Anti-inflammatory effects of microRNA-223 on sepsis-induced lung injury in rats by targeting the Toll-like receptor signaling pathway

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Abstract. The aim of the present study was to investigate the mediation of micro RNA (miR)-223 on the anti-inflammatory effect of the Toll-like receptor (TLR) signaling pathway on sepsis-induced lung injury in rats via negatively regulating the expression of interleukin (IL)-6. Sprague-Dawley rats were used in the present study. It was determined whether miR-223 is differentially expressed in the lung using reverse transcription-quantitative PCR techniques and the content of cytokines in bronchoalveolar lavage (BAL) fluid was detected. The protein expression levels of TLR4 and nuclear factor (NF)-κB p65 were examined by western blotting and the pathological changes in the lung tissues of the sepsis group were observed. Hematoxylin and eosin was used to stain the lung tissues. The alveoli in the sham group exhibited a normal structure and morphology. In the sepsis group, the alveoli of the lung tissues were surrounded by numerous neutrophils, the mesenchyme was swollen, regions of the alveolar wall exhibited fibrosis and the alveolar wall was thickened. Furthermore, in the sepsis group, miR-223 expression was increased in the lung tissues when compared with that in the sham group. The content of cytokines, IL-6 and IL-1β in the BAL fluid was significantly increased when compared with that of the sham group and TLR4 and NF-κB were also highly expressed. In addition, when compared with RAW264.7 cells that were overexpressing miR-223, the content of IL-6 and IL-1β in the supernatant and protein expression of TLR and NF-κB in cells were markedly decreased. Thus, it was demonstrated that miR-223 negatively regulated the expression of IL-6, mediating the TLR4/NF-κB signaling pathway and exerting an anti-inflammatory effect in sepsis-induced lung injury.

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Introduction

Sepsis is a very serious inflammatory response, often occurring due to the host's inability to fight off viral or bacteria infection, which leads to the loss of function and eventual failures of various organs (1). Among the numerous organs that are affected, lung tissue is often primarily affected, causing acute lung injury (ALI). ALI presents at the beginning of sepsis with an extremely high incidence rate (1). ALI involves multiple inflammatory signaling pathways that are gradually amplified. The cascade reaction of cytokines and inflammatory mediators ultimately leads to the destruction of pulmonary capillary endothelial cells and, in the most severe cases, results in the dysfunction of these cells and increases pulmonary capillary permeability, increasing the likelihood of pulmonary edema, causing inflammatory exudation, and eventually leading to acute respiratory distress syndrome (2). Toll-like receptors (TLRs) participate in the innate immune and inflammatory responses of the body. Previous studies have indicated that the knockout or targeted inhibition of the TLR4 gene significantly reduces the severity of ALI (3-5). TLR4 transmits signals following recognition of pathogen-associated molecular patterns, such as those produced by gram-negative bacteria, including endotoxin E and Lipid A from lipopolysaccharide (LPS) (6). LPS binds to TLR4 on the surface of monocytes, macrophages, neutrophils and other immune cells after entering the body. This signal is then transduced to the intracellular domain [Toll/interleukin (IL)-1 receptor region], which binds to IL-1, thus triggering the expression of nuclear factor (NF)-κB and various inflammatory cytokines and aggravating lung injury. The TLR4/NF-κB signaling axis influences the transmission of inflammatory signals from the extracellular environment to the cell membrane and cytoplasm and subsequently enters the nucleus, thus modulating the expression of target genes (7-9).

MicroRNAs (miRNAs/miRs) are extensively distributed and belong to RNA fragments that do not encode proteins (10,11). They participate in multiple physiological processes of the body and bind to the 3'-untranslated region of target mRNAs (12,13). Furthermore, it has been reported that miRNAs exert a regulatory effect in inflammatory responses (14). As regulatory elements of the immune

system and immune responses, miRNAs have attracted an increased amount of attention (14-16). miR-223 in particular has a crucial role in innate immunity, myeloid cell differentiation and cell homeostasis. Various targets of miR-223 are involved in pathways implicated in the pathogenesis of inflammation-related diseases (17). Previous studies have demonstrated that expression levels of miR-223 were increased in blood samples and inflamed lung samples (18,19). It has also been reported that miRNA expression can be induced by tumor necrosis factor-α, IL-1 and TLRs during the activation of lymphocytes and monocytes (16). The functions of miRNAs in endothelial cell activation, pro-inflammatory cytokine expression and heat shock protein remain elusive. However, to the best of our knowledge, there is currently little research on miR-223 in rats with sepsis-induced lung injury. Therefore, the aim of the present study was to reveal the anti-inflammatory mechanism of miR-223 in septic rats, thereby providing a novel method for the treatment of inflammatory responses in lung tissues.

Materials and methods

Materials. A total of 40 specific pathogen free male Sprague-Dawley rats (n=40; Hebei Province Animal Research Center; age, 8 weeks; weight, 240-260 g) were used in the cuuremt study. Rats were housed in a temperature-controlled room (21±2˚C) with a relative humidity of 30-40% and a 12 h light/dark cycle. All rats had free access to water and food. Mouse monocyte macrophage RAW264.7 cells (American Type Culture Collection), ELISA kit for cytokines (R&D Systems, Inc. cat. no. ML-Elisa-1458), phosphate buffered saline (EMD Millipore), bicinchoninic acid (Beyotime Institute of Biotechnology), antibodies (all 1:1,000; all from Abcam), RNA reverse transcription kit (Roche Diagnostics GmbH), hematoxylin and eosin (H&E) staining reagent (Beyotime Institute of Biotechnology) and TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.).

Research objects and grouping. In the present study, SD rats were divided into a sham group and a sepsis-induced lung injury (sepsis) group according to whether cecal ligation and puncture was performed. The RAW264.7 cells were stimulated with LPS and divided into the following groups, blank, LPS, LPS + control and LPS + mimic. The present study was approved by the Ethics Committee of the Fourth Central Hospital of Baoding City (Hebei, China).

Establishment of the rat model of sepsis-induced lung injury. Following anesthesia via intraperitoneal injection of pentobarbital sodium (dose, 40 mg/kg), rats underwent laparotomy via a linear abdominal incision. The cecum was punctured twice at different sites with an 18-gauge needle and gently pressed until the feces was squeezed out. The intestines were placed back in the abdomen and the abdominal incision was subjected to layered closure. Upon completion of surgery, saline (5 ml/100 g body weight) was injected subcutaneously into the rats in the sepsis and sham groups to replace the extracellular fluid that was isolated during peritonitis. The rats in the sham group underwent a sham surgery without ligation or puncture of the cecum.

Reverse transcription-quantitative PCR (RT-qPCR). Lung tissues and monocytes were homogenized in TRIzol® reagent and total RNAs were extracted according to the manufacturer's protocol. Subsequently, 3 μg RNAs were reverse transcribed into complementary deoxyribose nucleic acids (cDNAs) at 37˚C for 1 h using the RNA reverse transcription kit. The PCR mixture contained 0.5 μl Taq polymerases, 1 μl of each primer and 2 μl of each cDNA sample, with a final volume of 20 μl. In all amplifications, three repeated wells were set and quantitative changes in mRNA expression were evaluated by qPCR. qPCR was subsequently performed using the SYBR-Green Master kit (Roche Diagnostics). Quantitative analysis was performed using the ABI 7500 fluorescence PCR amplification instrument (Applied Biosystems; Thermo Fisher Scientific, Inc.). The reaction system volume was 25 μl. The thermocycling conditions for qPCR were as follows: Pre-denaturation at 95˚C for 5 min, denaturation at 95˚ for 30 sec, annealing at 60˚ for 45 sec, extension at 72˚ for 3 min for 35 cycles, and then a final extension at 72˚C for 5 min. PCR products were stored at 4˚C. The following primer pairs were used for qPCR: GAPDH: Forward, 5'-ACA GCAACAGGGTGGTGGAC-3' and reverse, 5'-TTTGAGGGT GCAGCGAACTT-3'; TLR4: Forward, 5'-AAGGCATGGCAT GGCTTACAC-3' and reverse, 5'-GGCCAATTTTGTCTCCAC AGC-3'; NF-κB: Forward, 5'-CCCAAACCTTGGCATCCTG-3' and reverse, 5'-CCGAACAACACTCAAATCC-3'; miR-223: Forward, 5'-UGUCAGUUUGUCAAAUACCCCAAAA-3' and reverse, 5'-UGUCAGUUUGUCAAAUACCCCAUUU-3'. Finally, Cq values were processed using the $2^{-\Delta\Delta Cq}$ method (20), with GAPDH serving as the control.

Observation of pathological changes following sepsis-induced lung injury via H&E staining. Pneumonectomy was performed 24 h after the rat model was established in the two groups. The trachea was fixed at 25˚C with 100% ethanol for 48 h at a distance of 20 mm from the lung. Following fixation, the paraffin-embedded tissue sections were cut into slices (thickness, ~5 μm) and histologically stained with methylene blue at 25˚C for 5 min. The slices were stored at -65˚C overnight prior to the experiment. At the beginning of the experiment, the sections were deparaffinized in 65, 70 and 90% xylene tanks at 25˚C, dehydrated in ethanol with five gradually increasing concentrations (60, 75, 90, 95 and 98%), gently washed with deionized water four times and dried in the air. The tissues were subsequently spread on a sterile glass slide and placed above an alcohol lamp for 15-30 sec to dry. Eosin Y dye solution was added dropwise for 3 min for cell plasma staining. Following cytoplasm staining, the dye solution was diluted with distilled water to terminate the staining. The stained tissue samples were cleaned with ethanol and added to the methylene blue dye solution dropwise for cell nucleus staining. After 60 sec, staining was ceased by diluting the dye solution with deionized water. A light microscope (IX70; Olympus) was used to observe cells under five randomly selected fields of view. Following staining, the lung was independently evaluated and scored by two experts. For each rat, the following characteristics of three different lobes were examined: Interstitial edema, hemorrhage and neutrophil infiltration.

Tissue protein extraction and western blot analysis. Cervical dislocation (following anesthesia with intraperitoneal injection

of pentobarbital sodium at a dose of 40 mg/kg) was used as the method of euthanasia. The tissues surrounding the lungs were dissected and stored in equal parts at -80˚C and were thawed prior to use. The RIPA (radioimmunoprecipitation assay) protein lysate (Beyotime Institute of Biotechnology) was mixed with the tissues evenly, swirled three times for 15 sec each time and centrifuged at 13,500 x g/min at 4˚C for 40 min. Total protein concentration was calculated by bicinchoninic acid (BCA) Protein Assay Kit (Beyotime Institute of Biotechnology). The protein concentration was obtained according to 450 mm absorbance and the samples were boiled for denaturation. Subsequently, the proteins were separated via SDS-PAGE and transferred to a polyvinylidene fluoride membrane (EMD Millipore). Then non-specific antigen sites were blocked with 5% bovine serum albumin (Sigma-Aldrich; Merck KGaA) at 25˚C for 1 h and incubated with TLR4 and NF-κB antibodies (1:1,000; cat. no. ab32536) and GAPDH (1:1,000; cat. no. ab8245) at 4˚C for 14 h. Finally, secondary antibodies (HRP-conjugated goat anti-rabbit IgG; 1:5,000; cat. no. ab6721) were added at 25˚C for 1 h. Immunoreactive bands were visualized using an enhanced chemiluminescence detection kit (Amersham; Cytiva). The gray value was analyzed using ImageJ software (version 1.38; National Institutes of Health).

Transfection of RAW264.7 cells stimulated by LPS with miR-223 control and mimic. RAW264.7 cells were evenly spread in a six-well plate with a total volume of 1 ml. LPS was added after 12-16 h of culture at 37˚C and, after 24 h of stimulation, the stimulated inflammatory cells were selected for seeding into 24-well plates at $1x10^5$ cells/well. Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) was used to perform the transfection. The cells were transfected with miR-223 mimic (20 μm) and miR-223 control (20 μm) when the confluency reached 70-80%. The sequences were as follows (Dojindo Molecular Technologies, Inc.): miR-223 mimic: Forward, 5'-CCUACGGAGUUACCA ACCUGGC-3' and reverse, 5'-AAGACUGGCCAGCAUUAU AGAC-3'; miR-223 control: Forward 5'- GCCAGGACGUUC GAGACGUCAG-3' and reverse, 5'-GCAGCUGCGACGUUA CCUUAGA-3'. The transfection reagent was added into the cells for 12 h of culturing at 37˚C and then replaced with a normal culture medium to observe the cell state after 48 h.

Detection of IL-6 and IL-1β levels using an ELISA kit. The supernatant from the blank, LPS, LPS + control and LPS + mimic groups was collected for analysis. Bronchoalveolar lavage (BAL) fluid was collected from the blank, LPS, LPS + control and LPS + mimic groups. The diluted standard substance was defrosted and diluted according to the 50% ratio gradient. Then 90 μl of standard substance and horseradish peroxidase (HRP)-labeled working solution were added to the standard well. ELISA plates (cat. no. 201303; Lianyungang Jinma Biotech Co., Ltd.) were coated with 100 μl/well of the diluted antigen for 1 h at 37˚C, after which plates were washed three times with PBS containing 0.05% Tween 20 and blocked with 200 μl 1% bovine serum albumin and 0.05% Tween 20 diluted in PBS for 2 h at 37˚C. The plate was cleaned five times and 90 μl HRP reaction substrate was added to each well in the dark. After 20 min, the enzymatic reaction was terminated, the optical density value was detected and the experimental data were recorded.

Statistical analysis. Experimental results were obtained and analyzed using Statistical Product and Service Solutions 17.0 software (SPSS, Inc.). Differences between two groups were analyzed by using a Student's t-test. Comparisons between three groups was performed using one-way ANOVA followed by the Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression levels of miR-223 and TLR4 and NF-κB proteins in rats with sepsis-induced lung injury. Compared with that in the sham group, the expression levels of miR-223 and NF-κB and TLR4 proteins were significantly higher than in the sepsis group ($P<0.05$), indicating that miR-223 is associated with the TLR/NF-κB signaling pathway (Fig. 1).

Levels of IL-6 and IL-1β in BAL fluid. Compared with the sham group, the sepsis group demonstrated significantly higher IL-6 and IL-1β content in the BAL fluid, indicating a strong inflammatory response in the sepsis group ($P<0.05$; Fig. 2).

Pathological changes in the alveoli of rats with sepsis-induced lung injury. H&E staining and microscopic observation of the lung tissue from the sham group indicated alveoli with a normal structure and few pathological changes. In the sepsis group, the alveoli were observed to be surrounded by a large number of neutrophils, the mesenchyme was swollen, part of the alveolar wall exhibited fibrosis and the alveolar wall was thickened (Fig. 3), suggesting that the inflammatory response of cells is enhanced following sepsis.

Changes in TLR4 and NF-κB expression following RAW264.7 cell transfection with the miR-223 mimic and control. Transfection was verified to be successful (Fig. 1B). Following LPS stimulation, the expression levels of miR-223 and TLR4 and NF-κB proteins in the cells of the LPS group were significantly increased when compared with the blank group ($P<0.05$). When compared with the other three groups, miR-223 expression in the LPS + mimic group was the highest (Fig. 4A). The protein expression levels of TLR4 and NF-κB in the LPS + mimic group were significantly reduced when compared with the LPS group and the LPS + control group ($P<0.05$) (Fig. 4B and C). qPCR revealed that the mRNA expression levels of TLR4 and NF-κB in the LPS + mimic group were significantly higher when compared with those in the blank group, but they were significantly decreased in the LPS + mimic group when compared with the LPS group and the LPS + control group ($P<0.01$; Fig. 4D). The results indicate that the inflammatory responses weaken after miR-223 is elevated and the anti-inflammatory effect of miR-223 is related to the inhibition of the TLR4/NF-κB pathway.

Changes in the content of IL-6 and IL-1β in the blank, LPS, LPS + control and LPS + mimic groups. Following LPS stimulation, the content of IL-6 and IL-1β in the cell supernatant increased significantly. Compared with that in

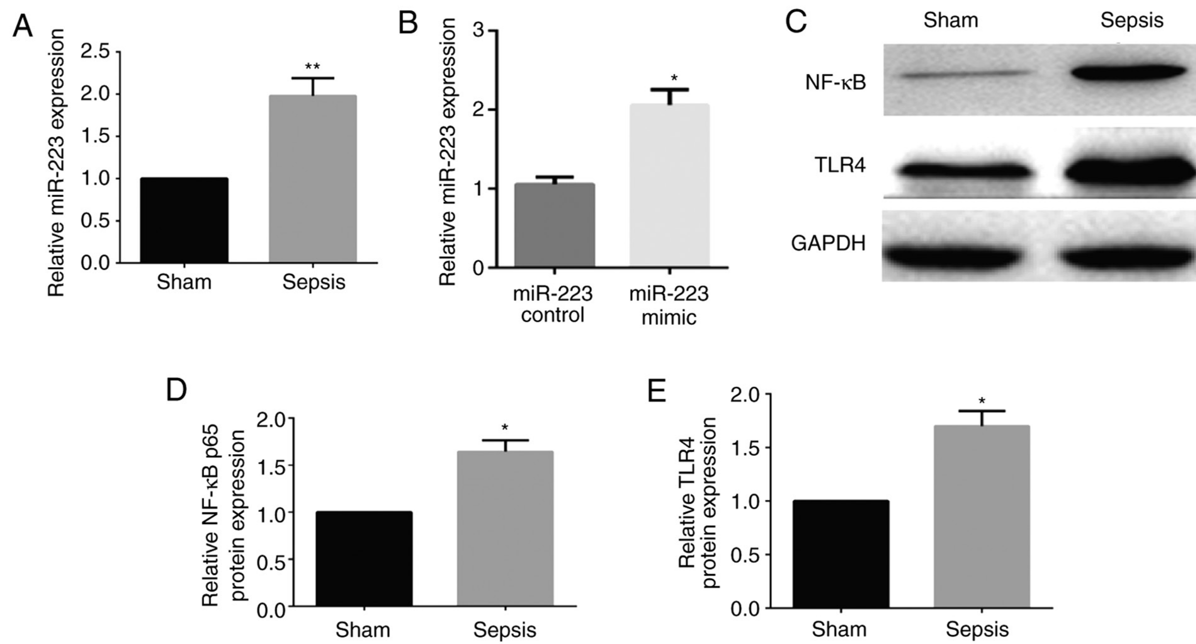

Figure 1. Expression levels of miR-223, TLR4 and NF-κB proteins in rats with sepsis-induced lung injury. (A) miR-223 expression in the sepsis group is significantly higher than that of the sham group. **P<0.01 vs. sham group. (B) The expression levels of miR-223 in miR-223-mimic were significantly higher than the miR-223-control group. *P<0.05 vs. miR-223 control. (C-E) Protein expression of TLR and NF-κB in the sepsis group is significantly higher compared with that in the sham group. Data are presented as the mean ± SD. *P<0.05 vs. sham group. miR, microRNA; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; TLR, Toll-like receptor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

the LPS group and the LPS + control group, the content of IL-6 and IL-1β in the supernatant of the LPS + mimic group was significantly decreased (P<0.05; Fig. 5), indicating that miR-223 inhibits the expression of IL-6 and plays an anti-inflammatory role.

Discussion

Inflammatory responses are a type of immune response and refer to a stress protection reaction in response to microorganism infection. However, a persistent inflammatory response can destroy healthy cells. Inflammatory responses exert vital effects in host defense and immune responses against bacterial infection (21). The purpose and regulation of inflammatory responses are inaccurate and they often become uncontrolled cascade reactions, which may cause collateral damage to tissues (22). Pulmonary infection imposes a heavy burden on public health worldwide and is the main cause of mortality in the USA (23). Infections caused by gram-negative bacteria are particularly concerning due to their increasing levels of antibiotic resistance (24). High mortality and morbidity rates following bacterial infection are usually caused by the imbalance of host defense capability between removing the infection and excessive inflammatory responses (leading to tissue damage) (25). Previous studies have shown that miR-223 is a small RNA specific to hematopoietic tissues, and that miR-223 modulates the inflammation of tissues and organs (26,27).

IL-6, a cytokine secreted by inflammatory cells, has various roles, and therefore its functions are more complex depending on the pathophysiology (28,29). Furthermore, data from animal models indicate that IL-6 plays a critical role in a variety of pathophysiological events (such as fever, acute liver reaction and the transition from acute inflammation to chronic inflammation) (30). However, the innate immune mechanism of IL-6 requires further investigation. TLRs are transmembrane glycoprotein families with two domains, among which IL-1R homologous cytoplasmic signal domain (Toll/IL-1R domain) can bind to IL to function as inflammatory cytokines, as well as to a variety of other bacterial and viral peptides (31). TLR predominantly exerts its effects via the TLR/NF-κB signaling pathway. As TLR ligands, bacteria and virus peptides usually activate MAPK, NF-κB and the interferon regulatory factor (IRF)-3/IRF-7 pathway after binding to TLRs. The final result is to promote the secretion of type I interferon and mediate the function of inflammatory cytokines, thus controlling the response to pathogens (31). Therefore, the role of IL-6 in inflammation may be associated with the TLR/NF-κB signaling pathway.

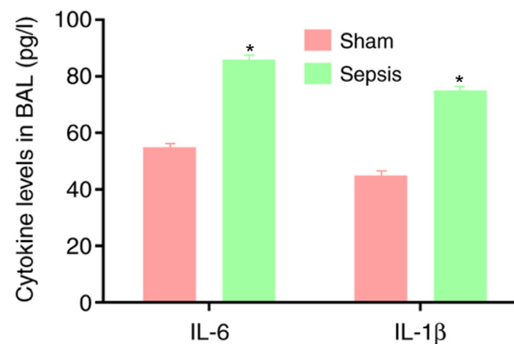

Figure 2. Changes in the content of cytokines, IL-6 and IL-1β in BAL fluid. IL-6 and IL-1β content in the sepsis group is significantly higher than that in the sham group. *P<0.05 vs. sham group. BAL, bronchoalveolar lavage; IL, interleukin; BAL, bronchoalveolar lavage.

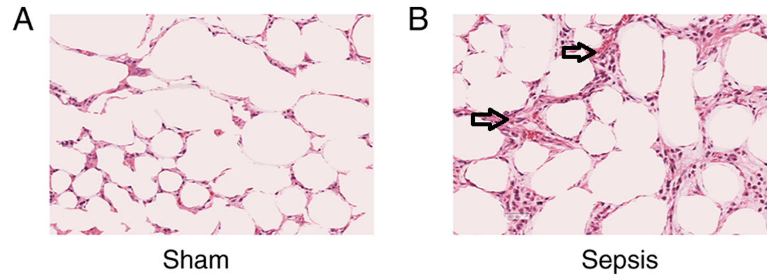

Figure 3. Hematoxylin and eosin-stained lung tissues from the sham and sepsis groups demonstrating pathological changes of the alveoli. (A) Alveoli from the lung tissues of the sham group exhibit a normal structure (magnification, x200). (B) In the sepsis group, the alveoli were observed to be surrounded by a large number of neutrophils. Additionally, the mesenchyme was swollen, with part of the alveolar wall exhibiting fibrosis. The alveolar wall was also generally thickened. (magnification, x200).

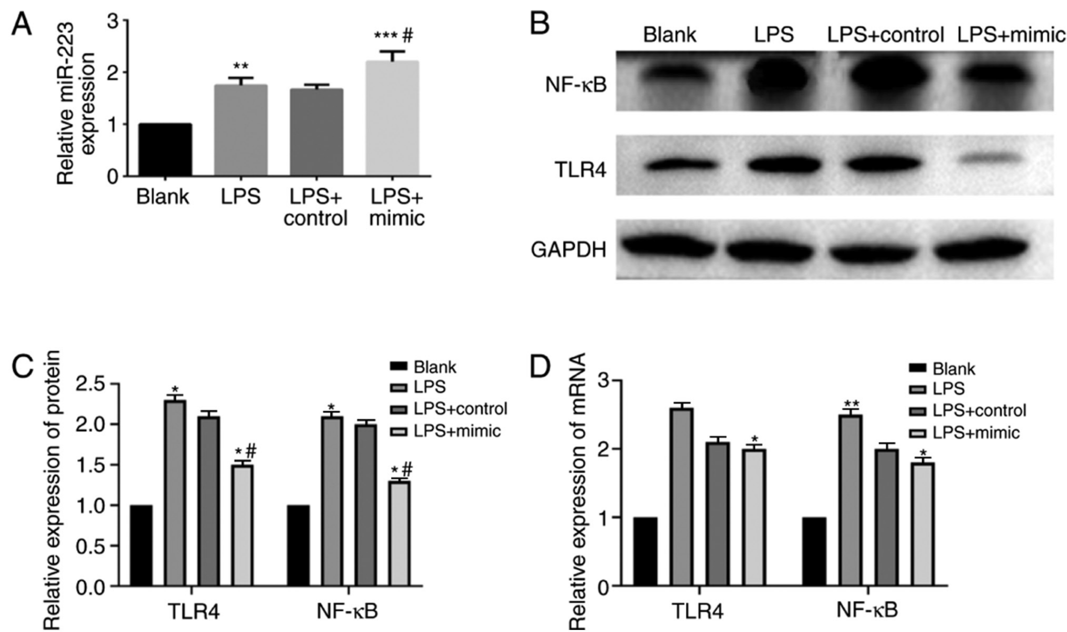

Figure 4. Changes in the expression levels of TLR4 and NF-κB in RAW264.7 cells following overexpression of miR-223. (A) Compared with that in the blank group, miR-223 expression in the LPS group is significantly increased. miR-223 expression in the LPS + mimic group was significantly increased when compared with that in the LPS group and that in the LPS + control group. **P<0.01 vs. blank group; #P<0.01 vs. LPS + control group; ***P<0.001 vs. the blank group. The data presented as the mean ± SD. (B) Compared with those in the blank group, the protein expression levels of TLR4 and NF-κB in LPS group were raised. (C) Compared with those in the LPS group and the LPS + control group, the protein expression levels of TLR4 and NF-κB in the LPS + mimic group were significantly decreased. *P<0.05 vs. blank group; #P<0.01 vs. LPS + control group. (D) Compared with the blank group, the LPS group exhibited significantly elevated mRNA expression levels of TLR4 and NF-κB. *P<0.05 vs. blank group; **P<0.01 vs. blank group When compared with the LPS group and the LPS + control group, the LPS + mimic group exhibited significantly decreased mRNA expression levels of TLR4 and NF-κB. TLR, Toll-like receptor; NF-κB, nuclear factor-κB; miR, microRNA; LPS, lipopolysaccharide.

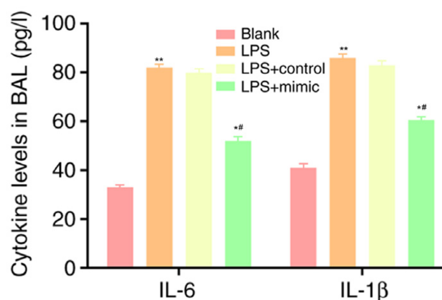

Figure 5. Changes in the content of IL-6 and IL-1β following the overexpression of miR-223. Compared with that in the blank group, the content of IL-6 and IL-1β in the LPS group is significantly increased. **P<0.01 vs. blank group. Compared with that in the LPS group and the LPS + control group, the content of IL-6 and IL-1β in the LPS + mimic group is significantly reduced. *P<0.05 vs. blank group; and #P<0.05 vs. LPS + control group.

In the present study, a lung injury model was established by cecal ligation and puncture. The expression levels of miR-223 and TLR4 and NF-κB proteins in the lung tissue cells from sham and sepsis groups were detected. The content of IL-6 and IL-1β secreted in BAL fluid was then examined via ELISA. The results indicated that the expression levels of miR-223 and TLR4 and NF-κB proteins were significantly increased. In addition, the content of cytokines, IL-6 and IL-1β, was significantly increased following lung injury. It can therefore be concluded that the presence of miR-223 may be associated with TLR4 and IL-6 following lung injury. Microscopic visualization of H&E-stained lung tissue showed alveoli with normal structures in the sham group. The pathological results demonstrated that following lung injury, the lung tissues were destroyed and the inflammatory responses of the body were enhanced.

The expression levels of miR-223 and TLR4 and NF-κB proteins in the lung tissues indicate that the content of miR-223 may be associated with TLR4 and IL-6 following lung injury. Subsequently, the regulatory mechanism among the three at the cellular level was investigated. RAW264.7 cells were stimulated with LPS to become inflammatory cells and were then transfected with miR-223 controls and mimics. Changes in the expression levels of miR-223 and TLR4 and NF-κB proteins were detected. Following LPS stimulation of the cells, the expression levels of miR-223 and TLR4 and NF-κB proteins were significantly increased. When compared with the LPS + control group and the LPS group, the LPS + mimic group presented significantly increased miR-223, but significantly decreased expression levels of IL-6 and TLR4 and NF-κB proteins and mRNAs.

Thus, it can be concluded that miR-223 negatively regulates the expression of IL-6 in cells and mediates the TLR4/NF-κB signaling pathway to play an anti-inflammatory role once the level of IL-6 is decreased. Whether IL-6 is one of the target genes of miR-223 was not biologically predicted and verified in the present study. Furthermore, the anti-inflammatory effect of miR-223 was not verified *in vivo*. However, the present study may provide a novel therapeutic method for relieving inflammatory responses in sepsis-induced lung injury.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

XM and DT designed the study and performed the experiments. XM and WL established the animal models, DT and BG collected the data, ZM and XZ analyzed the data, XM prepared the manuscript. All authors read and approved the final manuscript. XM and DT confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present study was approved by the Animal Ethics Committee of the Fourth Central Hospital of Baoding City Animal Center (Hebei, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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