Allergologia et immunopathologia
Sociedad Española de Inmunología Clínica, Alergología y Asma Pediátrica
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ORIGINAL ARTICLE

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DEPTOR alleviates LPS-induced inflammation and ER stress in WI-38 cells

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Received 8 April 2025; Accepted 3 June 2025
Available online 1 July 2025

KEYWORDS
endoplasmic reticulum stress;
DEPTOR;
inflammation;
LPS;
WI-38 cells

Abstract
Background: Pediatric pneumonia is a severe inflammatory condition frequently precipitated by bacterial endotoxins, such as lipopolysaccharide (LPS), which can elicit oxidative stress, endoplasmic reticulum (ER) stress, and apoptotic cell death. DEP domain-containing mTOR-interacting protein (DEPTOR), an endogenous inhibitor of mTOR signaling, has been implicated in the regulation of inflammation and ER homeostasis. However, its specific function in the pathogenesis of pneumonia remains poorly defined.
Methods: WI-38 human fetal lung fibroblast cells were employed to establish an *in vitro* model of LPS-induced inflammation. DEPTOR was overexpressed via plasmid transfection to examine its functional role. The impact of DEPTOR on pro-inflammatory cytokine release, oxidative and ER stress responses, apoptosis, and nuclear factor *kappa B* signaling was comprehensively evaluated using quantitative real-time polymerase chain reaction, Western blot analysis, enzyme-linked-immunosorbent serologic assay, flow cytometry, and biochemical assays.
Results: DEPTOR expression is significantly downregulated in LPS-stimulated WI-38 cells ($P < 0.01$). DEPTOR overexpression markedly suppresses LPS-induced pro-inflammatory cytokine production ($P < 0.01$), ameliorates oxidative and ER stress—as indicated by decreased lipid peroxidation and restoration of superoxide dismutase and *glutathione* levels ($P < 0.01$)—and inhibits apoptosis, reducing apoptotic cell percentages by over 10% ($P < 0.01$).
Conclusion: These results suggest that DEPTOR confers a protective role against LPS-induced cellular injury, supporting its potential as a promising therapeutic target for mitigating

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https://doi.org/10.15586/aei.v53i4.1380

Introduction

Globally, pneumonia remain one of the leading causes of morbidity and mortality among children aged <5 years.[1] It is characterized by clinical manifestations, such as persistent cough, fever, dyspnea, and respiratory distress. In severe cases, pediatric pneumonia may escalate into febrile seizures, respiratory failure, and cardiac dysfunction, emphasizing the urgent need for more effective and targeted therapeutic strategies.[2,3] Among the common etiological factors, Gram-negative bacteria and their endotoxins—particularly lipopolysaccharide (LPS)—are well-established mediators of acute lung injury (ALI) and potent inducers of systemic inflammation in both clinical and experimental pneumonia models.[4,5] The nuclear factor *kappa B (*NF-κB) signaling pathway plays a pivotal role in orchestrating inflammatory responses and is a critical regulator of pneumonia progression, necessitating the identification of novel molecular targets to modulate this pathway effectively.

Lung fibroblasts, while not primary immune effector cells, are essential components of pulmonary microenvironment. They actively contribute to inflammation by secreting cytokines (e.g., interleukin 6 [IL-6]) and participate in extracellular matrix remodeling during lung injury. Their ability to engage in crosstalk with immune and epithelial cells through paracrine signaling makes them an indispensable model for studying pneumonia-related inflammation. WI-38 human fetal lung fibroblast cells, derived from normal human lung tissue, serve as a robust *in vitro* model to investigate inflammatory responses and cytokine production relevant to pulmonary pathology.

Endoplasmic reticulum (ER) stress has emerged as a crucial pathological contributor to several inflammatory conditions, including pneumonia.[6] The ER is integral to protein synthesis, folding, and trafficking; however, perturbations in ER homeostasis lead to the accumulation of misfolded proteins, triggering the unfolded protein response (UPR).[7] While initially cytoprotective, chronic or overwhelming ER stress exacerbates inflammation and promotes apoptosis.[8] Recent studies have suggested that pharmacological or genetic attenuation of ER stress mitigates LPS-induced pulmonary inflammation and cellular injury, highlighting ER stress as a viable therapeutic target in pneumonia management.[9]

DEP domain-containing mammalian target of rapamycin (mTOR)-interacting protein (DEPTOR), a negative regulator of mTORC1 and mTORC2 complexes, plays a multifaceted role in regulating cellular processes, including proliferation, apoptosis, autophagy, and ER homeostasis.[10] In hepatic models, DEPTOR overexpression has been shown to alleviate inflammatory injury in alcoholic liver disease by suppressing mTORC1 signaling.[11,12] Furthermore, emerging data suggest that DEPTOR modulates immune responses via the mTOR/NF-κB axis and mitigates ER stress in conditions such as asthma and allergic rhinitis.[13] However, the precise role and mechanistic action of DEPTOR in LPS-induced pulmonary inflammation, particularly in lung fibroblasts, remain largely undefined.

In this study, we explore the functional role of DEPTOR in LPS-stimulated WI-38 cells, focusing on its regulation of inflammatory cytokines, oxidative stress, ER stress markers, and apoptosis, as well as its involvement in NF-κB signaling. Our aim is to determine the potential of DEPTOR as a novel therapeutic modulator in pediatric pneumonia and to provide mechanistic insights that could inform future clinical interventions.

Materials and Methods

Cell culture

Human lung fibroblast WI-38 cells were obtained from the *American Type Culture Collection* (ATCC; Manassas, VA, USA) and cultured in Dulbecco's modified eagle *medium* (DMEM) (Thermo Fisher Scientific, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Miami, FL, USA). Cells were maintained at 37°C in a humidified incubator with 5% CO_2. WI-38 cells were authenticated by short tandem repeat (STR) profiling and confirmed as free of mycoplasma (MycoAlert™; Lonza, Basel, Switzerland).

LPS treatment and plasmid transfection

To establish an inflammatory model, WI-38 cells were treated with LPS at a concentration of 10 μg/mL (ST1730; Beyotime Biotechnology, China) for 12 h. For gene overexpression studies, cells were transfected with a human DEPTOR overexpression plasmid (Addgene, MA, USA) or an empty vector control (pCDNA3.1) using Lipofectamine™ 3000 transfection reagent (Cat# L3000008; Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted using RNAiso Plus reagent (Cat# 9109; Takara, Japan) and reverse-transcribed using a cloning DNA (cDNA) synthesis kit (Cat# RR047A; Takara). qRT-PCR was performed using SYBR Green PCR Master Mix (Cat# RR820A; Takara) on a QuantStudio™ 5 RT-PCR system (Applied Biosystems, MA, USA). Primers were: tumor necrosis factor-α (TNF-α): F: CCTCTCTCTAATCAGCCCTCTG; R: GAGGACCTGGGAGTAGATGAG; IL-6: F: ACTCACC TCTTCAGAACGAATTG; R: CCATCTTTGGAAGGTTCAGGTTG; IL-1β: F: CAGCTACGAATCTCCGACCAC; R: GCCCAAGGC CACAGGTATTT.

Western blot analysis

Total protein was extracted and separated via sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), then transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, MA, USA). Membranes were blocked in 5% non-fat milk and incubated overnight at 4°C with primary antibodies targeting DEPTOR (Cat# ab126440; Abcam, Cambridge, UK), anti-p65 (ab32536), anti-p-p65 (ab76302), anti-nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha

(IκBα; ab32518), anti-phosphorylated (p)-IκBα (ab133462), anti-Bcl-2-associated X (Bax) (ab32503), anti-B-cell lymphoma-2 (Bcl-2; ab182858), anti-G-protein coupled receptor (GPCR)-87 (GPR87) (ab235569), anti-activating transcription factor 6 (ATF6; ab227830), anti-CHOP protein (ab11419), and anti-β-actin (ab8226). After washing, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Abcam), and signals were detected using enhanced chemiluminescence (ECL) substrates.

Enzyme-linked-immunosorbent serologic assay (ELISA)

Supernatants from treated WI-38 cells were collected, and the concentrations of TNF-α, IL-6, and IL-1β were quantified using ELISA kits (Beyotime Biotechnology, Shanghai, China) according to the manufacturer's protocols. Absorbance was measured at 450 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

Oxidative stress assay

Intracellular oxidative stress was assessed by measuring the levels of malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione (GSH) using commercially available assay kits (Beyotime). Specifically, MDA (Cat# S0131S), SOD (Cat# S0103), and GSH (Cat# S0053) assays were conducted in accordance with the manufacturer's instructions.

Apoptosis assay

Cell apoptosis was evaluated using an Annexin V-FITC/PI apoptosis detection kit (C1062M; Beyotime). Briefly, cells were harvested, washed with phosphate-buffered saline solution (PBS), and stained with Annexin V-FITC and propidium iodide (PI) in the dark. Apoptotic cells were then quantified by flow cytometry (FCM; FACSCanto II; BD Biosciences, NJ, USA). Data were analyzed using the FlowJo software.

Statistical analysis

All experiments were repeated at least thrice independently. Data were presented as mean ± standard deviation (SD) from three biological replicates (n = 3). Statistical comparisons between multiple groups were performed using one-way analysis of variance (ANOVA), followed by Tukey's *post hoc* test. Statistical analysis and graph plotting were carried out using the GraphPad Prism 9 software. A $P < 0.05$ was considered statistically significant.

Results

DEPTOR is downregulated in LPS-stimulated WI-38 cells

To explore the involvement of DEPTOR in LPS-induced lung inflammation, we first evaluated its expression levels in WI-38 human lung fibroblasts following the LPS treatment (10 μg/mL for 12 h). qRT-PCR results revealed a significant downregulation of DEPTOR mRNA in LPS-treated cells, compared with untreated controls (Figure 1A). Consistently, Western blot analysis confirmed a marked reduction in DEPTOR protein expression with LPS exposure, supported by densitometric quantification (Figure 1B). Subsequent transfection with a DEPTOR overexpression plasmid effectively restored both mRNA and protein levels, validating successful transgene expression and establishing a foundation for downstream functional assays (Figures 1A and 1B). These findings indicated that LPS stimulation suppressed DEPTOR expression in WI-38 cells, suggesting its potential role in inflammation-associated injury.

DEPTOR reduces LPS-induced pro-inflammatory cytokine production

To determine whether DEPTOR modulates inflammatory response in LPS-challenged fibroblasts, we quantified the mRNA and protein expression of key pro-inflammatory cytokines. qRT-PCR analysis demonstrated that LPS treatment significantly upregulated the transcription of TNF-α,

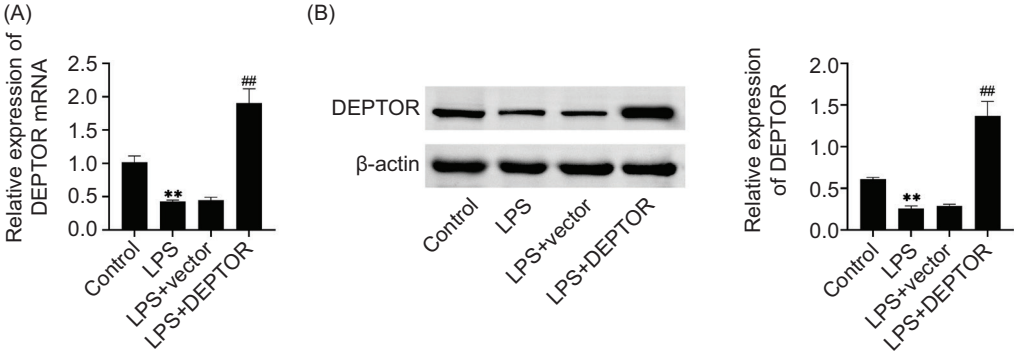

Figure 1 DEPTOR is downregulated in WI-38 cells upon LPS stimulation. (A) qRT-PCR analysis of DEPTOR mRNA levels in WI-38 cells treated with LPS (10 μg/mL, 12 h), with or without DEPTOR overexpression. (B) Representative Western blot images and the corresponding quantification of DEPTOR protein levels in each group. Data are presented as mean ± SD (n = 3). **P < 0.01 vs. control group; ##P < 0.01 vs. LPS+vector group (one-way ANOVA with Tukey's *post hoc* test).

IL-6, and IL-1β, while these elevations were only marginally affected by vector transfection (Figure 2A). Notably, DEPTOR overexpression significantly attenuated the mRNA levels of these cytokines. ELISA assays further validated this trend at protein level, revealing that DEPTOR markedly reduced the secretion of TNF-α, IL-6, and IL-1β into the culture medium (Figure 2B). These data collectively demonstrated that DEPTOR exerted anti-inflammatory effects by suppressing LPS-induced cytokine expression and secretion in WI-38 cells.

DEPTOR alleviates LPS-induced oxidative stress and ER stress

To evaluate the antioxidant potential of DEPTOR, we assessed key markers of oxidative stress. LPS exposure significantly increased MDA levels, indicative of elevated lipid peroxidation, while significantly reducing SOD and GSH levels, reflecting impaired antioxidant defense (Figure 3A). DEPTOR overexpression significantly reversed these changes, restoring SOD and GSH levels and decreasing MDA accumulation.

Furthermore, we examined markers of ER stress. Western blot analysis showed that LPS markedly upregulated the expression of GPR87, ATF6, and CHOP proteins compared to controls (Figure 3B). Vector transfection had no effect on this induction; however, DEPTOR overexpression significantly downregulated all three ER stress markers. These findings indicated that DEPTOR mitigated both oxidative and ER stress induced by LPS exposure, supporting its cytoprotective function in inflamed lung fibroblasts.

DEPTOR inhibits LPS-induced apoptosis in WI-38 cells

To assess whether DEPTOR influences cell survival under inflammatory conditions, we analyzed apoptosis using Annexin fluorescein isothiocyanate (V-FITC)/propidium iodide (PI) staining flow cytometry. LPS significantly increased the percentage of apoptotic cells, a response that persisted in the LPS+vector group (Figure 4A). In contrast, DEPTOR overexpression significantly decreased the proportion of apoptotic cells, demonstrating its anti-apoptotic effect.

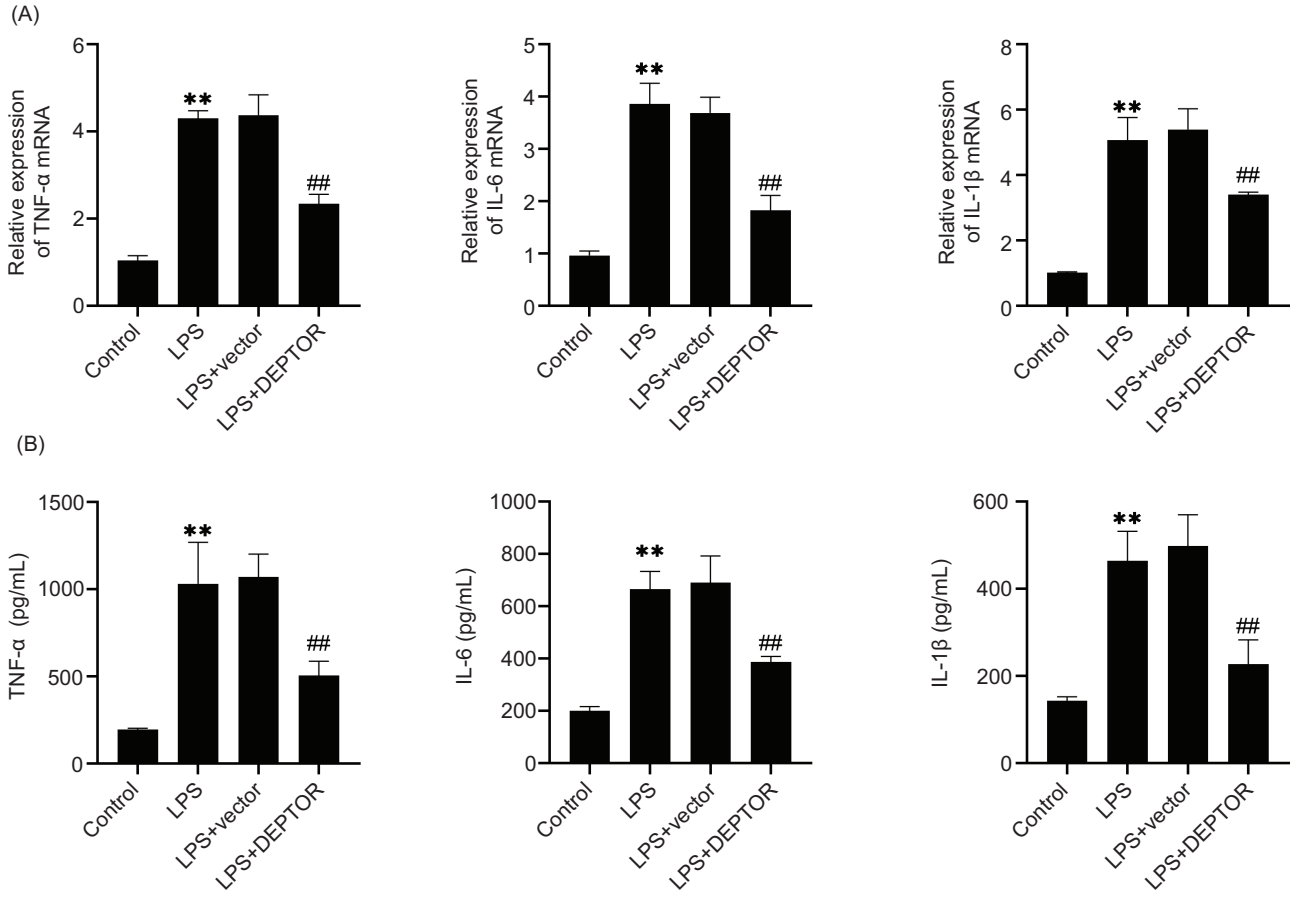

Figure 2 DEPTOR's overexpression reduces LPS-induced pro-inflammatory cytokine production in WI-38 cells. (A) mRNA expression levels of TNF-α, IL-6, and IL-1β were measured by qRT-PCR in WI-38 cells after LPS stimulation (10 μg/mL, 12 h) with or without DEPTOR overexpression. (B) Protein levels of TNF-α, IL-6, and IL-1β were assessed by ELISA under the same treatment conditions. Data are presented as mean ± SD (n = 3). **P < 0.01 vs. control group; ##P < 0.01 vs. LPS+vector group (one-way ANOVA with Tukey's *post hoc* test).

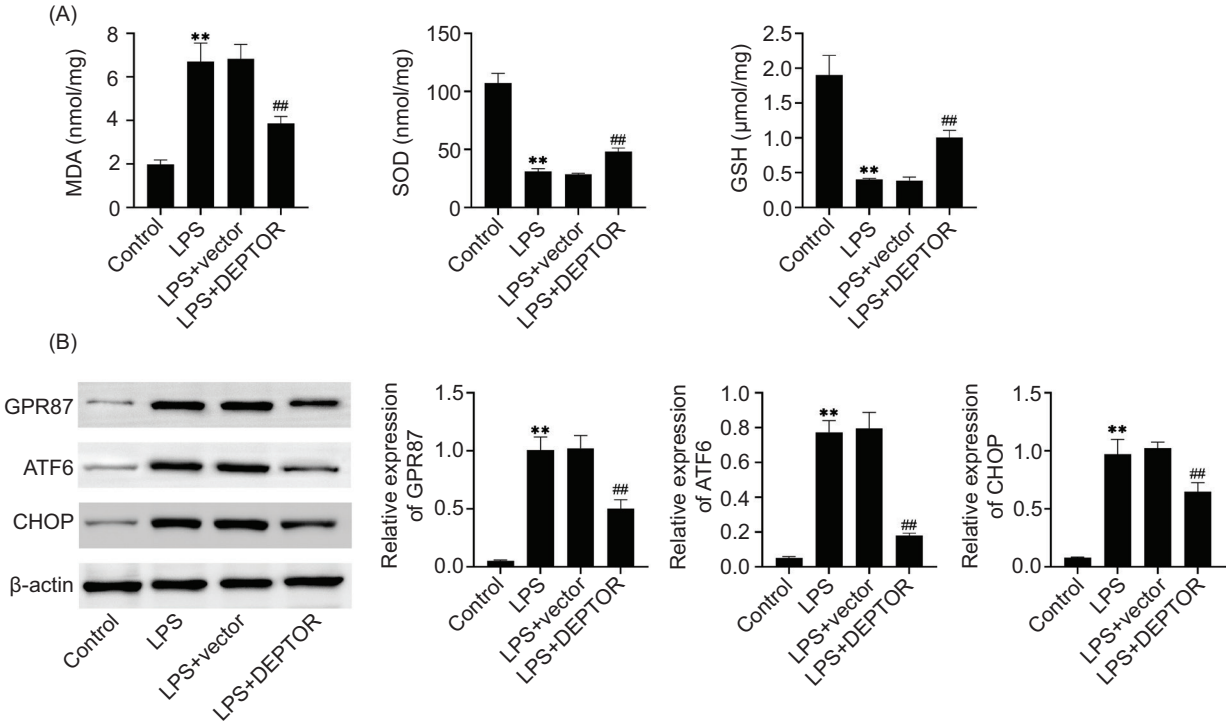

Figure 3 DEPTOR alleviates LPS-induced oxidative stress and endoplasmic reticulum stress in WI-38 cells. (A) Oxidative stress markers were measured following LPS treatment (10 μg/mL, 12 h), with or without DEPTOR overexpression. (B) ER stress markers GPR87, ATF6, and CHOP were evaluated by Western blot analysis and quantified. Data are presented as mean ± SD (n = 3). **P < 0.01 vs. control group; ##P < 0.01 vs. LPS+vector group (one-way ANOVA with Tukey's *post hoc* test).

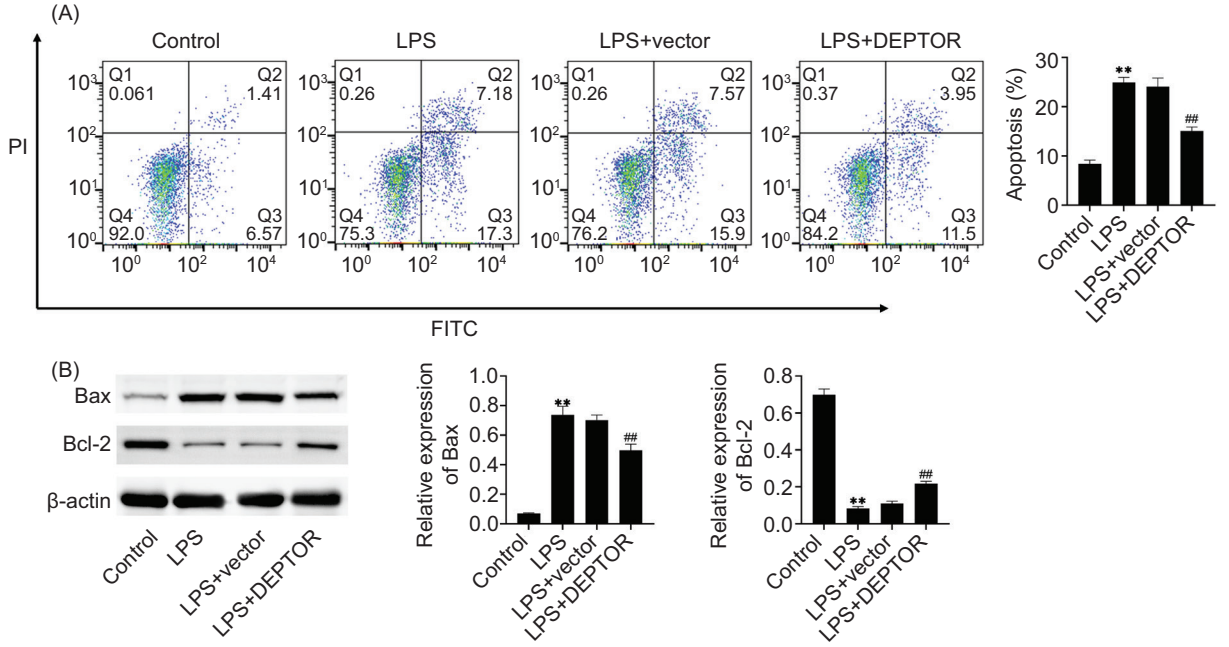

Figure 4 DEPTOR attenuates LPS-induced apoptosis in WI-38 cells. (A) Apoptosis of WI-38 cells was evaluated by flow cytometry using Annexin V-FITC/PI staining after LPS stimulation (10 μg/mL, 12 h) with or without DEPTOR overexpression. The percentage of apoptotic cells (Q2+Q3 quadrants) was quantified. (B) Expression levels of pro-apoptotic Bax and anti-apoptotic Bcl-2 proteins were assessed by Western blot analysis and quantified. β-actin served as the internal control. Data are presented as mean ± SD (n = 3). **P < 0.01 vs. control group; ##P < 0.01 vs. LPS+vector group (one-way ANOVA with Tukey's *post hoc* test).

Western blot analysis further revealed that LPS treatment upregulated the pro-apoptotic protein Bax and downregulated the anti-apoptotic protein Bcl-2 (Figure 4B). DEPTOR overexpression reversed these alterations, restoring the Bax/Bcl-2 balance in favor of cell survival. These results suggested that DEPTOR protected WI-38 cells from LPS-induced apoptosis by modulating key regulators of apoptotic signaling.

DEPTOR suppresses NF-κB pathway activation in LPS-induced WI-38 cells

To elucidate the underlying mechanism of DEPTOR's anti-inflammatory function, we investigated the NF-κB signaling pathway. LPS stimulation led to a substantial increase in the phosphorylation levels of p65 and IκBα, consistent with NF-κB pathway activation, and concurrently reduced total IκBα protein, indicating its degradation (Figure 5). However, in cells overexpressing DEPTOR, phosphorylation of both p65 and IκBα was significantly suppressed, and the total level of IκBα was restored partially. These data indicate that DEPTOR inhibited LPS-induced activation of the NF-κB pathway, providing mechanistic insight into its anti-inflammatory and cytoprotective effects in pulmonary fibroblasts.

Discussion

Pneumonia remains a significant global health burden, particularly among children aged <5 years, where it accounts for substantial morbidity and mortality.[14] Despite advances in antimicrobial and supportive therapies, complications, such as ALI and systemic inflammation, persist as serious clinical challenges. Among the primary pathogenic stimuli, LPS, a component of Gram-negative bacterial cell walls, plays a central role in activating inflammatory cascades and tissue damage in pneumonia.[15] While macrophages and epithelial cells are often the focus of acute inflammatory research, fibroblasts also contribute meaningfully to pulmonary inflammation through cytokine secretion and modulation of tissue remodeling. Although this study employed WI-38 lung fibroblasts, future investigations comparing DEPTOR's role across multiple lung cell types—including macrophages and alveolar epithelial cells—are warranted to obtain a more comprehensive understanding. LPS-stimulated WI-38 cells serve as a well-established *in vitro* model for evaluating lung inflammation and injury mechanisms.[16] Our findings confirm that LPS induces robust inflammatory and cellular stress responses in these cells, including elevated cytokine expression, oxidative damage, ER stress activation, and apoptosis. Notably, overexpression of DEPTOR significantly ameliorated these pathological changes, suggesting its potential function as a protective modulator in inflammatory lung injury.

The role of ER stress in the pathogenesis of pneumonia has gained increasing attention.[17] ER stress results from the accumulation of misfolded or unfolded proteins in the ER lumen, leading to activation of unfolded protein response (UPR), which initially acts to restore homeostasis.[18] However, persistent ER stress may exacerbate inflammation and induce cell apoptosis.[18] Previous studies have highlighted ER stress as a key intermediary linking infection-induced cellular dysfunction to inflammatory tissue damage in lung diseases.[19] In our study, LPS significantly upregulated the expression of GPR87, ATF6, and CHOP, all of which are classical markers of ER stress. DEPTOR overexpression markedly reversed this upregulation, supporting its ability to mitigate ER stress. Concurrently, DEPTOR restored antioxidant capacity, evidenced by increased levels of SOD and GSH and a reduction in MDA, underscoring its broader role in maintaining cellular redox and ER homeostasis under inflammatory conditions.

DEPTOR is an endogenous inhibitor of mTOR complex,[20] a central regulator of cell growth, metabolism, autophagy, and stress responses.[12] In disease contexts, DEPTOR expression and activity are often altered, influencing outcomes in metabolic disorders, cancer, and inflammation.[20] Recent studies have revealed that DEPTOR downregulation is associated with increased inflammatory responses and

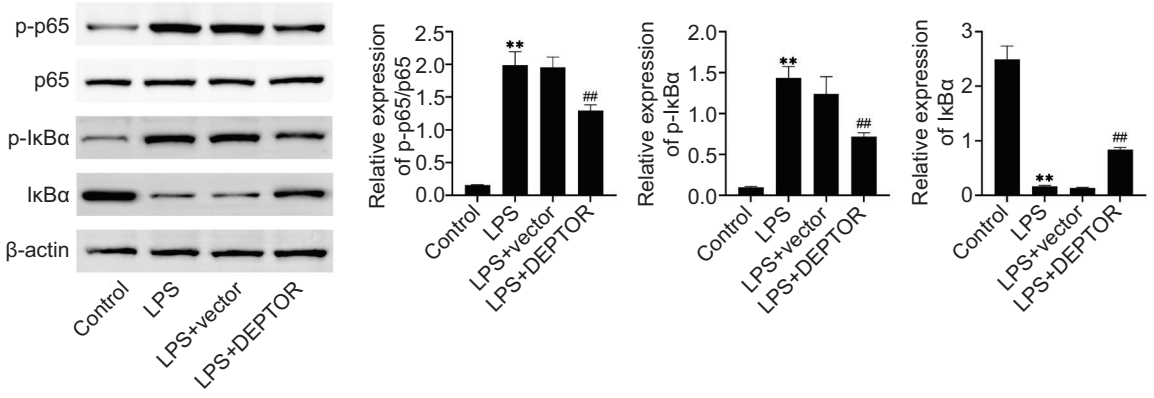

Figure 5 DEPTOR modulates the NF-κB signaling pathway in LPS-stimulated WI-38 cells. Western blot analysis was performed to assess the levels of phosphorylated p65 (p-p65), total p65, phosphorylated IκBα (p-IκBα), and total IκBα in WI-38 cells treated with LPS (10 μg/mL, 12 h), with or without DEPTOR overexpression. Data are presented as mean ± SD (n = 3). **P < 0.01 vs. control group; ##P < 0.01 vs. LPS+vector group (one-way ANOVA with Tukey'sn Reference 24.

tissue injury.[10] Consistent with earlier reports, we observed significant downregulation of DEPTOR at both mRNA and protein levels following LPS exposure in WI-38 cells. Overexpression of DEPTOR restored its expression and conferred anti-inflammatory effects, including suppressed production of TNF-α, IL-6, and IL-1β, reduced ER and oxidative stress, and diminished apoptosis. These observations reinforce the critical function of DEPTOR as a protective mediator in inflammation and suggest its therapeutic relevance in pneumonia.

The NF-κB signaling pathway is a central regulator of inflammation and immune responses, and its overactivation contributes to the pathogenesis of pneumonia.[21] Additionally, NF-κB has been shown to modulate ER stress responses and promote apoptosis under pathological conditions.[22] In lung injury, persistent NF-κB activation contributes to cytokine storm, tissue damage, and potentially respiratory failure.[23,24] In our study, LPS induced marked activation of NF-κB pathway, as evidenced by increased phosphorylation of p65 and IκBα, and degradation of total IκBα protein. Notably, DEPTOR overexpression significantly inhibited p65 and IκBα phosphorylation and partially restored IκBα protein levels, suggesting suppression of NF-κB activation. Given the parallel reduction in pro-inflammatory cytokines and ER stress markers, our findings support the hypothesis that NF-κB acts as a critical axis linking LPS stimulation to cellular injury, and that DEPTOR mitigates this injury by disrupting NF-κB signaling.

Previous research has suggested that DEPTOR regulates inflammatory and stress pathways through mTOR-NF-κB signaling cross-talk.[11] As a negative regulator of both mTORC1 and mTORC2, DEPTOR may attenuate NF-κB activity by modulating upstream kinase signaling events.[12] In our model, DEPTOR overexpression inhibited NF-κB activation without affecting total p65 expression, pointing to suppression of pathway activation, rather than protein synthesis. The restored IκBα expression further suggests stabilization of inhibitory complex, potentially preventing nuclear translocation of p65. While these findings implicate DEPTOR in NF-κB suppression, further validation using NF-κB reporter assays and immunofluorescence for p65 nuclear translocation is necessary to confirm the mechanistic relationship. Furthermore, since DEPTOR's canonical function involves inhibition of mTORC1/2, future studies should assess downstream mTOR targets (e.g., p-S6K1 and p-4EBP1) to confirm involvement of mTOR pathway in this context.

This study has several limitations that warrant consideration. First, our experiments were conducted using a single human lung fibroblast cell line (WI-38), which, although widely used for studying pulmonary inflammation, does not fully recapitulate the complexity of lung tissue responses *in vivo*. The lung microenvironment comprises diverse cell types, including epithelial cells, macrophages, and endothelial cells, whose interactions are essential in mediating the pathophysiology of pneumonia. To address this limitation, future studies should incorporate more physiologically relevant models, such as primary human lung fibroblasts, alveolar epithelial cells, and advanced 3D lung organoid cultures. Organoid systems can mimic the architecture and function of lung tissue more closely than monolayer cultures and are utilized in follow-up work to assess DEPTOR's regulatory role in a three-dimensional (3D) inflammatory microenvironment.[25]

As a mechanistic *in vitro* study, our focus was on elucidating the molecular functions of DEPTOR via overexpression strategies. However, the effects of DEPTOR knockdown or genetic ablation remain unexplored. Future research can employ gene silencing and CRISPR–Cas9-based knockout models to better delineate the bidirectional role of DEPTOR in regulating inflammatory signaling and cellular stress. Additionally, while our data indicate a protective role for DEPTOR in LPS-induced injury, these findings need to be validated *in vivo*. Animal studies using LPS-induced ALI mouse models are planned to investigate whether DEPTOR modulates inflammation, ER stress, and tissue damage in a whole-organism context.

Moreover, future experiments should include dose-response and time-course analyses to better characterize the kinetics of DEPTOR's regulatory effects under various inflammatory intensities and durations. Co-culture systems involving lung fibroblasts and alveolar epithelial cells can be used to simulate cellular interactions within the lung environment. From a translational perspective, therapeutic strategies aimed at enhancing DEPTOR activity, such as adeno-associated virus (AAV)-mediated gene delivery or pharmacological modulation of the mTOR pathway using small-molecule inhibitors, hold promise and could be tested in preclinical models. Furthermore, DEPTOR-targeted approaches should be evaluated in genetically engineered models and patient-derived cells to assess their potential clinical relevance.

Conclusion

Our study demonstratestudy demonstrates that DEPTOR expression is suppressed following LPS stimulation in WI-38 cells, and that its overexpression effectively attenuates pro-inflammatory cytokine production, oxidative stress, ER stress, and apoptosis. These findings identify DEPTOR as a novel modulator of LPS-induced cellular injury and support its potential as a therapeutic target for the treatment of inflammatory lung diseases, such as pediatric pneumonia. Nonetheless, further *in vivo* and translational studies are necessary to confirm DEPTOR's clinical applicability and therapeutic feasibility.

Availability of Data and Materials

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

Author Contributions

Xiangxiang Shi designed the study, completed the experiment and supervised data collection. Jin Ding analyzed and interpreted the data. Bihe Zeng prepared the manuscript for publication and reviewed draft of the manuscript. All authors had read and approved the final manuscript.

Conflict of Interests

The authors stated that there was no conflict of interest to disclose.

Funding

Not applicable.

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