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ORIGINAL ARTICLE

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Knocking down TNFAIP1 alleviates inflammation and oxidative stress in pediatric pneumonia through PI3K/Akt/Nrf2 pathway

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Abstract

Background: Pneumonia is an acute respiratory infection with increasing global incidences. Children are more susceptible to pneumonia than adults, and its incidences grow extremely high during peak seasons. Thus, it is necessary to investigate the pathogenesis and molecular mechanism of childhood pneumonia.

Methods: This study examined the role of tumor necrosis factor alpha-inducible protein 1 (TNFAIP1) in lipopolysaccharide (LPS)-induced pneumonia mice. After LPS exposure, lung function, TNFAIP1 activation, infarction volume, oxidative stress, lung tissue apoptosis ratio, and inflammatory response were assessed by immunohistochemistry staining, hematoxylin and eosin staining, Western blot analysis, terminal deoxynucleotidyl transferase dUTP nick end labeling assay, and enzyme-linked-immunosorbent serologic assay, respectively. The mechanism of TNFAIP1 regulating phosphoinositide 3-kinases (PI3K)-protein kinase B (Akt)-nuclear factor erythroid 2-related factor 2 (Nrf2) pathway was analyzed by Western blot analysis.

Results: TNFAIP1 expression was enhanced in the LPS-induced pneumonia mice but was negatively correlated with the LPS-induced lung injury. Silencing TNFAIP1 alleviated inflammatory response, production of reactive oxygen species (ROS), and cellular apoptosis in LPS-induced pneumonia. Moreover, PI3K/Akt/Nrf2 signaling pathways were predominantly involved in the TNFAIP1-mediated lung injury, which also played a role in the process of LPS-induced pneumonia.

Conclusion: This study suggested that TNFAIP1 acted as a negative regulator of acute pneumonia by attenuating inflammatory response, production of ROS, and cellular apoptosis via PI3K/Akt/Nrf2 pathway. The findings suggested that TNFAIP1 is a potential candidate for pneumonia therapy.

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Introduction

Pneumonia is an acute respiratory infection with increasing global incidences. Children are more susceptible to pneumonia than adults, and the incidences grow extremely high during peak seasons.¹ Pathogens or microorganisms that cause pneumonia trigger the release of inflammatory factors in the immune system.^{2,3} In addition, pulmonary infection often causes the infection of respiratory tract with clinical manifestations of pneumonia, bronchitis, etc.⁴ In severe cases, the infection accumulates in multiple organs, leading to complications, such as encephalitis, myocarditis, and hepatitis.⁵ Activated neutrophils propagate inflammation and injury by producing reactive oxygen species (ROS) and proteolytic enzymes.⁶ Oxidative stress is involved in the pathogenesis of numerous lung diseases through modulating the signaling pathways that control lung inflammation response.⁷ Exploring the status of oxidative stress and release of inflammatory factor in children with acute pneumonia could determine their possible role in pediatric pneumonia-induced lung injury.^{7,8} However, the clinical treatment of pediatric pneumonia is still complex and the pathogenesis remains unclear. Thus, it is necessary to explore the molecular mechanism of pediatric pneumonia. Tumor necrosis factor alpha-inducible protein 1 (TNFAIP1) is a BTB-domain (also known as POZ domain) protein that plays a critical role in cell motility, DNA synthesis, and cellular apoptosis.⁹⁻¹¹ Previous study has shown that the knockdown of TNFAIP1 could prevent bis(2-ethylhexyl) phthalate-induced neurotoxicity by activating the cAMP-response element binding protein (CREB) pathway.¹² Furthermore, TNFAIP1 mediates production of ROS and inflammatory response in myocardial ischemia/reperfusion injury by regulating Akt/GSK-3 β /nuclear factor erythroid 2-related factor 2 (Nrf2).¹³ However, the function of TNFAIP1 in pediatric pneumonia has not been reported. Therefore, this study aimed to explore the function and specific mechanism of TNFAIP1 in pediatric pneumonia. The *in vitro* and *in vivo* studies demonstrated that TNFAIP1 was up-regulated in lung tissues and pneumonia cells of lipopolysaccharide (LPS)-induced pneumonia mice. Knockdown of TNFAIP1 could improve pneumonia cell viability, reduce cell apoptosis, and suppress pro-inflammatory factor secretion and oxidative stress to alleviate LPS-induced acute lung injury. Mechanistic studies also indicated that TNFAIP1 could regulate PI3K/Akt/Nrf2 pathway.¹⁴

Methods

Animal model

All animal experiment procedures were approved by the Anhui Provincial Children's Hospital and were conducted in accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male C57BL/6 mice (1 week old, weight 4-5 g) were purchased from Shanghai SLAC Laboratory Animal Co. Ltd. (Shanghai, China) and housed with 12-h light and dark cycle per day at 22°C. Mice were treated intraperitoneally with saline or 2-mg LPS/kg. Mice were randomly divided into two groups,

with 10 animals in each of the following groups: LPS+AAV shTNFAIP1 and LPS+AAV. Short hairpin RNA (shRNA) was used to silence TNFAIP1 expression. Adeno-associated virus (AAV) vectors carrying shRNA targeting mouse TNFAIP1 (AAV shTNFAIP1) and control AAV-CMV-EGFP (AAV-control) were obtained from Taitool Biotechnology Co. Ltd. (Shanghai, China). The mice were anesthetized with isoflurane (3.0% for induction and 1.5% for maintenance). The AAV and AAV shTNFAIP1 were bilaterally injected into the lung region. Mice in each group were treated intraperitoneally with saline or 2-mg/kg LPS. After experiments, animals were sacrificed at 8 h post-injection, and plasma and lungs were stored for further experiments.

Western blot analysis

The lung tissues were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer. Proteins from lysates were separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose membranes (Bio-Rad, CA, USA). After being blocked with 5% skimmed milk, all membranes were incubated with primary antibodies overnight at 4°C against TNFAIP1 (1:2000, PA5-80141; Thermo Fisher Scientific, CA, USA), phosphatidylinositol 3-kinase (PI3K, 1:8000, ab191606; Abcam, MA, USA), phospho (p)-PI3K (1:2000, D-4; Santa Cruz Biotechnology, Santa Cruz, USA), protein kinase B (Akt [PKB/Akt], 1:8000, sc-81434; Santa Cruz Biotechnology), p-Akt (1:2000, ab18206; Abcam), nuclear factor erythroid 2-related factor 2 (Nrf2, 1:5000, ab31163; Abcam), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:10,000, ab8245; Abcam). Reactivity was determined using horseradish peroxidase (HRP)-conjugated secondary antibodies, and visualized using enhanced chemiluminescence.

Immunohistochemistry

The lung tissues from mice were frozen and sliced into 35- μ m-thick sections. All lung sections were permeabilized with 0.2% Triton X-100 and blocked with 1% bovine serum albumin (BSA) for 1 h at room temperature. Then, all sections were incubated overnight with primary anti-TNFAIP1 (1:200, ab196659; Abcam) antibody at 4°C. Next, lung sections were incubated with Alexa-conjugated secondary antibody (1:500; Abcam) for 90 min at room temperature. Lung sections were mounted on a glass slide and covered with 4',6-diamidino-2-phenylindole (DAPI)-containing mounting solution. Fluorescence microscope was used to capture the complex images of pneumonia's pathogenesis.

Hematoxylin and eosin (H&E) staining

Lung tissues were collected from each group and stained with Harris' hematoxylin solution for 6 h at 70°C. Then, the tissues were differentiated twice by 10% acetic acid and 85% ethanol. Next, the tissues were put in a saturated lithium carbonate solution for 12 h. Finally, the sections were stained with eosin Y ethanol solution, and all images of slices were captured with a microscope.

Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay

Lung samples of mice were collected after experiments. The lung tissue sections were fixed with 4% paraformaldehyde. After washing with phosphate-buffered saline solution (PBS), the sections were stained with the TUNEL assay kit (Abcam) according to manufacturer's instructions. The staining was analyzed under a microscope and apoptotic index was calculated as a ratio of apoptotic cell number/total cell number in each field.

Enzyme-linked-immunosorbent serologic assay (ELISA)

Inflammatory cytokines, including tumor necrosis factor α (TNF- α), interleukin 1 β (IL-1 β), and IL-6 in serum or lung tissues of each group were measured using ELISA according to the manufacturer's protocol (R&D Systems, MN, USA). Levels of oxidative-related productions, including erythrocyte malondialdehyde (MDA) and the activities of superoxide dismutase (SOD), glutathione (GSH), and catalase (CAT) in the lung tissues of each group were measured using ELISA according to the manufacturer's protocol at room temperature.

Statistics

All experiments were conducted in triplicate and analyzed using GraphPad Prism Software 7 (La Jolla, CA, USA). Quantitative data are presented as mean \pm Standard deviation (SD). One-way analysis of variance (ANOVA) and Student's *t*-test were used to make a comparison. $P < 0.05$ was considered as statistically significant.

Results

TNFAIP1 expression is elevated in LPS-induced pneumonia mice

In order to identify changes in TNFAIP1 expression in acute pneumonia, this study established a pneumonia mice model by LPS injection, and inflammatory response of the lungs was assessed. There was an enhanced expression of TNFAIP1 in the lung tissues of LPS-induced pneumonia mice (Figure 1A). Immunohistochemistry staining revealed that TNFAIP1 was expressed in the lung tissues of mice after LPS induction (Figure 1B). H&E staining confirmed that pulmonary edema in the LPS treatment group was more prominent than in the sham group (Figure 1C). Therefore, the results confirmed that TNFAIP1 expression is enhanced in the LPS-induced pneumonia mice.

Knockdown of TNFAIP1 alleviates LPS-induced lung injury in mice

In order to determine whether TNFAIP1 was related to LPS-induced acute pneumonia, we constructed

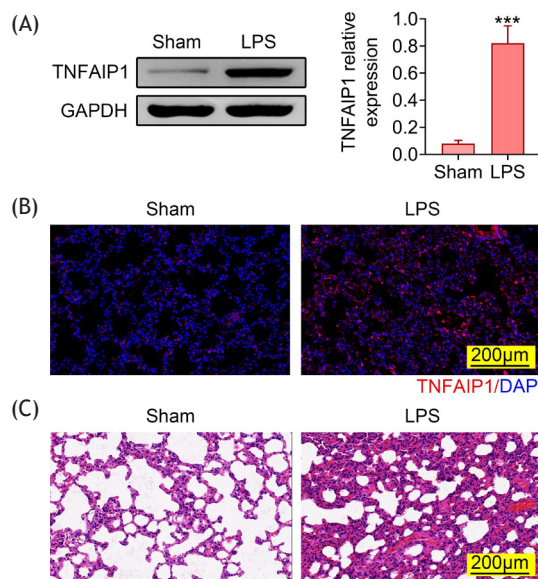


Figure 1 The expression of TNFAIP1 in LPS-induced pneumonia mice. (A) The TNFAIP1 expression was determined by Western blot analysis. (B) The expression of TNFAIP1 in lung tissues was evaluated by immunohistochemistry staining. (C) The pulmonary edema was measured by H&E staining. *** $P < 0.005$ vs sham. Data are expressed as mean \pm SD.

adeno-associated viruses to knockdown the expression of TNFAIP1 (AAV shTNFAIP1 and AAV were presented as a sham group), and injected intratracheally into mice. The Western blot analysis showed that the level of TNFAIP1 was obviously raised in lung tissues of the pneumonia mice in the LPS treatment group, while knockdown of TNFAIP1 in LPS+AAV shTNFAIP1 group decreased the TNFAIP1 expression (Figure 2A). The immunohistochemistry staining suggested that the expression of TNFAIP1 was obviously decreased in LPS+AAV shTNFAIP1 group (Figure 2B). The H&E staining revealed LPS-induced structural injury of lung tissues in mice, accompanied with shrinking of the alveoli and damage of lung tissues. Conversely, LPS+AAV shTNFAIP1 could remarkably alleviate the above-mentioned pathological changes induced by LPS (Figure 2C). Moreover, the present study also evaluated apoptosis rate with a TUNEL assay in the lung tissues of LPS-induced mice after AAV shTNFAIP1 treatment. As expected, LPS promoted the rate of apoptosis in lung tissues, which was reversed by AAV shTNFAIP1 transfection (Figures 2D and 2E). These results showed that TNFAIP1 protected LPS-induced lung injury in pneumonia mice.

TNFAIP1 levels correlate positively with inflammation in LPS-induced pneumonia mice

In order to further explore the role of TNFAIP1 in LPS-induced inflammatory response, the protective effect of silenced TNFAIP1 on inflammation in the lung tissue of mice after LPS exposure was determined. The expressions of inflammation-related factors (IL-6, IL-1 β , and TNF- α) in serum and lung tissues were all remarkably induced after LPS exposure, and the release of IL-6, IL-1 β , and TNF- α was

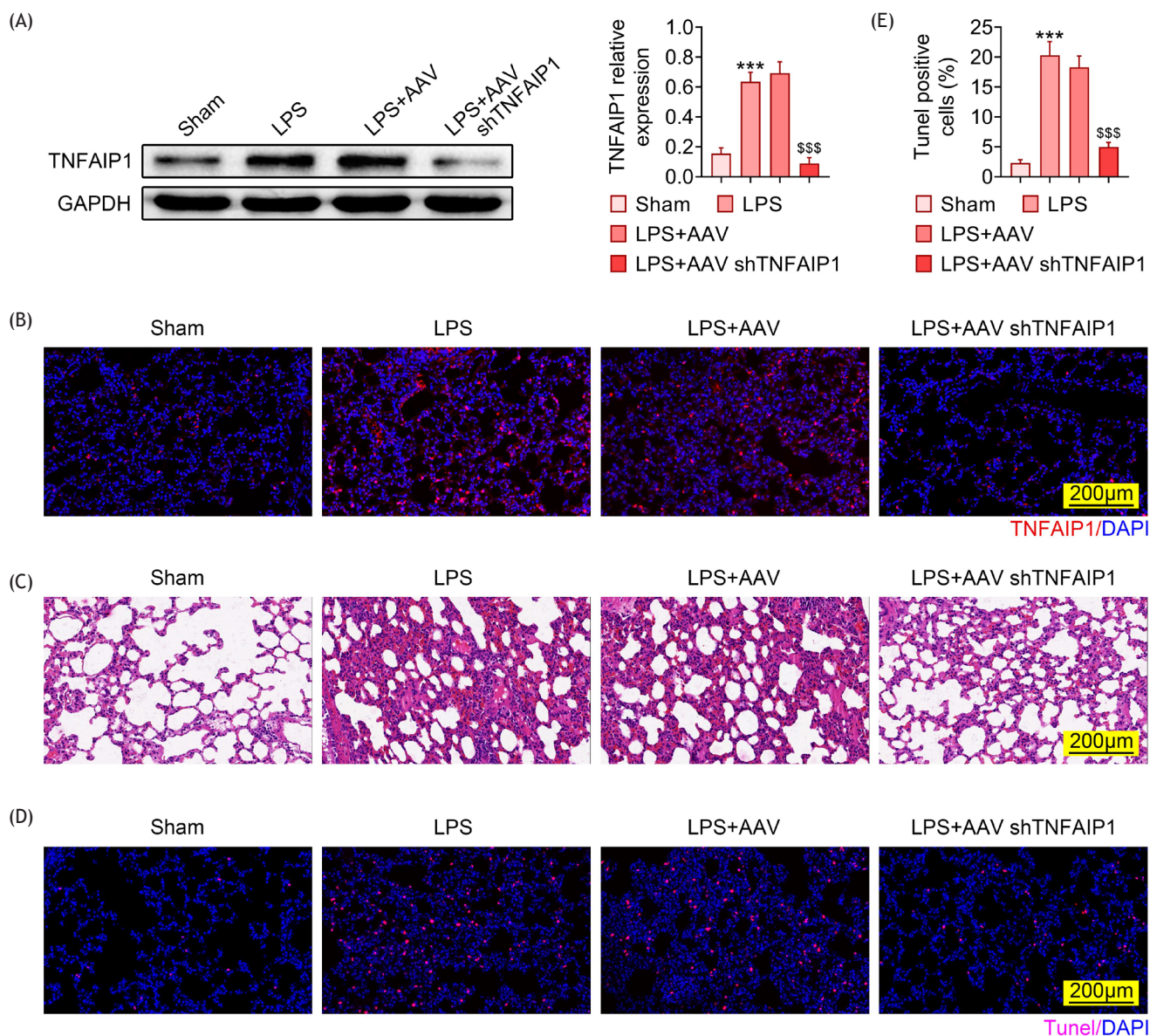


Figure 2 Effects of TNFAIP1 on LPS-induced lung injury in mice. (A) The mice were injected with AAV or AAV-shTNFAIP1 before LPS treatment. The TNFAIP1 expression was determined by Western blot analysis. (B) The expression of TNFAIP1 in lung tissues was evaluated by immunohistochemistry staining. (C) The pulmonary edema was measured by H&E staining. (D) Cell apoptosis of lung tissues was evaluated by TUNEL assay. *** $P < 0.005$ vs sham. \$\$\$ $P < 0.005$ vs LPS+AAV. Data are expressed as mean \pm SD.

obviously attenuated after TNFAIP1 knockdown (Figures 3A and 3B). The results indicated that silenced TNFAIP1 alleviated inflammatory response by inhibiting the secretion of inflammatory cytokine in LPS-induced pneumonia.

Knockdown of TNFAIP1 attenuated LPS-induced oxidative stress

Next, we explored the effect of TNFAIP1 knockdown on regulating LPS-induced oxidative stress. The expression of ROS-related proteins was determined by ELISA. The results of ELISA are shown in Figure 4. The expressions of SOD, GSH, and CAT in lung tissues were remarkably decreased after exposure to LPS, while the activation of MAD was

enhanced after LPS exposure. However, the abnormal expressions of these antioxidant enzymes and biomolecules in pneumonia mice lung tissues were ameliorated by the knockdown of TNFAIP1 (Figure 4). These results suggested that ROS production was enhanced by exposure to LPS. Downregulation of TNFAIP1 by shTNFAIP1 significantly inhibited oxidative stress.

TNFAIP1 interacted with PI3K/Akt/Nrf2 pathway

In order to investigate the potential molecular mechanism underlying the biofunction of TNFAIP1, the present study explored PI3K/Akt/Nrf2 pathway after TNFAIP1 knockdown. Western blot analysis was applied to identify the role of

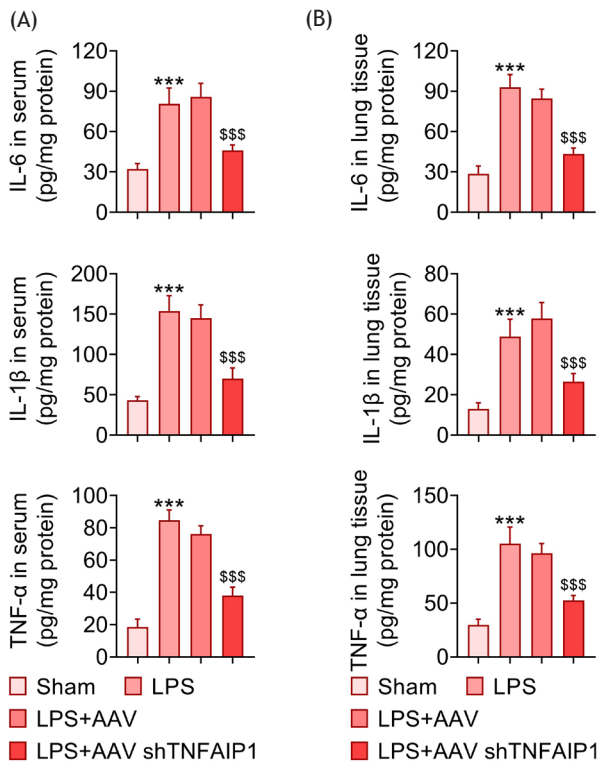


Figure 3 Effect of TNFAIP1 on LPS-induced inflammatory response in mice. (A) The expression level of inflammatory cytokines, including IL-6, IL-1 β , and TNF- α , in serum was measured by commercial ELISA kit. (B) The expression level of inflammatory cytokines, including IL-6, IL-1 β , and TNF- α , in lung tissues were measured by commercial ELISA kit. *** $P < 0.005$ vs sham. \$\$\$ $P < 0.005$ vs LPS+AAV. Data are expressed as mean \pm SD.

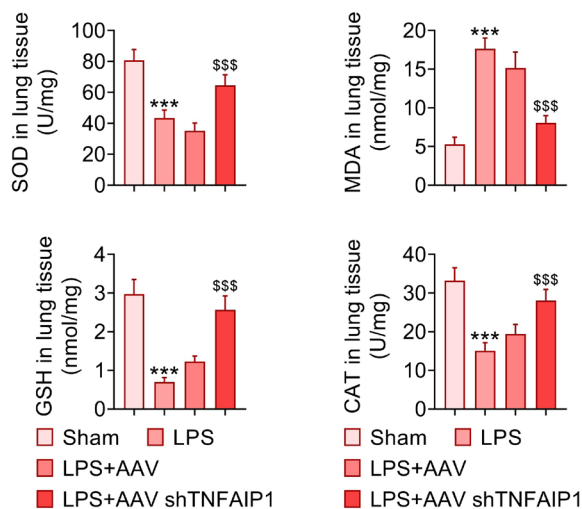


Figure 4 Effect of TNFAIP1 on LPS-induced oxidative stress in mice. The expression levels of oxidative stress-related protein expressions, including SOD, MDA, GSH, and CAT, in serum were measured by commercial ELISA kit. *** $P < 0.005$ vs sham. \$\$\$ $P < 0.005$ vs LPS+AAV. Data are expressed as mean \pm SD.

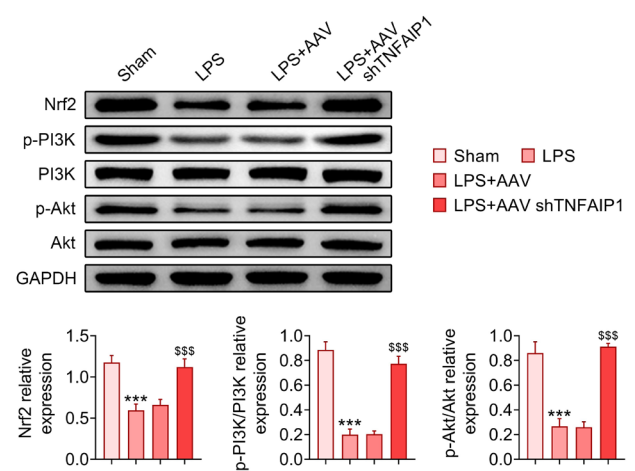


Figure 5 TNFAIP1 modulated LPS-induced pneumonia in mice via regulating PI3K/Akt/Nrf2 pathway. The protein expressions of p-AKT, p-PI3K, and Nrf2 in pneumonia mice lung tissues were examined by Western blot analysis. *** $P < 0.005$ vs sham. \$\$\$ $P < 0.005$ vs LPS+AAV. Data are expressed as mean \pm SD.

TNFAIP1 silencing in PI3K/Akt/Nrf2 signaling pathway to modulate the expression of p-AKT, p-PI3K, and Nrf2 in pneumonia mice lung tissues. As illustrated in Figure 5, the expression levels of p-AKT, p-PI3K, and Nrf2 were suppressed after LPS exposure. In contrast, the levels of p-AKT, p-PI3K, and Nrf2 in the lung tissues of LPS+AAV shTNFAIP1 group were obviously enhanced, compared to the LPS and LPS+AAV group. These results demonstrate that PI3K/Akt/Nrf2 signaling pathway is predominantly involved in the TNFAIP1-mediated lung injury, which also plays a role in LPS-induced pneumonia.

Discussion

Pneumonia is a widespread infectious disease of children with increasing global incidences and mortality.¹⁵ The pathogenesis of pneumonia is complex, which severely affects the functioning of lung tissues, and it is often related to inflammatory response and impaired functioning of lung tissues.

Pneumonia is most commonly characterized by inflammation-induced alveolar capillary membrane injury and secretion of inflammatory cytokines, which contribute to lung tissue dysfunction.^{16,17} Therefore, it's urgent to explore new and beneficial therapeutic targets for pneumonia. This study aimed to investigate whether TNFAIP1 is a specific target that plays a protective effect in LPS-induced pneumonia mice.

The results of the present study showed that TNFAIP1 was highly expressed in LPS-induced pneumonia mice. Knockdown of TNFAIP1 attenuated the damage of LPS on lung tissues and decreased apoptosis ratio, oxidative stress, and inflammatory response, and triggered the activation of PI3K/Akt/Nrf2 signaling pathway. Mechanistically, the silencing of TNFAIP1 occurred against LPS-induced lung injury via PI3K/Akt/Nrf2 signaling pathway.

The expression of TNFAIP1 is enhanced in cells in response to multiple detrimental stimuli and acts as a crucial inflammation regulator by modulating nuclear factor kappa B (NF- κ B) activity.¹⁸ The expression level of TNFAIP1 was enhanced in the neurons upon amyloid-beta, formaldehyde, or di-(2-ethylhexyl) phthalate treatment.¹³ TNFAIP1 reportedly promotes neurotoxicity and is overexpressed in the brain of Alzheimer's disease *in vivo*.¹⁹ A previous study indicated that the enhanced TNFAIP1 expression promoted myocardial ischemia/reperfusion injury.¹³ In pneumonia, inflammatory response and cytokines play a critical role in the progression of pathogenesis.

A recent study has reported that the suppression of TNFAIP1 attenuated NF- κ B activation and inhibited the secretion of proinflammatory cytokines in the myocardial tissue.¹³ Although TNFAIP1 has been shown to induce anti-inflammatory effect in the progression of sepsis,²⁰ the anti-inflammatory mechanism of TNFAIP1 in pneumonia remains unclear. Multiple animal studies have reported that treatment with LPS triggers an acute inflammatory response and causes early histopathological injury in the lung.^{21,22} Thus, we used the LPS-induced pneumonia animal model to study pulmonary inflammation. The results showed that silencing of TNFAIP1 attenuated the production of pro-inflammatory cytokines, such as IL-6, IL-1 β , and TNF- α , in LPS-induced pneumonia lung tissues. TNFAIP1 acts as a crucial regulator for production of ROS.¹⁹ Oxidative stress is caused by the imbalance of oxidation and is associated with the pathophysiology of pulmonary inflammation.²³ In SH-SY5Y cells, upregulation of TNFAIP1 could enhance the amyloid-beta-induced ROS production.¹⁹ In agreement with the finding, this study indicated that knockdown of TNFAIP1 effectively alleviated the LPS-induced oxidative stress.

PI3K/Akt/Nrf2 signaling has a key role in regulating cell survival, cellular apoptosis, oxidative stress, and inflammatory response in numerous diseases.^{24,25} The Nrf2 signaling pathway is an antioxidant intracellular defense system that effectively protects cells against oxidative injury.²⁶ The activation of PI3K/Akt signaling pathway has been associated with suppressing oxidative stress and attenuating apoptosis in endothelial cells.²⁷ However, it remains unknown whether TNFAIP1 regulates PI3K/Akt/Nrf2 pathway to attenuate inflammatory and oxidative stress in pneumonia. Therefore, this study investigated the cross-regulation between TNFAIP1 silencing and PI3K/Akt/Nrf2 signaling pathway in the lungs of pneumonia mice. LPS-induced pneumonia mice exhibited a significant downregulation of pulmonary Nrf2, PI3K, and Akt phosphorylation. However, the knockdown of TNFAIP1 could reverse this effect. These findings suggested the involvement of PI3K/Akt/Nrf2 signaling pathway in protecting effects of TNFAIP1 on LPS-induced lung injury. In addition, it is supported by the studies showing the effect of TNFAIP1 silencing on PI3K/Akt/mTOR pathway in LPS-induced pneumonia.

The present study has some limitations. It focused on TNFAIP1/PI3K/Akt/Nrf2 pathway, and the other molecular mechanisms involved in the protective effect of TNFAIP1 are still unknown. It should be noted that the pathological mechanism of pneumonia is a complicated procedure, and this study was conducted on mice; hence, the results should be confirmed in clinical studies in the future.

Conclusion

The results in this study suggested that TNFAIP1 acted as a negative regulator of pneumonia by attenuating oxidative stress, inflammatory response, and cellular apoptosis via PI3K/Akt/Nrf2 pathway. These findings suggest that TNFAIP1 can be explored as a potential therapeutic target for pneumonia therapy.

Availability of Data and Materials

All data generated or analyzed in this study are included in this published article. The datasets used and/or analyzed are available from the corresponding author on reasonable request.

Competing interests

The authors stated that they had no conflict of interest to disclose.

Ethical approval

Ethical approval was obtained from the Ethics Committee of Anhui Provincial Children's Hospital (Approval No. 2021-155).

Author Contributions

Jing Chen designed the study, completed the experiment, and supervised data collection. Mengtian Zhao analyzed and interpreted the data. Wei Fang and Chaojun Du prepared and reviewed the draft of the manuscript for publication. All authors read and approved the final manuscript.

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