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Ubiquitin specific peptidase 25 alleviates acute lung injury and suppresses the inflammatory response in lung epithelial cells

Zhengzhen Xu[1], Qingrong Peng[1], Yanli Wang[2], Xiaobing Chen[2] and Jinyan Zhang[1]

[1] *Emergency Room Nursing Unit, The First People's Hospital of Lianyungang, Lianyungang, Jiangsu, P.R. China*
[2] *Emergency Department, The First People's Hospital of Lianyungang, Lianyungang, Jiangsu, P.R. China*

Abstract. As COVID-19 spreads over the world, the treatment of acute lung injury (ALI) has attracted much attention. Considering ubiquitin-specific protease (USP) 25 has been relevant to inflammation, this article focused on its role in ALI and its regulatory mechanism. Lipopolysaccharide (LPS) was applied to separately stimulate mice and human lung epithelial cells to establish *in vivo* and *in vitro* ALI models. To discover the effects of USP25 overexpression on mouse, lung pathology, inflammatory factor levels, edema, number of inflammatory cells, and downstream protein levels were evaluated. USP25 overexpression in mice could alleviate LPS-induced lung tissue lesions and edema, and reduce inflammatory factors and inflammatory cells. It also inhibited the levels of downstream TRAF6, MAPK pathway-related proteins, and Fos Proto-Oncogene (FOS) *in vivo*. Furthermore, BEAS-2B cells were transfected with TNF receptor-associated factor 6 (TRAF6) plasmids to study the role of TRAF6 in the regulatory mechanism of USP25. TRAF6 overexpression was found to reverse the functions of USP25 overexpression on cells. In conclusion, USP25 reduced ALI and inhibited inflammation in lung epithelial cells *via* regulating TRAF6/MAPK/FOS signaling.

Key words: Ubiquitin specific peptidase 25 — TNF receptor-associated factor 6 — MAPK — Inflammation — Acute lung injury

Introduction

Acute lung injury (ALI) and its aggravated form, acute respiratory distress syndrome (ARDS), are acute respiratory diseases that frequently occur in severely ill patients with a high mortality rate (Li et al. 2020). Especially the new type of coronavirus pneumonia (COVID-19) is raging around the world, the treatment of ALI/ARDS has attracted more attention (He et al. 2021). ALI is a systemic inflammatory syndrome with complicated pathogenesis and regulation mechanisms, and is difficult to treat (Wang RH et al. 2020). The obvious symptoms of ALI are severe dyspnea and hypoxemia caused by insufficient lung oxygen. The patients' clinical manifestations are shortness of breath, increased breathing effort, and refractory hypoxemia (Mokrá 2020). Mechanical ventilation and promoting the disappearance of inflammation are frequently employed to curb the development of ALI at present (Goligher et al. 2016). In addition, when effective comprehensive care is implemented for patients with ALI during the treatment period, the patients' discomfort is greatly reduced, which positively affects the lung function (Mowery et al. 2020).

Ubiquitin is an evolutionarily highly conserved polypeptide expressed by eukaryotes. Its major function is to mark proteins that need to be broken down. After the protein is modified by ubiquitination, it will be recognized and degraded by the proteasome. This process is an important approach for protein degradation in the body (Zheng and Shabek 2017; Asmamaw et al. 2020). However, due to the presence of the deubiquitinating enzyme (DUB), the ubiquitination process is reversible (Mevissen and Komander 2017). Most

Correspondence to: Jinyan Zhang, Emergency Room Nursing Unit, The First People's Hospital of Lianyungang, 6 Zhenhua East Road, Lianyungang, Jiangsu, 222002, P.R. China
E-mail: zhangjy07@163.com

DUBs can release ubiquitin from the substrate protein, edit the ubiquitin chain and dispose of the ubiquitin precursor (Suresh et al. 2020). Ubiquitin-specific protease (USP) is the largest class of DUB with diverse structure (Basters et al. 2018). Therein, USP25 is a protein involved in inflammatory regulation (Gersch et al. 2019). A previous study revealed that mice deficient in USP25 were more sensitive to septic shock, which is relevant to the increase of pro-inflammatory cytokines (Zhong et al. 2013). Meanwhile, another study indicated that USP25 overexpression reduced the production and secretion of inflammatory factors in macrophages induced by sepsis (Ding et al. 2017). However, to the best of our knowledge, whether USP25 affects the inflammatory response in ALI has not been disclosed.

Lipopolysaccharide (LPS), a component of the cell wall of gram-negative bacteria, is one of the most intensively studied immunogenic stimuli. It has been proven to induce systemic inflammation and sepsis (Wang Z et al. 2020). Therefore, LPS has been widely used to induce ALI in animal models to simulate human ALI (D'Alessio 2018).

In the present study, LPS was applied to separately stimulate mice and human lung epithelial cells to establish *in vivo* and *in vitro* ALI models. According to the KEGG pathway, USP25 targets TNF receptor-associated factor 6 (TRAF6), thereby inhibiting the MAPK family and downstream signal FOS. The purpose of this paper was to study the role of USP25 in the inflammatory response in ALI, as well as its regulatory pathways, to provide a novel therapeutic target for ALI.

Materials and Methods

Mice handling

Animal experiments were approved by the animal care and use committees of The First People's Hospital of Lianyungang and carried out in strict compliance with the National Institutes of Health guidelines. A total of 30 male C57BL/6 mice (6–8 weeks; Cyagen Biosciences, Suzhou, China) were adaptively housed in an environment with a natural light/dark cycle, ~55% humidity, and free access to diet at 25°C for a week.

To discover the effect of USP25 on ALI, the expression of USP25 in mice was promoted by lentivirus infection. Mice were randomly divided into three groups: control, lentiviruses expressing USP25 (Lv-USP25), and its negative control (Lv-vector; Genechem, Shanghai, China). As shown in Figure 1A, mice were intravenously administered with Lv-vector or Lv-USP25 (5×10^7 plaque forming unit/mouse) for 5 days before intratracheal injection of LPS (5 mg/kg). After LPS induction for 24 h, blood was collected from the orbital venous plexus. The mice were euthanized by cervical dislocation, and lung tissues were collected.

Cell culture and transfection

Human normal lung epithelial BEAS-2B cells (Beyotime, Shanghai, China) were cultured in DMEM (Gibco, United

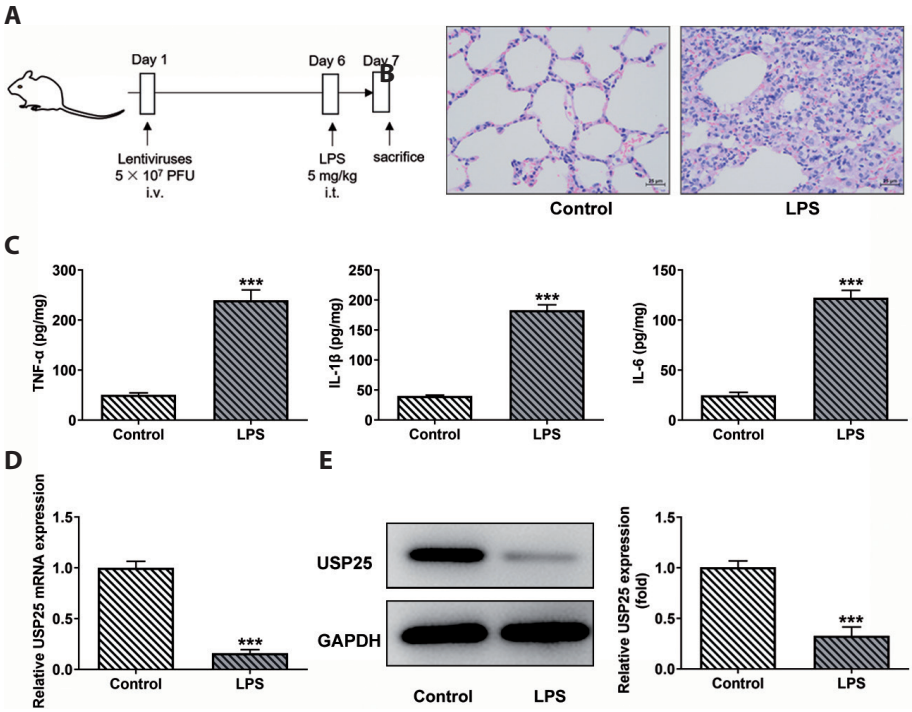

Figure 1. USP25 expression was declined in the lung tissue of ALI mouse models. **A.** Mice handling procedures. **B.** Pathological changes of lung tissue in the LPS group were determined using H&E staining. **C.** The levels of inflammatory factors in the serum of mice were measured using ELISA assay. The expression level of USP25 in the lung tissue was assessed using RT-qPCR (**D**) and Western blotting (**E**). *** $p < 0.001$ *vs*. control. PFU, plaque forming unit; i.v., intravenous; LPS, lipopolysaccharide; i.t., intratracheal.

States) supplemented with 10% FBS (Invitrogen, CA, United States), 1% penicillin/streptomycin (Invitrogen) in 5% CO_2 atmosphere at 37°C.

To discover the roles of USP25 and TRAF6, cells were divided into different groups and separately transfected with plasmids overexpressing USP25 (Ov-USP25), TRAF6 (Ov-TRAF6), or the negative controls (Ov-NC; Vector-Builder, Guangzhou, China). The operation was carried out with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After cells were cultured stably, these cells were stimulated with LPS for 24 h and collected. The cells without LPS treatment were used as the control group.

Histological examination and lung injury scoring

Hematoxylin-eosin (H&E) staining for lung tissue was performed using an H&E staining kit (#C0105, Beyotime) according to the manufacturer's instructions. Briefly, the lower lobe of the right lung tissue was fixed in 4% paraformaldehyde for 24 h, dehydrated with gradient alcohol, permeabilized with xylene, embedded in paraffin, and cut into 4-μm slices. After drying the sections, they were deparaffinized in xylene and rehydrated in gradient alcohols followed by being stained with hematoxylin solution for 5 min. Next, the sections were washed with distilled water and stained with eosin for 30 s. Then they were soaked with gradient alcohol and xylene. The lung injury scoring was performed according to the criterion as previously described (Wang et al. 2021).

Assessment of pulmonary edema

The left lung of the mouse was peeled off, and the water and blood on the surface of the lung tissue were absorbed with filter paper, and then the wet weight (W) of the left lung was weighed. After drying in an oven at 70°C for 72 h, the dry weight (D) was weighed, and the W/D ratio was calculated.

Enzyme-linked immunosorbent (ELISA) and cell counting

The neck and chest of the mouse were dissected, the trachea and right lung lobes were ligated, and a puncture needle was inserted into the upper end of the trachea, lavage was performed with 0.2 ml PBS three times, and the alveolar lavage fluid from the left lung was collected. The collected lavage fluid was centrifuged at 1500 r/min at 4°C for 10 min. The levels of TNF-α, IL-1β, and IL-6 in the supernatant and serum were determined using assay kits (Thermo Fisher Scientific, Shanghai, China) according to the manufacturer's instructions. The precipitated cells were used for cell counting with a cell counter (Countstar, Shanghai, China).

Western blotting

Cultured cells and the apical lobe of the right lung tissue were lysed in RIPA lysis buffer (Beyotime). After the determination of protein concentration with a BCA method, proteins (25 μg/lane) were separated by SDS-PAGE, transferred onto PVDF membranes, and the membranes were blocked with 5% skimmed milk at room temperature for 2 h. Subsequently, the membranes were incubated with primary antibodies at 4°C overnight followed by an HRP-conjugated antibody at 37°C for 1.5 h. The blots were developed with an ECL reagent (Millipore) for 2 min, and quantified using ImageJ v1.8 software (National Institutes of Health). The antibodies are listed in Table 1.

Reverse transcription-quantitative PCR (RT-qPCR)

Total RNA was extracted from the lung tissue and cultured cells using TRIzol® reagent (Invitrogen). RNA was reverse

Table 1. Antibodies used for Western blotting

Antibody	Catalog number	Host	Dilution ratio	Company
USP25	GTX33576	Rabbit	1:1,000	GeneTex
TRAF6	Orb543461	Rabbit	1:1,000	Biorbyt
p-p38 MAPK	AM063	Mouse	1:1,000	Beyotime
p38 MAPK	AM065	Mouse	1:1,000	Beyotime
p-JNK	Ab124956	Rabbit	1:5,000	Abcam
JNK	Ab179461	Rabbit	1:1,000	Abcam
p-ERK1/2	Ab278538	Rabbit	1:5,000	Abcam
ERK1/2	Ab184699	Rabbit	1:10,000	Abcam
FOS	GTX25794	Rabbit	1:500	GeneTex
HRP anti-rabbit lgG	A0208	Goat	1:1,000	Beyotime
HRP anti-mouse lgG	A0216	Goat	1:1,000	Beyotime

transcribed into cDNA using a PrimeScript RT reagent kit (Takara, Japan) and RT-qPCR was performed using a QuantiTect SYBR Green PCR Kit (Qiagen) according to the product manual. The amplification conditions were as follows: 95°C for 10 min, 40 cycles of 95°C for 30 s, 64°C for 34 s, and 72°C for 30 s. Relative mRNA expression was normalized to GAPDH and calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). The primers are listed in Table 2.

Statistics analysis

All experimental data are presented as mean ± standard deviation (SD) and experiments were performed in triplicate. The statistical method of unpaired Student's *t*-test and one-way ANOVA followed by Tukey's *post hoc* test was used to analyze the data in Graph Pad Prism 8.0 software. $p < 0.05$ represents a significant difference.

Table 2. Primer sequences used for RT-qPCR analysis

Gene	Sequence (5'–3')
Musculus	
USP25	F: GTTTAGCCATGGGGTGGACA
	R: CAAAAATAGATCGGTGGTAACATGA
TNFα	F: GCTGAGCTCAAACCCTGGTA
	R: CGGACTCCGCAAAGTCTAAG
IL-1β	F: TGTGAAATGCCATTTGA
	R: GGTCAAAGGTTTGGAAGCAG
IL-6	F: CCAGTTGCCTTCTTGGGACTG
	R: CAGGTCTGTTGGGAGTGGTATCC
GAPDH	F: GGGTCCCAGCTTAGGTTCATC
	R: TACGGCCAAATCCGTTCACA
Homo sapiens	
USP25	F: CCCTCTCTCCCTTCCCCAAA
	R: TCATTGCCAGGAAGTGCTGT
TRAF6	F: GCGCACTAGAACGAGCAAG
	R: TATGAACAGCCTGGGCCAAC
TNFα	F: TGGGATCATTGCCCTGTGAG
	R: GGTGTCTGAAGGAGGGGGTA
IL-1β	F: GGCTGCTCTGGGATTCTCTT
	R: ATTTCACTGGCGAGCTCAGG
IL-6	F: GTCCAGTTGCCTTCTCCCTGG
	R: CCCATGCTACATTTGCCGAAG
GAPDH	F: GGAGCGAGATCCCTCCAAAAT
	R: GGCTGTTGTCATACTTCTCATGG

F, forward primer; R, reverse primer.

Results

USP25 expression is declined in the lung tissue of ALI mouse models

Pathological changes in lung tissue in the LPS group were determined using H&E staining. The alveolar morphology of the control group was normal, with slender walls, whereas the alveolar compartment of the LPS group was widened, interstitial fibrous tissue proliferated, accompanied by a large number of inflammatory cell infiltration, and some alveolar cavities narrowed (Fig. 1B). The levels of inflammatory factors in the serum of mice in the LPS induction group were also measured. The levels of TNF-α, IL-1β, and IL-6 were all significantly elevated in the LPS group compared with the control group (Fig. 1C). The expression level of USP25 in the lung tissue was then assessed with RT-qPCR and Western blotting. The results indicated that, in contrast to the control group, the mRNA and protein levels of USP25 in the LPS group were markedly declined (Fig. 1D and E).

USP25 overexpression attenuates the pathological damage of lung tissue

To explore the specific role of USP25 in the lung, the mice were intravenously administrated with Lv-vector or Lv-USP25. The levels of USP25 in the lung tissue of different groups were detected using RT-qPCR and Western blotting. The results revealed that the level of USP25 was up-regulated in the mice pretreated with Lv-USP25 (Fig. 2A and B). The inflammatory features of the lungs in this group were reduced, which were manifested by the diminution of the alveolar wall and the decrease of inflammatory cell infiltration. In addition, the result of lung injury scoring displayed that the score of the LPS group was sharply increased, whereas that of the Lv-USP25 was declined compared with the Lv-vector group (Fig. 2C). Moreover, the degree of pulmonary edema in mice was assessed. The W/D ratio was significantly increased in the LPS, and Lv-USP25 treatment reversed the upward trend, indicating that USP25 overexpression could alleviate pulmonary edema in mice (Fig. 2D).

USP25 overexpression attenuates the inflammation in the BALF and the lung tissue

The numbers of neutrophils and macrophages in the BALF were counted separately. Compared with the control group, the number of these two types of cells and total cells in the LPS group was significantly increased, and USP25 overexpression reduced the numbers of these cells (Fig. 3A). The levels of the inflammatory factors in

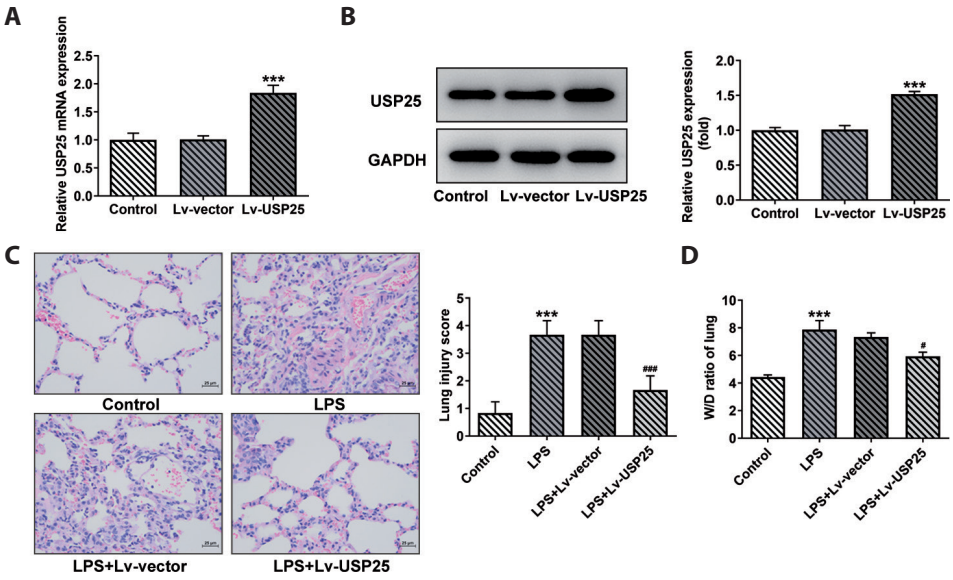

Figure 2. USP25 overexpression attenuates the pathological damage of lung tissue. The levels of USP25 in the lung tissues of different groups were detected using RT-qPCR (**A**) and Western blotting (**B**). **C.** The degree of lung injury was scored. **D.** The degree of pulmonary edema in mice was assessed with a W/D (wet/dry weight) ratio. *** $p < 0.001$ *vs.* control or Lv-vector; # $p < 0.05$, ### $p < 0.001$ *vs.* LPS+Lv-vector.

the BALF and the lung tissue were subsequently measured using ELISA kits and RT-qPCR, respectively. The levels of TNF-α, IL-1β, and IL-6 in the BALF or the lung tissue were all significantly elevated in the LPS group compared with the control group, and their levels in the Lv-USP25 group were less increased (Figs. 3B and 4A). Moreover, the expression levels of proteins related to the TRAF6/MAPK/Fos Proto-Oncogene (FOS) cascade signaling were determined using Western blotting. The protein expression levels of TRAF6, p-p38 MAPK, p-JNK, p-ERK1/2, and FOS were significantly increased in the LPS group and declined in the Lv-USP25 group (Fig. 4B).

USP25 overexpression attenuates the inflammation via TRAF6/MAPK/FOS signaling in vitro

BEAS-2B cells were subjected to LPS, and the level of USP25 in the cells was determined using RT-qPCR and Western blotting. The level was down-regulated in the LPS group (Fig. 5A and B). To study the mediated role of TRAF6 in the regulation of USP25, the cells were transfected with TRAF6 overexpression plasmid, and the overexpressed level of TRAF6 was confirmed using RT-qPCR (Fig. 5C). Afterward, the expression levels of TRAF6, p-p38 MAPK, p38 MAPK, p-JNK, JNK, and FOS in each group were

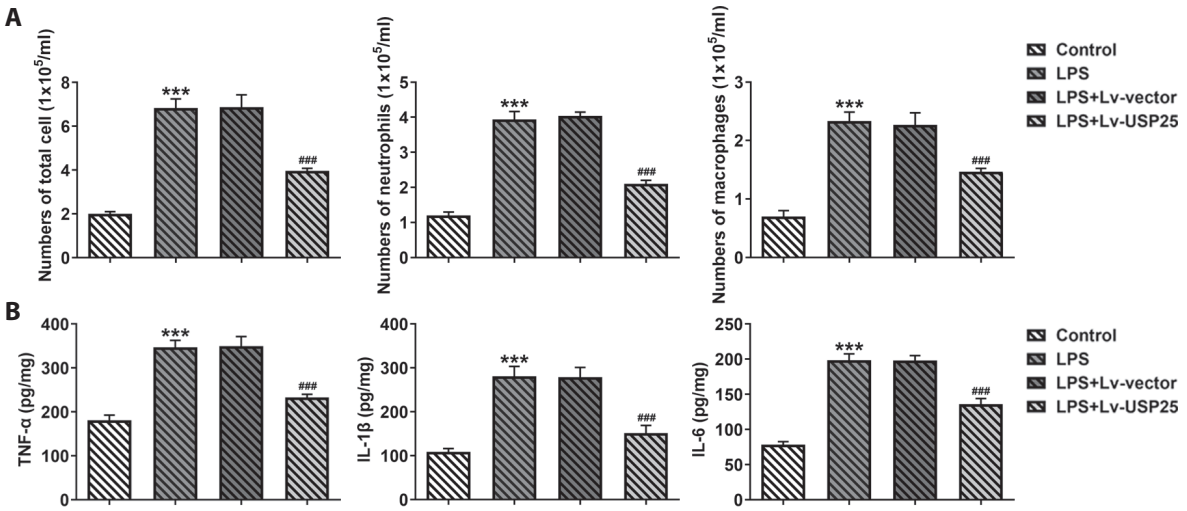

Figure 3. USP25 overexpression attenuates the inflammation in the BALF. **A.** The numbers of neutrophils, macrophages, and total cells in the BALF were counted. **B.** The levels of the inflammatory factors in the BALF were measured using ELISA kits. *** $p < 0.001$ *vs.* control; ### $p < 0.001$ *vs.* LPS+Lv-vector.

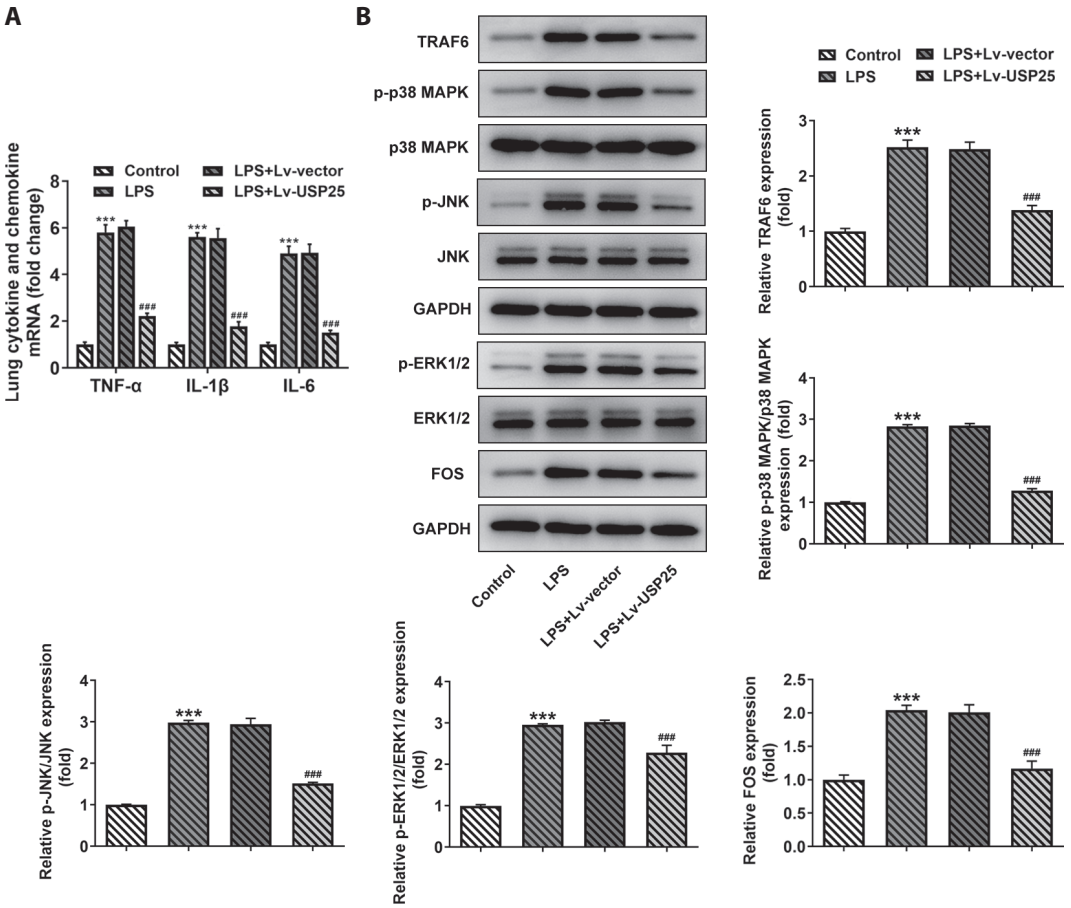

Figure 4. USP25 overexpression attenuates the inflammation in the lung tissue. **A.** The levels of the inflammatory factors in the lung tissue were measured using RT-qPCR. **B.** The expression levels of proteins related to the TRAF6/MAPK/FOS cascade signaling were determined using Western blotting. *** $p < 0.001$ *vs*. control; ### $p < 0.001$ *vs*. LPS+Lv-vector.

determined using Western blotting. The levels of TRAF6, p-p38 MAPK, p-JNK, and FOS were increased in the LPS group compared with the control group, and declined under the additional Ov-USP25 treatment, then partly elevated when Ov-TRAF6 was co-transfected with Ov-USP25 (Fig. 5D). This indicated that TRAF6 overexpression could block the inhibitory regulation of USP25 to the MAPK/FOS signaling. Furthermore, the Western blotting result revealed that TRAF6 overexpression also broke the suppressive effect of USP25 on the levels of TNF-α, IL-1β, and IL-6 in the cells (Fig. 5E).

Discussion

Although the current clinical treatment of ALI is mostly limited to mechanical ventilation and drug therapy (Mokra et al. 2019), along with nursing care and dietary conditioning in plenty of countries (Matthay et al. 2020), considerable novel and potential methods are entering our field of vision. In recent years, with the advancement of molecular biology research technology and in-depth research on the pathogenesis of ALI, stem cell therapy, biologically targeted therapy, and gene therapy are actively being developed and on trial (Mei et al. 2016; Monsel et al. 2016). This article also focuses on USP25, a potential therapeutic target, and investigates its potential role in ALI.

It is recognized that LPS can be applied to establish the ALI model. Therefore, this article used LPS to induce ALI in mice, and the successful establishment of the model was confirmed by pathological analysis. The down-regulated level of USP25 was detected in the lung tissue of the models. Then the mice were stimulated to overexpress USP25 with lentivirus fluid, and it was found that USP25 overexpression could slow down the pathological damage of lung tissue. Another study found that cigarette smoke extract could suppress the expression of USP25 in lung epithelial cells, facilitating Pseudomonas aeruginosa

bacterial infection (Long et al. 2020). These indicate that USP25 is vital in lung organ resistance to various traumas or infections.

Afterward, we counted the number of neutrophils and macrophages in the mouse BALF, as well as the levels of inflammatory factors. The results demonstrated that the number of neutrophils and macrophages increased sharply in the model group, and the degree of increase was significantly reduced in the mice overexpressing USP25. We believe that USP25 can alleviate the damage of LPS to alveolar capillaries, reduce the increase in the number of neutrophils and macrophages in the lung, and then reduce the release of vast cytokines. When these pro-inflammatory cytokines are inhibited, the inflammatory response cascade caused by LPS will no longer be amplified (Meng et al. 2018). Moreover, ALI is a disease in which neutrophils dominate.

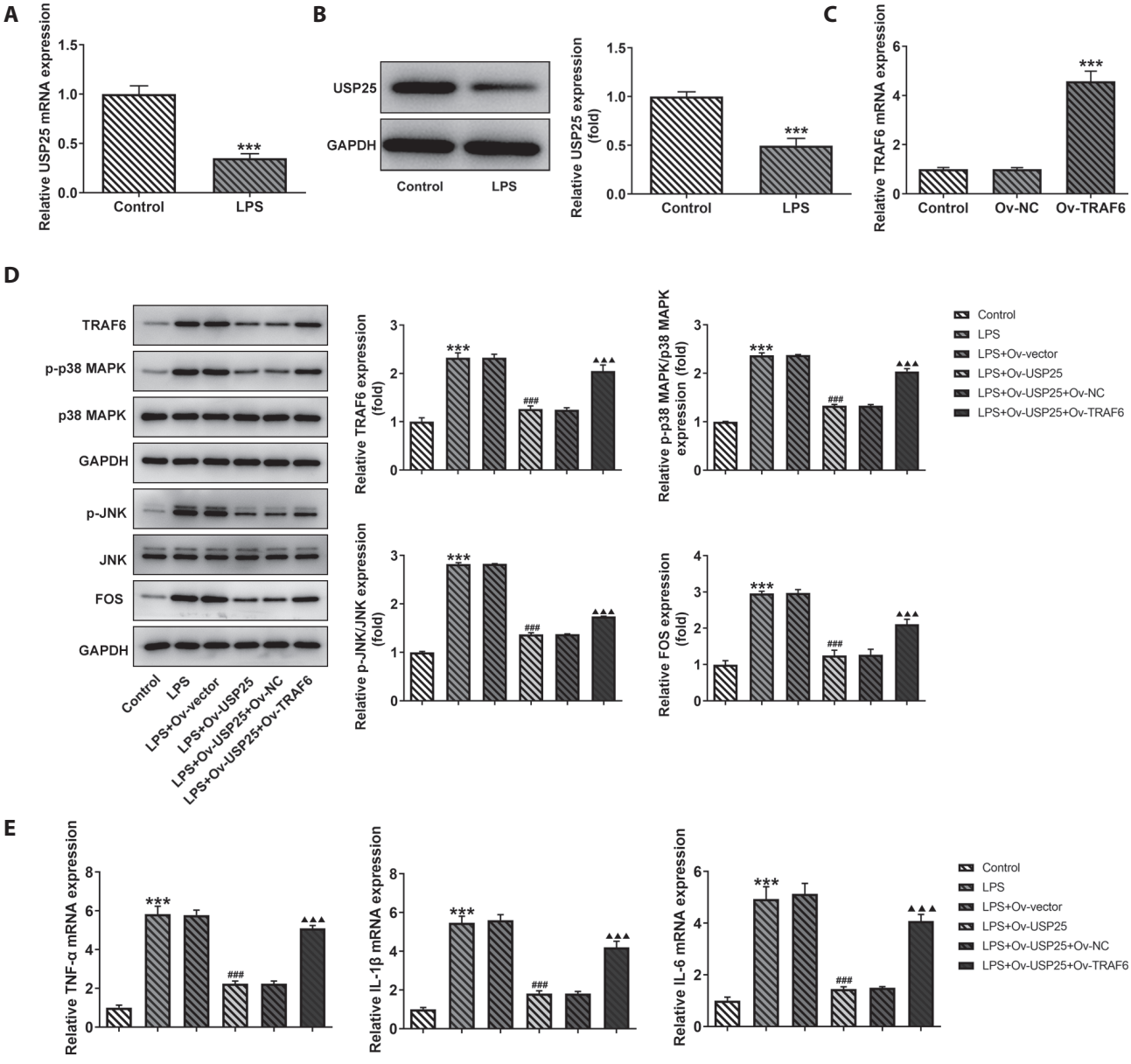

Figure 5. USP25 overexpression attenuates the inflammation *via* TRAF6/MAPK/FOS signaling. The level of USP25 in the cells was determined using RT-qPCR (**A**) and Western blotting (**B**). **C.** The overexpressed level of TRAF6 was confirmed using RT-qPCR. **D.** The expression levels of TRAF6/MAPK/FOS cascade signaling in the cells were determined using Western blotting. **E.** The levels of the inflammatory factors in the cells were measured using Western blotting. *** $p < 0.001$ *vs.* control or Ov-NC; ### $p < 0.001$ *vs.* LPS+Ov-vector; $^{\triangle\triangle\triangle}$ $p < 0.001$ *vs.* LPS+Ov-USP25+Ov-NC.

Neutrophils account for 70% of the total number of circulating white blood cells in the human body. Their life span is approximately 7–10 h in circulation and can be extended to ≥48 h when stimulated by inflammation (Park et al. 2019). Previous studies have revealed that the number of activated neutrophils in the BALF of patients with ALI increases, and it is correlated with the degree of lung injury and the increase in mortality (Mokra and Kosutova 2015; Song et al. 2019; Gustine and Jones 2021).

According to the KEGG pathway, it is shown that USP25 can target the expression of TRAF6, thereby inhibiting the MAPK family and downstream signal FOS. Hence, we determined TRAF6, MAPK-related signals, and the protein expression level of FOS in lung tissue. The results indicated that USP25 could significantly reduce the levels of these proteins. Later, BEAS-2B cells were transfected with TRAF6 overexpression plasmids, and it was found that TRAF6 overexpression could increase the levels of MAPK signal-related proteins and FOS in the cells, and increase the levels of intracellular inflammatory factors. These results suggested that USP25 achieved the inhibition of ALI *via* the TRAF6/MAPK/FOS pathway. Up to now, there are few investigations on USP25/TRAF6, with the majority of them focusing on innate immunity. According to a certain research, TRAF6 degradation is involved in the feedback regulation of USP25, USP25-deficient mice are more susceptible to virus infection (Lin et al. 2015). TRAF6 is a ubiquitin ligase (Wang J et al. 2020), and USP25 inhibits ubiquitination followed by negatively regulating the MAPK pathway. And abundant researches indicate that the MAPK pathway is associated with the development of ALI (Nie et al. 2019; Ko et al. 2020; Wang Y et al. 2020). Nevertheless, more research is required on the regulation mechanism of USP25 in ALI.

In summary, USP25 alleviates ALI and inhibits the release of inflammatory factors in lung epithelial cells *via* regulating TRAF6/MAPK/FOS signaling. It is hoped that the findings of this article can provide novel direction and promote the development of treatments for ALI.

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Ethics approval and consent to participate. This study was approved by the Institutional Animal Care and Use Committee of The First People's Hospital of Lianyungang.

Availability of data and materials. The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflict of interests. The authors declare that they have no competing interests.

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