

MicroRNA-223 attenuates LPS-induced inflammation in an acute lung injury model via the NLRP3 inflammasome and TLR4/NF- κ B signaling pathway via RHOB

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Abstract. Acute lung injury (ALI) and the more severe acute respiratory distress syndrome are common and complex inflammatory lung diseases. MicroRNAs (miRs) have emerged as novel gene regulatory molecules, serving a crucial role in a variety of complex diseases, including ALI. In the present study, the anti-inflammatory action of miR-223 on inflammation in ALI was demonstrated and the possible mechanism was further examined. In lipopolysaccharide-induced ALI, the expression of miR-223 was reduced compared with that in the control normal group. An *in vitro* model was used to analyze the effect of miR-223 downregulation on an ALI model, which increased inflammation, and induced the activation of the NACHT, LRR and PYD domains-containing protein 3 (NLRP3) inflammasome and Toll-like receptor 4 (TLR4)/nuclear factor (NF)- κ B signaling pathway via rho-related GTP-binding protein RhoB (RHOB). In addition, the overexpression of miR-223 reduced inflammation and suppressed the NLRP3 inflammasome and TLR4/NF- κ B signaling pathway via RHOB in the *in vitro* model. Furthermore, TLR4 inhibitor or NLRP3 inhibitor reduced the pro-inflammatory effect of miR-223 downregulation in ALI. In conclusion, the results of the present study indicated that miR-223 functioned as a biological indicator by regulating inflammation in ALI, and may represent a novel potential therapeutic target and prognostic marker of ALI.

Introduction

Acute lung injury (ALI)/acute respiratory distress syndrome (ARDS) refers to the acute progressive respiratory insufficiency or respiratory failure caused by various factors, including trauma, blood transfusion and infection (1); this is a common respiratory crisis, pathologically characterized by pulmonary edema, pulmonary hemorrhage and severe respiratory impairment (1). The mortality rate of ALI/ARDS may be as high as 30-50% (2). Due to the undefined pathogenesis, there are no effective therapeutic drugs in clinical practice at present (2). Supportive treatment remains the principal treatment option (3). A series of ALI animal models have been established to simulate the status of ALI caused by diverse pathogenesis (3). Among them, the majority of studies have focused on lipopolysaccharide (LPS), one of the primary causative factors of ALI caused by bacterial infections, particularly Gram-negative bacteria (3). As the active component of bacterial endotoxin, LPS is an important pathogenic factor in ALI (4). In LPS-induced ALI animal models, the excessive inflammatory reaction of neutrophils leads to extensive damage and even mortality (4).

The NACHT, LRR and PYD domains-containing protein 3 (NLRP3) inflammasome is a multiprotein large-cytoplasmic complex, which is composed of NLRP3, apoptosis-associated speck-like proteins and pro-caspase-1 (5). NLRP3 serves a critical role in regulating the relevant components of the inflammatory complex, including apoptosis-associated speck-like proteins and pro-caspase-1 (6). It has been reported that in acute injury, the stimulated intracellular reactive oxygen species may activate the NLRP3 inflammasome and caspase-1, and promote the cleavage and maturation of pro-interleukin (IL)-1 β and pro-IL-18. As a result, inflammatory cytokines are released, resulting in damage to lung tissue (7,8).

Alterations in microRNA (miRNA/miR) expression are associated with immune responses, inflammatory signaling pathways and the pathogenesis of inflammatory lung diseases, including ALI. Therefore, miRNAs are novel promising therapeutic targets (9). miRNA-induced changes in gene expressions are generally modest (9). However, the results may affect the expression of a large number of subsequent genes, which

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further affect multiple biological processes. Therefore, it is feasible to utilize miRNAs as markers (10). miRNA expression has been verified to be dynamic, which reflects alterations in the internal and external environments, and cell signals (10). Therefore, it is promising to use miRNAs as biomarkers to represent a specific disease state or the underlying pathophysiological processes at different stages. In addition, miRNAs may be detected in a variety of specimens, including tissue, blood or other body fluids (11). miRNAs are also fairly stable and are virtually unaffected by errors in sample handling, making miRNAs more attractive biomarkers (11). Intriguingly, miRNAs in plasma have been used as biomarkers to diagnose and monitor a variety of inflammatory lung diseases, including ALI. Neudecker *et al* (12) suggested that miRNA-223 deficiency was associated with severe lung inflammation. In the present study, the anti-inflammatory effect of miRNA-223 on inflammation in ALI, and the possible mechanism, was demonstrated.

Materials and methods

Mice and histopathological assay. Male C57BL/6 mice (5-6 weeks; 18-20 g) were obtained from Shandong University Laboratory Animal Center (Jinan, China). All mice were housed at 22-23°C, 55-60% humidity, on a 12-h light/dark cycle with free access to food/water. All mice were randomly assigned to two groups: Control and ALI mice. All ALI model mice were injected with 35 mg/kg pentobarbital sodium [intraperitoneal (i.p.)] and injected with LPS at 5 mg/kg (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) into the chest. After 1 day, all mice were injected with 35 mg/kg pentobarbital sodium and sacrificed via decollation. Lung tissue was acquired and washed with PBS, and fixed with 4% paraformaldehyde for 24 h at room temperature. The lung tissue was dehydrated using 100-75% ethyl alcohol for 5 min at 4°C, and cut into 5-μm sections. Lung tissue sections were stained with hematoxylin and eosin (HE) for 5 min at room temperature, and were finally examined under a light microscope (Nikon Eclipse TE2000-U; Nikon Corporation, Tokyo, Japan) at x100 magnification. The experimental procedures in the present study were performed with the approval of Binzhou Medical University Hospital (Liaocheng, China).

Cytokine detection. Serum samples were centrifuged at 1,000 x g for 10 min and used to measure TNF-α (cat. no. H052), IL-1β (cat. no. H002), IL-6 (cat. no. H007) and IL-18 (cat. no. H0015) levels using ELISA kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Cells were lysed with radioimmunoprecipitation assay buffer for 15 min and protein concentrations in the extracts were measured by bicinchoninic acid assay. Proteins (10 μg) were centrifuged at 1,000 x g for 10 min and collected to measure TNF-α, IL-1β, IL-6 and IL-18 levels using ELISA kits.

Measurement of miRNA and mRNA expression. Total RNA was extracted from lung tissues or cells using TRIzol reagent, according to the manufacturer's instructions (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). cDNA was synthesized using a qScript cDNA Synthesis kit (QuantaBio, Beverly, MA, USA) at 37°C for 60 min and at

82°C for 5 sec. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis was executed using a SYBR Green Detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) on a 7500 real-time PCR systems (Applied Biosystems; Thermo Fisher Scientific, Inc.). Primer sequences were as follows: miR-223 forward, 5'-GTGCAGGGTCCGAGGT-3' and reverse, 5'-CGGGCTGTCAGTTTGTCA-3'; U6 forward, 5'-GCTTCGGCAGCACATATACTAAAAT-3' and reverse, 5'-CGCTTCACGAATTTGCGTGTCA-3'. The PCR conditions were 95°C for 30 sec, followed by 40 cycles of 95°C for 20 sec, 60°C for 30 sec and 72°C for 30 sec. Analysis of relative gene expression data was performed using the $2^{-\Delta\Delta C_q}$ method (13).

Microarray analysis. Isolated RNA was cleaned up using an RNeasy Mini kit (Qiagen, Inc., Valencia, CA, USA) and biotin-labeled cRNA was produced by metal-induced hydrolysis at 94°C and hybridized onto the Affymetrix Human Genome U133 Plus 2.0 Array (Affymetrix; Thermo Fisher Scientific, Inc.) at 45°C for 16 h. Fluidic Station-450 and GeneChip were performed using the Affymetrix GeneChip Scanner 7G (Affymetrix; Thermo Fisher Scientific, Inc.). Data were analyzed using GeneSpring GX 10 software (Silicon Genetics; Agilent Technologies, Inc., Santa Clara, CA, USA).

Cell culture and transfection. Lung adenocarcinoma A549 cells were obtained from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China), and maintained in Dulbecco's modified Eagle's medium (high glucose; Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.). A549 cells were treated with 100 ng LPS and 2 mM adenosine 5'-triphosphate (ATP) for 4 h for the ALI model. miRNA-223, anti-miRNA-223, RHOB plasmid and negative mimics were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). A549 cells (1x10⁶ cells/ml) were co-transfected with 0.1 μg miRNA-223 (5'-UGUCAGUUUGUCAAUACCCCA-3'), anti-miRNA-223 (5'-TGTCAGTTTGTCAAA TACCCCAT-3'), RHOB plasmid (5'-CGCTCATGGAGGCCATCCGC-3' and 5'-CTGCAATGCTATGAGGGC-3'; Sangon Biotech Co., Ltd.) and negative mimics (5'-AGGUCGACUACGGGUCAAUC-3') using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). After 6 h, transfected cells were treated with 100 ng LPS (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and 2 mM ATP (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 4 h for the ALI model.

Luciferase reporter gene assay. TargetScan (http://www.targetscan.org/vert_61/), online bioinformatics software, concluded that NLRP3 is a downstream target gene of miR-223. A549 cells were co-transfected with miRNA-223, anti-miRNA-223, pMIR-REPORT-RhoB-3'-untranslated region (UTR) luciferase reporter plasmid (Huayueyang Biotechnology Co., Ltd., Beijing, China) and pMIR-REPORT-NLRP3-3'-UTR (Huayueyang Biotechnology Co., Ltd.) luciferase reporter plasmid using Lipofectamine 2000. A total of 24 h after transfection, cells were treated with 100 ng LPS and 2 mM ATP for 4 h, and luciferase activity was measured using the Dual-luciferase reporter assay kit (Promega

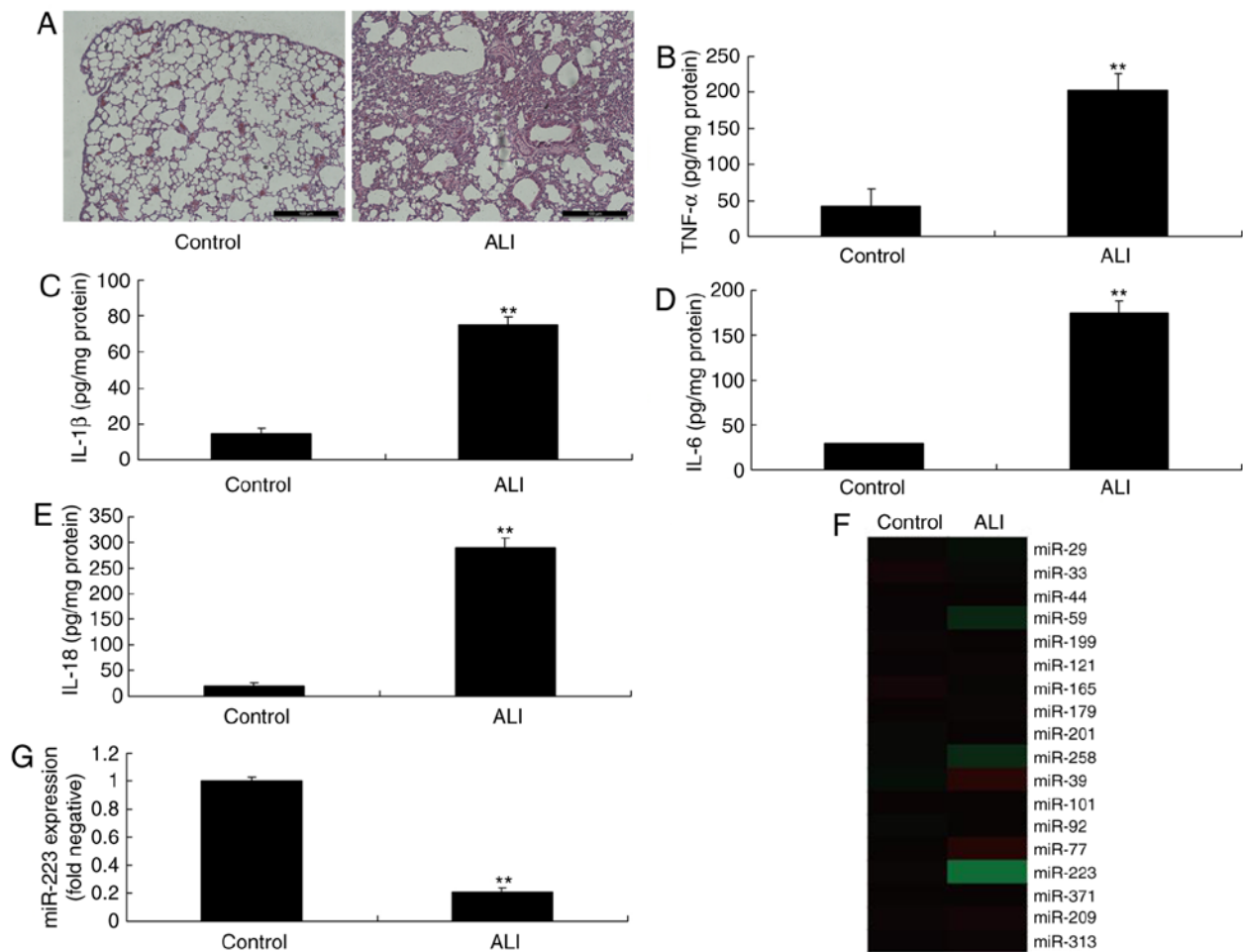


Figure 1. miR-223 expression in ALI mice. (A) Hematoxylin and eosin staining of lung tissue. (B) TNF- α , (C) IL-1 β , (D) IL-6 and (E) IL-18 were assayed. (F) Gene chip and (G) reverse transcription-quantitative polymerase chain reaction analysis for miR-223 expression in ALI mice. Scale bar, 100 μ m. **P<0.01 vs. control. ALI, acute lung injury; miR, microRNA; IL, interleukin; TNF- α , tumor necrosis factor- α .

Corporation, Madison, WI, USA) in comparison with *Renilla* luciferase.

Western blot analysis. Cells were lysed with radioimmunoprecipitation assay buffer for 15 min and protein concentrations in the extracts were measured by bicinchoninic acid assay. Proteins (50 μ g) were separated by electrophoresis on 10% SDS-polyacrylamide gels and 3% polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were blocked with 5% bovine serum albumin (BSA; Beyotime Institute of Biotechnology, Haimen, China) in TBS at 37°C for 1 h and incubated with the indicated antibodies: Toll-like receptor 4 (TLR4; 1:2,000; cat. no. ab13556; Abcam, Cambridge, UK), nuclear factor (NF)- κ B (1:2,000; cat. no. 8242; Cell Signaling Technology, Inc., Danvers, MA, USA), NLRP3 (1:2,000; cat. no. 13158; Cell Signaling Technology, Inc.), caspase-1 (1:1,000; cat. no. 3866; Cell Signaling Technology, Inc.), RHOB (1:2,000; cat. no. 2098; Cell Signaling Technology, Inc.) and GAPDH (1:5,000; cat. no. 5174; Cell Signaling Technology, Inc.) overnight at 4°C. Following three washes with TBS with Tween-20 (TBST) for 15 min, the membranes were incubated with anti-rabbit horseradish peroxidase-coupled secondary antibodies (cat. no. 7074; 1:5,000; Cell Signaling Technology, Inc.) at room

temperature for 1 h. The membrane was washed with TBST and detected by enhance chemiluminescence (Pierce; Thermo Fisher Scientific, Inc.), and quantified using Image Lab 3.0 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Immunofluorescence staining. Cells (1×10^5 cells/ml) were washed with PBS and fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. Cells were permeabilized with 0.1% Triton X-100 in PBS for 15 min at room temperature and blocked with 5% BSA in PBS for 1 h. Subsequently, the cells were incubated with anti-RHOB (1:100; cat. no. 2098; Cell Signaling Technology, Inc.) overnight at 4°C. The cells were washed with PBS, and incubated with goat anti-rabbit IgG-CFL 555 secondary antibodies (cat. no. sc-362272; 1:100; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 1 h at room temperature. Cells were stained with DAPI for 15 min in the dark at room temperature and washed with PBS. Cells were examined under a Zeiss LSM 510 confocal microscope (Carl Zeiss AG, Oberkochen, Germany).

Statistical analysis. All the experiments were performed at least three times independently (n=3) using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). A Student's t-test was used to determine statistical significance between two groups. One-way

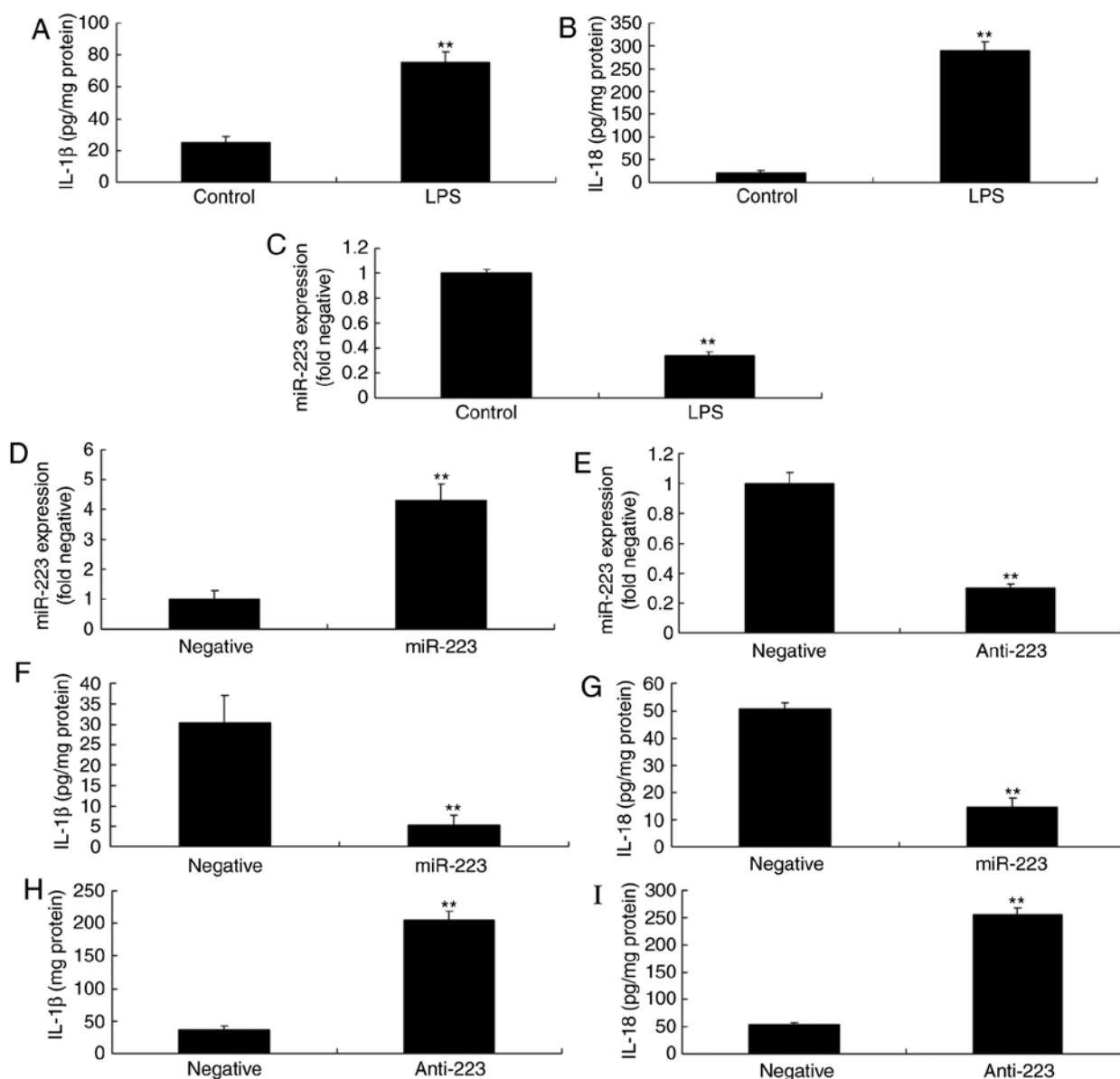


Figure 2. miR-223 expression in ALI *in vitro*. (A) IL-1 β , (B) IL-18, (C) miR-223 expression was assessed *in vitro* in the ALI model induced by LPS. miR-223 expression in cells transfected with (D) miR-223 and (E) anti-miR-223. IL-1 β expression in cells transfected with (F) miR-223 and (H) anti-miR-223. IL-18 expression in cells transfected with (G) miR-223 and (I) anti-miR-223. **P<0.01 vs. respective control. ALI, acute lung injury; miR, microRNA; LPS, lipopolysaccharide; IL, interleukin.

analysis of variance and Tukey's post hoc test were performed for parametric multivariable analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

miRNA-223 expression in ALI mice. To investigate the function of miR-223 in ALI mice, the alterations in miR-223 was analyzed in ALI mice. As presented in Fig. 1A, HE staining indicated that pulmonary alveoli were damaged in ALI mice compared with control mice. The levels of TNF- α , IL-1 β , IL-6 and IL-18 were increased in ALI mice, in comparison with the control group (Fig. 1B-E). A gene chip assay was used to analyze the alterations in miRNAs, which revealed that miR-223 expression was decreased in ALI mice compared with the control group (Fig. 1F). The RT-qPCR assay demonstrated

that miR-223 expression was decreased in ALI mice compared with the control group (Fig. 1G). Therefore, miR-223 expression may be associated with inflammation in ALI.

miR-223 expression in ALI *in vitro*. Moreover, the levels of IL-1 β and IL-18 were increased in A549 cells following treatment with LPS, compared with the control group (Fig. 2A and B). miR-223 expression was reduced in A549 cells following treatment with LPS, compared with the control group (Fig. 2C). To analyze the function of miR-223 in ALI, miR-223 and anti-miR-223 mimics were used to increase or inhibit the expression of miR-223 in A549 cells treated with LPS, compared with a negative control group (Fig. 2D and E). Overexpression of miR-223 reduced the levels of IL-1 β and IL-18 in A549 cells following treatment with LPS, compared with the negative control group (Fig. 2F and G).

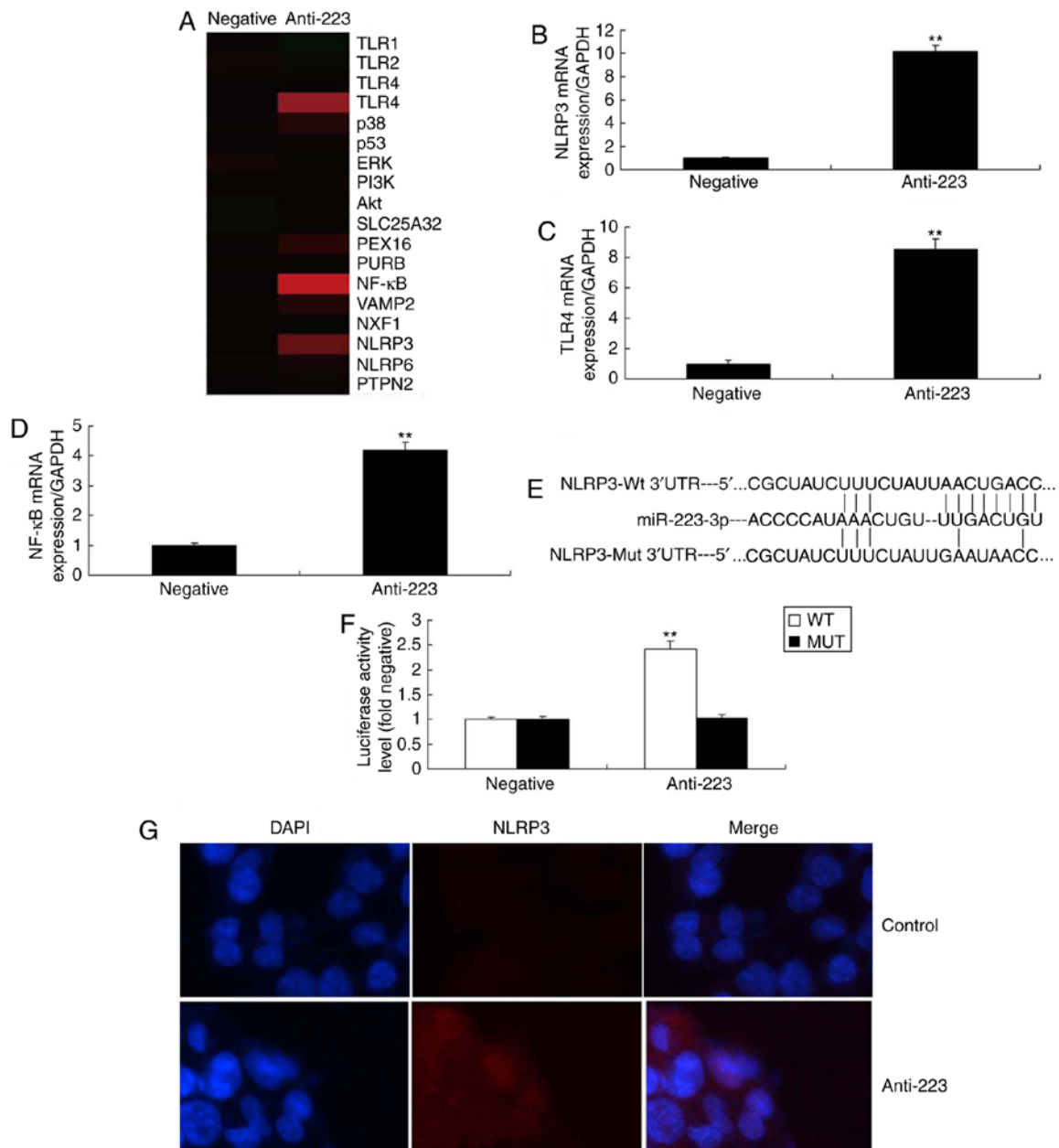


Figure 3. miR-223 regulates NLRP3 protein expression in acute lung injury *in vitro*. (A) Gene chip assay of the signaling pathway. (B) NLRP3, (C) TLR4 and (D) NF-κB mRNA expression. (E) Predicted miR-223 binding sites in the NLRP3 3'-UTR. (F) Dual-luciferase reporter gene assay. (G) Immunofluorescence for NLRP3 protein expression. Magnification, x200. **P<0.01 vs. respective control. UTR, untranslated region; NLRP3, NACHT, LRR and PYD domains-containing protein 3; miR, microRNA; TLR4, Toll-like receptor 4; NF-κB, nuclear factor-κB; WT, wild type; MUT, mutant.

Downregulation of miR-223 promoted the levels of IL-1β and IL-18 in A549 cells treated with LPS, compared with the negative control group (Fig. 2H and I). Together, these results demonstrated that miR-223 regulated inflammation in ALI, while its mechanism required investigation.

miRNA-223 regulates NLRP3 protein expression in ALI *in vitro*. In order to investigate the mechanism of action of miR-223 in ALI, a gene chip assay was used to measure the alterations in miR-223 in ALI *in vitro* following inhibition of miR-223. Consequently, inhibition of miR-223 induced the gene expression of TLR4, NF-κB and NLRP3 in ALI *in vitro* compared with the negative control (Fig. 3A). Downregulation of miR-223 also increased the mRNA expression of TLR4,

NF-κB and NLRP3 in ALI *in vitro*, compared with the negative control group (Fig. 3B-D). The 3'-UTR of NLRP3 mRNA is a target site of miR-223 in various species (Fig. 3E). The relative luciferase activity of the wild-type 3'-UTR following downregulation of miR-223 was increased, in comparison with the control group (Fig. 3F). As presented in Fig. 3G, immunofluorescence demonstrated that the downregulation of miR-223 induced NLRP3 protein expression compared with the control group. These results indicated that miR-223 may regulate pro-inflammatory cytokine expression by targeting NLRP3.

miRNA-223 regulates the TLR4 and NLRP3 signaling pathway in ALI *in vitro*. Downregulation of miR-223 induced

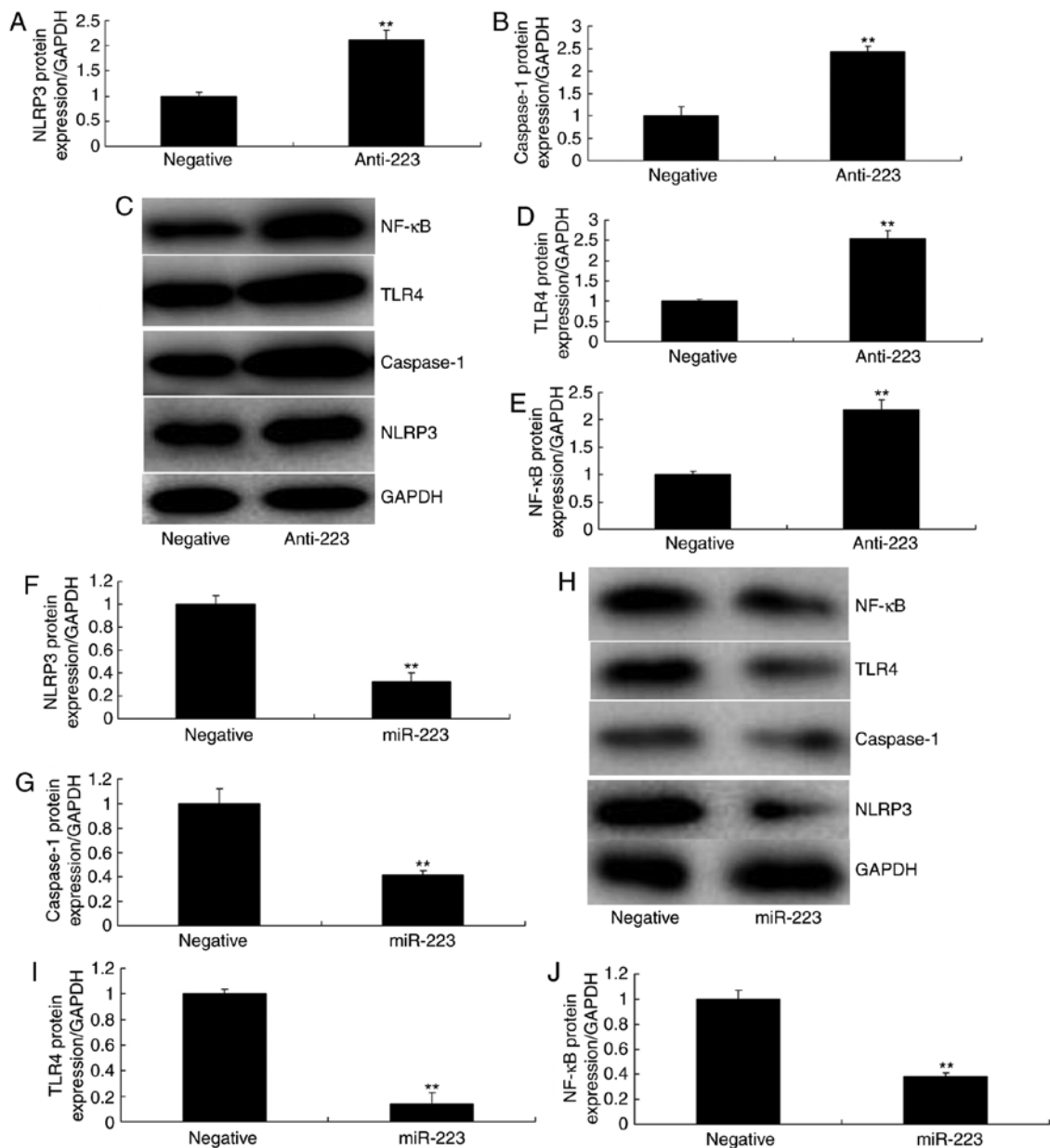


Figure 4. miR-223 regulates the TLR4 and NLRP3 signaling pathway in acute lung injury *in vitro*. (A) NLRP3 and (B) caspase-1 protein expression was quantified following downregulation of miR-223. (C) Western blot analysis of NLRP3, caspase-1, TLR4 and NF-κB. (D) TLR4 and (E) NF-κB protein expression was quantified following downregulation of miR-223. (F) NLRP3 and (G) caspase-1 protein expression was quantified following upregulation of miR-223. (H) Western blot analysis of NLRP3, caspase-1, TLR4 and NF-κB. (I) TLR4 and (J) NF-κB protein expression was quantified following upregulation of miR-223. ** $P < 0.01$ vs. negative control. NLRP3, NACHT, LRR and PYD domains-containing protein 3; miR, microRNA; TLR4, toll-like receptor 4; NF-κB, nuclear factor-κB.

the protein expression of TLR4, NF-κB, NLRP3 and caspase-1 in ALI *in vitro*, compared with the negative control group (Fig. 4A-E). Overexpression of miR-223 suppressed the protein expression of TLR4, NF-κB, NLRP3 and caspase-1 in ALI *in vitro*, compared with the negative control group (Fig. 4F-J). Taken together, these results demonstrated that miR-223 may regulate the TLR4 and NLRP3 signaling pathway in ALI *in vitro*.

Inhibition of TLR4 reduces the pro-inflammatory effect of anti-miR-223 in ALI *in vitro*. To elucidate the mechanism of action of miR-223 in ALI *in vitro*, a TLR4 inhibitor was used to decrease the protein expression of TLR4 in ALI

in vitro. The inhibition of TLR4 did not affect the protein expression of NLRP3 and caspase-1, but suppressed that of TLR4 and NF-κB in ALI *in vitro* following miR-223 downregulation, compared with miR-223 downregulation alone (Fig. 5A-E). The inhibition of TLR4 reduced the levels of IL-1β and IL-18 in ALI *in vitro* following miR-223 downregulation, in comparison with miR-223 downregulation alone (Fig. 5F and G). Taken together, these results demonstrated that TLR4 is required to maintain the proinflammatory effect of anti-miR-223 in ALI.

Inhibition of NLRP3 reduces the pro-inflammatory effect of anti-miR-223 in ALI *in vitro*. To further elucidate the role

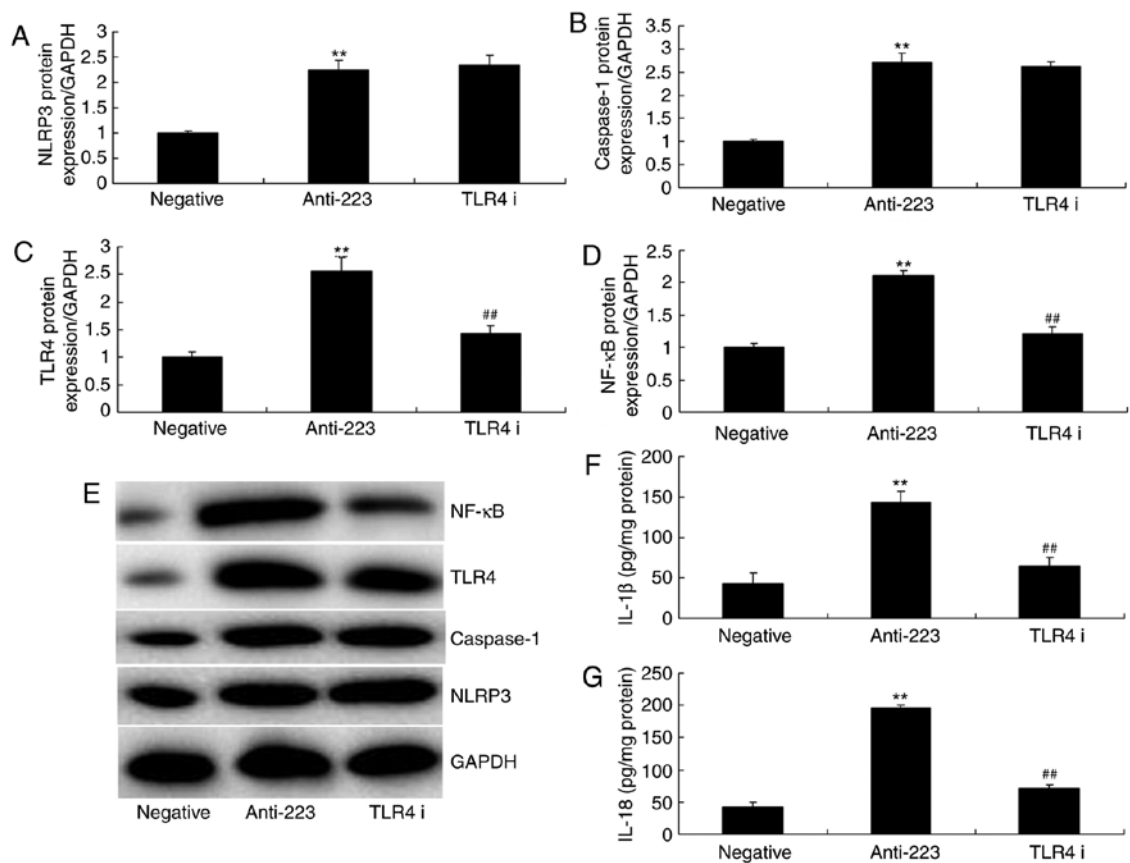


Figure 5. Inhibition of TLR4 reduces the pro-inflammatory effect of anti-miR-223 on inflammation in acute lung injury *in vitro*. (A) NLRP3, (B) caspase-1, (C) TLR4 and (D) NF-κB protein expression was quantified. (E) Western blot analysis of NLRP3, caspase-1, TLR4 and NF-κB protein expression. (F) IL-1β and (G) IL-18 were analyzed. **P<0.01 vs. negative control; ##P<0.01 vs. anti-223 group. TLR4 i, TLR4 inhibitor and anti-miR-233 combined; NLRP3, NACHT, LRR and PYD domains-containing protein 3; miR, microRNA; TLR4, toll-like receptor 4; NF-κB, nuclear factor-κB; IL, interleukin.

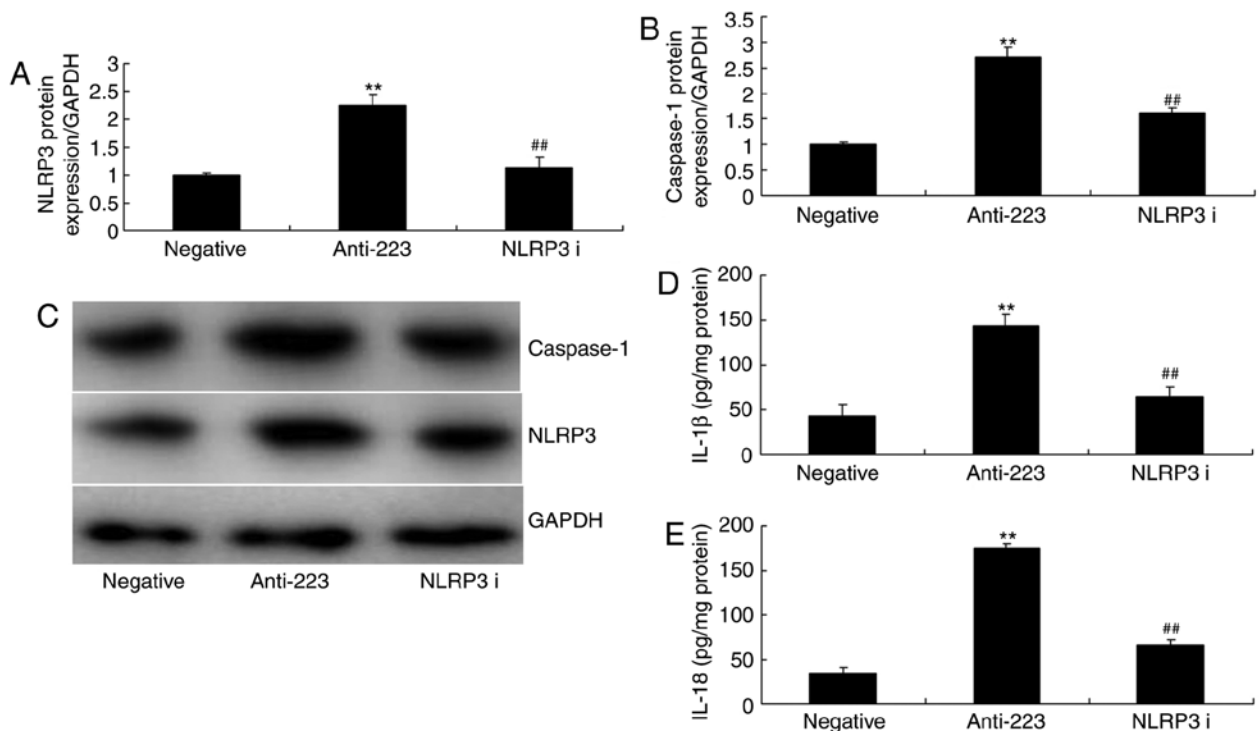


Figure 6. Inhibition of NLRP3 reduces the pro-inflammatory effect of anti-miR-223 on inflammation in acute lung injury *in vitro*. (A) NLRP3 and (B) caspase-1 protein expression was quantified. (C) Western blot analysis of NLRP3 and caspase-1 protein expression. (D) IL-1β and (E) IL-18 were analyzed. **P<0.01 vs. negative control; ##P<0.01 vs. anti-223 group. NLRP3, NACHT, LRR and PYD domains-containing protein 3; NLRP3 i, NLRP3 inhibitor and anti-miR-233 combined; miR, microRNA; IL, interleukin.

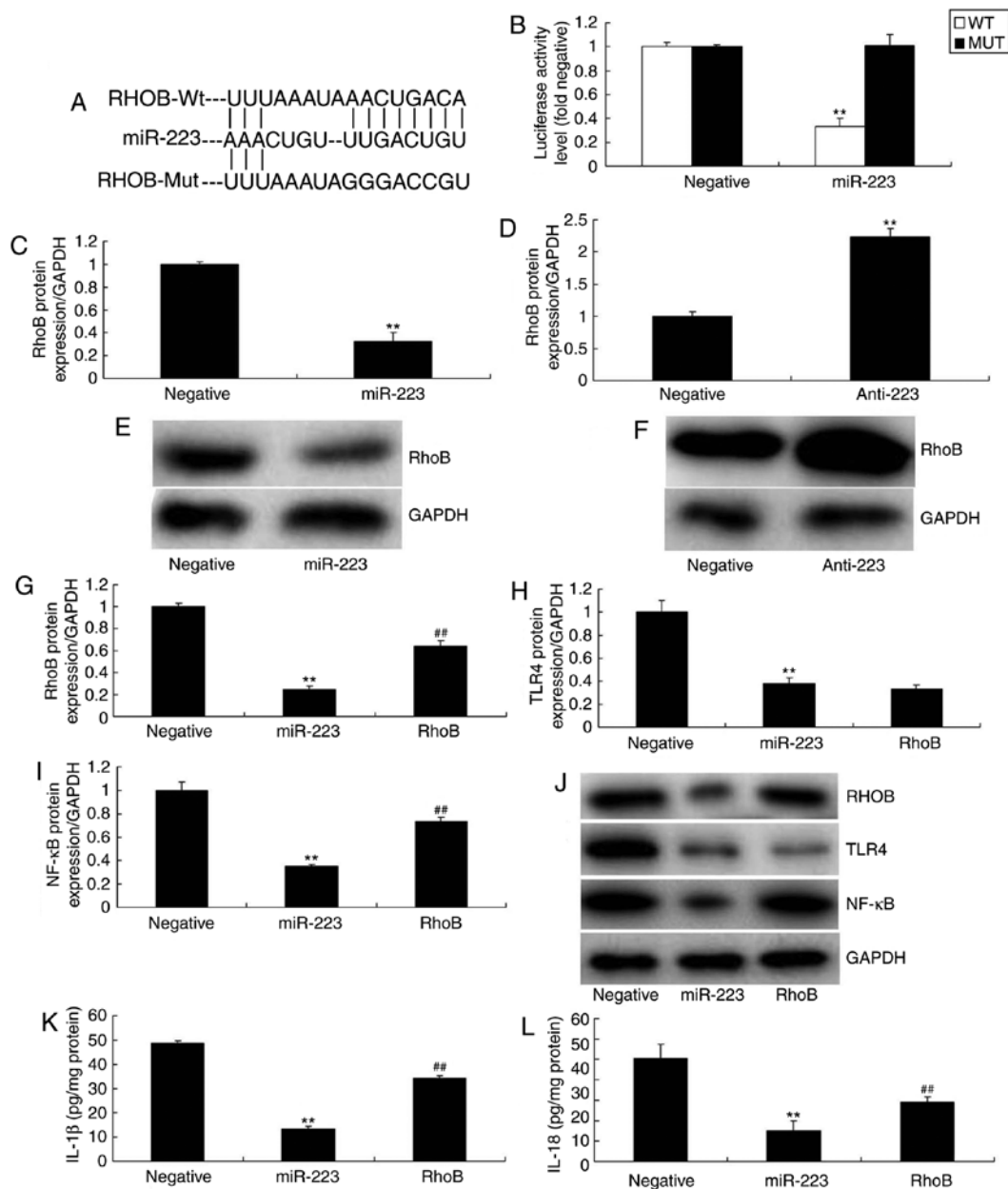


Figure 7. miR-223 regulates the TLR4 signaling pathway in acute lung injury *in vitro* via RHOB. (A) Predicted miR-223 binding sites in the RHOB 3'-untranslated region. (B) Dual-luciferase reporter gene assay. RHOB protein expression was quantified in cells with (C) miR-223 upregulation and (D) miR-223 downregulation. Western blot analysis of RHOB protein expression in cells with (E) miR-223 upregulation and (F) miR-223 downregulation. (G) RHOB, (H) TLR4 and (I) NF-κB protein expression was quantified. (J) Western blot analysis of RHOB, TLR4 and NF-κB. (K) IL1β and (L) IL-18 were analyzed. **P<0.01 vs. negative control; ##P<0.01 vs. miR-223 group. RHOB, rho-related GTP-binding protein RhoB; miR, microRNA; TLR4, toll-like receptor 4; NF-κB, nuclear factor-κB; IL, interleukin.

of NLRP3 in the pro-inflammatory effect of anti-miR-223 in ALI *in vitro*, an NLRP3 inhibitor was used to decrease the protein expression of NLRP3. As a result, the inhibition of NLRP3 suppressed the protein expression of NLRP3 and caspase-1 in ALI *in vitro* following treatment with anti-miR-223, compared with treatment with anti-miR-223 alone (Fig. 6A-C). The inhibition of NLRP3 reduced the levels of IL-1β and IL-18 in ALI *in vitro* following treatment with anti-miR-223, compared with treatment with anti-miR-223 alone (Fig. 6D and E). Taken together, these results demonstrated that NLRP3 is required to maintain the proinflammatory effect of anti-miR-223 in ALI, mediated by IL-1β and IL-18.

miRNA-223 regulates the TLR4 signaling pathway in ALI in vitro via RHOB. Prior to determining the role of miR-223 in RHOB-activated inflammation, the 3'-UTR of RHOB mRNA was revealed to be a target site of miR-223 in various species (Fig. 7A). The relative luciferase activity of the wild-type 3'UTR in miR-223 was decreased compared with the control group (Fig. 7B). Overexpression of miR-223 suppressed the protein expression of RHOB, while downregulation of miR-223 induced RHOB protein expression in ALI *in vitro*, compared with the negative control group (Fig. 7C-F). A RHOB plasmid was utilized to induce the protein expression of RHOB, which did not affect TLR4 protein expression in ALI *in vitro* following treatment with anti-miR-223, compared

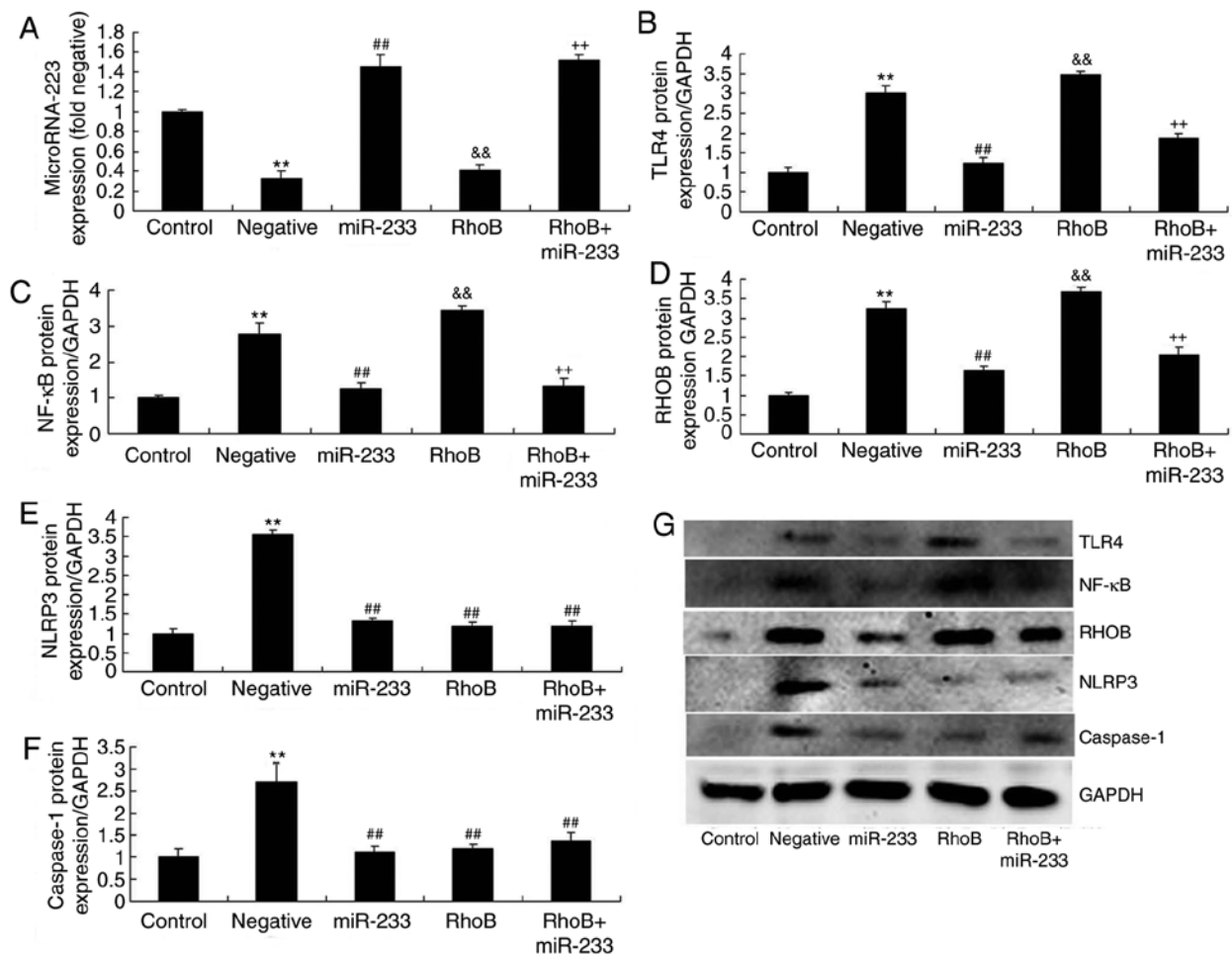


Figure 8. Activation of RHOB reduces the effects of miR-223 on inflammation in acute lung injury *in vitro*. (A) miR-223, (B) TLR4, (C) NF-κB, (D) RHOB, (E) NLRP3 and (F) caspase-1 protein expression was quantified. (G) Western blot analysis. ** $P < 0.01$ vs. control group; ## $P < 0.01$ vs. negative control; && $P < 0.01$ vs. miR-223 group; ++ $P < 0.01$ vs. RhoB group. miR, microRNA; RHOB, rho-related GTP-binding protein RhoB, rho-related GTP-binding protein RhoB; TLR4, toll-like receptor 4; NF-κB, nuclear factor-κB; NLRP3, NACHT, LRR and PYD domains-containing protein 3.

with treatment with anti-miR-223 alone (Fig. 7G-I). The activation of RHOB reduced the pro-inflammatory effect of anti-miR-223 on the levels of IL-1 β and IL-18 in ALI *in vitro* following treatment with anti-miR-223, compared with treatment with anti-miR-223 alone (Fig. 7J and K).

Activation of RHOB reduces the effects of miR-223 on inflammation in ALI *in vitro*. To further study the role of RHOB in the effects of miR-223 on inflammation in ALI *in vitro*, RHOB plasmid was used to induce RHOB protein expression in ALI following overexpression of miR-223. RHOB plasmid did not affect miR-223 expression in the ALI model, compared with the negative group (Fig. 8A). As presented in Fig. 8B-G, RHOB plasmid induced RHOB, TLR4 and NF-κB protein expression, and did not affect NLRP3 and caspase-1 protein expression in ALI following overexpression of miR-223, compared with overexpression of miR-223 alone.

Discussion

ALI/ARDS is a common and important rapidly progressive inflammatory lung disease. It is estimated that there are 190,000 new ALI cases in the USA annually, with approxi-

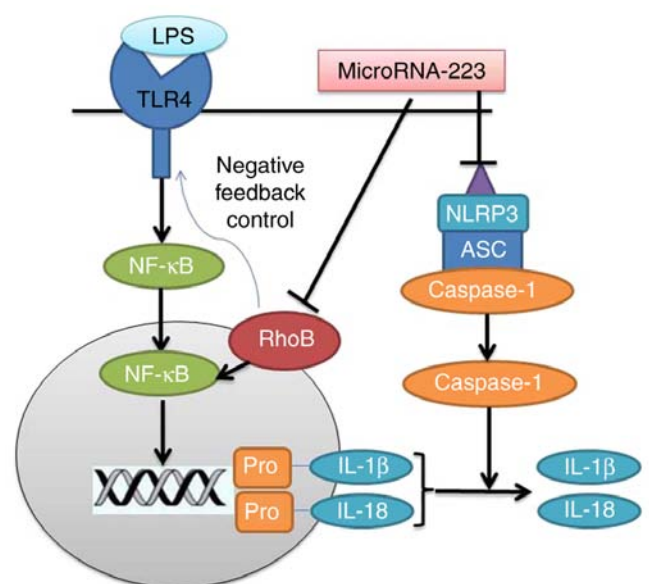


Figure 9. MicroRNA-223 attenuates LPS-induced inflammation in acute lung injury model via the NLRP3 inflammasome. LPS, lipopolysaccharide; RHOB, rho-related GTP-binding protein RhoB; TLR4, Toll-like receptor 4; NF-κB, nuclear factor-κB; NLRP3, NACHT, LRR and PYD domains-containing protein 3; IL, interleukin; ASC, PYD and CARD domain containing.

mately 75,000 mortalities (2). In China, the incidence of ALI/ARDS is even higher, with a mortality rate of 52% (14). In recent years, scientists have noticed that miRNAs serve vital roles in multiple biological processes and signal transduction pathways of ALI/ARDS (14). In the present study, it was identified that miRNA-223 expression was decreased in ALI mice. Neudecker *et al* (12) suggested that miRNA-223 deficiency was associated with severe lung inflammation.

ALI/ARDS is damage to alveolar epithelial cells and capillary endothelial cells due to a variety of direct causes (including pneumonia and pulmonary contusion) and indirect causes (including adenosis and trauma) (15). This leads to diffuse edema of the pulmonary interstitium and alveoli, subsequently triggering acute hypoxemia. ALI/ARDS is not considered a simple lung disease or inflammatory disease (15,16). Instead, it is a systemic inflammatory response in the lung tissue, according to current opinion (17). In cases of ALI/ARDS, neutrophils, macrophages and endothelial cells release a large quantity of proinflammatory cytokines, chemokines and inflammatory mediators. This leads to damage to the lung epithelial cells and pulmonary vascular endothelial cells, increased alveolar permeability, and exudation of inflammatory cells, proteins and water. Additionally, this results in reduced lung tissue volume, decreased pulmonary compliance, disorders of gas diffusion, exchange and metabolism, and eventual mortality attributed to respiratory failure. The present study demonstrated that the downregulation of miRNA-223 promoted IL-1 β and IL-18 levels in A549 cells treated with LPS, and the downregulation of miRNA-223 promoted TLR4 and NF- κ B protein expression in A549 cells treated with LPS. Wang *et al* (18) indicated that miRNA-223 negatively regulated the activation of the TLR4/NF- κ B pathway in macrophages.

In the occurrence and development of ALI, the release of proinflammatory mediators and apoptosis serve an important role (19). Disorders of the secretion of cytokines, inflammatory chemokines and other associated proteins promote the inflammatory response and accelerate the development of lung injury (20). Previous studies have confirmed that the NLRP3 inflammasome serves an important role in this process (21,22). It not only activates caspase-1 and inflammatory factors, but also promotes the maturation and secretion of inflammasomes (21,22). Therefore, the regulatory approach targeting NLRP3 inflammasomes may be of great importance for the treatment of ALI. The NLRP3 inflammasome is a multiprotein large-cytoplasmic complex that is composed of NLRP3, apoptosis-related spot-like proteins and pro-caspase-1 (23). NLRP3 serves a critical role in regulating the relevant components of the inflammation complex, including apoptosis-related point-like proteins and pro-caspase-1 (23). Recent studies have confirmed that activation of the inflammasome induces the division of pro-IL-1 β and pro-IL-18 (21,22). Furthermore, it results in increased alveolar permeability, subsequently leading to pulmonary edema and lung injury (21,22). In the present study, miRNA-223 negatively regulated the TLR4/NF- κ B signaling pathway via RHOB in ALI. Downregulation of miRNA-223 induced TLR4, NF- κ B, NLRP3 and caspase-1 protein expression in ALI *in vitro*. Yang *et al* (24) reported that miRNA-223 regulates inflammation and brain injury via feedback to NLRP3 inflammasome after intracerebral hemor-

rhage. Zeng *et al* (25) demonstrated that miRNA-223 attenuates hypoxia-induced vascular remodeling through RHOB/myosin light chain 9 in pulmonary arterial smooth muscle.

In conclusion, the present study demonstrated that miRNA-223 reduced inflammation, suppressed the NLRP3 inflammasome, and negatively regulated the TLR4/NF- κ B signaling pathway via RHOB in ALI (Fig. 9). miRNA-223 is involved in the activation of the TLR4/NF- κ B signaling pathway via RHOB, and the NLRP3 inflammasome may be a therapeutic target for the treatment of inflammation in ALI. Therefore, miRNA-223 serves as a potent positive regulator of inflammation in ALI.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

ZZ designed the study. YY, KL and TY performed the experiments. ZZ and YY analyzed the data. ZZ wrote the manuscript.

Ethics approval and consent to participate

The experimental procedures in the present study were performed with the approval of Binzhou Medical University Hospital (Binzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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