGGCA GCACATACTAAAAT-3', and 5'-CGTTTCGAGGATTTG CCGTGTC-3'.

Dual-luciferase reporter assay. We created the TLR-4 3'‑UTR luciferase reporter construct using the psiCHECK luciferase reporter vector and named it TLR-4-WT (5'...CGAACCUCAAUAAGCUC...3'). We created the
TLR-4 3’-UTR luciferase reporter construct with miR-21 (5’-AGU UGU AGU CAG ACU AUU CGA U-3’) target site mutation using the miR-21-binding site mutation primers and named it TLR-4-MUT (5’…CGA AACCUCAAAGAUCU…3’). The target sites were showed in bold. Then, we co-transfected cells with TLR-4-WT or TLR-4-MUT and miR-21. The relative luciferase activity was measured using the dual-luciferase reporter assay system twenty-four hours later.

Western blot analysis. Western blotting was performed to detect the changes in expression of TLR-4 and the intra-nuclear NF-κBp65 protein in different groups of NR8383 cells. In short, 50 µg of total protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 12%) from the cellular protein extracts and transferred to nitrocellulose membranes (EMD Millipore, Billerica, MA, USA). After blocking with Tris-buffered saline containing Tween-20 (TBS-T; 0.1%) and blocking buffer (nonfat dried milk, 5%) at room temperature for 2 h, the membranes were incubated with primary antibodies (diluted in blocking buffer, 1:200) against TLR-4 and the intra-nuclear NF-κBp65 protein from the cellular protein extracts and transferred to nitrocellulose membranes (EMD Millipore, Billerica, MA, USA). After blocking with Tris-buffered saline containing Tween-20 (TBS-T; 0.1%) and blocking buffer (nonfat dried milk, 5%) at room temperature for 2 h, the membranes were incubated with primary antibodies (diluted in blocking buffer, 1:200) against TLR-4 and the intra-nuclear NF-κBp65 protein at room temperature overnight. Subsequently, the membranes were further incubated with secondary antibodies (diluted in blocking buffer, 1:2,000) at room temperature for 1 h and then washed with TBS-T six times. Finally, the membranes were exposed to an enhanced chemiluminescence kit, and the results were recorded on X-ray film. GAPDH was used as a control. All experiments were repeated in triplicate.

Results

LPS caused pro-inflammation and upregulation of miR-21 in the model of ALI. LPS was used to induce ALI in rats to study the inflammatory response in ALI. Eight hours after challenge with or without LPS, the lung tissues of the rats were collected for histological analysis. As shown in Fig. 1A, LPS administration caused obvious obstruction of the tubular lumen, infiltration of inflammatory cells and impairment. TNF-α is one of the most commonly used inflammation markers of ALI. The TNF-α level was approximately 9-fold higher in ALI rats than in the control group. The mRNA expression of TNF-α in the AMs of ALI rats was also significantly increased (about 4-fold) compared with that of the control group, as shown in Fig. 1B. In addition, IL-6 and IL-1β, two other inflammatory cytokines that play vital roles in lung injury, had markedly increased expression levels after LPS stimulation (Fig. 1C and D). In contrast, the expression level of IL-10 (an anti-inflammatory cytokine) was significantly decreased in rats after LPS treatment (Fig. 1E). According to information from previous studies on the upregulation of miR-21 in macrophages after LPS administration, miR-21 expression was measured by qRT-PCR 8 h after LPS treatment. As shown in Fig. 1F, the miR-21 expression level increased approximately 3.6-fold in AMs from lung tissues in ALI rats compared to the control group. Therefore, LPS-induced ALI displayed histological impairments of the lungs, pro-inflammatory responses and upregulation of miR-21.
increased in a time-dependent manner (Fig. 2A), while the expression of TNF-α mRNA peaked 6 h after the LPS treatment (Fig. 2B). In the meantime, the concentrations of the proinflammatory cytokines IL-6 and IL-1β were gradually increased after LPS administration and lasted for at least 12 h (Fig. 2C and D). However, the expression level of IL-10 was significantly decreased after LPS treatment (Fig. 2E). An increase in miR-21 expression after LPS treatment was also observed, and a significant 4.2-fold increase in miR-21 expression was detected 12 h after LPS administration (Fig. 2F). Due to the mismatch between the early peak in TNF-α expression and the delayed increase in miR-21 expression after LPS challenge in AMs, we propose the hypothesis that miR-21 may participate in the negative regulation of ALI inflammatory responses.

Expression of miR-21 regulates inflammatory responses in ALI. To confirm the underlying role of miR-21 in the regulation of inflammation in ALI, NR8383 cells transfected with the miR-21 mimic or the anti-miR-21 inhibitor were investigated. After transfection, significant increases and decreases in miR-21 expression were observed in miR-21 mimic- and anti-miR-21 inhibitor-transfected cells, respectively, compared to the single LPS-treated group (Fig. 3A). Subsequently, the effects of miR-21 overexpression or downregulation on the expression of TNF-α, IL-6, IL-1β and IL-10 after LPS treatment in NR8383 cells were investigated. As shown in Fig. 3B-E, NR8383 cells overexpressing miR-21 showed significant suppression of pro-inflammatory cytokines and increased levels of anti-inflammatory cytokines after exposure to LPS. In contrast, downregulation of miR-21 promoted the inflammatory responses of NR8383 cells after LPS exposure. Taken together, it demonstrated that miR-21 could negatively regulate LPS-induced inflammatory responses.

miR-21 inhibited inflammation induced by LPS by targeting the TLR4-NF-κB pathway. According to previous research, TLR-4 is a common receptor of LPS, and its downstream signaling effector, NF-κB, plays a crucial role in the inflammatory responses in ALI. The potential targeting interaction between miR-21 and TLR-4 was verified by the dual-luciferase reporter assays. The result showed that the luciferase activity was significantly decreased after co-transfection with TLR-4-WT and miR-21 but not after co-transfection with TLR-4-MUT and miR-21 (Fig. 4A), indicating a targeting interaction between miR-21 and TLR-4. To illuminate the underlying mechanism of miR-21-regulated inflammatory events in AMs, the TLR4-NF-κB pathway was investigated. As shown in Fig. 4B-D, transfection with miR-21 mimics markedly inhibited the expression of TLR-4 and intra-nuclear NF-κB p65. Similarly, downregulation of miR-21 significantly increased TLR-4 expression and the activation of NF-κB p65 in LPS-treated AMs. Therefore, these results demonstrated that miR-21 could impede the activation of the TLR4-NF-κB pathway induced by LPS in AMs.

Discussion

ALI is a frequent, severe complication following sepsis in patients with critical illness, and it is characterized by widespread lung inflammation and high pulmonary vascular permeability (8,9,17,18). LPS administration is a classical method to create ALI models in vivo (6,19-21). In the present work, we chose intra-tracheal instillation of LPS for ALI induction over intravenous administration to avoid potential systemic injuries. The NR8383 cells used in the present study to illuminate the underlying role of miR-21 in the modulation of LPS-induced inflammation were derived from normal rat macrophages, which have the typical features of phagocytosis and inflammatory cytokines secretion.
LPS is transferred by the LPS-binding protein and binds to macrophages via TLR-4 and CD14; subsequently, NF-κB translocates into the nucleus, the production of pro-inflammatory cytokines (such as TNF-α, IL-6, and IL-1β) is activated and the level of anti-inflammatory cytokines (like IL-10) is decreased (9,18,19,22). In our ALI model, the
concentrations of TNF-α, IL-6 and IL-1β in the BALF were obviously increased 8 h after LPS administration, which was also verified histopathologically. In contrast, the level of IL-10 was reduced, facilitating the pro-inflammatory events. Taken together, LPS can activate AMs and induce ALI.

Many studies have shown that miRNAs participate in the regulation of the inflammatory response (6,17,23). Various miRNAs such as miR-9, miR-147 and miR-132 have been reported to have an inhibitory effect on inflammatory responses (24-28). Studies from Sheedy et al showed that miR-21 inhibits the pro-inflammatory mediator PDCD4, blocking NF-kB activation and impeding the inflammatory response (29). However, the role of miR-21 in the inflammatory responses of ALI have not been extensively studied. In the present study, the early expression of TNF-α was suppressed by the increased expression of miR-21 after LPS challenge in AMs. In addition, according to previous research, the TLR-4 (a common receptor of LPS) and NF-κB pathway plays a crucial role in the inflammatory responses in ALI (13,29,30). Therefore, we proposed the hypothesis that miR-21 may play a vital role in the negative regulation of ALI inflammation responses. The mechanism by which miR-21 affects the TLR-4-NF-κB signaling pathway needs to be investigated. The expression level of miR-21 was manipulated in LPS-stimulated NR8383 cells, and the secretion of pro/anti-inflammatory cytokines, the expression of TLR-4 and the intra-nuclear NF-κB p65 level were measured. The results suggested that miR-21 could relieve LPS-induced inflammation by inhibiting the TLR-4-NF-κB pathway.

However, ALI is a complex pathological process involving many miRNAs and target genes. The main limitation of this study is the sole focus on the TLR-4-NF-κB pathway. The underlying relationship between this pathway and other related pathways needs further investigations.

In summary, our research investigated the potential role of miR-21 in the regulation of inflammation in LPS-induced ALI. In the present study, LPS exposure induced a high expression level of miR-21 both in vivo and in NR8383 cells. Further analysis revealed that miR-21 participated in the negative regulation of inflammatory responses in LPS-stimulated NR8383 cells through the TLR-4-NF-κB signaling pathway, indicating the vital role of miR-21 in the progression of ALI. Therefore, this study has provided further insight into the molecular mechanism of ALI progression.

Acknowledgements

The present study was supported by the Basic Medical Center of the Traditional Chinese Medical Hospital of Zhiji.

Funding

No funding was received.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

W-DZ designed the study and revised the manuscript. JX drafted the manuscript. The experiments were performed by JX, MZ and T-MZ. Y-HZ and KS analyzed and processed the data.

Ethics approval and consent to participate

All animal experiments were approved by the Animal Committee of the Traditional Chinese Medical Hospital of Zhiji.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.