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Knockdown of ITIH4 reduces inflammatory damage and apoptosis of A549 cells induced by *Mycoplasma pneumoniae* through NLRP3 inflammation

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Abstract
Background: Mycoplasma pneumoniae (MP) is a leading cause of community-acquired respiratory infections in pediatric patients. This study aimed to investigate whether the pro-inflammatory function of inter-alpha-trypsin inhibitor heavy chain (ITIH4) contributes to the pathogenesis of MP-induced pneumonia.
Method: A549 cells were stimulated with MP to model pneumonia in vitro. ITIH4 expression was knocked down in A549 cells using lentiviral transfection. Cell viability was measured using the Cell Counting Kit-8 (CCK-8) assay, while cell apoptosis was assessed via flow cytometry. The concentrations of pro-inflammatory (IL-6, TNF-α) and anti-inflammatory (IL-10) cytokines were quantified using enzyme-linked immunosorbent assay (ELISA). Western blotting was conducted to detect apoptosis-related proteins and components of the NLRP3 inflammasome.
Result: MP stimulation led to increased ITIH4 expression in A549 cells, and knockdown of ITIH4 prevented the MP-induced reduction in cell viability. Moreover, ITIH4 knockdown reduced the release of inflammatory cytokines in response to MP and significantly decreased MP-induced apoptosis. In addition, ITIH4 knockdown inhibited activation of the NLRP3 inflammasome, while reactivation of NLRP3 reversed the protective effects associated with ITIH4 knockdown.
Conclusion: ITIH4 knockdown alleviates MP-induced inflammatory damage and cell death in A549 cells by inhibiting activation of the NLRP3 inflammasome.

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Introduction

Mycoplasma pneumoniae (MP) is a recognized pathogen that can infect both the upper and lower respiratory tracts, particularly in children.[1] It has been identified as the cause of approximately 40% of community-acquired pneumonia cases in children over 5 years of age and accounts for 30% of all pneumonia cases, with up to 18% of affected children requiring hospitalization.[2,3] Despite advancements in diagnostic techniques, current treatments remain insufficient to completely eradicate the pathogen from the respiratory tract.[4] In addition, growing evidence indicates that excessive inflammation plays a critical role in the progression of MP-induced pneumonia. The overproduction of cytokines exacerbates damage to respiratory epithelial cells, resulting in cilia loss, vacuole formation, and cell shedding, which collectively intensify lung injury.[5]

A proteomics study has indicated that elevated levels of the protein inter-alpha-trypsin inhibitor heavy chain (ITIH4) are associated with immune responses in childhood pneumonia.[6] ITIH4 is a plasma protein synthesized by the liver, belonging to the inter-α-inhibitor/ITIH family, and has been implicated in various inflammatory diseases.[7] For instance, ITIH4 has been reported as a potential inflammatory marker in bloodstream infections, particularly those caused by bacteria.[8] It has also been associated with susceptibility, disease activity, and inflammation levels in inflammatory bowel disease.[9] In patients with sepsis, changes in serum ITIH4 levels over time have been linked to inflammation, multi-organ injury, and an increased risk of mortality.[10] Nonetheless, the role of ITIH4 in MP-induced pneumonia and its underlying mechanisms remains poorly understood and requires further investigation.

To explore this, the present study employed lentiviral transfection to knock down ITIH4 in A549 cells and examined its effects on MP-induced inflammation and apoptosis, potentially offering insights into new biological targets and therapeutic approaches.

Methods

Mycoplasma culture

MP strain 29342 (American Type Culture Collection) was cultured for three days at 37°C with 5% CO_2 in mycoplasma medium (CM403). To determine the number of viable MP, serial 10-fold dilutions were prepared and plated on mycoplasma agar.

Cell culture and infection

The A549 cell line, derived from human alveolar cell carcinoma, was selected due to its widespread use as an in vitro model for type II pulmonary alveolar epithelial cells and its characteristic features of human alveolar epithelial cells. A549 cells were obtained from the Shanghai Cell Bank, Chinese Academy of Sciences, and cultured at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco). To establish the infection model, 10 mL of cell culture medium (containing approximately 1×10^7 CFU per 10^6 A549 cells) was mixed with 1 mL of MP, and the mixture was incubated for 24 hours.[11]

Cell transfection

Short hairpin RNA (shRNA) targeting ITIH4 (shITIH4) and the corresponding negative control (shCtrl) were purchased from Shanghai Gene Pharmaceutical Biotechnology Co., Ltd. The lentivirus was transfected into A549 cells according to the manufacturer's instructions. Successfully transfected cells were selected for further analysis. Additionally, the full-length human NLRP3 gene was cloned into the pLVX-puro vector (Clontech). pLVX-NLRP3 (oeNLRP3) and the packaging plasmid were subsequently transfected into A549 cells.

RT-PCR

RT-qPCR was performed following the protocol outlined in previous studies.[12] The primer sequences used were as follows: *ITIH4*: 5'-GGTCTCATCCCGATTTGC-3' (forward), 5'-TGATCCCTGGGTAGGTCAT-3' (reverse); *GAPDH*: 5'-GAAGGTGAAGGTCGGAGT-3' (forward), 5'-GAAGATGGT GATGGGATTTC-3' (reverse).

Cell Counting Kit-8 (CCK-8) assay

A549 cells were seeded at a density of 1×10^4 cells/well in a 96-well culture plate. After 24 hours of incubation, 10 μL of CCK-8 reagent (Beyotime, China) was added to each well, and the plate was incubated for one hour. The optical density was then measured at 450 nm using a microplate reader (Thermo Fisher Scientific, MA, USA).

Enzyme-linked immunosorbent assay (ELISA)

The levels of tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, IL-10, IL-4, and IL-13 in the supernatants were measured using ELISA kits (Nanjing Jiancheng). The optical density was measured at 450 nm using a microplate reader (Thermo Fisher Scientific, MA, USA).

Flow cytometry

A549 cells (3×10^5) were cultured for 24 hours before being harvested from 6-well plates. Each cell suspension was then treated with 5 μL of fluorescently labeled Annexin V (Annexin V-FITC) and propidium iodide (PI) reagent (Shanghai Hengfei Biotechnology Co., Ltd.), followed by incubation in the dark for 15 minutes. Flow cytometry was subsequently performed to determine the rate of cell apoptosis using a flow cytometer (Beckman, USA). The data were analyzed using the Cell Quest software (version 5.1; BD Biosciences).

Western blot assay

Cellular proteins were isolated using a total protein extraction kit (Takar, Japan), and protein concentration

was determined using the BCA method (Beyotime, China). The proteins were separated by SDS-PAGE and transferred to a 0.45 μm PVDF membrane, which was then blocked with 5% skim milk. After incubation with specific primary antibodies—ITIH4 (ab180139; Abcam), NLRP3 (ab263899; Abcam), ASC (ab283684; Abcam), Caspase 1 (ab207802; Abcam), IL-1β (ab283818; Abcam), BAX (ab32503; Abcam), BCL-2 (ab182858; Abcam), cleaved-caspase-3 (ab32042; Abcam), caspase-3 (ab32351; Abcam), and GAPDH (ab9485; Abcam)—secondary antibodies conjugated to horseradish peroxidase (HRP) (Santa Cruz, USA) were added. Protein bands were then visualized using enhanced chemiluminescence (Beyotime, China) according to the manufacturer's instructions.

Statistical analysis

All experiments were performed with at least three biological replicates. Data distribution was tested for normality using the Shapiro-Wilk test. Statistical comparisons between two groups were performed using the Student's t-test, while comparisons among more than two groups were analyzed using analysis of variance (ANOVA) followed by Tukey's post hoc test. A *P*-value of less than 0.05 was considered statistically significant. Data are expressed as mean ± standard deviation (SD).

Results

ITIH4 is upregulated in A549 cells induced by MP infection

Western blot analysis revealed that MP infection significantly increased ITIH4 expression in A549 cells (Figure 1A), suggesting that ITIH4 may play a role in the pathogenesis of MP-induced pneumonia.

ITIH4 knockdown alleviates inflammatory damage in A549 cells induced by MP infection

A549 cells transfected with shITIH4 exhibited significantly reduced ITIH4 expression following lentiviral transfection (Figure 2A,B). The CCK-8 assay showed that MP infection decreased A549 cell viability, and this reduction was reversed upon ITIH4 knockdown (Figure 2B). Additionally, MP induced the release of pro-inflammatory cytokines (IL-6, IL-1β, TNF-α) and suppressed the anti-inflammatory cytokine IL-10; these effects were reversed following ITIH4 knockdown (Figure 2C). Together, these findings suggest that ITIH4 contributes to MP-induced inflammatory damage in A549 cells.

ITIH4 knockdown reduces apoptosis in A549 cells induced by MP infection

Flow cytometry analysis revealed that MP infection induced apoptosis in A549 cells, and ITIH4 knockdown significantly reduced this apoptotic response (Figure 3A). Moreover, MP infection increased the expression of pro-apoptotic proteins, including BAX and cleaved-caspase-3, while decreasing the expression of the anti-apoptotic protein BCL-2. These changes were reversed by ITIH4 knockdown (Figure 3B), suggesting that ITIH4 plays a role in regulating apoptosis in A549 cells during MP infection.

ITIH4 knockdown inhibits NLRP3 inflammasome activation in A549 cells

The NLRP3 inflammasome plays a crucial role in MP-induced pneumonia.[13] Western blot analysis showed that MP infection activated the NLRP3 inflammasome in A549 cells, as indicated by increased expression of NLRP3, ASC, cleaved-caspase-1, and IL-1β. In contrast, ITIH4 knockdown inhibited activation of the NLRP3 inflammasome (Figure 4), indicating that ITIH4 modulates inflammasome activity in response to MP infection.

NLRP3 overexpression reverses the effects of ITIH4 knockdown

To further confirm the role of the NLRP3 inflammasome in the anti-inflammatory and anti-apoptotic effects of ITIH4 knockdown, NLRP3 was overexpressed in A549 cells to reactivate the inflammasome (Figure 5A). The results

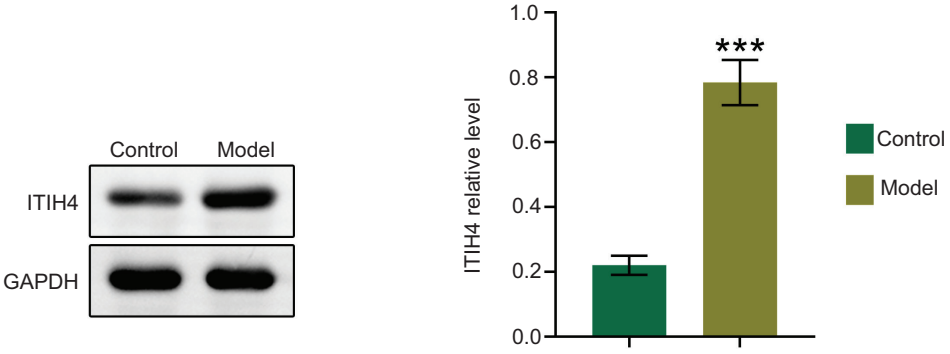

Figure 1 Upregulation of ITIH4 in A549 cells induced by *Mycoplasma pneumoniae* (MP) infection. ITIH4 expression in A549 cells was assessed by western blotting. Data are presented as mean ± SD. ***$p < 0.001$ vs Control group. n=3.

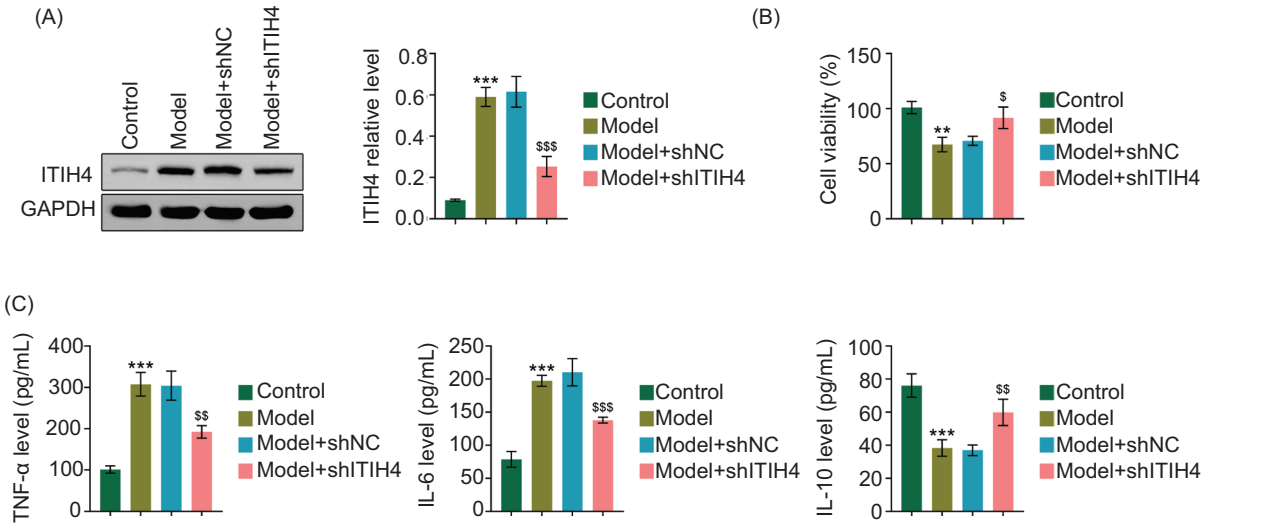

Figure 2 ITIH4 knockdown alleviates inflammatory damage in A549 cells induced by MP infection. (A) RT-qPCR analysis of ITIH4 mRNA expression in A549 cells. (B) Western blot analysis of ITIH4 protein expression in A549 cells. (C) CCK-8 assay assessing cell viability. (D) ELISA analysis of IL-1β, IL-6, IL-10, and TNF-α levels in the culture supernatant. Data are presented as mean ± SD. $^{**}p < 0.01$, $^{***}p < 0.001$ vs Control group. $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ vs model+shNC group. n=3.

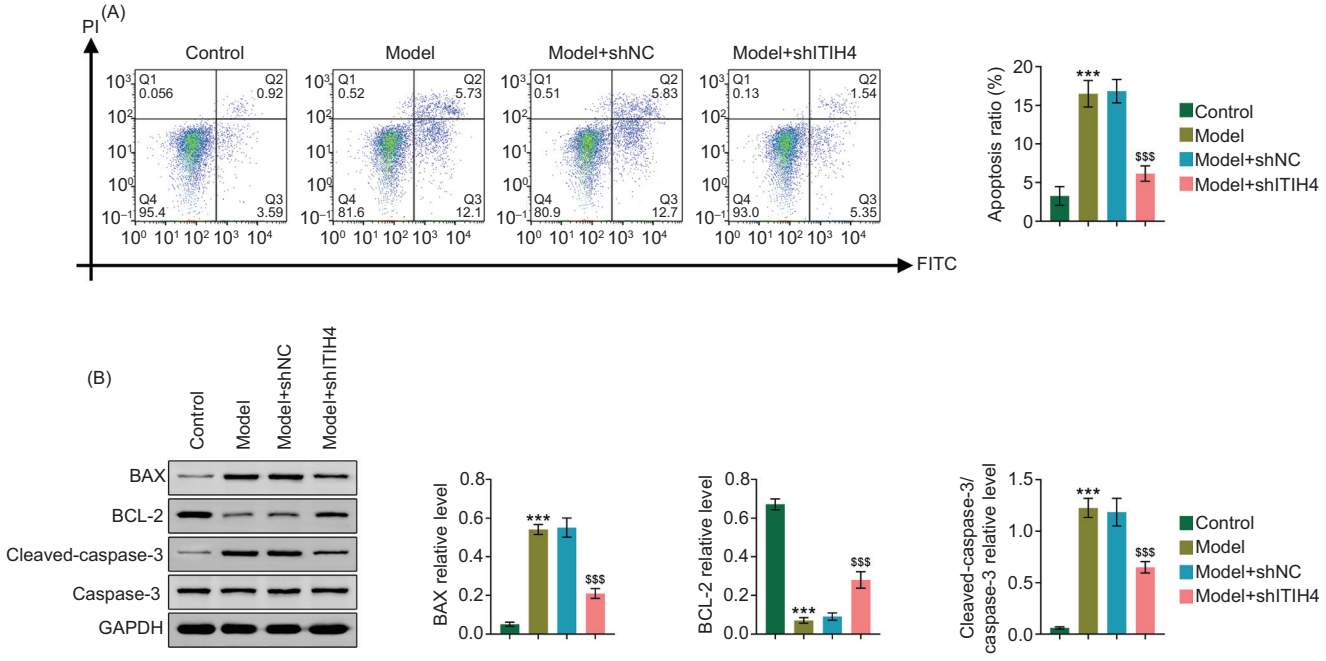

Figure 3 ITIH4 knockdown reduces apoptosis in A549 cells induced by MP infection. (A) Flow cytometry analysis of apoptosis rate in A549 cells. (B) Western blot analysis of apoptosis-related proteins (BAX, BCL-2, cleaved caspase-3, and caspase-3) in A549 cells. Data are presented as mean ± SD. $^{***}p < 0.001$ vs Control group. $^{\$\$\$}p < 0.001$ vs model+shNC group. n=3.

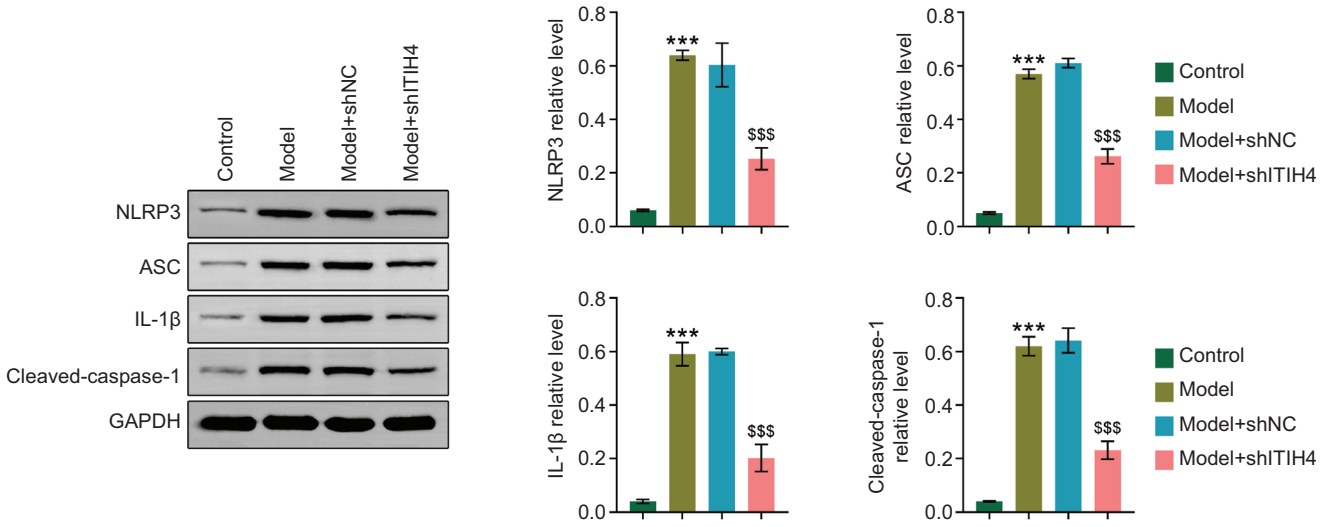

Figure 4 ITIH4 knockdown inhibits NLRP3 inflammasome activation. Western blot analysis of NLRP3, ASC, caspase-1, and IL-1β protein expression in A549 cells. Data are presented as mean ± SD. ***$p < 0.001$ vs Control group. $^{\$\$\$}p < 0.001$ vs model+shNC group. n=3.

demonstrated that reactivation of the NLRP3 inflammasome reversed the protective effects of ITIH4 knockdown, restoring MP-induced inflammatory damage and apoptosis in A549 cells (Figure 5B-E). These findings suggest that ITIH4 knockdown alleviates MP-induced inflammatory damage and apoptosis in A549 cells by inhibiting activation of the NLRP3 inflammasome.

Discussion

Although MP is a common pathogen responsible for respiratory tract infections, particularly in children, the precise mechanisms underlying MP infection remain poorly understood.[14] ITIH4 has been implicated in various inflammatory disorders, with elevated levels associated with bacterial infections, Th2 cell-specific cytokines, pro-inflammatory cytokines, and the severity of asthma exacerbations in children.[8,15] In the present study, we found that MP infection induced upregulation of ITIH4 expression in A549 cells, consistent with the findings reported by Cheng et al.[6] Our results further suggest that ITIH4 knockdown attenuates MP-induced inflammatory damage and apoptosis in A549 cells by inhibiting activation of the NLRP3 inflammasome.

Innate immunity serves as the body's first line of defense against infection, with inflammation playing a central role in this response. Two key pro-inflammatory cytokines, TNF-α and IL-6, are essential to the pathophysiology of lung damage induced by MP infection. TNF-α, produced by monocytes and macrophages, is recognized as a critical mediator of lung injury during MP infection and has been proposed as a reliable biomarker for its diagnosing.[16,17] IL-10, a pleiotropic cytokine with anti-inflammatory properties, suppresses the production of several pro-inflammatory cytokines, including TNF-α and IL-6.[18] While the inflammatory response is crucial for combating infection, excessive inflammation can trigger apoptosis—a form of programmed cell death that maintains tissue homeostasis. However, excessive apoptosis can cause significant tissue damage, particularly in the lungs.[19] Furthermore, ITIH4 expression in alveolar epithelial cells has been associated with inflammation and apoptosis induced by air pollution.[20] In this study, our findings suggest that ITIH4 knockdown alleviates MP-induced inflammation and apoptosis, underscoring its role in regulating these processes during MP infection.

The inflammasome, a multiprotein complex, plays a critical role in the host's defense against invasive infections by promoting the release of pro-inflammatory molecules. Among the various types, the NLRP3 inflammasome is considered the most prominent. This complex is composed of NLRP3, ASC, and caspase-1.[21,22] Previous studies have emphasized the crucial role of NLRP3 in regulating inflammation and modulating innate immune responses during MP infection.[23] Consistent with these findings, our study confirmed that MP infection activates the NLRP3 inflammasome, resulting in the production of several inflammatory mediators. Furthermore, we demonstrated that ITIH4 knockdown alleviates inflammation and apoptosis by inhibiting NLRP3 inflammasome activation.

This study had several limitations. First, it primarily relied on in vitro models, such as A549 cells. While these models offer valuable insights into the cellular mechanisms underlying MP infection, they do not fully replicate the complex physiological and pathological conditions of the in vivo environment. Therefore, further validation using in vivo models is necessary to confirm the relevance and applicability of these findings. Second, recent studies have demonstrated that MP infection is associated with elevated immunoglobulin E (IgE) levels, which are closely linked to a Th2-dominant immune response.[24,25] Future research should explore the role of ITIH4 and its potential relationship with the Th2 immune response in MP-induced pneumonia, utilizing in vivo models to gain a more comprehensive

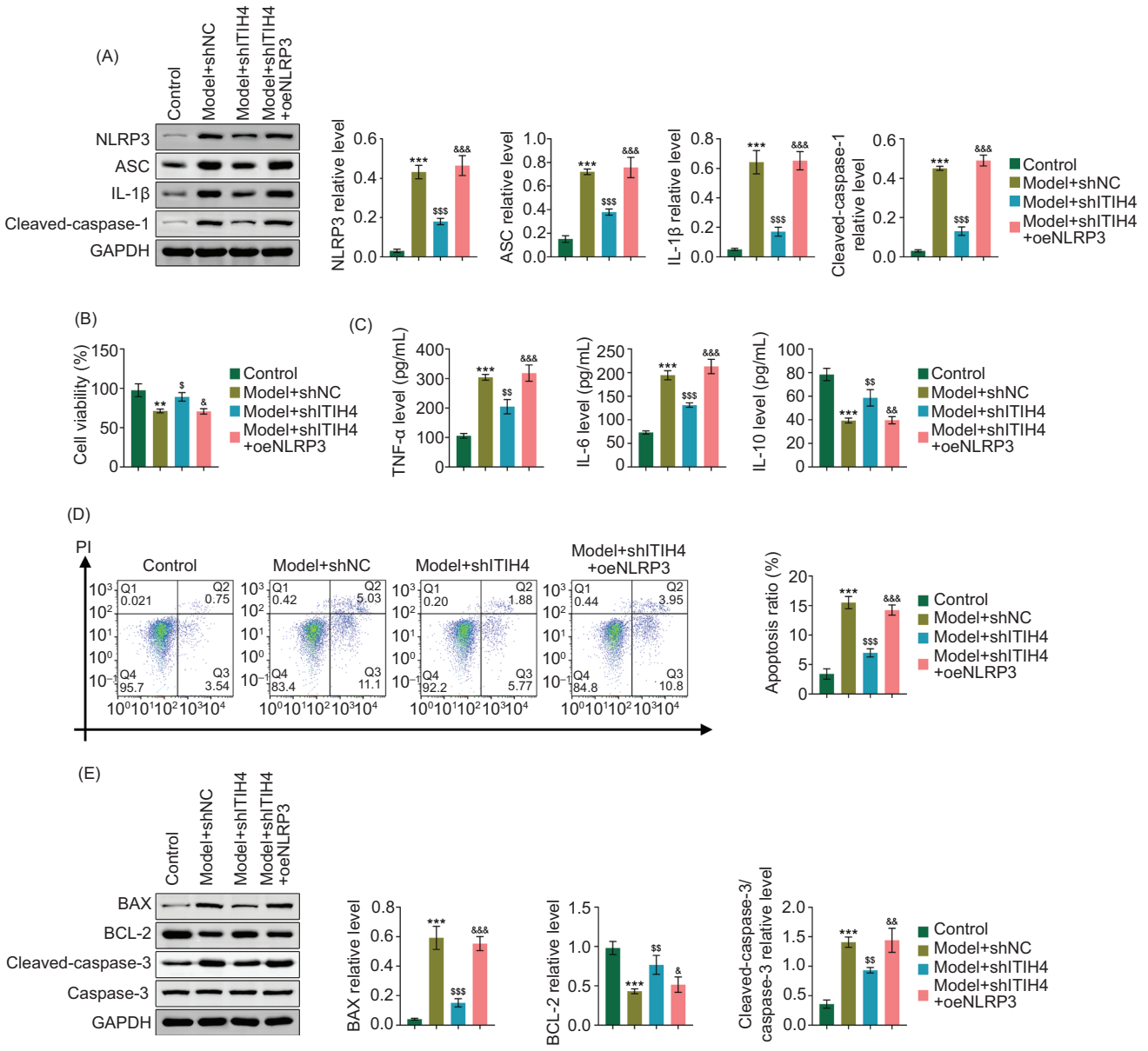

Figure 5 NLRP3 overexpression reverses the effects of ITIH4 knockdown. (A) Western blot analysis of NLRP3, ASC, caspase-1, and IL-1β protein expression. (B) CCK-8 assay to assess cell viability. (C) ELISA analysis of IL-1β, IL-6, IL-10, and TNF-α levels in the culture supernatant. (D) Flow cytometry analysis of apoptosis rate. (E) Western blot analysis of apoptosis-related proteins (BAX, BCL-2, cleaved caspase-3, and caspase-3). Data are presented as mean ± SD. ***$p < 0.001$ vs Control group. $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ vs model+shNC group. $^{\&}p < 0.05$, $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$ vs model+sh ITIH4 group. n=3.

understanding of its role in host immunity and its broader implications in pneumonia pathogenesis.

Conclusion

In conclusion, our findings indicate that ITIH4 knockdown alleviates MP-induced apoptosis and inflammatory damage in A549 cells by inhibiting the NLRP3 inflammasome pathway, highlighting ITIH4's potential as a therapeutic target and a candidate for developing ITIH4-based biomarkers. Further research is required to validate its utility as a diagnostic and prognostic marker for pneumonia.

Availability of Data and Materials

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

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

Authors Contribution

All authors contributed to the study conception and design. Material preparation and the experiments were performed by ZZ. Data collection and analysis were performed by YZ. The first draft of the manuscript was written by BZ and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Conflicts of Interest

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

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