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CITED2 alleviates lipopolysaccharide-induced inflammation and pyroptosis in - human lung fibroblast by inhibition of NF- κ B pathway

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Abstract

Background: Pneumonia, a severe infectious respiratory disease, is one of the leading causes of mortality and morbidity in children. Cbp/P300 interacting transactivator with Glu/Asp-rich carboxy-terminal domain 2 (CITED2) functions as a transcription cofactor, and plays critical roles in the development of embryonic and extra-embryonic tissues, including fetal lung maturation. The present study investigates the role of CITED2 in infantile pneumonia.

Methods: The human fetal lung fibroblasts (MRC-5 and WI-38) were treated with lipopolysaccharides to induce cytotoxicity, and the cell viability was detected by MTT. Inflammation was evaluated by ELISA, and western blot was used to investigate the pyroptosis.

Results: CITED2 was down-regulated in lipopolysaccharide-treated MRC-5/WI-38 cells. The over-expression of CITED2 protected MRC-5 and WI-38 cells from lipopolysaccharide-induced cytotoxicity by increasing the cell viability and decreasing LDH expression. CITED2 reduced the expression of TNF- α , IL-6, IL-1 β in lipopolysaccharide-treated MRC-5/WI-38 cells. Lipopolysaccharide stimulated pyroptosis in MRC-5 and WI-38 cells through the up-regulation of NLRP3, GSDMD-N, caspase-1, IL-1 β and IL-18. However, CITED2 down-regulated the expression of NLRP3, GSDMD-N, caspase-1, IL-1 β , and IL-18 protein in lipopolysaccharide-treated MRC-5/WI-38 cells. CITED2 also down-regulated the protein expression of p-p65 in lipopolysaccharide-treated MRC-5/WI-38 cells.

Conclusion: CITED2 exhibited anti-inflammatory effect on lipopolysaccharide-treated human lung fibroblasts and reduced pyroptosis through inactivation of NF- κ B pathway.

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Introduction

Pneumonia, an inflammatory disease affecting lungs, is caused due to infections from fungal, bacterial, or viral pathogens.¹ Pneumonia is a leading cause of mortality and morbidity in infants and children.² Infantile pneumonia, also known as primary atypical pneumonia, is a lung infection caused by mycoplasma pneumoniae, and the prevalence and incidence of it has been on the rise in recent years.² Oral antibiotics are widely used in the management of infantile pneumonia. The high recurrence rate and severe complications - including encephalitis, myelitis, and nephritis - still threaten the life and health of infants.³ Therefore, innovative interventions are indispensably needed for the management of infantile pneumonia.

Fungal, bacterial, or viral pathogens binds to lung fibroblasts and induces severe inflammatory responses.⁶ The release of pro-inflammatory cytokines stimulates infiltration of leukocytes, inhibit the microvascular function, and promote necrosis and apoptosis, thus leading to the development of infantile pneumonia.⁷ It was found that traditional herbs, such as *Urtica dioica*, have exerted anti-inflammatory effect against diabetic rats.^{4,5} Therefore, the anti-inflammatory strategies are considered to be promising for the intervention of infantile pneumonia.⁸

CITED2 is a non-DNA-bound transcription coactivator, and plays a key role in the development of embryonic and extra-embryonic tissues, such as heart⁹ and lens¹⁰ morphogenesis. CITED2 was involved in stem cell differentiation,¹¹ and played a role in tumorigenesis through the regulation of autophagy, migration, differentiation, apoptosis, and proliferation.¹² CITED2 was also associated with cardiomyocyte apoptosis during the development of congenital heart abnormality.¹³ The deletion of CITED2 gene in mice resulted in heart malformations, neural tube abnormalities, adrenal dysplasia, left and right pattern malformations and placental defects, thus leading to the death of embryos in the middle and late stages of pregnancy.¹⁴ Moreover, CITED2 was essential for the activation of PPAR γ , and promoted the expression of anti-inflammatory genes in macrophages, thus protecting the host against inflammatory insults.¹⁵ CITED2 also mediated the maturation of fetal lung through regulation of *Cebpa*.¹⁶ CITED2 deficiency induced recruitment of macrophages and neutrophils to lipopolysaccharide-induced lung inflammatory sites, and the over-expression of CITED2 reduced the lipopolysaccharide-induced activation of inflammatory responses in macrophages (CITED2 limits pathogenic inflammatory gene programs in myeloid cells). However, the role of CITED2 in infantile pneumonia remains unknown.

In this study, the effects of CITED2 on inflammation and pyroptosis of lipopolysaccharide-treated human fetal lung fibroblasts were investigated.

Materials and Methods

Cell culture and treatment

Human fetal lung fibroblasts (MRC-5 and WI-38) were cultured in Dulbecco's modified Eagle's medium (Thermo

Fisher Scientific, Waltham, MA, USA) with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA). The cells were then treated with 1, 2, 5, 10, or 20 $\mu\text{g}/\text{mL}$ lipopolysaccharide (Sigma-Aldrich) for 12 hours to induce pneumonia.

Cell transfection and viability assays

MRC-5 and WI-38 cells were treated with 10 $\mu\text{g}/\text{mL}$ lipopolysaccharide for 12 hours, and then transfected with pcDNA empty vector (NC) or pcDNA-CITED2 (CITED2) (Ribio, Guangzhou, China) by using Lipofectamine 2000 (Life Technologies, Grand Island, NY, USA) for another 24 hours. To detect cell viability, MRC-5 and WI-38 cells were seeded into 96-well plates, treated with lipopolysaccharide, and then transfected with pcDNA vectors. The cells were then treated with MTT solution (Beyotime, Beijing, China) for four hours. The medium was replaced, and dimethyl sulfoxide was added. The absorbance at 450 nm was measured by microplate reader (Thermo Fisher Scientific).

qRT-PCR

MRC-5 and WI-38 cells were lysed using TRIzol kit (Life Technologies). The isolated RNAs were then synthesized into cDNAs using MultiscribeTM Reverse transcription Kit (Applied Biosystems, Foster City, CA, USA). The mRNA expression of CITED2 was detected by PreTaq II kit (Takara, Dalian, China) with following primers: CITED2 (Forward: 5'-CCGCCCAATGTCATAGACACTGATTTTC-3' and Reverse: 5'-ATTTCTTTTCAGCCGCGAGGTTAACC-3'); GAPDH (Forward: 5'-CGCTAACATCAAATGGGGTG-3', Reverse: 5'-TTGCTGACAATCTTGAGGGAG-3') was used as the internal control. The expression level of CITED2 was calculated using $2^{-\Delta\Delta Ct}$ method and was normalized to GAPDH.

ELISA

Supernatants of cultured medium of MRC-5 and WI-38 cells were harvested, and the production of LDH, TNF- α , IL-1 β , IL-6 were detected by ELISA kits (Abcam, Cambridge, UK).

Western blot

MRC-5 and WI-38 cells were lysed in RIPA buffer (Beyotime), and the cell lysates were subjected to 10% SDS-PAGE. The protein samples were transferred onto nitrocellulose membranes, and then blocked in 5% bovine serum albumin. The membranes were probed with specific antibodies: anti-CITED2 and anti- β -actin (1:2000), anti-NLRP3 and anti-GSDMD-N (1:2500), anti-caspase-1 and anti-IL-1 β (1:3000), anti-IL-18 (1:3500), anti-p-p65 and anti-p65 (1:4000), and anti-p-I κ B α and anti-I κ B α (1:4500). The membranes were then washed and incubated with horseradish peroxidase-conjugated secondary antibody (1:5000). Immunoreactivities were visualized using enhanced chemiluminescence (Sigma-Aldrich), and the blots of proteins were quantified by ImageJ using β -actin as the internal reference. All the antibodies were acquired from Abcam.

Statistical analysis

All the data with at least triple replicates were expressed as mean \pm SEM, and analyzed by student's t test or one-way analysis of variance with post-hoc analysis under SPSS software. The normality and homogeneity of the data were investigated by Shapiro-Wilk and Levene's test respectively. $p < 0.05$ was considered as a statistically significant difference.

Results

CITED2 was reduced in lipopolysaccharide-treated MRC-5/WI-38 cells.

To induce pneumonia, MRC-5 and WI-38 cells were treated with 1, 2, 5, 10, or 20 $\mu\text{g/mL}$ lipopolysaccharide for 12 hours. The mRNA (Figure 1A) and protein (Figure 1B) expression of CITED2 was reduced in MRC-5 and WI-38 cells by lipopolysaccharide treatment in a dosage dependent way. Lipopolysaccharide (5, 10, or 20 $\mu\text{g/mL}$) reduced the cell viability (Figure 1C), but it did not affect the cell viability of MRC-5 and WI-38 cells at the dose of 1 or 2 $\mu\text{g/mL}$ (Figure 1C). Therefore, 10 $\mu\text{g/mL}$ lipopolysaccharide was used to induce the pneumonic model.

CITED2 enhanced the cell viability of lipopolysaccharide-treated MRC-5/WI-38 cells.

MRC-5 and WI-38 cells were treated with 10 $\mu\text{g/mL}$ lipopolysaccharide, and then transfected with pcDNA-CITED2. Transfection with pcDNA-CITED2 enhanced the protein expression of CITED2 (Figure 2A). Over-expression of

CITED2 increased the cell viability of lipopolysaccharide-treated MRC-5/WI-38 cells (Figure 2B). Moreover, CITED2 attenuated the lipopolysaccharide-treated increase of LDH in MRC-5 and WI-38 (Figure 2C), suggesting the protective effect of CITED2 against lipopolysaccharide-induced cytotoxicity in MRC-5 and WI-38 cells.

CITED2 inhibited the lipopolysaccharide-induced inflammation in MRC-5/WI-38 cells

Lipopolysaccharide induced inflammatory response in MRC-5 and WI-38 cells through the up-regulation of TNF- α , IL-1 β , IL-6 protein (Figure 3). However, the over-expression of CITED2 reduced the expression of TNF- α , IL-1 β , and IL-6 (Figure 3), and exerted anti-inflammatory effect on lipopolysaccharide-treated MRC-5/WI-38 cells.

CITED2 inhibited the lipopolysaccharide-induced pyroptosis in MRC-5/WI-38 cells

Lipopolysaccharides also induced the pyroptosis in MRC-5 and WI-38 cells through up-regulation of NLRP3, GSDMD-N, caspase-1, IL-1 β , and IL-18 (Figure 4). However, the over-expression of CITED2 reduced those protein expression (Figure 4), thus suppressing lipopolysaccharide-induced pyroptosis in MRC-5/WI-38 cells.

CITED2 mediated NF- κ B pathway in lipopolysaccharide-treated MRC-5/WI-38 cells

Lipopolysaccharides enhanced p-I κ B α and p-p65 expression in MRC-5 and WI-38 cells (Figure 5). However, p-I κ B α and p-p65 in lipopolysaccharide-treated MRC-5/WI-38 were

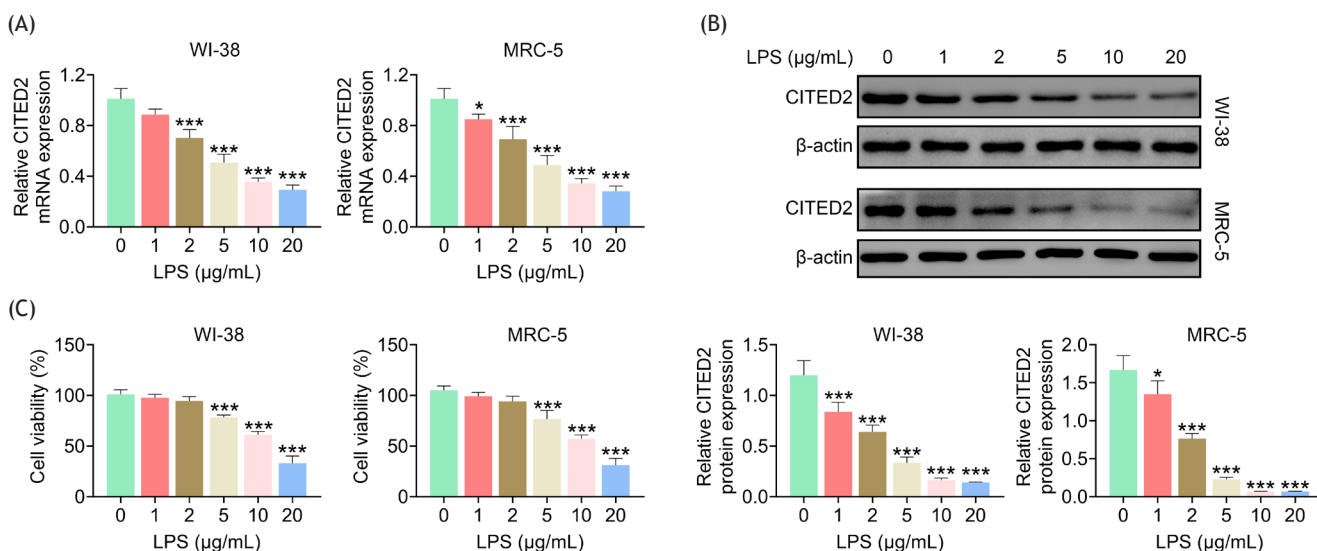


Figure 1 CITED2 was reduced in lipopolysaccharide-treated MRC-5/WI-38 cells; (A) The mRNA expression of CITED2 was reduced in lipopolysaccharide-treated MRC-5/WI-38 cells in a dosage dependent way; (B) The protein expression of CITED2 was reduced in lipopolysaccharide-treated MRC-5/WI-38 cells in a dosage dependent way; (C) Lipopolysaccharide (5, 10, or 20 $\mu\text{g/mL}$) reduced cell viability of MRC-5 and WI-38 cells, while lipopolysaccharide (1 or 2 $\mu\text{g/mL}$) did not affect the cell viability of MRC-5 and WI-38 cells. * $p < 0.05$, *** $p < 0.001$.

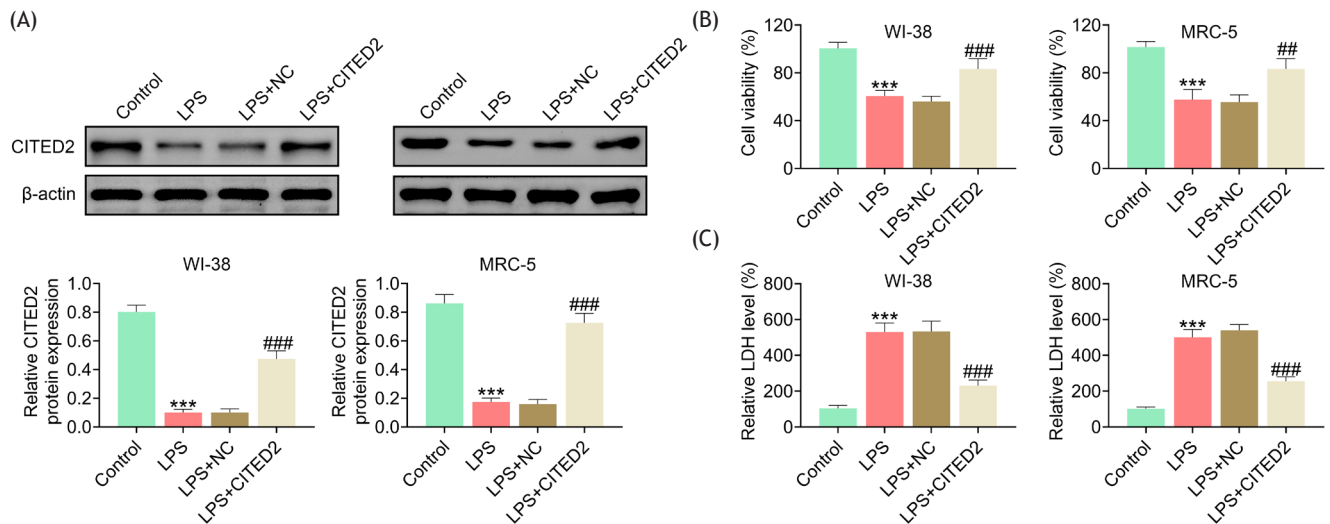


Figure 2 CITED2 enhanced cell viability of lipopolysaccharide-treated MRC-5/WI-38 cells; (A) Transfection with pcDNA-CITED2 up-regulated the protein expression of CITED2 in lipopolysaccharide-treated MRC-5/WI-38 cells; (B) Over-expression of CITED2 increased cell viability of lipopolysaccharide-treated MRC-5/WI-38 cells; (C) Over-expression of CITED2 decreased LDH release of lipopolysaccharide-treated MRC-5/WI-38 cells. *** vs. control $p < 0.001$. ### vs. LPS+NC $p < 0.001$. Lipopolysaccharide: LPS.

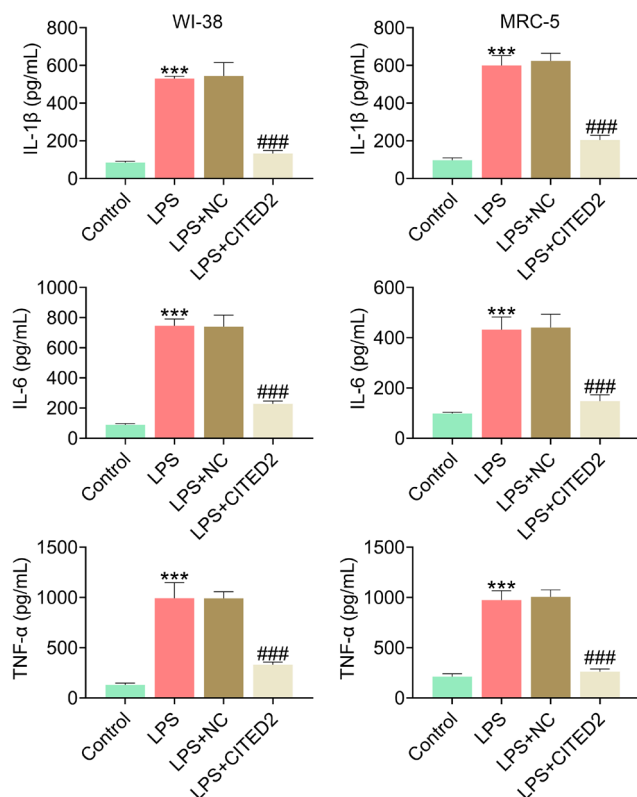


Figure 3 CITED2 inhibited lipopolysaccharide-induced inflammation in MRC-5/WI-38 cells. Over-expression of CITED2 reduced TNF- α , IL-1 β , IL-6 in lipopolysaccharide-treated MRC-5/WI-38 cells. *** vs. control $p < 0.001$. ### vs. LPS+NC $p < 0.001$.

decreased by the ectopic expression of CITED2 (Figure 5), revealing that CITED2 repressed the activation of NF- κ B pathway to protect MRC-5 and WI-38 from lipopolysaccharide-induced inflammatory injury.

Conclusion

Inflammation is implicated in the pathogenesis of infantile pneumonia, and the inhibition of the inflammatory response contributes to the amelioration of infantile pneumonia.¹⁷ In the present study, the anti-inflammatory effect of CITED against infantile pneumonia was identified.

Lipopolysaccharide functions as an endotoxin, binds to the lung fibroblasts and induces severe inflammatory responses.⁷ Lipopolysaccharide-treated lung fibroblasts were widely used as the cell model of pneumonia, and was applied to investigate the role of therapeutic agents for infantile pneumonia.¹⁸ The results of this study showed that the treatment with lipopolysaccharide decreased the cell viability of human fetal lung fibroblasts, enhanced the release of LDH and secretion of TNF- α , IL-1 β , IL-6, suggesting that the pneumonic cell model was well established.

A previous study has shown that the expression of CITED2 was induced by biological stimuli such as lipopolysaccharide, and the loss of CITED2 showed hypoplasia in fetal liver development.¹⁹ CITED2 was found to be down-regulated in the lipopolysaccharide-induced pneumonic cell model in this study. The over-expression of CITED2 enhanced the cell viability and reduced LDH release in lipopolysaccharide-treated MRC-5/WI-38 cells, demonstrating the protective effect of CITED2 against lipopolysaccharide-induced cytotoxicity in lung fibroblasts. However, the effect of CITED2 on cell apoptosis of lipopolysaccharide-treated MRC-5/WI-38 cells remains unknown. The deficiency of CITED2 in lipopolysaccharide-treated macrophages up-regulated the expression of genes involved in pro-inflammatory signaling pathways.²⁰ CITED2 restrained the recruitments of macrophage and neutrophil to the lungs, and protected the host against inflammatory insults.²⁰ Over-expression of CITED in this study also attenuated lipopolysaccharide-induced increase of TNF- α , IL-1 β , IL-6 in MRC-5 and WI-38, thus exhibiting anti-inflammatory effect on infantile pneumonia. Moreover, lipopolysaccharide

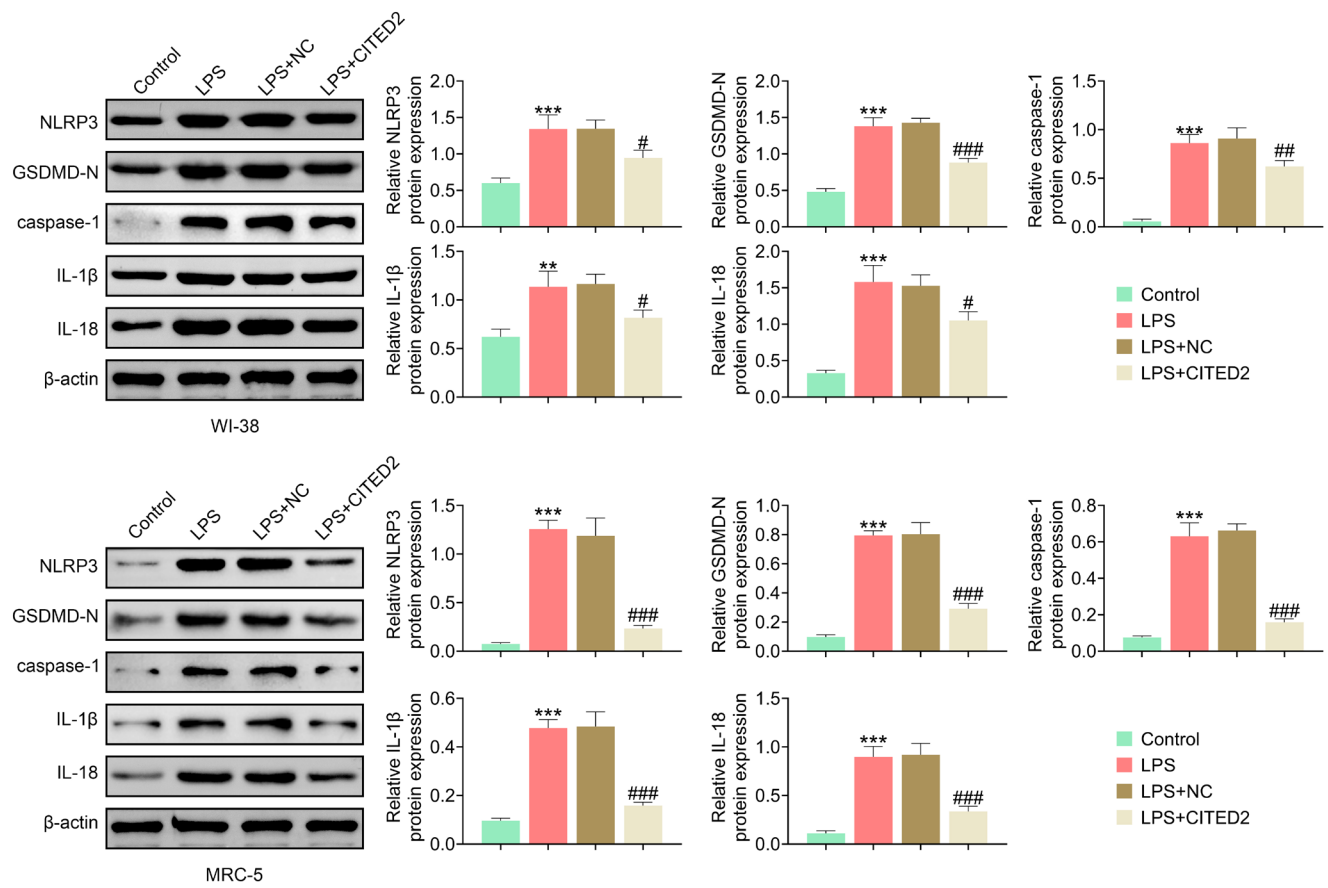


Figure 4 CITED2 inhibited lipopolysaccharide-induced pyroptosis in MRC-5/WI-38 cells Over-expression of CITED2 reduced the protein expression of NLRP3, GSDMD-N, caspase-1, IL-1 β and IL-18 in lipopolysaccharide-treated MRC-5/WI-38 cells. **, *** vs. control $p < 0.01$, $p < 0.001$. #, ### vs. LPS+NC $p < 0.05$, $p < 0.001$.

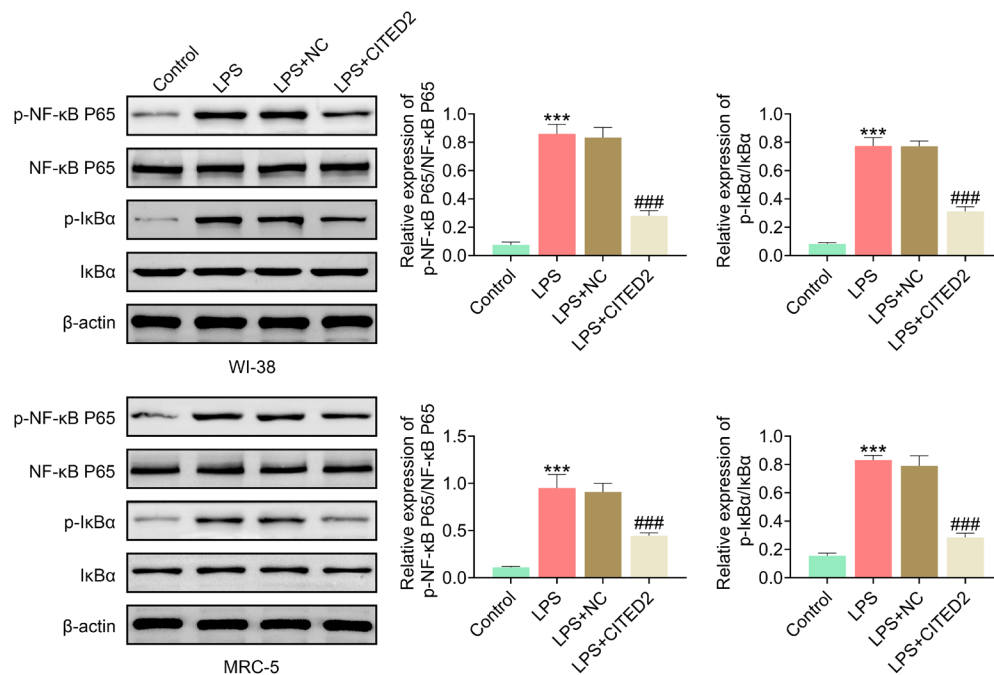


Figure 5 CITED2 mediated NF- κ B pathway in lipopolysaccharide-treated MRC-5/WI-38 cells Over-expression of CITED2 reduced the protein expression of p-I κ B α and p-p65 in lipopolysaccharide-treated MRC-5/WI-38 cells. *** vs. control $p < 0.001$. ### vs. LPS+NC $p < 0.001$.

also induced oxidative stress in bronchial epithelioid cells, and aggravated the inflammatory injury in infantile pneumonia.²¹ CITED2 was repressed by hyperglycemia-induced oxidative stress in cultured embryos. CITED2 is essential for the homeostasis of endoplasmic reticulum stress and formation of neural tube defects during development of an embryo.²² Therefore, CITED2 might also exert anti-oxidant effect on lipopolysaccharide-treated MRC-5/WI-38 cells.

Pyroptosis is initiated by cleavage of GSDMD to produce GSDMD-N, and then promotes the secretion of IL-1 β and IL-18.²³ Lipopolysaccharide induces the activation of NLRP3 inflammasome, and promotes the production of caspase-1 to stimulate the maturation of GSDMD-N, IL-1 β , and IL-18.²³ Lipopolysaccharide-induced pyroptosis in lung fibroblasts was associated with the development of pneumonia.²⁴ The over-expression of CITED2 reduced lipopolysaccharide-induced protein expression of NLRP3, GSDMD-N, caspase-1, IL-1 β , and IL-18 in MRC-5/WI-38 cells to inhibit the pyroptosis.

NF- κ B signaling regulated the expression of genes involved in cell survival, apoptosis, and secretion of inflammatory cytokines. The abnormal activation of NF- κ B was associated with inflammatory diseases.²⁵ Lipopolysaccharide induced the activation of NF- κ B in lung fibroblasts, and facilitated the inflammatory injury in infantile pneumonia.²⁶ CITED2 has been shown to interact with p300, dissociate p65 from p300, and restrain p65 binding to the target promoters.²⁷ The overexpression of CITED2 weakened lipopolysaccharide-induced recruitment of NF- κ B p65 to target promoters, thus inhibiting the transcriptional activity of NF- κ B.²⁰ This study also found that CITED2 over-expression attenuated lipopolysaccharide-induced increase of p-I κ B α and p-p65 in MRC-5 and WI-38 cells.

Thus, CITED2 restrained lipopolysaccharide-induced inflammation and pyroptosis in human lung fibroblasts through the inactivation of NF- κ B pathway. Therefore, CITED2 might be a potential target for infantile pneumonia. However, the effect of CITED2 in animal model of pneumonia should be investigated further for findings.

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Competing Interests

The authors state that there are no conflicts of interest to disclose.

Ethics Approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Contribution of Authors

Xuzhong Zhang Wei Chen and Wei Liu designed the study and carried it out. Donge Li and Wei Shen supervised the data collection, analyzed the data, interpreted the data, prepared the manuscript for publication, and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

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