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Knockdown of EPSTI1 alleviates lipopolysaccharide-induced inflammatory injury through regulation of NF-κB signaling in a cellular pneumonia model

Adilijiang Kari, Zhihua M, Zhayidan Aili, Ayinuerguli Adili, Nadire Hairula, Abulaiti Abuduhaer*

Pediatric Intensive Care Unit, First Affiliated Hospital of Xinjiang Medical University, Urumqi, China

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KEYWORDS
epithelial-stromal interaction 1; inflammation; nuclear factor κ-light-chain-enhancer of activated B cells; pneumonia

Abstract
Background: Although early diagnosis, antibiotic therapies, corticosteroid application, and health care services are conventional managements for pneumonia, antibiotic resistance and adverse reactions remain as limitations for pneumonia treatment.
Objectives: The study attempted to evaluate the potential role of EPSTI1 against pneumonia and reveal its underlying mechanism.
Methods: Lipopolysaccharide (LPS) (5, 10, and 20 μg/mL) was applied in WI-38 cells to establish the *in vitro* pneumonia model. Knockdown of epithelial-stromal interaction 1 (EPSTI1) was performed by transfection with EPSTI1 siRNA (siEPSTI1) into LPS-treated cells. Cell Counting Kit-8 assays were implemented to measure cell viability, and apoptotic cells were detected using flow cytometry. Interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α) were quantified using enzyme-linked immunosorbent assay (ELISA). Immunoblotting and quantitative real-time polymerase chain reaction (qRT-PCR) were conducted to quantify EPSTI1 expression, and proteins related to nuclear factor κ-light-chain-enhancer of activated B cell (NF-κB) signaling, including p-p65, p65, p-IκBα, and IκBα.
Results: EPSTI1 was highly expressed in LPS-treated WI-38 cells. Cell apoptosis was promoted, and cell viability was inhibited after being exposed to LPS. Besides, IL-1β, IL-6, and TNF-α were dramatically upregulated. Knockdown of EPSTI1 restored cell viability, inhibited cell apoptosis, and attenuated expressions of proinflammatory factors. Additionally, knockdown of EPSTI1 visibly decreased the increased ratios of p-p65/p65 and p-IκBα/IκBα induced by LPS. Knockdown of EPSTI1 alleviated inflammatory injury through the inactivation of the NF-κB pathway.
Conclusions: These results provided promising management in preventing pneumonia in patients.

***Corresponding author**: Abulaiti·Abuduhaer, Pediatric Intensive Care Unit, First Affiliated Hospital of Xinjiang Medical University, No. 137 Liyushan South Road, Xinshi District, Urumqi, Xinjiang 830054, China. *Email address*: abulati1077@163.com

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Introduction

Pneumonia is a lower respiratory tract disease infected by pathogens and manifested as inflammatory responses.[1] The overall mortality of pneumonia was 2.9%–8.0% in China depending on different regions.[2] Early diagnosis, appropriate antibiotic therapies, and the use of corticosteroids and health care services remain as conventional managements for pneumonia.[1,3] However, due to antibiotic resistance and adverse reactions, the optimal management of pneumonia is a 'hot topic'.

Epithelial-stromal interaction 1 (EPSTI1), a gene mapped to human chromosome 13q13.3, was first discovered as a stromal fibroblast gene in breast cancer.[4] Previous studies have demonstrated that EPSTI1 plays pivotal roles in ovarian tumorigenesis,[5] lung squamous cell cancers,[6] and prostate cancer development.[7] Besides, EPSTI1 expression is remarkably elevated in alveolar epithelial type II cells from COPD patients.[8] Recently, EPSTI1 was highly expressed in peripheral blood and bronchoalveolar lavage fluid of patients with COVID-19.[9] EPSTI1 is implicated in diseases related to inflammatory signaling,[10] and excessive EPSTI1 induces the hyperactivation of B cell by regulating NF-κB signaling in primary Sjögren's syndrome.[11] Unfortunately, the involvement of EPSTI1 in pneumonia remains unclear.

The inflammatory response is certainly related to acute and chronic diseases, and NF-κB is essential not only in the proinflammatory phase but also in the stage of anti-inflammatory gene expression and inducing apoptosis.[12] Oxidative stress and the NF-κB pathway participate in the development of influenzal pneumonia, while inactivation of the NF-κB pathway could attenuate the replication of influenza A virus.[13] In children with pneumonia, inhibition of the NF-κB pathway alleviates the inflammatory responses.[14] This study attempted to investigate whether EPSTI1 was involved in pneumonia through regulating the NF-κB pathway. Therefore, lipopolysaccharide (LPS) was applied to establish a cellular pneumonia model in WI-38 cells, and knockdown of EPSTI1 was subsequently performed, followed by determining the involvement of NF-κB signaling.

Methods

Cell culture and in vitro pneumonia model induction

Human fetal lung fibroblast WI-38 cells obtained from the American Type Culture Collection (ATCC; Rockville, Maryland, USA) were maintained in a DMEM medium (Gibco, Grand Island, NY, USA), supplemented with 10% fetal bovine serum, 100 μg/mL streptomycin, and 100 U/mL penicillin (Invitrogen, Carlsbad, CA, USA) at 37°C with 5% CO_2. When cells were grown to 80% confluence, WI-38 cells at a density of 2×10^5 cells/well were placed in six-well plates and cultured for 48 h at 37°C. Afterward, LPS (5, 10, and 20 μg/mL) was applied to WI-38 cells and then incubated for 24 h.

SiRNA transfection

For knockdown of EPSTI1, EPSTI1 siRNA and a negative control (siNC) were bought from GenePharma (Shanghai, China), which were subsequently transfected into WI-38 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After 48 h of transfection, cells were harvested for further experiments.

Cell viability

WI-38 cells (5×10^3 cells/well) were placed in 96-well plates. After 24 h of LPS stimuli or 48 h of siEPSTI1 transfection, cell viability was assessed using a CCK-8 assay kit (Beyotime Biotechnology, Jiangsu Province, China) according to the manufacturer's instructions. The optical density was read at 450 nm using a microplate reader (Thermo Scientific, San Jose, CA, USA).

Cell apoptosis

Cells were collected after 48 h of transfection, rinsed with PBS, and then resuspended using 100 μL of buffer. Subsequently, 10 μL of Annexin V-FITC was applied to cells in the dark for 30 min, followed by addition of 5 μL of propidium iodide for 15 min. Cell apoptosis was detected using FACScan flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA).

ELISA

Cells (1×10^5 cells/well) were placed in 24-well plates for 24 h, and the culture supernatant was obtained. The levels of IL-6 (ab178013, Abcam, Cambridge, MA, USA), IL-1β (ab214025, Abcam, Cambridge, MA, USA), and TNF-α (ab181421, Abcam, Cambridge, MA, USA) were determined using ELISA kits according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA).

RT-qPCR

For total RNA extraction, TRIzol reagent (LanGang biological, China) was implemented in LPS-treated or siEPSTI1-transfeted WI-38 cells. First-strand cDNA was synthesized using a SuperScript III Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA). Next, qRT-PCR was conducted using a SYBR® Premix Ex Taq™ II kit (TaKaRa, Japan) on an ABI 7300 real-time PCR detection system (Applied Biosystems, Foster City, CA, USA) at 95°C for 5 min, followed by 40 cycles at 94°C for 30 s, 56°C for 90 s, and 72°C for 60 s. The primers for EPSTI1 were 5'-ACCCGCAATAGAGTGGTGAAC-3' (forward) and 5'-GCTATCAAGGTGTATGCACTTGT-3' (reverse). The gene expression was quantified using the $2^{-\Delta\Delta Ct}$ method.[15]

Immunoblotting analysis

Total proteins were obtained from LPS-treated or siEPSTI1-transfeted WI-38 cells by RIPA lysis buffer. Proteins were electrophoresed on 10% SDS-PAGE, transferred onto poly(vinylidene difluoride) membranes, and then blocked with 5%

nonfat milk, followed by incubation with antibodies against EPSTI1 (1:200, sc-100657, Santa Cruz, CA, USA), B-cell lymphoma-2 (Bcl-2, 1:100, sc-166943, Santa Cruz, CA, USA), Bcl-2 associated x protein (Bax, 1:200, sc-7480, Santa Cruz), cleaved-caspase 3 (1:500, ab32042, Abcam), cleaved-caspase 9 (1:500, ab2324, Abcam), p-p65 (1:500, ab31624, Abcam), p65 (1:1000, ab32536, Abcam), p-IκBα (1:200, sc-8404, Santa Cruz), and IκBα (1:200, sc-1643, Santa Cruz) at 4°C overnight. Afterward, membranes were incubated with peroxidase-conjugated goat anti-rabbit IgG antibodies (1:2000, ab205718, Abcam) for 2 h. Blots were visualized with an enhanced chemiluminescence kit (Millipore, Billerica, MA, USA).

Statistical analysis

Data were analyzed using SPSS 17.0 software (Chicago, IL, USA) and exhibited as mean ± SD. ANOVA was applied for comparison between multiple groups, followed by Tukey's post hoc tests. The value of p was set to 0.05.

Results

EPSTI1 was highly expressed in LPS-treated WI-38 cells

After being exposed to LPS, the expression of EPSTI1 in WI-38 cells was quantified using RT-qPCR and immunoblotting. As presented in Figure 1A,B, 10 μg/mL and 20 μg/mL LPS dramatically promoted EPSTI1 expression in WI-38 cells (P < 0.01 or 0.001). LPS stimuli remarkably inhibited the viability of WI-38 cells in a dose-dependent manner. Collectively, EPSTI1 was downregulated by LPS stimuli in a dose-dependent manner.

Knockdown of EPSTI1 repressed the apoptosis of LPS-treated WI-38 cells

To investigate whether EPSTI1 was implicated in the inhibitory effect of LPS (10 μg/mL) on cell survival, siEPSTI1 was subsequently transfected into LPS-treated WI-38 cells. Figure 2A,B displays that LPS induced a significant increase in EPSTI1 expression compared to the control (P < 0.001), while siEPSTI1 remarkably suppressed the level of EPSTI1 compared to siNC (P < 0.01). After treatment of LPS for 24h, the viability of WI-38 cells was significantly inhibited compared to that of the control (P < 0.001), which was dramatically increased after siEPSTI1 transfection (P < 0.05) (Figure 2C). LPS also caused a higher apoptotic rate of WI-38 cells than the control (P < 0.001), while siEPSTI1 suppressed the LPS-induced apoptotic rate compared to that of transfection of siNC (P < 0.001) (Figure 2D). Besides, immunoblotting portrayed that Bax, cleaved caspase 3, or cleaved caspase 9 were significantly upregulated, but Bcl-2 was downregulated after LPS treatment compared with those of the control (P < 0.001). However, transfection with siEPSTI1 reversed these results (Figure 2D). Taken together, knockdown of EPSTI1 repressed the apoptosis of LPS-treated WI-38 cells.

Knockdown of EPSTI1 suppressed inflammatory responses in LPS-treated WI-38 cells

Subsequently, the levels of IL-6, IL-1β, and TNF-α were determined by ELISA, and Figure 3A shows that 10 μg/mL LPS induced distinct increases in IL-6, IL-8, and TNF-α as compared to those of the control (P < 0.001), whereas siEPSTI1 remarkably suppressed these inflammatory cytokines as compared to those of siNC (P < 0.01 or 0.05).

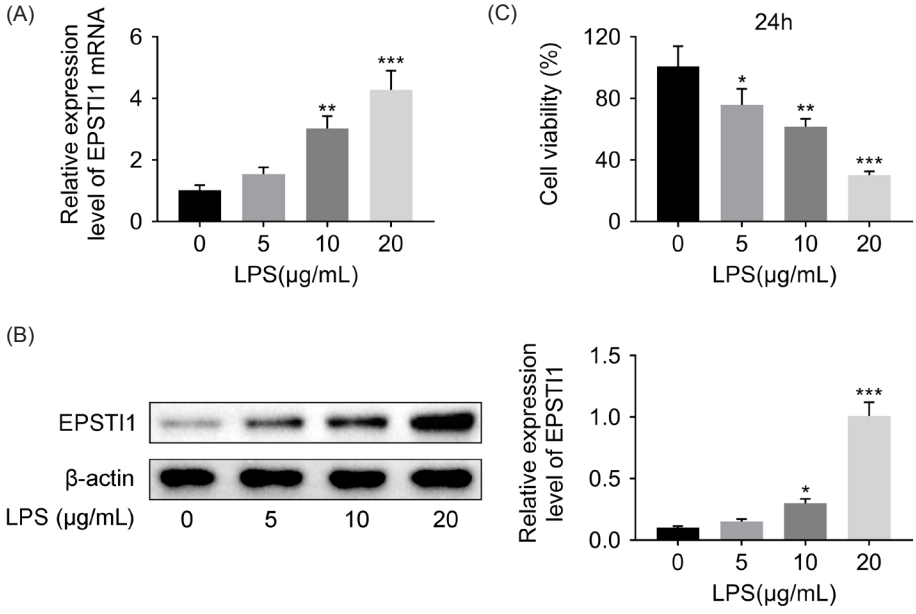

Figure 1 EPSTI1 was highly expressed in LPS-treated WI-38 cells. (A) EPSTI1 expression was quantified by RT-qPCR after different concentrations of LPS (5, 10, 20 μg/mL) in WI-38 cells. (B) Protein level of EPSTI1 was determined by western blot analysis when stimulated by LPS in WI-38 cells. (C) Viability of LPS-treated WI-38 cells was assessed using CCK-8 assay. *P < 0.05, **P < 0.01, and ***P < 0.001 versus untreated WI-38 cells.

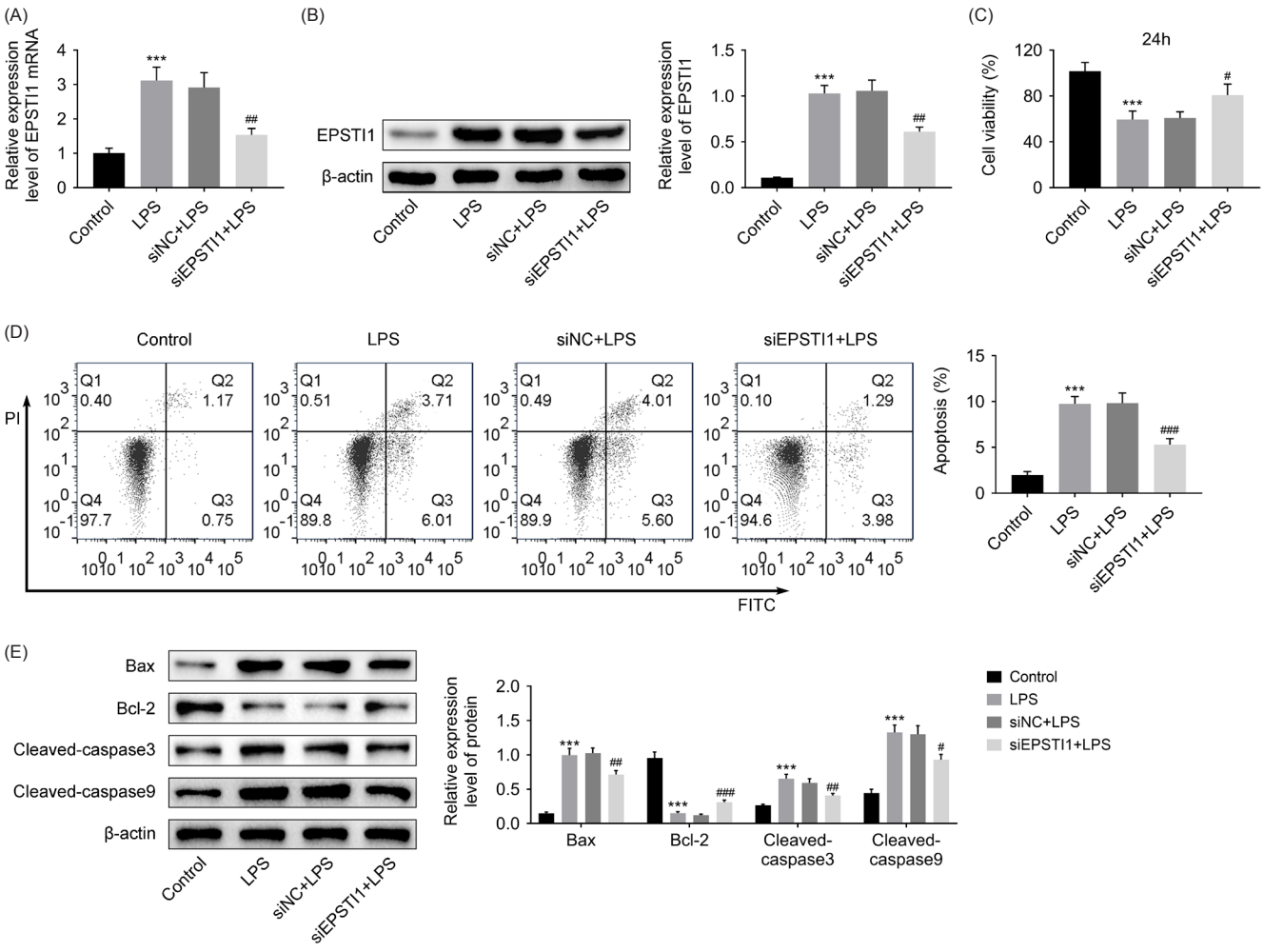

Figure 2 Knockdown of EPSTI1 inhibited apoptosis after LPS treatment in WI-38 cells. (A) EPSTI1 expression was assessed by RT-qPCR after transfecting siEPSTI1 into LPS-treated (10 μg/mL) WI-38 cells. (B) Protein level of EPSTI1 was determined by western blot analysis after transfecting siEPSTI1 into LPS-treated WI-38 cells. (C) Cell viability was assessed after transfecting siEPSTI1 into LPS-treated WI-38 cells. (D) Cell apoptosis was measured after transfecting siEPSTI1 into LPS-treated WI-38 cells. (E) Western blotting was implemented to determine the protein levels of Bax, Bcl-2, cleaved caspase 3, and cleaved caspase 9 after transfecting siEPSTI1 into LPS-treated WI-38 cells. $^{***}P < 0.001$ versus the control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ versus the siNC+LPS group.

Similarly, immunoblotting displayed that siEPSTI1 visibly inhibited LPS-induced inflammatory responses as compared to the siNC group (Figure 3B). Collectively, knockdown of EPSTI1 suppressed inflammatory responses in LPS-treated WI-38 cells.

Knockdown of EPSTI1 inactivated the NF-κB signaling pathway

Furthermore, proteins related to NF-κB signaling were quantified to investigate the mechanism underlying the regulatory effects of EPSTI1 on LPS-treated WI-38 cells. Figure 4 reveals that LPS remarkably elevated the ratios of p-p65/p65 and p-IκBα/IκBα as compared to the control ($P < 0.001$). However, transfection with siEPSTI1 visibly reduced ratios of p-p65/p65 and p-IκBα/IκBα as compared to those of siNC ($P < 0.01$). Together, knockdown of EPSTI1 inactivated NF-κB signaling.

Discussion

In the current study, EPSTI1 was highly expressed after being exposed to LPS, while cell viability was suppressed after LPS treatment . Knockdown of EPSTI1 repressed cell apoptosis and alleviated inflammatory responses in LPS-treated WI-38 cells. In addition, knockdown of EPSTI1 inactivated the NF-κB signaling pathway.

Pneumonia is a complex disorder caused by pathogenic microorganisms and manifests as local and systemic inflammatory responses. Specially, bacterial and viral co-infection is frequently observed in pediatric pneumonia.[16] LPS is a structure of Gram-negative bacteria, and accordingly,

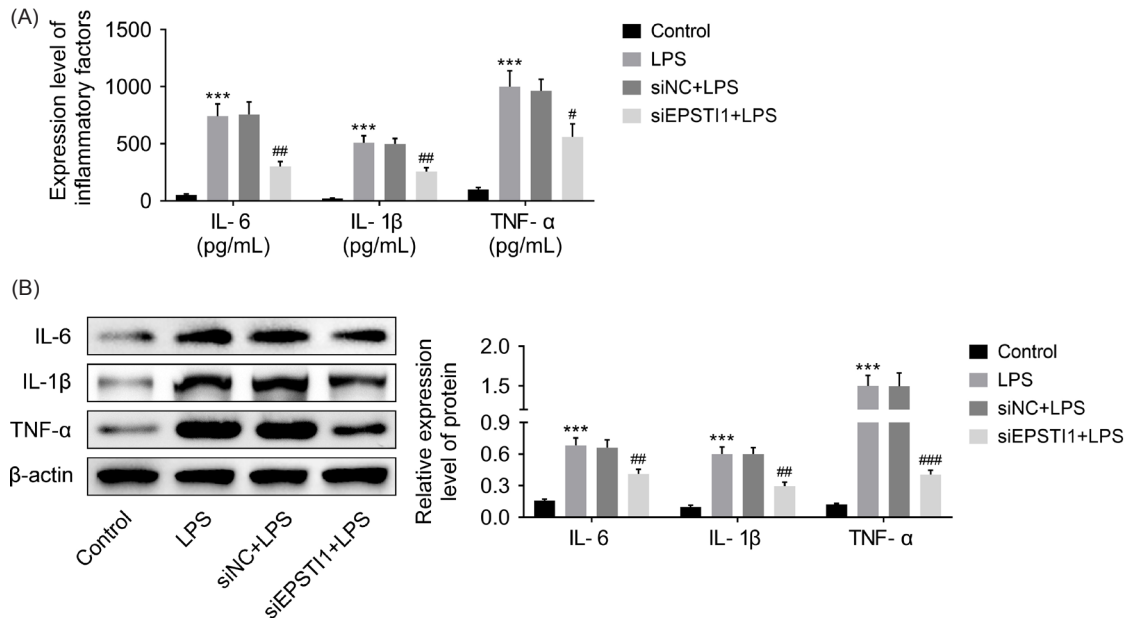

Figure 3 Knockdown of EPSTI1 suppressed inflammatory responses in LPS-treated WI-38 cells. (A) ELISA was implemented to determine the levels of IL-6, IL-1β, and TNF-α after transfecting siEPSTI1 into LPS-treated WI-38 cells. (B) Protein levels of IL-6, IL-1β, and TNF-α were quantified by immunoblotting, followed by transfection of siEPSTI1 into LPS-treated WI-38 cells. $^{***}P < 0.001$ versus the control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ versus the siNC+LPS group.

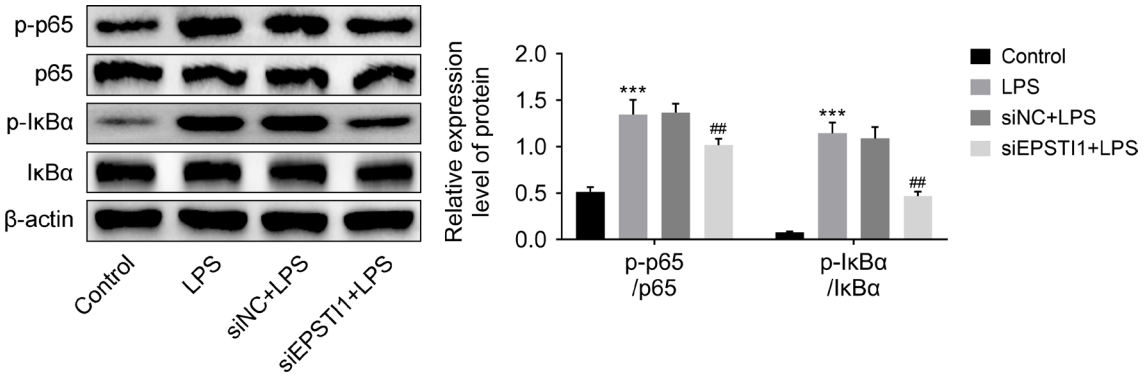

Figure 4 Knockdown of EPSTI1 inactivated the NF-κB signaling pathway. Western blot analysis was employed to determine the protein levels of p-p65, p65, p-IκBα, and IκBα, and the ratios of p-p65/p65 and p-IκBα/IκBα were represented by histograms. $^{***}P < 0.001$ versus the control group. $^{\#\#}P < 0.01$ versus the siNC+LPS group.

pneumonia caused by Gram-negative bacteria was modeled *in vitro*. Application of LPS can result in remarkable inflammatory reactions and apoptotic cells in WI-38 cells.[17] In this study, when LPS was administrated into WI-38 cells, the apoptotic rate was increased while cell viability was significantly inhibited. Besides, the inflammatory products including IL-6, IL-1β, and TNF-α were upregulated. These findings implied that the cellular pneumonia model was induced successfully.

Elevated circulating proinflammatory products, including IL-6 and TNF-α, were observed in most patients with community-acquired pneumonia (CAP), and IL-6 concentration was associated with the severity.[18] It has been reported that the serum level of IL-6 was an independent factor for pneumococcal infection among children with CAP.[19] Additionally, aberrant TNF-α has been shown to contribute to the development of lung cancer, COPD, or pneumonia, while elevated IL-6 concentration might be considered a potential biomarker for COPD progression.[20] Recently, IL-1β was highly expressed in endothelial cells of pulmonary tissues. Similarly, IL-6 and TNF-α both have significant immunoreactivities in COVID-19 specimen, suggesting that a cytokine storm might be triggered after infection.[21] A multicenter study demonstrated that for children with severe COVID-19, IL-1β, IL-6, and chemokine interferon-inducible protein-10 were dramatically increased, which could independently predict severe pneumonia in children with COVID-19.[22] In consistence with the above results, IL-6, IL-1β, and TNF-α were significantly upregulated in LPS-treated WI-38 cells, but knockdown of EPSTI1 obviously

suppressed inflammatory responses, indicating that IL-6, IL-1ß, and TNF-α contributed to the inflammatory responses while inhibiting EPSTI1 alleviated LPS-caused inflammation.

EPSTI1 gene is well-known as an important participant in cancer development and progression, as well as in some aspects of tumor immune interaction.[23] Interestingly, a previous study confirmed that EPSTI1 was implicated in the regulation of inflammatory responses in macrophages. The level of EPSTI1 was upregulated in LPS or interferon γ-treated macrophages, whereas knockdown of EPSTI1 provoked the differentiation of M2-type macrophages and polarization of M1 type. Besides, the silence of EPSTI1 could suppress proinflammatory products through inhibiting Stat1 and p65 nuclear localization and phosphorylation, which indicated that EPSTI1 might function as a regulator in the treatment of inflammatory disorders.[24] This study revealed that EPSTI1 was overexpressed after LPS stimuli, while knockdown of EPSTI1 attenuated the proinflammatory cytokines and inhibited apoptosis in WI-38 cells. These findings suggested that elevated EPSTI1 might be implicated in the development of pneumonia and provided a potentially important role of EPSTI1 in pneumonia.

Previous study has confirmed that EPSTI1 triggered NF-κB signaling and its nuclear translocation via interacting with valosin-containing protein (VCP) and inducing NF-κB inhibitor alpha degradation.[10] Meanwhile, the result also identified that EPSTI1 could inhibit cell apoptosis.[10] A recent study identified that EPSTI1 was increased in primary Sjögren's syndrome B cells, and it activated the NF-κB pathway and p-p65. However, inhibition of EPSTI1 dramatically decreased p-p65 expression and inactivated the NF-κB pathway.[11] Moreover, EPSTI1 promoted IκBα degradation and activated NF-κB signaling through interaction with VCP, whereas deficiency of EPSTI1 remarkably suppressed breast cancer cell invasion.[25] In the present study, LPS dramatically increased the ratios of p-p65/p65 and p-IκBα/IκBα, but knockdown of EPSTI1 significantly decreased these ratios. Collectively, knockdown of EPSTI1 inactivated NF-κB signaling.

In summary, the results identified that knockdown of EPSTI1 attenuated LPS-caused inflammatory injury in WI-38 cells through regulation of NF-κB signaling, which provided a potential target for the management of pneumonia at the cellular level.

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