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Inhibition of KLF6 reduces the inflammation and apoptosis of type II alveolar epithelial cells in acute lung injury

Qingbin Chen^a, Zhen Jia^a, Changjing Qu^{b*}

^aDepartment of Anesthesiology, Qinghai University Affiliated Hospital, Xining, Qinghai, China

^bDepartment of Critical Medicine, Yangpu Hospital, Tongji University School of Medicine, Shanghai, China

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KEYWORDS

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Abstract

Background: The development of acute lung injury (ALI) into a severe stage leads to acute respiratory distress syndrome (ARDS). The morbidity and mortality of ALI and ARDS are very high.

Objective: This study is aimed to explore the effect of Krüppel-like factor 6 (KLF6) on lipopolysaccharide (LPS)-induced type II alveolar epithelial cells in ALI by interacting with cysteine-rich angiogenic inducer 61 (CYR61).

Material and Methods: ALI mice model and LPS-induced type II alveolar epithelial cells were conducted to simulate ALI *in vivo* and *in vitro*. The messenger RNA (mRNA) and protein expression of KLF6 in lung tissues were detected by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and Western blot analysis. Pathological changes in lung tissues were observed by hematoxylin and eosin (H&E) staining. The viability and KLF6 expression of A549 cells treated with different concentrations of LPS were detected by cell counting kit-8 (CCK-8) assay, RT-qPCR, and Western blot analysis. After indicated treatment, the viability and apoptosis of A549 cells were analyzed by CCK-8 and TUNEL assays, and the inflammation factors of A549 cells were detected by Enzyme-linked-immunosorbent serologic assay, RT-qPCR, and Western blot analysis. The combination of KLF6 and CYR61 was determined by chromatin immunoprecipitation (ChIP)-PCR and dual-luciferase reporter assay.

Results: KLF6 expression was increased in lung tissues of ALI mice and LPS-induced A549 cells. Interference with KLF6 improved the viability, reduced the inflammatory damage, and promoted the apoptosis of LPS-induced A549 cells. In addition, KLF6 could bind to CYR61. Interference with KLF6 could decrease CYR61 expression in LPS-induced A549 cells. LPS also enhanced the TLR4/MYD88 signaling pathway, which was reversed by KLF6 interference. The above phenomena in LPS-induced A549 cells transfected with Si-KLF6 could be reversed by overexpression of CYR61.

Conclusion: Inhibition of KLF6 promoted the viability and reduced the inflammation and apoptosis of LPS-induced A549 cells, which was reversed by CYR61.

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*Corresponding author: Changjing Qu, Department of Critical Medicine, Yangpu Hospital, Tongji University School of Medicine, No. 450, Tengyue Road, Shanghai 200090, China. Email address: qucjing27@163.com

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Introduction

Acute lung injury (ALI), especially its severe form, known as acute respiratory distress syndrome (ARDS), is an inflammatory lung disease characterized by pulmonary edema, endothelial and epithelial injury, and neutrophil infiltration.^{1,2} Clinically, the primary treatment methods of treatment for ALI and ARDS are still mechanical ventilation and liquid tube management, and relatively effective drug treatment is still lacking.³ Owing to the rapid onset and poor prognosis of ALI and ARDS, the morbidity and mortality of both are very high, leading to the overall mortality of 40%.^{1,4} Therefore, it is crucial to find an effective method to alleviate ALI in spite of mechanical intends.

Involvement of Krüppel-like factor 6 (KLF6) has been shown in promoting inflammation.^{5,6} Previous studies have proved that KLF6 expression is increased in serum of patients with acute kidney injury and in lipopolysaccharide (LPS)-induced HK2 cells,⁷ and in periodontal membrane stem cells in LPS-induced periodontitis⁸ as well as acute liver injury. KLF6 is also anticipated in inducing acute liver injury.⁹ These studies indicate that KLF6 is involved in organ injury, although its role in LPS-induced lung injury has not been studied yet. Studies have indicated that high glucose can induce increased expression of KLF6 in human type II alveolar epithelial cells and bronchial epithelial cells,¹⁰ and interference with KLF6 can reduce the production of inducible nitric oxide synthase (iNOS) and apoptosis of type II epithelial cells induced by virus.¹¹ The Human Protein Atlas database (<https://www.proteinatlas.org/>) indicates that KLF6 is widely expressed in lung tissues and cells. Therefore, this study investigated the expression of KLF6 in LPS-induced lung tissues and cells, and whether it could alleviate injury in LPS-induced lung cells after interference.

Materials and methods

ALI mice model

A total of 10 C57BL/6J male mice (8 weeks old, weight 20–30 g) were conventionally adapted for 1 week. The animals were anesthetized by injecting 100 mg/kg ketamine and 10 mg/kg xylazine intraperitoneally. Then mice were intranasally instilled with sterile saline (Control group; $n = 5$) and LPS, 100 $\mu\text{g}/\text{mL}$ (LPS group; $n = 5$) at the volume of 1 $\mu\text{L}/\text{g}$ body weight.^{12–14} Mice were euthanized and lung tissues were collected after inducing inflammation for 6 h. The present experiment was approved and supervised by the Animal Care and Use Committee and the Animal Ethics Committee of Yangpu Hospital.

Histopathological Analysis

The lung tissues were fixed in 4% paraformaldehyde solution for 24 h at 4°C, cut into 5- μm thick sections, and stained with hematoxylin and eosin (H&E) for 2–3 min. The lung tissues were examined under optical

microscope (Eclipse E100, magnification, $\times 100/\times 400$; Nikon Corporation, Japan). The alveolar congestion was graded according to the following scale: 0: no damage, 1: mild damage, 2: moderate damage, 3: severe damage, and 4: maximal damage.¹⁵

Cell culture and LPS induction

Human A549 cells were obtained from the American Type Culture Collection (ATCC, USA). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin at 37°C in 5% carbon dioxide (CO_2).

CCK-8 assay

A549 cells were seeded in 96-well plates (1.0×10^4 cells/well) and cultured for 24 h. Then cells were induced in LPS at different concentrations (0, 20, 40, 60, and 80 $\mu\text{g}/\text{mL}$) for 24 h. After exposure period, 10- μL CCK-8 reagent was added into each well after 24h, and incubated for 2 h. The absorbance was measured with a multifunctional microplate reader at 450 nm.

Cell transfection

The Si-KLF6 and corresponding negative controls (Si-NC) were bought from Guangzhou Ruibo Biotechnology Co. Ltd. Plasmid cloning DNA (pcDNA3) and recombinant plasmid pc-CYR61 were designed and synthesized from Shanghai Gene Pharmaceutical Co. Ltd. (Shanghai, China). A549 cells were inoculated into 24-well plates at a density of 5×10^4 cells/well and cultured for 48 h. When confluence was about 80%, cells were transfected using the Lipofectamine 3000 kit (Invitrogen) and collected for analysis after 48 h.

Reverse transcription-quantitative Polymerase chain reaction (RT-qPCR)

Total RNA in lung tissues and A549 cells was extracted with TRIzol® reagent (Thermo Fisher Scientific, USA). Complementary DNA (cDNA) was synthesized using PrimeScript RT Reagent kit (Takara, Japan) and subjected to real-time PCR using a SYBR premix Ex Taq™ II PCR kit (Takara, Japan). The relative expression levels of KLF6, tumor necrosis factor- α (TNF- α), interleukin (IL)-1 β , IL-6, and CYR61 messenger RNA (mRNA) were calculated using the $2^{-\Delta\Delta\text{Ct}}$ method with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as internal reference.¹⁶

Western blot analysis

Total proteins in lung tissues and A549 cells were isolated using radioimmunoprecipitation assay (RIPA) buffer (Beyotime, China), and bicinchoninic acid (BCA) method was expended to detect protein concentration. Every

50- μ g protein was loaded per lane and separated by sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) electrophoresis, followed by transfer to polyvinylidene fluoride (PVDF) membrane (EMD Millipore, Germany). Non-fat milk, 5%, was used to block the membrane for 1 h, and then primary antibodies were added to the membrane for overnight incubation at 4°C. The horseradish peroxidase (HRP)-conjugated goat anti-rabbit Immunoglobulin G (IgG) secondary antibody was added to the membrane for incubation at room temperature for 1 h. The protein bands were viewed by ECL kit (GENVIEW) and observed by a Clinx ChemiScope 6000 (Clinx Science Instruments) imaging system. The bands' density was quantified by ImageJ 1.8.0 (National Institutes of Health, USA). The primary antibodies included were KLF6, TNF- α , IL-1 β , IL-6, B-cell lymphoma-2 (Bcl-2), Bcl-2-associated X (Bax), cleaved caspase 3, cysteine-rich angiogenic inducer 61 (CYR61), toll-like receptor 4 (TLR4), Myeloid differentiation primary response 88 (MYD88), and GAPDH.

Enzyme-linked-immunosorbent serologic assay (ELISA)

After treatment, the culture medium of A549 cells was collected and centrifuged at 1000 \times g for 20 min. The levels of TNF- α , IL-1 β , and IL-6 in supernatants were analyzed by Human TNF- α ELISA kit (Cat. No. PT518; Beyotime, China), Human IL-1 β ELISA kit (Cat. No. PI305; Beyotime, China), and Human IL-6 ELISA kit (Cat. No. PI330; Beyotime, China) using spectrophotometer (BioTek, USA) to measure absorbance at 450 nm.

Terminal deoxynucleotidyl transferase dUTP nick end labelling assay (TUNEL) assay

After treatment, A549 cells were fixed with 4% paraformaldehyde at room temperature for 30 min and permeabilized with PBS containing 0.3% Triton X-100 for 5 min, also at room temperature. A549 cells were then treated with TUNEL mixture, and DNA fragmentation was determined by TUNEL assay. Fluorescent images were taken using fluorescence microscope (Carl Zeiss, Germany), and cell apoptosis was analyzed by the ImageJ 1.8.0 software.

Chromatin immunoprecipitation (ChIP)-PCR

Lipopolysaccharide-induced A549 cells, 1×10^7 , were cross-linked with 1% formaldehyde for 10 min at room temperature. The immunoprecipitation was performed with antibodies specific to KLF6 or IgG as a negative control to generate antigen-antibody complexes. Afterwards, the complexes were collected by protein A agarose beads (Merck Millipore, Germany), and DNA was eluted from the immunoprecipitated complexes on agarose beads. DNA fragments obtained by ChIP were detected by PCR Mix (Thermo Fisher Scientific, USA) according to the manufacturer's instructions.

Dual-luciferase reporter (DLR) assay

The wild type (WT) or mutant (MUT) 3'-UTRs of CYR61 were subcloned into the pGL3 vector. The plasmid containing CYR61-3'-UTR-WT/CYR61-3'-UTR-MUT, as well as Si-KLF6/Si-NC, was co-transfected into A549 cells using Lipofectamine[®] 3000 (Invitrogen, USA) and cultured at 37°C with 5% CO₂ for 48 h. The luciferase activity was calculated by the DLR assay (Promega, USA).

Statistical analysis

The experimental data were expressed as mean \pm SD and statistically analyzed by the GraphPad Prism 8.0 (GraphPad Software Inc.). One-way ANOVA with Tukey's *post hoc* test was used for comparisons among multiple groups, or unpaired Student's *t*-test was used for comparisons between two groups. $P < 0.05$ was considered as statistically significant.

Results

KLF6 expression was increased in LPS-induced lung injury

The mRNA and protein expression of KLF6 in lung tissues of ALI mice were increased compared with that in control group (Figures 1A and B). Pathological changes in lung tissues of ALI mice, such as alveolar congestion, hemorrhage, and infiltration of inflammatory cells, were observed by H&E staining. These changes were observed after induction of LPS for 6 h (Figure 1C).

KLF6 expression was increased in LPS-induced type II alveolar epithelial cells

A549 cells were treated with different concentrations (0, 20, 40, 60, and 80 μ g/mL) of LPS for 24 h; cell viability decreased at induction of 40, 60, and 80 μ g/mL LPS (Figure 2A). The elevated mRNA and protein expression of KLF6 in LPS-induced A549 cells were also observed at 40, 60, and 80 μ g/mL LPS induction (Figures 2B and C).

Interference with KLF6 improved viability and reduced inflammatory damage in LPS-induced type II alveolar epithelial cells

When A549 cells were transfected with Si-KLF6-1 or Si-KLF6-2, down-regulated mRNA and protein expression of KLF6 in A549 cells was observed. As transfection effect of Si-KLF6-1 was higher, Si-KLF6-1 was chosen for the subsequent experiment (Figures 3A and B). LPS induction suppressed the viability of A549 cells, which was improved by interference with KLF6 (Figure 3C). ELISA indicated that LPS promoted the levels of TNF- α , IL-1 β , and IL-6 and suppressed Si-KLF6 transfection (Figure 3D). Similarly, the mRNA and protein expressions of TNF- α , IL-1 β , and IL-6 were increased

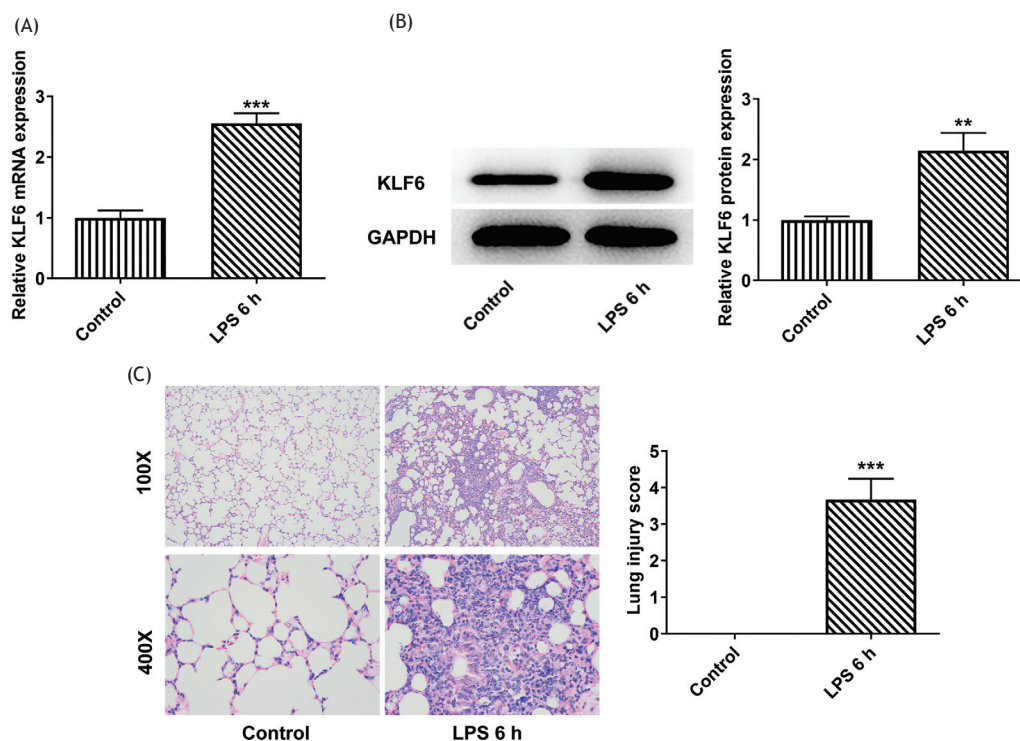


Figure 1 KLF6 expression was increased in LPS-induced lung injury. (A) The mRNA expression and (B) protein expression of KLF6 in lung tissues of ALI mice were detected by RT-qPCR and Western blot analysis. (C) Pathological changes in the lung tissues of ALI mice were observed by H&E staining. ** $P < 0.01$ and *** $P < 0.001$ vs. control group.

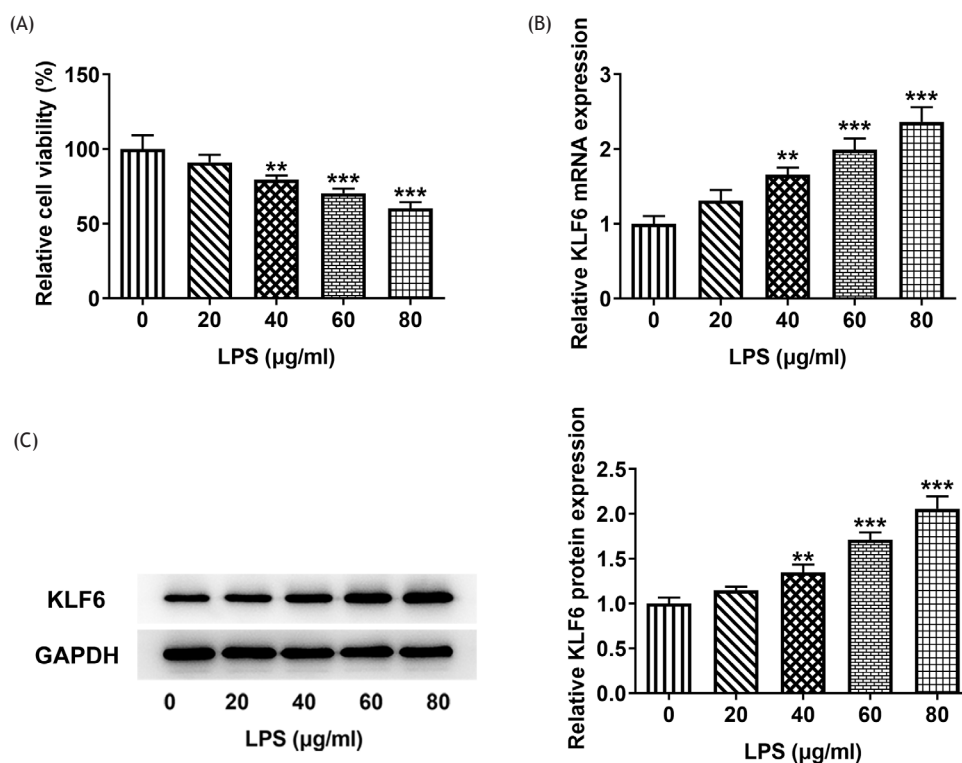


Figure 2 KLF6 expression was increased in LPS-induced type II alveolar epithelial cells. (A) The viability of A549 cells treated with different concentrations of LPS was detected by CCK-8 assay. (B) The mRNA expression and (C) protein expression of KLF6 in A549 cells treated with different concentrations of LPS were detected by RT-qPCR and Western blot analysis. ** $P < 0.01$ and *** $P < 0.001$ vs. 0- $\mu\text{g/ml}$ group.

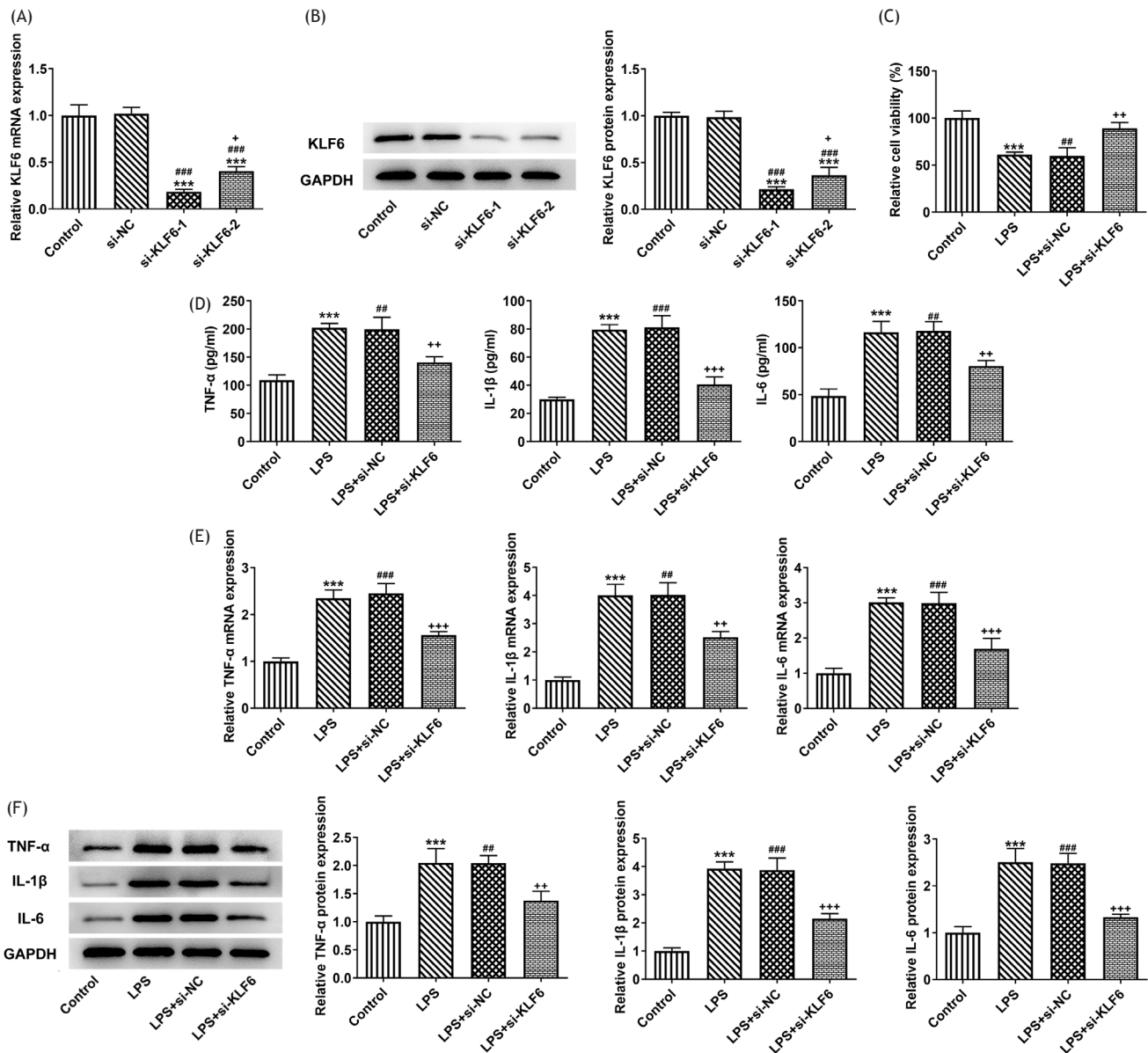


Figure 3 Interference with KLF6 improved viability and reduced inflammatory damage in LPS-induced type II alveolar epithelial cells. (A) The mRNA expression and (B) protein expression of KLF6 in A549 cells transfected with Si-KLF6-1 or Si-KLF6-2 were detected by RT-qPCR and Western blot analysis. *** $P < 0.001$ vs. control group. ### $P < 0.001$ vs. Si-NC group. * $P < 0.05$ vs. Si-KLF6-1 group. (C) The viability of LPS-induced A549 cells transfected with Si-KLF6 was analyzed by CCK-8 assay. (D) The levels, (E) mRNA expression, and (F) protein expressions of TNF- α , IL-1 β , and IL-6 in LPS-induced A549 cells transfected with Si-KLF6 were determined by assay kits and RT-qPCR. *** $P < 0.001$ vs. control group. ## $P < 0.01$ and ### $P < 0.001$ vs. LPS group. ** $P < 0.01$ and *** $P < 0.001$ vs. LPS + Si-NC group.

in LPS group but decreased after Si-KLF6 transfection (Figures 3E and F).

Interference with KLF6 alleviated LPS-induced apoptosis of type II alveolar epithelial cells

The apoptosis of A549 cells was increased after LPS induction, which was reversed by KLF6 interference (Figures 4A and B). LPS also inhibited the expression of Bcl-2 and promoted the expression of Bax and cleaved caspase 3, but interference by KLF6 weakened the effect of LPS on the expression of apoptosis-related proteins (Figure 4C).

Interference with KLF6 inhibited the expression of CYR61 in LPS-induced type II alveolar epithelial cells

The JASPAR (Japan Automotive Software Platform and Architecture) database predicted that KLF6 could bind to CYR61 promoter (Figure 5A). The mRNA and protein expression of CYR61 were up-regulated by induction of LPS but down-regulated by KLF6 interference in LPS-induced A549 cells (Figures 5B and C). The results of CHIP-PCR and DLR assay demonstrated the combination of KLF6 and CYR61 (Figures 5D and E).

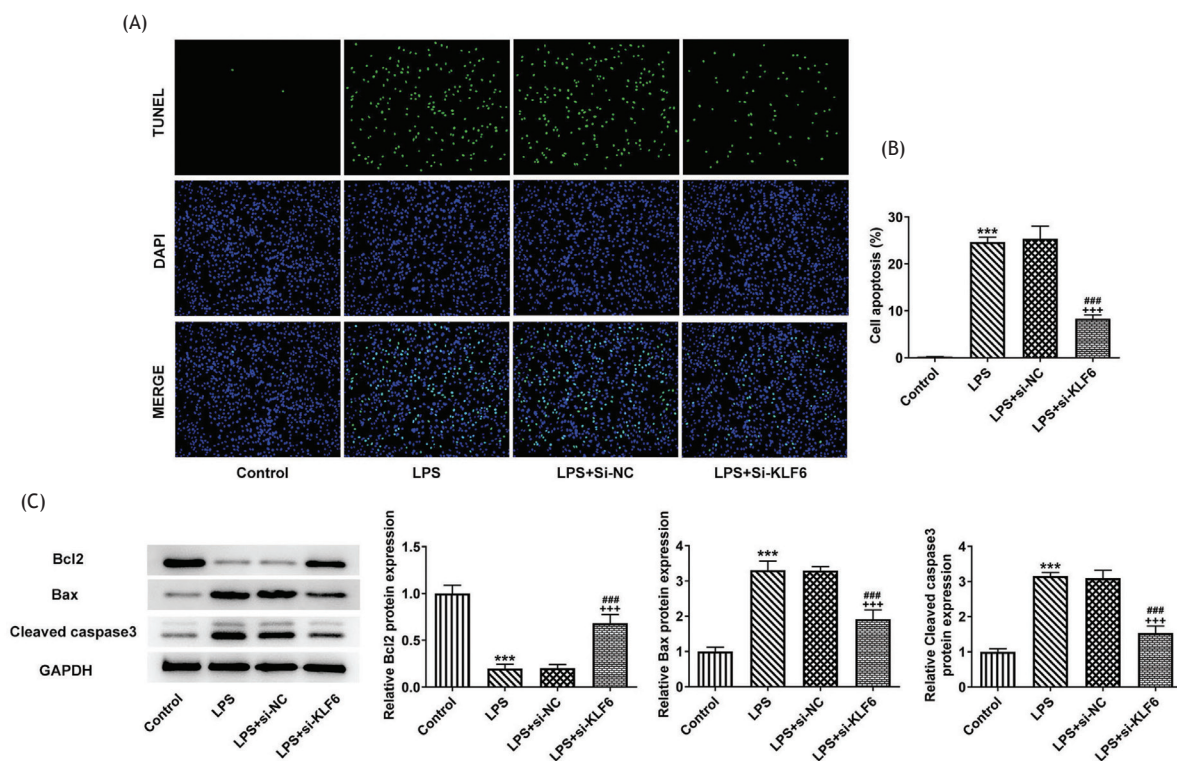


Figure 4 Interference with KLF6 improved the viability and reduced the inflammatory damage in LPS-induced type II alveolar epithelial cells. (A, B) The apoptosis of LPS-induced A549 cells transfected with Si-KLF6 was analyzed by TUNEL. (C) The expression of apoptosis-related proteins in LPS-induced A549 cells transfected with Si-KLF6 was detected by Western blot analysis. *** $P < 0.001$ vs. control group. ### $P < 0.001$ vs. LPS group. *** $P < 0.001$ vs. LPS + Si-NC group.

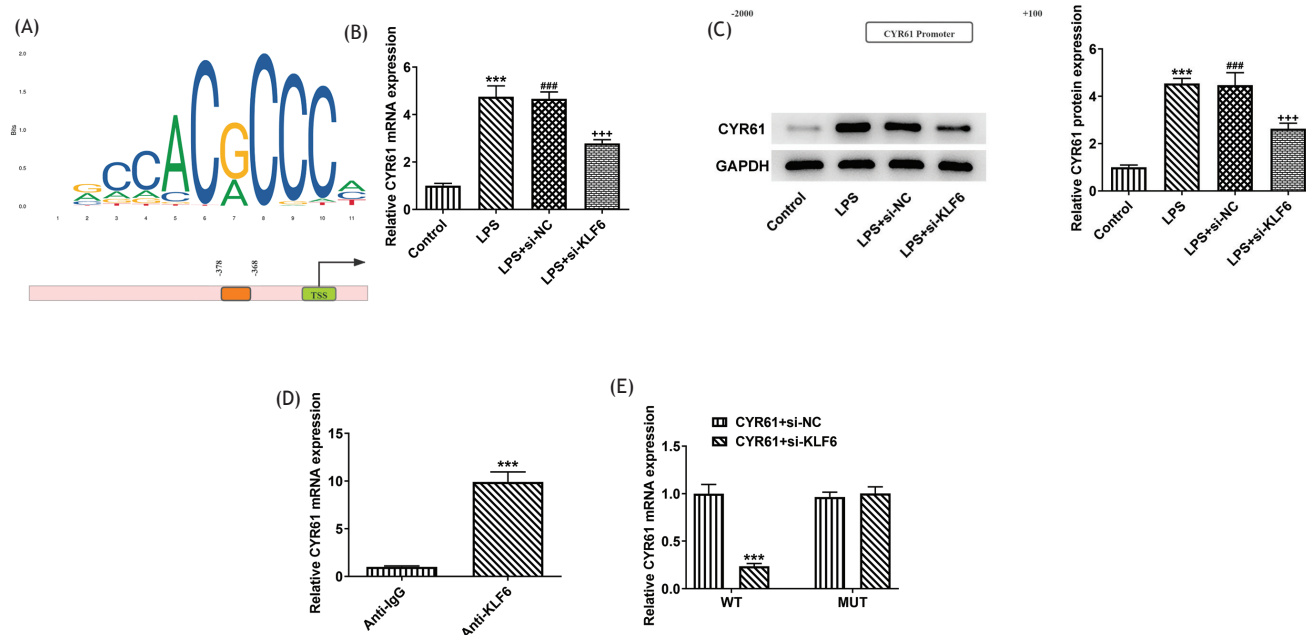


Figure 5 Interference with KLF6 inhibited the expression of CYR61 in LPS-induced type II alveolar epithelial cells. (A) JASPAR database predicated that KLF6 could bind to CYR61 promoter. (B) The mRNA expression and (C) protein expression of CYR61 in LPS-induced A549 cells transfected with Si-KLF6 were detected by RT-qPCR and Western blot analysis. *** $P < 0.001$ vs. control group. ### $P < 0.001$ vs. LPS group. *** $P < 0.001$ vs. LPS + Si-NC group. (D) The binding of KLF6 to the promoter region of CYR61 in LPS-induced A549 cells was verified by CHIP-PCR. *** $P < 0.001$ vs. anti-IgG group. (E) The binding of KLF6 to the promoter region of CYR61 in LPS-induced A549 cells was also determined by DLR assay. *** $P < 0.001$ vs. CYR61 + Si-NC group.

CYR61 overexpression reversed the inhibitory effect of interfering KLF6 on LPS-induced type II alveolar epithelial cell viability and inflammatory injury

After A549 cells were transfected with Ov-CYR61, the mRNA and protein expression of CYR61 were up-regulated

(Figures 6A and B). Overexpression of CYR61 weakened the effect of interfering KLF6 on LPS-induced A549 cell viability (Figure 6C). As shown in Figures 6D-F, the levels of mRNA and protein expressions of TNF- α , IL-1 β , and IL-6 were increased by overexpression of CYR61 in LPS-induced A549 cells transfected with Si-KLF6.

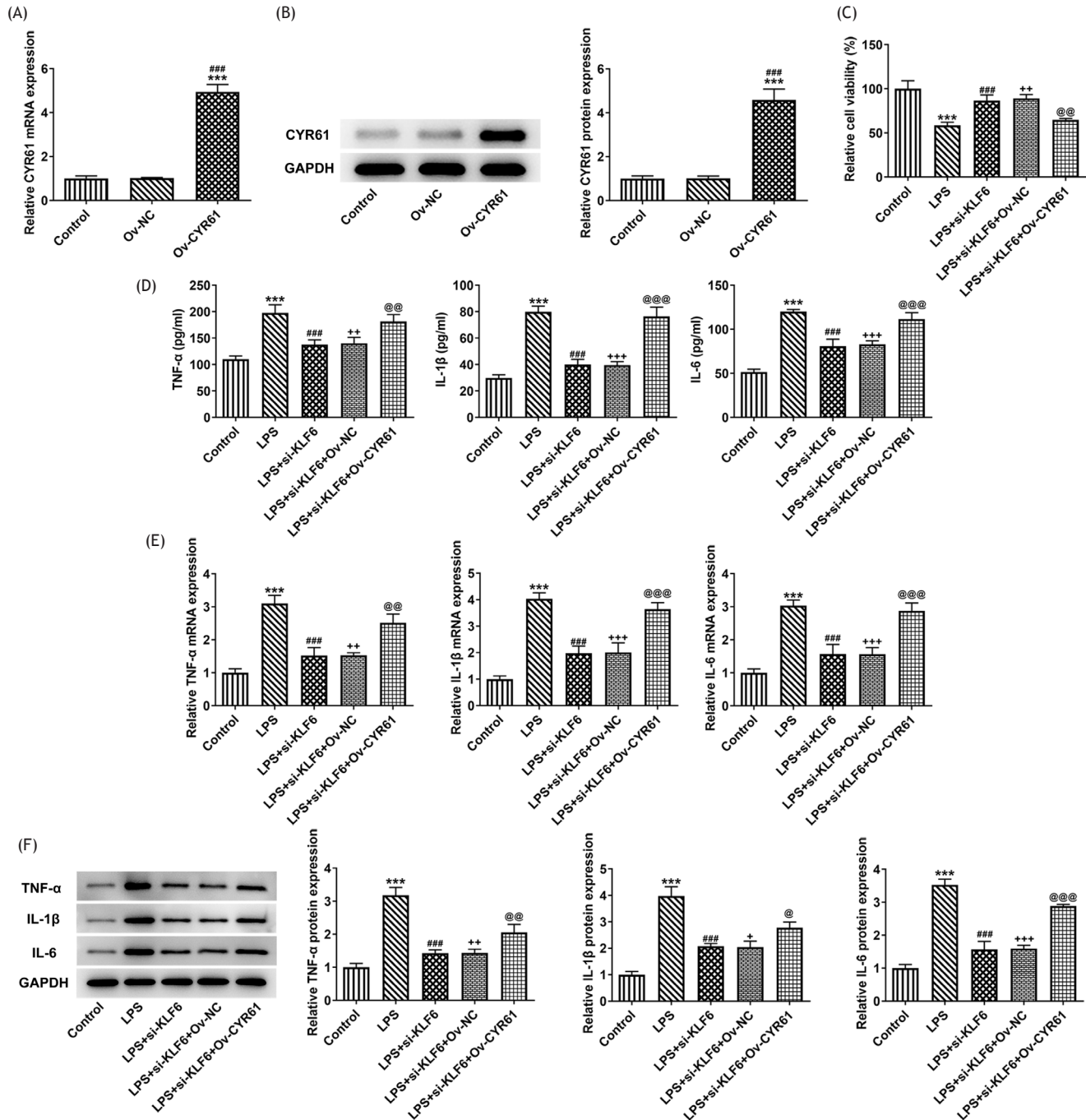


Figure 6 Overexpression of CYR61 reversed the inhibitory effect of interfering KLF6 on LPS-induced type II alveolar epithelial cell viability and inflammatory injury. (A) The mRNA expression and (B) protein expression of CYR61 in A549 cells transfected with Ov-CYR61 were detected by RT-qPCR and Western blot analysis. (C) The viability of LPS-induced A549 cells transfected with Si-KLF6 and Ov-CYR61 was analyzed by CCK-8 assay. (D) The levels of (E) mRNA expression, and (F) protein expressions of TNF- α , IL-1 β , and IL-6 in LPS-induced A549 cells transfected with Si-KLF6 and Ov-CYR61 were determined by assay kits and RT-qPCR. ***P < 0.001 vs. control group. ###P < 0.001 vs. LPS group. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. LPS + Si-KLF6 group. @P < 0.05, @@P < 0.01, and @@@P < 0.001 vs. LPS + Si-KLF6 + Ov-NC group.

CYR61 overexpression reversed the inhibitory effect of interfering KLF6 on apoptosis and TLR4/MYD88 signaling pathway of LPS-induced type II alveolar epithelial cells

Overexpression of CYR61 promoted the apoptosis of LPS-induced A549 cells transfected with Si-KLF6 (Figures 7A and B). It decreased the expression of Bcl-2 and increased the expression of Bax and cleaved caspase 3 in LPS-induced A549 cells transfected with Si-KLF6 (Figure 7C). Induction of LPS enhanced the expressions of TLR4 and MYD88, which were reversed by Si-KLF6 transfection, and overexpression of

CYR61 improved the expressions of both TLR4 and MYD88 in LPS-induced A549 cells transfected with Si-KLF6 (Figure 7D).

Discussion

The prevention and treatment strategy of ALI should be primarily considered for inhibiting the overexpression of inflammatory mediators.^{17,18} The role of inflammatory dysregulation in the pathogenesis of acute lung injury has been widely recognized, which is primarily related to the release of pro-inflammatory factors and the imbalance of

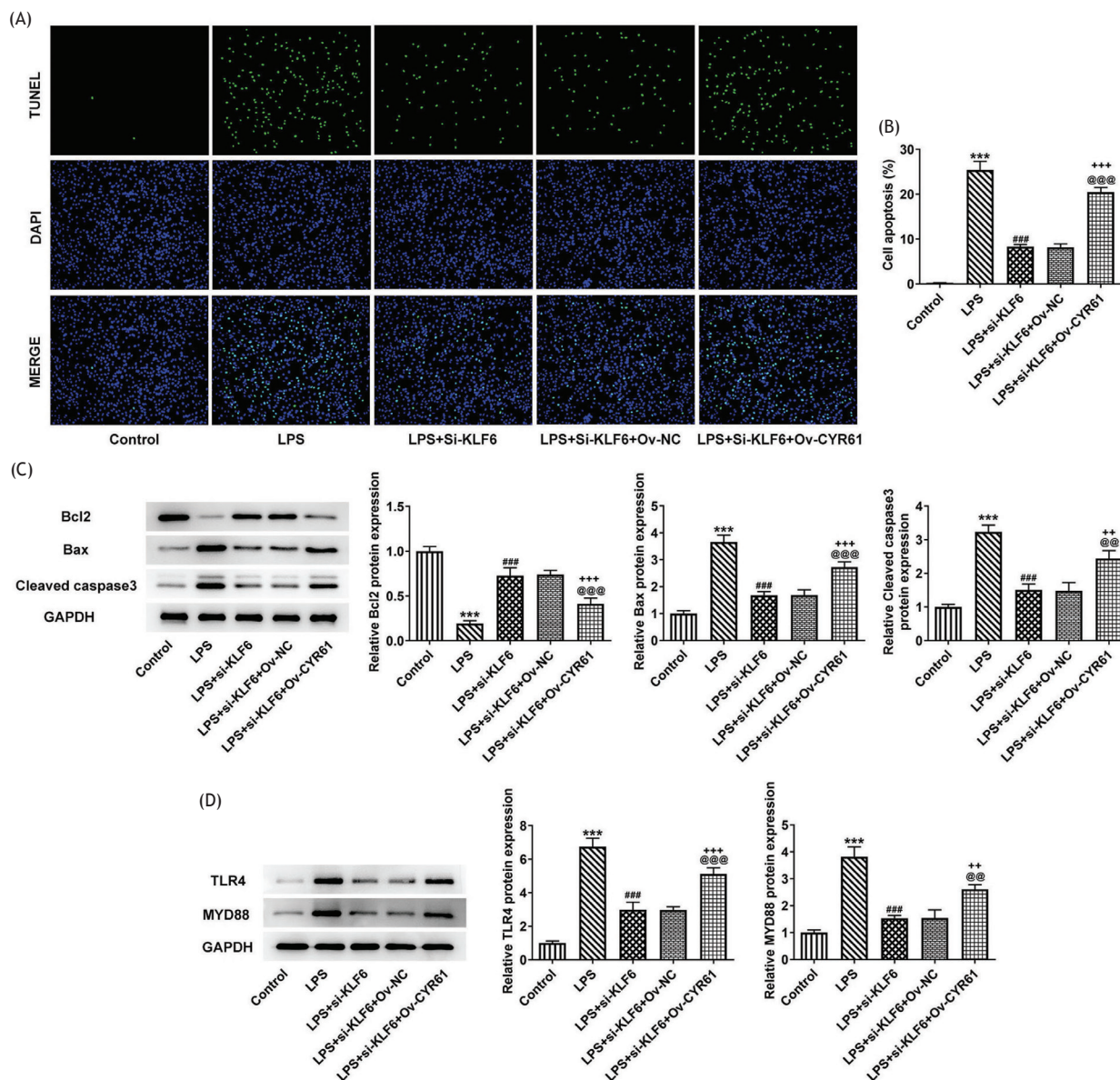


Figure 7 Overexpression of CYR61 reversed the inhibitory effect of interfering KLF6 on the apoptosis and TLR4/MYD88 signaling pathway of LPS-induced type II alveolar epithelial cells. (A, B) The apoptosis of LPS-induced A549 cells transfected with Si-KLF6 and Ov-CYR61 was analyzed by TUNEL. (C) The expression of apoptosis-related proteins in LPS-induced A549 cells transfected with Si-KLF6 and Ov-CYR61 was detected by Western blot analysis. (D) The expression of TLR4/MYD88 signaling pathway in LPS-induced A549 cells transfected with Si-KLF6 and Ov-CYR61 was detected by Western blot analysis. ***P < 0.001 vs. control group. ###P < 0.001 vs. LPS group. **P < 0.01 and ***P < 0.001 vs. LPS + Si-KLF6 group. @@P < 0.01 and @@@P < 0.001 vs. LPS + Si-KLF6 + Ov-NC group.

inflammatory factors.¹⁹⁻²¹ Although many kinds of treatment strategies, such as cytokine therapy, stem cell treatment, and hormone treatment, are accessible for ALI, the incidence and death rate of ALI are still high.²²⁻²⁴

Lipopolysaccharide is the primary pathogenic factor of ALI induced by the cell wall of Gram-negative bacteria. In this study, abdominal injection of LPS caused inflammatory changes in the lung tissues of mice to establish the septic model of ALI. We observed that KLF6 expression was increased in lung tissues of ALI mice and LPS-induced A549 cells. KLF6 is closely related to inflammatory regulation. KLF6 can promote macrophage inflammation by up-regulating Bcl-2 transcription by binding *PR/SET Domain 1 (PRDM1)* gene.²⁵ KLF6 promotes pro-inflammatory gene expression and functions by repressing miR-223 expression in macrophages.²⁶ KLF6 is highly expressed in kidney ischemia-reperfusion (IR) injury and can promote inflammation in acute kidney injury (AKI).²⁷ In this study, we down-regulated the expression of KLF6 in LPS-induced A549 cells and observed that down-regulated KLF6 could improve the viability, reduce the inflammatory response, and decrease the apoptosis of LPS-induced A549 cells.

The Coexpedia database (<https://www.coexpedia.org/>) was used to predict the co-expressed genes of KLF6, and CYR61 was found as a co-expressed gene of KLF6. This study also used ChIP-PCR and DLR assay to confirm the interaction between KLF6 and CYR61. One study indicated that CYR61 is present in the lungs of mice and humans during the acute inflammatory phase of lung injury, with higher concentrations in patients with increased markers of severity; the stated paper also suggested that alveolar epithelial cells could secrete CYR61 expression.²⁸ Other studies have shown that overexpression of CYR61 can induce accelerated lung injury,²⁹ and LPS can also induce the expression of CYR61 in bronchial epithelial cells,³⁰ indicating that the expression of CYR61 increases in lung injury. Here, expression of CYR61 was found to be up-regulated in LPS-induced A549 cells. Overexpression of CYR61 could weaken the protective effect of interference with KLF6 on LPS-induced A549 cells to suppress the viability, and promote the inflammatory response and apoptosis of LPS-induced A549 cells.

A recent study has shown that CYR61 can promote the expression of TLR4,³¹ while TLR4 is widely involved in promoting acute lung injury.³²⁻³⁴ The stated study indicated that TLR4/MYD88 signaling pathway was activated by induction of LPS, which was suppressed by the interference of KLF6. Consistent with the previous study,³¹ overexpression of CYR61 promoted the expressions of TLR4 and MYD88 in LPS-induced A549 cells transfected with Si-KLF6.

Conclusion

Inhibition of KLF6 could improve the viability, reduce the inflammatory response, and decrease the apoptosis of LPS-induced A549 cells, which was reversed by overexpression of CYR61. In addition, KLF6 is involved in regulating TLR4/MYD88 signaling pathway through CYR61.

Competing interests

The authors had no competing interests to declare.

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