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Knockdown of THBS1 inhibits inflammatory damage and oxidative stress in in vitro pneumonia model by regulating NF-κB signaling pathway

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Abstract
Pneumonia is an acute lower respiratory tract infection in children and the elderly. Recent studies have identified the significance of thrombospondin1 (THBS1) in inflammation. Nonetheless, the specific mechanisms by which THBS1 operates in pneumonia remain unclear. We treated 16HBE cells as an in vitro pneumonia model with lipopolysaccharide (LPS) and conducted a series of experiments to examine markers of inflammation and oxidative stress. LPS induces an increase in THBS1 expression in 16HBE cells. Knockdown of THBS1 reversed LPS-induced release of inflammatory factors [tumor necrosis factor α (TNF-α), interleukin (IL)-6, IL-1β]. Knocking down THBS1 reversed the LPS-induced increase in ROS and MDA and the decrease in SOD and GSH-Px. Inhibition of LPS led to the reversal of NF-κB pathway activation in response to LPS. Suppression of THBS1 hindered the NF-κB signaling cascade, resulting in a decrease in inflammation and oxidative stress triggered by LPS.

Introduction

Pneumonia, an acute infection affecting the lower respiratory tract, is associated with elevated levels of illness and death in both the young and the elderly. Historically, pneumonia has been a lethal infectious condition that has claimed the lives of older individuals. Because of weakened immune systems, impaired mucociliary function, diminished fever response, and varying degrees of cardiac dysfunction, the elderly population is more susceptible to contracting pneumonia.[1-3] In spite of advancements in treatment, diagnosis, and prevention, pneumonia continues to be the world's most common cause of infection-related mortality.[4,5] It is essential to understand the regulatory mechanisms underlying the inflammatory process to identify more effective targets for treating pneumonia.

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Proteomic study indicates that thrombospondin1 (THBS1) is a diagnostic marker for pneumonia and uncovers possible age-related prognostic indicators in sepsis caused by pneumonia.[6] THBS1, a versatile group of proteins found in the extracellular matrix, plays a crucial role in various biological processes such as tumor initiation, wound repair, and embryonic growth, as well as tissue restructuring. Studies suggest that THBS1 functions by suppressing the body's natural process of forming new blood vessels. In addition, THBS1 interacts with multiple cell receptors to produce specific physiological outcomes.[7,8] Studies have shown that USF2 knockdown can reduce THBS1 expression, leading to the inhibition of the transforming growth factor β (TGF-β) signaling pathway and reduction in pyroptosis in sepsis-induced acute kidney injury.[9] During acute episodes of chronic liver failure, THBS1 also increases systemic inflammation and the severity of the disease.[10] Moreover, THBS1 plays a role in reducing IL-36 gamma-induced neutrophilic inflammation during a Pseudomonas aeruginosa lung infection.[11] THBS1, however, has been very rarely been linked to pneumonia, and its exact mechanism is unknown.

In this work, we used lipopolysaccharide (LPS) stimulation to create an in vitro model of pneumonia before examining the role and mechanism of THBS1 on LPS-induced inflammatory damage in 16HBE cells.

Methods

Cell culture and LPS treatment

Originating from the BeNa Culture Collection in China, the 16HBE cell line is immortalized human bronchial epithelial cells that are cultured in high glucose Dulbecco's modified Eagle medium (DMEM-H, Gibco, Zug, Switzerland) supplemented with 10% (FBS, Gibco) and 1% antibiotics (Solarbio, Beijing, China). On July 15, STR analysis confirmed that the cells were free of contamination. The temperature of the culture is kept at 37°C, with a CO_2 level of 5%. When the cells reach a confluence of approximately 70%–80%, they were treated with LPS at a concentration of 5 μg/mL[12] (LPS, Sigma-Aldrich, Castle Hill, Australia) for 24h.

Cell transfection

Small interfering RNA (siRNA), negative control, and the THBS1 plasmid were all acquired from Shanghai GenePharma Co., Limited. To initiate cell seeding, place 1×10^5 cells into a six-well plate and cultivate them until a 60%–90% confluence is reached. Then, the cells were transfected with Beyotime's Lipo8000TM transfection reagent (Shanghai, China). The transfection mixture, consisting of 2500 ng plasmid or 37.5 pmol siRNA, was mixed with 4μL of Lipo8000 and incubated at 37°C. Experimental procedures were conducted 48 hours after transfection.

Detection of cell viability

Cell viability was determined by CCK-8 assay (Dojindo, Kumamoto, Japan) following the manufacturer's protocol and measuring the quantity of the formazan dye. The absorbance was measured at OD 450 nm using a ChroMate 4300 microplate reader (Awareness Technology, Palm City, FL, USA).

Enzyme-linked immunosorbent assay (ELISA)

The production of tumor necrosis factor alpha (TNF-α, P16599), IL-6 (EH2IL6), and ILβ(P10749) was measured according to the manufacturer's instructions using equivalent human ELISA kits (Invitrogen, Basel, Switzerland). The absorbance of each plate was measured using a ChroMate 4300 microplate reader, with the primary wavelength set at 450 nm and the backup reference wavelength at 620 nm.

Detection of oxidative stress indicators

Using the fluorescent probe dichlorodihydrofluorescein diacetate (DCFH-DA), intracellular reactive oxygen species (ROS) were quantified. After adding 10 μM DCFH-DA to the growth media and placing the cells in a dark incubator, they have to be incubated for 30 minutes at 37°C. Subsequently, the fluorescence intensity was measured following three washes with PBS using a fluorescence spectrophotometer, where the excitation and emission wavelengths were set at 488 and 535 nm, respectively. As previously mentioned, measurements of Malondialdehyde (MDA) levels, superoxide dismutase (SOD) activity, and glutathione peroxidase (GSH-Px) activity were made. The pertinent kits were provided by the Nanjing Jiangcheng Bioengineering Institute (Nanjing, China).

Western blot analysis

The total protein extraction was achieved by treating cells with RIPA lysis buffer (Beyotime, Nanjing, China). Measurement of the total protein content was done using a BCA protein assay kit (Beyotime, Nanjing, China). The standardized total protein levels of each sample (ranging from 20 to 40 μg) were separated using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis. After the separation process, the proteins were transferred onto a polyvinylidene fluoride membrane from Millipore, USA, and left at room temperature for an hour to equilibrate before being blocked with 5% skim milk. Following three rounds of TBST washes, the membranes were incubated overnight at 4°C with primary antibodies. After three additional washes, the membrane was then exposed to affinipure goat anti-rabbit IgG secondary antibody at room temperature for 1 hour (peroxidase-conjugated; ZB-5301, ZSGB-BIO, Beijing, China). The BeyoECL Star kit (Beyotime, Nanjing, China) was used to detect chemiluminescent signals. Image J 1.43 software was used to perform densitometric analysis. GAPDH served as an internal control. The primary antibodies used were THBS1 (ab267388, 1:5000), p65 (1:1000, ab32536), p-p65 (1:1000, ab76302), IκB-α (1:1000, ab230341), p-IκB-α (1:1000, ab65211) and GAPDH (ab9485, 1:1000).

Statistical analysis

Version 16.0 of the SPSS software was utilized for all statistical analyses. To express data, mean ± SD was used. The *t*-test was used to compare the two sets of data. Data from three or more groups were compared using one-way analysis of variance, followed by Tukey's post hoc test. *P*-values less than 0.05 were considered to be statistically significant.

Results

THBS1 expression is increased in in vitro pneumonia models

We opted to use LPS-induced 16HBE as an in vitro model to investigate the function of THBS1 on pneumonia. The results of the study showed that the LPS group exhibited a notably elevated level of THBS1 in contrast to the control group (Figure 1). These results indicate that the upregulation of THBS1 expression plays a crucial role in pneumonia.

Knockdown of THBS1 inhibits 16HBE inflammatory damage

Initially, we were able to effectively inhibit THBS1 expression by 50% in 16HBE cells (Figure 2A). Second, we measured the production of proinflammatory cytokines and cell viability to assess the impact of LPS on 16HBE cells. The cell viability in the control group was high. LPS decreased cell viability in comparison to the control, according to the CCK-8 assay (Figure 2B). The cells in the control group showed little inflammatory response. The levels of pro-inflammatory cytokines (TNF-α, IL-6, and IL-1β) increased in 16HBE cells after LPS treatment, according to ELISA results (Figure 2C). All of the aforementioned findings suggested that LPS caused inflammation in 16HBE cells. Remarkably, LPS-induced inflammatory damage was dramatically reduced in 16HBE cells upon THBS1 knockdown (Figure 2B, C). This demonstrates the significant involvement of THBS1 in the inflammatory injury associated with pneumonia.

Knockdown of THBS1 inhibits 16HBE oxidative stress

In order to ascertain how THBS1 affects oxidative stress in pneumonia, we assessed the amounts of ROS, MDA, SOD, and GSH-Px in cells. As demonstrated in Figure 3, LPS suppressed the levels of SOD and GSH-Px while markedly raising the levels of MDA and ROS in 16HBE cells compared with the control group. Knocking down THBS1 significantly mitigates the oxidative stress induced by LPS, indicating

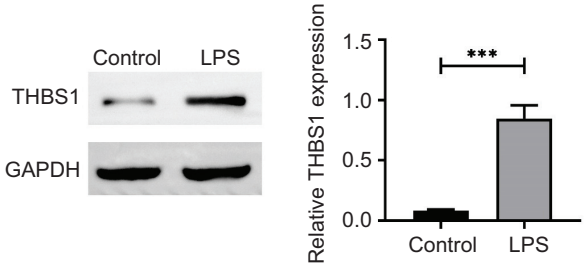

Figure 1 THBS1 expression is increased in in vitro pneumonia models. Expression of THBS1 in 16HBE cells before and after LPS treatment. Values are presented as mean ± SD. ***p < 0.001 versus control group. n=3.

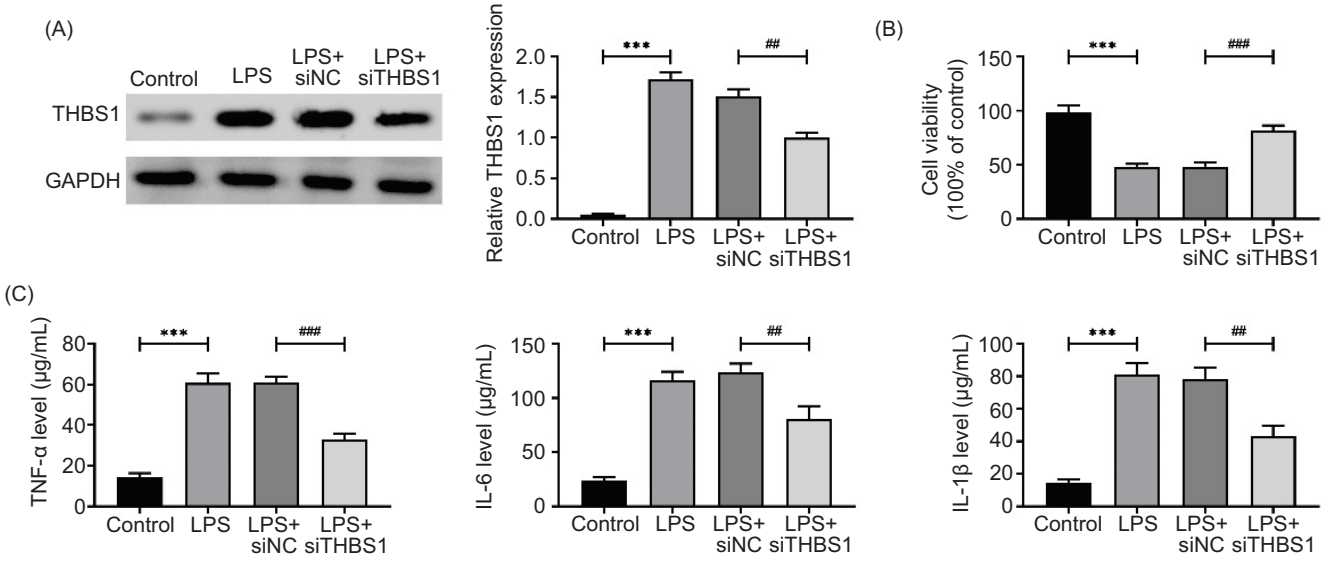

Figure 2 Knockdown of THBS1 inhibits 16HBE inflammatory damage. (A) THBS1 protein expression in 16HBE cells before and after transfection with siTHBS1. (B) CCK8 experiment to detect the survival rate of 16HBE cells. (C) ELISA detects the content of inflammatory factors in cell supernatants. Values are presented as mean ± SD. ***p < 0.001 versus control group. ##p < 0.01, ###p < 0.001 versus LPS+siNC group. n =3.

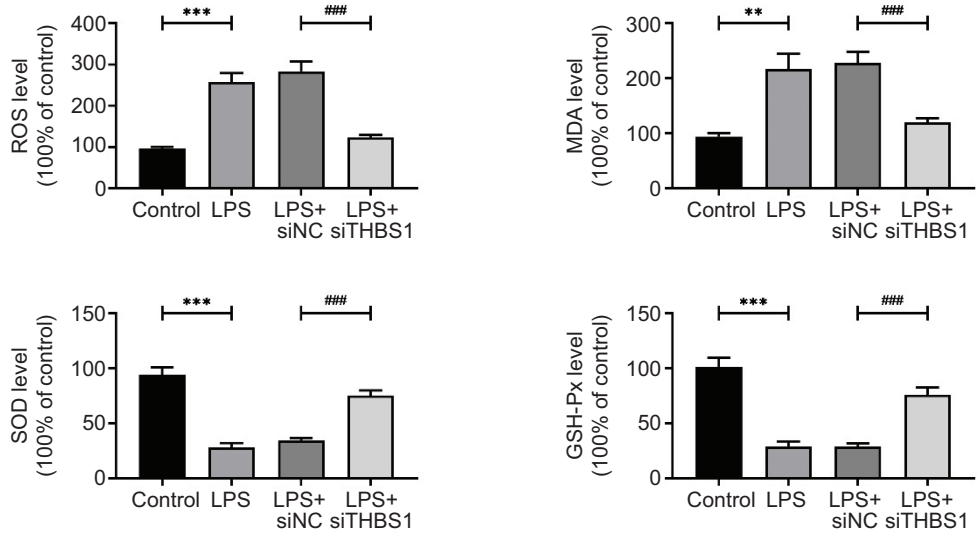

Figure 3 Knockdown of THBS1 inhibits 16HBE oxidative stress. DCFH-DA measures intracellular redox indicators (ROS, MDA, SOD, and GSH-Px). Values are presented as mean ± SD. **p < 0.01, ***p < 0.001 versus control group. ###p < 0.001 versus LPS+siNC group. n =3.

the crucial involvement of THBS1 in the oxidative stress response in pneumonia.

Knocking down THBS1 inhibits the NF-κB pathway

By means of Western blotting analysis, we successfully detected the protein levels of p65, p-p65, IκB-α, and p-IκB-α in 16HBE cells. The findings demonstrated that p-p65 and p-IκB-α expression were suppressed with THBS1 knockdown during inflammatory activation (Figure 4). It has been postulated that THBS1 activates the NF-κB pathway in cases of pneumonia.

Discussion

Pneumonia is a major global health issue, given its substantial morbidity and significant financial burden. The condition can develop and deteriorate because of an exaggerated inflammatory response, especially in the elderly population.[13] The outer membrane of Gram-negative bacteria, also known as LPS, has been widely employed in the development of in vitro pneumonia models because of its capacity to induce inflammatory responses.[14] Our findings in this investigation confirmed that LPS caused oxidative stress and inflammatory damage in 16HBE cells, which manifested as decreased levels of pro-inflammatory cytokines

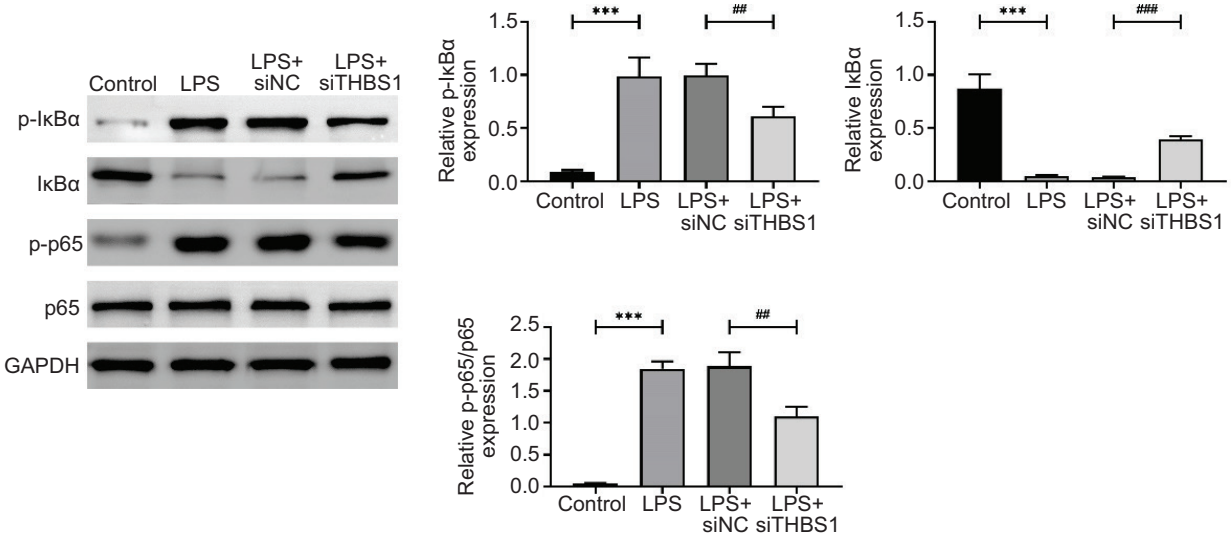

Figure 4 Knocking down THBS1 inhibits the NF-κB pathway. Western blotting is employed to detect p65, p-p65, IκB-α, and p-IκB-α protein expression in 16HBE cells. Values are presented as mean ± SD. ***p < 0.001 versus control group. ##p < 0.01, ###p < 0.001 versus LPS+siNC group. n =3.

(IL-6, TGF-α, and IL-1β) and suppression of cell viability. The levels of MDA and ROS increased, although GSH-Px and SOD levels decreased. By blocking the NF-κB pathway, knocking down THBS1 can reduce oxidative stress and inflammatory damage brought on by LPS in 16HBE cells.

Pneumonia has been associated with inflammation of the lung for a long time.[15] While research has shown the anti-inflammatory effects of THBS1 in the development of COVID-19 and the consequences of sepsis, its specific anti-inflammatory role in pneumonia remains to be fully understood.[16,17] In the current investigation, reduction of THBS1 also decreased LPS-induced 16HBE cell levels of TNF-α, IL-6, and IL-1β, which is consistent with published findings showing that THBS1 suppresses the production of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β in sepsis complications. As a result, it is evident that decreasing THBS1 levels can lower the incidence of pneumonia by inhibiting the production of inflammatory cytokines.

The production of ROS is crucial for inflammation.[18] Under normal circumstances, the levels of ROS will increase while the activity of the antioxidant system decreases, resulting in an immediate onset of oxidative imbalance known as oxidative stress. Under pathological conditions, on the other hand, ROS levels will fall and antioxidant system activity will rise.[19] Oxidative stress plays a crucial role in the development of inflammatory conditions like inflammation of the lung.[20] This investigation confirmed the impact of THBS1 on oxidative stress by measuring ROS and antioxidant enzyme levels (such as MDA, GSH-Px, and SOD). As indicated in the research findings, the presence of LPS in pneumonia resulted in a significant decline in SOD and GSH-Px levels, while causing an elevation in the concentrations of ROS and MDA. The decrease in SOD and GSH-Px activities, as well as the generation of MDA and ROS, were effectively alleviated through the suppression of THBS1 expression.

Through the NF-κB pathway, ROS control inflammatory responses.[21] IκBα is phosphorylated by the active IκB kinase complex (IKK), which causes IκBα to be ubiquitinated and degraded. Following liberation from the p65-p50/IκBα complex, the p65-p50 subunit migrates to the nucleus, thereby promoting the secretion of proinflammatory cytokines.[22] To evaluate the influence of THBS1 on the NF-κB signaling pathway post exposure to LPS, the expression levels of IκB-α and p65 were examined in 16HBE cells. The results indicated that depletion of THBS1 led to a reduction in the phosphorylation levels of IκB-α and p65. This implies that by controlling the NF-κB signaling pathway, THBS1 reduces inflammation in pneumonia.

This article has certain limitations, that is, it has not been verified in in vivo and clinical experiments. In the future, we will further conduct in-depth research in vivo and clinically. Our study is also important for future in vivo research and clinical applications.

Conclusion

To sum up, our research indicates that in vitro pneumonia models exhibit increased expression of THBS1. Knocking down THBS1 blocks the NF-κB signaling pathway, thereby reducing inflammation and oxidative damage brought on by LPS. Our results add to our understanding of the pathophysiology of pneumonia and the role of THBS1.

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Not applicable.

Ethics Approval

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

Consent to Participate Statement

Written informed consent was obtained from legally authorized representative(s) for anonymized patient information to be published in this article.

Data Availability

The authors declare that all data supporting the findings of this study are available within the paper and any raw data can be obtained from the corresponding author upon request.

Authors Contributions

Fang Wang and Rixi Xie designed the study, analyzed the data, interpreted the data, and prepared the manuscript for publication and reviewed the draft of the manuscript. Fang Wang supervised the data collection. Both authors have read and approved the manuscript.

Conflicts of Interests

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

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