

Ulinastatin ameliorates LPS-induced pulmonary inflammation and injury by blocking the MAPK/NF- κ B signaling pathways in rats

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Abstract. Ulinastatin, a urinary trypsin inhibitor (UTI) is commonly used to treat patients with acute inflammatory disease. However, the underlying mechanisms of its anti-inflammatory effect in acute lung injury (ALI) are not fully understood. The present study aimed to investigate the protective effect of UTI and explore its potential mechanisms by using a rat model of lipopolysaccharide (LPS)-induced ALI. Rats were treated with 5 mg/kg LPS by intratracheal instillation. The histological changes in LPS-induced ALI was evaluated using hematoxylin and eosin staining and the myeloperoxidase (MPO) activity was determined using ELISA. The wet/dry ratio (W/D ratio) of the lungs was used to assess the severity of pulmonary edema and Evans blue dye was used to evaluate the severity of lung vascular leakage. The results demonstrated that LPS administration induced histological changes and significantly increased the lung W/D ratio, MPO activity and Evans blue dye extravasation compared with the control group. However, treatment with UTI attenuated LPS-induced ALI in rats by modifying histological changes and reducing the lung W/D ratio, MPO activity and Evans blue dye extravasation. In addition, LPS induced the secretion of numerous pro-inflammatory cytokines in bronchoalveolar lavage fluid (BALF), including tumor necrosis factor- α , interleukin (IL)-6, IL-1 β and interferon- γ ; however, these cytokines were strongly reduced following treatment with UTI. In addition, UTI was able to reduce cellular counts in BALF, including neutrophils and leukocytes. Western blotting demonstrated that UTI significantly

blocked the LPS-stimulated MAPK and NF- κ B signaling pathways. The results of the present study indicated that UTI could exert an anti-inflammatory effect on LPS-induced ALI by inhibiting the MAPK and NF- κ B signaling pathways, which suggested that UTI may be considered as an effective drug in the treatment of ALI.

Introduction

Acute lung injury (ALI) is a progressive syndrome, which is the primary cause of morbidity and mortality in various types of disease, including acute pulmonary embolism, heart/lung transplantation, and ventilator and pulmonary thrombosis-induced lung injury (1-3). An acute inflammatory response and an increase in pulmonary microvascular permeability are common pathological changes observed in ALI, which cause a loss of epithelial barrier function and induce an influx of protein-rich edema fluid (4). ALI combined with severe injury or infection in patients induces the activation of pro-inflammatory signaling pathways and overexpression of inflammatory mediators, which causes a systemic inflammatory response that eventually leads to multi-organ failure, severe shock and death (5). Sepsis syndrome is a primary cause of ALI and is associated with its development (6). Inflammation and sepsis caused by Gram-negative bacteria are primarily due to lipopolysaccharide (LPS) release from the bacterial outer membrane (7). LPS-induced ALI is characterized by lung edema, disruption of endothelial and epithelial barrier integrity, extensive neutrophil infiltration and the release of inflammatory mediators (8). A previous study demonstrated that LPS serves a crucial role in regulating acute damage to the respiratory epithelium during sepsis (9). Numerous natural or synthetic chemicals have been extensively reported to impair the inflammatory responses in sepsis-induced ALI, including phyllyrin, kaempferol and farrerol (5,10,11). Phyllyrin has a protective effect against LPS-induced ALI by reducing the release of pro-inflammatory cytokines, including tumor necrosis factor- α (TNF- α), interleukin (IL)-1 β and IL-6 (5). However, the transfer of these findings to clinical treatment remains limited. It is therefore necessary to determine a novel therapeutic intervention in the treatment of sepsis-induced ALI.

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Ulinastatin, a urinary trypsin inhibitor (UTI), is a multi-valent Kunitz-type serine protease inhibitor found in urine, plasma and most organs (12). UTI can reduce the release of various types of inflammatory factor from neutrophils and inhibit neutrophil elastase activity (13). A previous study reported that UTI is used to treat numerous severe diseases, such as pancreatitis, shock and disseminated intravascular coagulation (14-16). Furthermore, UTI can reduce the inflammatory response in an animal model of ALI (17). However, the anti-inflammatory mechanism of UTI in ALI remains unclear. A previous study revealed that the NF- κ B and MAPK signaling pathways have crucial roles in the development of LPS-induced ALI (10). UTI may therefore attenuate ALI-induced inflammation responses via the MAPK and NF- κ B signaling pathways.

The present study established a rat model of LPS-induced ALI and investigated the therapeutic effect of UTI on rats with ALI. In addition, the molecular mechanism underlying the anti-inflammatory effects of UTI on ALI were further explored.

Materials and methods

Animals. Adult healthy male Sprague-Dawley (SD) rats (250-300 g) were obtained from the Experimental Animal Center of Zhongshan Hospital, Fudan University. All animals were housed in a light-controlled (12:12 light-dark cycle), temperature-controlled ($24\pm 2^{\circ}\text{C}$) and humidity-controlled (50%) room with free access to food and water. Rats underwent an acclimatization period of at least 1 week prior to experimental manipulation. All experimental protocols were approved by the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (IRB approval no. 15-000387). Sodium pentobarbital (50 mg/kg intraperitoneal) was used for anesthesia before each operation, and all efforts were made to minimize animal suffering. Sacrifice was performed by intraperitoneal injection of sodium pentobarbital overdose (200 mg/kg) followed by cervical dislocation, and mortality was confirmed by observation (18).

Rat model of LPS-induced ALI. Male SD rats were randomly divided into four groups ($n=6$ per group) as follows: i) Control group, which received only normal saline; ii) UTI group, which received 20,000 U/kg UTI; iii) LPS group, which received 5 mg/kg LPS by intratracheal instillation; and iv) LPS + UTI group, which received LPS plus 20,000 U/kg UTI (Guangdong Techpool Bio-pharma Co, Ltd.). LPS from *Escherichia coli* 055:B5 (5 mg/kg to induce ALI; Sigma-Aldrich) and normal saline were intratracheally administered as previously described (19). Then, 30 min following LPS administration, rats received UTI (20,000 U/kg) by intraperitoneal injection. These doses of drugs were determined based on preliminary experiments and previous studies (20-22). Rats were sacrificed with sodium pentobarbital by intraperitoneal injection (200 mg/kg; Sigma-Aldrich) 24 h following LPS administration, according to the Guide for Care and Use of Laboratory Animals. The bronchoalveolar lavage fluid (BALF) samples were subsequently collected and used for cell counting. The

lower lung tissues were fixed with 4% paraformaldehyde at 4°C for 24 h. Other parts of the lung tissue were stored at -80°C until further use.

Histological analysis. Lung tissue samples were fixed in 4% paraformaldehyde neutral buffer solution for 24 h, dehydrated in a graded ethanol series (30-100%; v/v), embedded in paraffin, and sliced into 5- μm thick sections. Following hematoxylin (2 g/l; 10 min at room temperature) and eosin (5 g/l; 30 sec at room temperature) (H&E) staining, pathological changes in the lung tissues were observed using light microscopy (magnification, $\times 200$; BXM; Olympus Corporation). Histological scoring was performed blindly according to the following four parameters: i) Presence of hemorrhage; ii) infiltration; iii) alveolar congestion, aggregation of neutrophils in the airspace or vessel wall; and iv) thickness of the alveolar wall/hyaline membrane formation. The severity of inflammation was graded from 0 to 1 as previously described (22).

Assessment of Evans blue extravasation. Evans blue dye (Sigma-Aldrich; Merck KGaA) extravasation was used to evaluate the pulmonary barrier permeability. Evans blue dye (20 mg/kg in 1 ml saline) was injected into the tail vein of rats 30 min before anesthesia in all groups. Normal saline was immediately injected into the right ventricle until it effused clear fluid from the left atrium at the end of the experiment. The right middle lung lobe was collected and dried at 60°C for 72 h. Tissues were sliced and incubated with formamide (3 ml/100 mg; Sigma-Aldrich; Merck KGaA) at room temperature for 24 h, samples were then centrifuged at $500 \times g$ for 10 min (4°C) for Evans blue dye extraction. The optical density of the centrifugal supernatant (Evans blue extravasation) was measured using spectrophotometry at 620 nm. Evans Blue dye concentration was determined via a standard curve and was expressed as μg of Evans Blue dye per 100 mg of lung tissue.

Lung wet/dry ratio determination. The severity of pulmonary edema was evaluated by assessing the lung wet/dry ratio (W/D ratio). The inferior lobe of the right lung was removed, rinsed briefly in PBS, blotted and then weighed to obtain the wet weight. The tissue was then placed in an incubator at 60°C for 72 h to obtain the dry weight. The W/D ratio was eventually calculated.

Assessment of lung MPO activity. It has been reported that MPO activity can be used as a marker of neutrophil activation (23). Thus, MPO activity was examined in the present study. Lung tissues were collected 24 h following LPS administration and homogenized in HEPES (pH 8.0) containing 0.5% cetyltrimethylammonium bromide and underwent three freeze-thaw cycles. Subsequently, the homogenate was centrifuged ($12,000 \times g$) at 4°C for 30 min. An ELISA kit (cat. no. ab105136; Abcam) was used to determine the myeloperoxidase (MPO) activity. Absorbance was measured at 460 nm on a microplate reader (Model 550; Bio-Rad Laboratories, Inc.). The concentration of total protein from the lung tissues was determined using the bicinchoninic acid method. The MPO activity of the homogenates supernatants was expressed as units per gram of total protein (U/g).

BALF and cell counting. Following anesthesia with sodium pentobarbital (50 mg/kg intraperitoneal), rats were sacrificed by intraperitoneal injection of sodium pentobarbital overdose (200 mg/kg) followed by cervical dislocation and BALF was collected by performing at least three lavages with 1 ml PBS (pH 7.2) following cannulation of the upper part of the trachea. The fluid recovery rate was >90%. BALF samples were kept on ice and BALF was centrifuged (700 x g) at 4°C for 5 min. The supernatants were stored at -80°C for analysis of cytokine concentrations. Subsequently, 50 µl PBS was used to resuspend the cell pellet and BALF cells were stained with 0.4% trypan blue dye (Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature for 3 min and total cells were counted under a light microscope. BALF cells were fixed using 4% paraformaldehyde at 4°C for 24 h on slides and stained with 0.5% H&E for 30 sec at room temperature for differential random counting (24). Subsequently, total cells, neutrophils and leukocytes were counted in a double-blind manner using a hemocytometer, according to morphology (25).

Cytokine measurement. Following BALF centrifugation for 4 min at 3,000 x g, BALF supernatant was collected and stored at -80°C prior to measuring cytokines. The following ELISA assay kits were used: TNF-α (cat. no. BMS607-3FIVE; Thermo Fisher Scientific, Inc.), IL-6 (cat. no. RAB0308; Sigma-Aldrich; Merck KGaA), IL-1β (cat. no. RAB0274; Sigma-Aldrich; Merck KGaA) and interferon-γ (IFN-γ; cat. no. MIF00; R&D Systems, Inc.), according to the manufacturer's instructions.

Extraction of lung nuclear proteins. Lung tissues were collected from each group and immediately stored in liquid nitrogen until homogenization. Frozen lung tissue (50 mg) was homogenized using a Precellys 24 bead-based tissue homogenizer in 0.5 ml ice-cold buffer A [1.5 mM MgCl₂, 10 mM HEPES (pH 7.9), 10 mM KCl, 0.5 mM dithiothreitol (DTT), 1% Nonidet P-40, 0.1 mM Na₂EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 125 µg/ml aprotinin, 25 µg/ml pepstatin A and 0.5 µg/ml leupeptin]. Cell debris were removed by centrifugation at 700 x g for 30 sec. Supernatant was then collected and placed on ice for 5 min, and centrifuged for 10 min at 5,000 x g. Cytoplasmic proteins were collected in the supernatant and the pellet was resuspended in cold buffer B (0.5 ml) [1.5 mM MgCl₂, 20 mM HEPES (pH 7.9), 0.42 mM NaCl, 0.5 mM DTT, 20% glycerol, 0.5 mM PMSF, 125 µg/ml aprotinin, 25 µg/ml pepstatin A and 0.5 µg/ml leupeptin], and kept on ice for 30 min. The nuclear fraction from the supernatant was obtained by centrifugation at 12,000 x g for 2 min, the pellet was collected and stored at -70°C.

Western blotting. The lower lobe of the right lung was collected and frozen at -80°C. Total proteins from lung tissues were isolated using RIPA buffer (Sigma Aldrich; Merck KGaA) and their concentrations were measured using bicinchoninic acid assay. Proteins (20 µg) were separated on 12% SDS-PAGE, and transferred onto polyvinylidene difluoride membranes (EMD Millipore). Following blocking with 5% non-fat milk at 4°C overnight, membranes were incubated at 4°C overnight with primary antibodies against ERK1/2 (cat. no. 353097; 1:1,000), p-ERK1/2 (cat. no. 8146; 1:1,000), JNK (cat. no. 2855;

1:1,000), phosphorylated (p-)JNK (cat. no. 2055; 1:1,000), p38 MAPK (cat. no. 16005; 1:1,000), p-p38 MAPK (cat. no. 9102; 1:1,000), p-NF-κB (Ser536)-specific p65 (cat. no. 3031; 1:1,000), IκBα (cat. no. 3025; 1:1,000), p-(Ser32/Ser36)-specific IκBα (cat. no. 9246; 1:1,000), which were all obtained from Cell Signaling Technology, Inc. β-actin (cat. no. 2832; 1:1,000; Sigma-Aldrich; Merck KGaA), incubated overnight at 4°C, was used as an internal control. Membranes were then incubated with horseradish peroxidase-conjugated secondary antibody (cat. no. ab205719; 1:2,000; Abcam) for 1 h at room temperature. The protein bands were measured using the ECLPlus Detection System (GE Healthcare) and analyzed using AlphaImager software version 2000 (ProteinSimple). Experiments were repeated at least three times, and the values obtained for the relative intensity were used for statistical analysis.

Statistical analysis. All statistical analysis was conducted using SPSS 14.0 software (SPSS, Inc.). Data are presented as the mean ± standard deviation. Statistical analyses were evaluated by Student's t-test (comparison between two groups) or one way ANOVA to make multiple-group comparisons, followed by the post hoc Tukey's test. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of UTI treatment on rats with LPS-induced ALI. To evaluate the potential of UTI to treat ALI, the rat model of LPS-induced ALI treated with 20,000 U/kg UTI was established. The results demonstrated that LPS induced lung inflammatory responses, including thickening of the alveolar walls and notable interstitial infiltration of inflammatory cells; however, UTI treatment distinctly reversed these phenomena (Fig. 1A). Furthermore, an Evans blue dye assay was performed to evaluate the severity of lung vascular leakage. As presented in Fig. 1B, Evans blue dye extravasation from the lung vasculature was significantly increased in the LPS group compared with the control group (P<0.01). However, treatment with UTI significantly reduced Evans blue dye extravasation compared with the LPS group (P<0.01; Fig. 1B). To further evaluate the effect of UTI on LPS-induced lung edema, pulmonary edema was assessed by calculating the lung W/D ratio. Rats who received LPS by intratracheal instillation exhibited a higher W/D ratio compared with the controls. However, UTI administration significantly decreased the W/D ratio in the LPS + UTI group compared with the LPS group (P<0.01; Fig. 1C). Furthermore, MPO activity is considered a key indicator of neutrophil migration into the lung (26). Therefore, the MPO activity was used to assess the accumulation of activated neutrophils in the lung tissues. The results revealed that MPO activity in the LPS group was significantly higher compared with the control group; however, MPO activity in LPS + UTI group was significantly lower than in the LPS group (P<0.01; Fig. 1D). In addition, the lung W/D ratio and MPO activity exhibited no differences in the lung tissues between the control and UTI groups. These data indicated that UTI administration may attenuate LPS-induced ALI in rats.

UTI reduces inflammatory cytokine expression in rats with LPS-induced ALI. Upregulation of inflammatory cytokines is an important feature of ALI. Therefore, the levels of TNF-α,

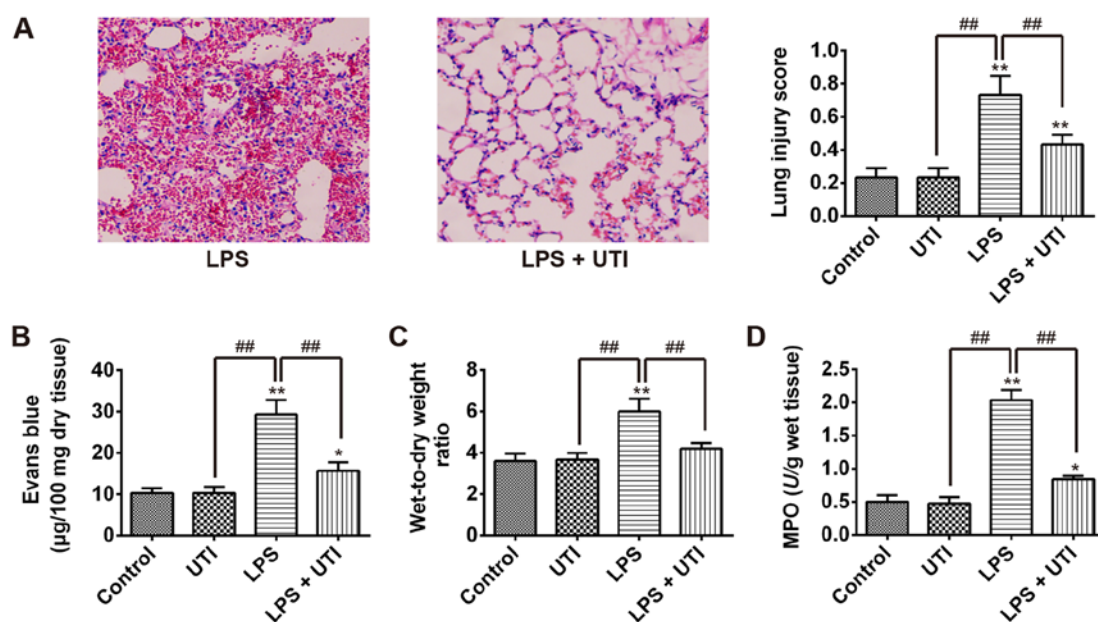


Figure 1. Effects of UTI treatment in rats with LPS-induced ALI. (A) Hematoxylin and eosin staining and lung injury score system were used to evaluate histological changes in LPS-induced ALI (magnification, x200). (B) The Evans blue dye was used to evaluate the severity of the lung vascular leakage in rats with LPS-induced ALI following UTI treatment. (C) The lung wet/dry weight ratio and (D) MPO activity were measured at 24 h following LPS administration. Data are presented as the mean \pm standard deviation (n=6 rats/group). *P<0.05, **P<0.01 vs. control; ##P<0.01. ALI, acute lung injury; LPS, lipopolysaccharide; MPO, myeloperoxidase; UTI, urinary trypsin inhibitor.

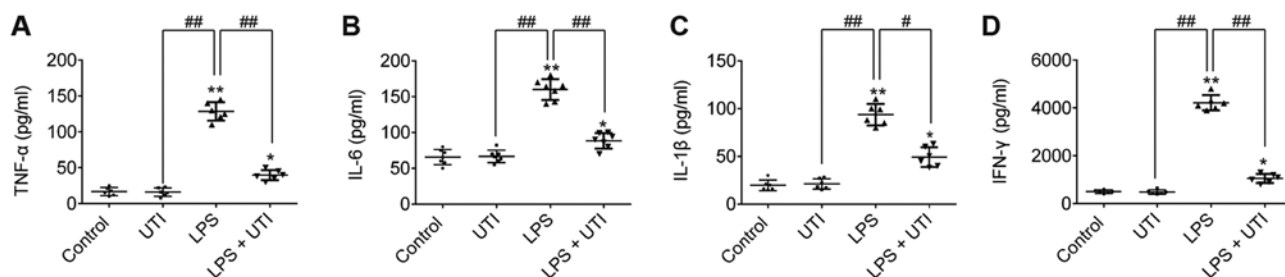


Figure 2. UTI reduces inflammatory cytokine expression in rat LPS-induced ALI. Rats were sacrificed at 24 h following LPS administration, and BALF was collected. The (A) TNF- α , (B) IL-6, (C) IL-1 β and (D) IFN- γ levels in BALF were measured by ELISA. Data are presented as the mean \pm standard deviation (n=6 rats/group). *P<0.05, **P<0.01 vs. control; ##P<0.01. ALI, acute lung injury; BALF, bronchoalveolar lavage fluid; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; MPO, myeloperoxidase; UTI, urinary trypsin inhibitor; TNF, tumor necrosis factor.

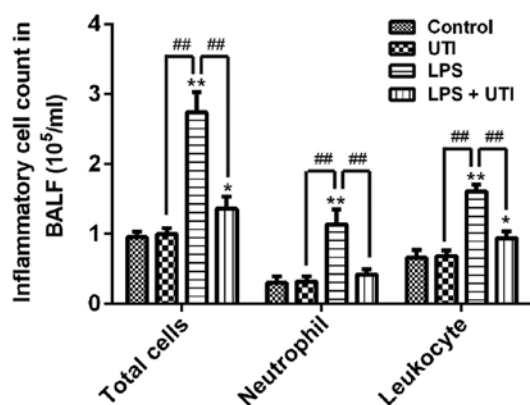


Figure 3. UTI reduces cell counts in BALF. At 30 min following LPS administration, rats were intraperitoneally injected with UTI (20,000 U/kg) and BALF was collected. Total cells, neutrophils and leukocytes were measured. Data are presented as the mean \pm standard deviation (n=6 rats/group). *P<0.05, **P<0.01 vs. control; ##P<0.01. ALI, acute lung injury; BALF, bronchoalveolar lavage fluid; LPS, lipopolysaccharide; MPO, myeloperoxidase; UTI, urinary trypsin inhibitor.

IL-6, IL-1 β and IFN- γ were determined in the BALF by ELISA. The TNF- α , IL-6, IL-1 β and IFN- γ levels in the BALF were significantly increased in the LPS group compared with the control group (P<0.01; Fig. 2). However, UTI treatment significantly decreased the TNF- α , IL-6, IL-1 β and IFN- γ levels compared with the LPS group (P<0.01; Fig. 2). These results suggested that UTI may reduce TNF- α , IL-6, IL-1 β and IFN- γ expression in rats with LPS-induced ALI.

UTI reduces cell counts in BALF. Neutrophils are the main inflammatory cells found in ALI, and serve important roles in the development of ALI (25). To further investigate the anti-inflammatory effects of UTI, the total cells, neutrophils and leukocytes were measured in BALF. The results demonstrated that total cells, neutrophils and leukocytes were significantly increased in BALF following LPS administration compared with the control group (P<0.01; Fig. 3). However, UTI treatment significantly decreased the total cells, neutrophils and leukocytes in BALF from the LPS + UTI group. These

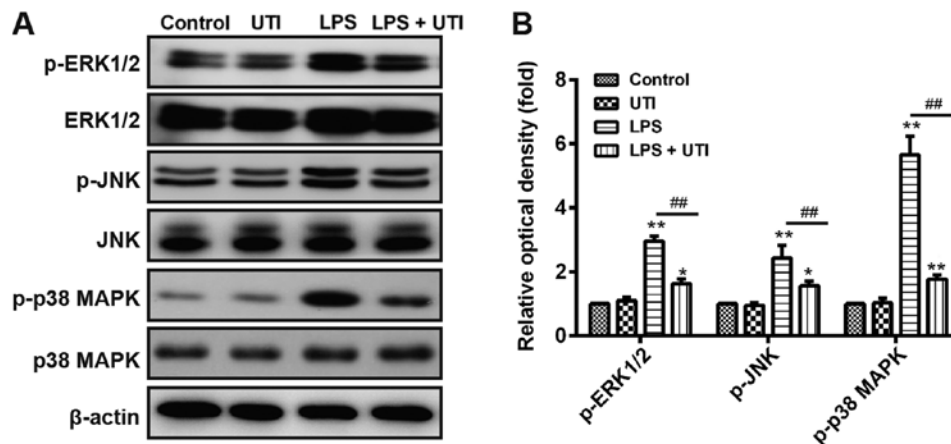


Figure 4. Effect of acute lung injury on the LPS-induced phosphorylation of ERK1/2, JNK and p38 MAPK in lung tissues. (A) The rats were intraperitoneally injected with UTI after the LPS administration and western blot analysis was used to detect ERK1/2, JNK and p38 MAPK in collected lung tissues. (B) Qualification of immunoblots was performed using ImageJ software. Protein level quantification was normalized to the internal control. β -actin was used as an internal control. The data are presented as the mean \pm standard deviation ($n=6$ rats/group). * $P<0.05$, ** $P<0.01$ vs. control; ## $P<0.01$. LPS, lipopolysaccharide; UTI, urinary trypsin inhibitor.

data indicated that UTI may reduce total cells, neutrophils and leukocytes in the lungs of rats with LPS-induced ALI.

UTI inhibits the MAPK signaling pathway in rats with LPS-induced ALI. MAPK signaling pathways serve crucial roles in LPS-induced ALI pathogenesis. Notably, it was reported that LPS stimulation can result in the phosphorylation of MAPKs, including in the ERK, JNK and p38 MAPK pathways (27). A previous study revealed that UTI inhibits LPS-induced TNF- α and subsequent IL-1 β and IL-6 secretion by macrophages, via suppression of MAPK signaling pathways, including JNK, ERK1/2 and p38 *in vitro* (28). UTI may therefore have an anti-inflammatory effect in rats with LPS-induced ALI through repression of MAPK signaling pathways. To further investigate this mechanism, the rats from the present study were intraperitoneally injected with UTI following LPS administration, and western blot analyses were conducted to detect the expression of ERK1/2, JNK and p38 MAPK in lung tissues. As presented in Fig. 4, LPS administration significantly increased the expression of p-ERK1/2, p-JNK and p-p38 MAPK in the LPS group compared with the control group ($P<0.01$). Conversely, p-ERK1/2, p-JNK and p-p38 MAPK levels were significantly decreased in the lung tissues of rats with LPS-induced ALI that were treated with UTI. However, there was no significant difference between the UTI group and the control group. These results indicated that UTI may exert an anti-inflammatory effect on LPS-induced ALI by suppressing the MAPK signaling pathways.

UTI inhibits the NF- κ B signaling pathway in rats with LPS-induced ALI. NF- κ B p65 has a crucial role in the inflammatory response (10). To further explore the anti-inflammatory mechanism of UTI in LPS-induced ALI, the effect of UTI on NF- κ B activation in lung tissues was evaluated by western blotting. The results demonstrated that NF- κ B expression in the nuclear extracts of the LPS group was significantly higher compared with that in the control group; however, UTI treatment significantly decreased the NF- κ B p65 protein level in nuclear extracts from the LPS + UTI group compared with

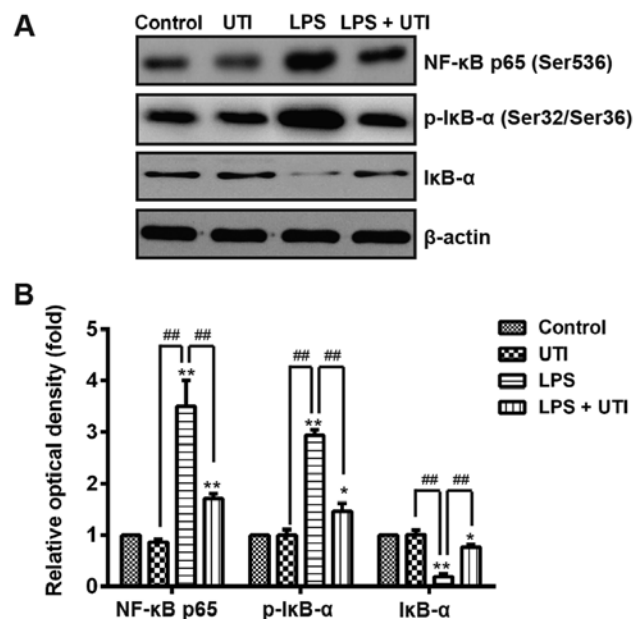


Figure 5. Effects of UTI on NF- κ B activation in LPS-treated rats. Rats were intraperitoneally injected with UTI following LPS administration, and cytosolic and nuclear extracts were fractionated from the lung tissues. (A) NF- κ B p65 (Ser536), I κ B- α and p-I κ B- α (Ser32/Ser36) protein levels in the cytosolic and nuclear fractions were detected using western blotting. β -actin was used as an internal control. (B) Protein level quantification was normalized to the internal control using ImageJ. The data are presented as the mean \pm standard deviation ($n=6$ rats/group). * $P<0.05$, ** $P<0.01$ vs. control; ## $P<0.01$. ALI, acute lung injury; LPS, lipopolysaccharide; MPO, myeloperoxidase; UTI, urinary trypsin inhibitor; p-, phosphorylated.

the LPS group ($P<0.01$; Fig. 5). Conversely, LPS significantly inhibited I κ B- α expression in cytosolic extracts compared with the control group, whereas UTI treatment significantly induced I κ B- α expression in the LPS + UTI group compared with the LPS group ($P<0.01$; Fig. 5). Furthermore, the results demonstrated that LPS significantly upregulated p-I κ B- α compared with the control group, whereas UTI treatment inhibited p-I κ B- α expression in the LPS + UTI group compared with the LPS group ($P<0.01$; Fig. 5). These results suggested that

UTI may suppress the NF- κ B signaling pathway by blocking I κ B- α degradation in the lungs of rats with LPS-induced ALI.

Discussion

The present study investigated the protective effects of UTI in rats with LPS-induced ALI and explored the molecular mechanism of the anti-inflammatory effects of UTI in ALI. The results demonstrated that UTI treatment reduced pathological changes, and decreased lung vascular leakage, pulmonary edema and MPO activity in lung tissues from rats with LPS-induced ALI. Conversely, UTI reduced the levels of inflammatory cytokine expression, neutrophils and leukocytes in BALF from rats with LPS-induced ALI. In addition, the results revealed that the anti-inflammatory effect of UTI on LPS-induced ALI may be mediated by NF- κ B and MAPK signaling pathway suppression.

LPS, which is a main component of the outer membrane of Gram-negative bacteria, induces the upregulation of numerous inflammatory cytokines that are considered principal components in sepsis-induced ALI (29). A model of LPS-induced ALI was therefore established by infusing LPS (5 mg/kg) into the rat trachea. The results revealed significant lung injury following LPS administration, particularly histopathological changes and increases in vascular leakage, the W/D ratio and MPO activity, which was consistent with previous studies (29,30). Conversely, UTI treatment reversed the histopathological changes, and decreased vascular permeability, the W/D ratio and MPO activity in rats with LPS-induced ALI. These results suggested that UTI treatment may attenuate LPS-induced ALI in rats.

ALI is closely associated with various inflammatory mediators. Numerous studies have reported that TNF- α , IL-6 and IL-1 β are the most important inflammatory mediators in the early development of inflammation (31-34). A previous study demonstrated that a complex network of inflammatory cytokines serves a crucial role in amplifying, mediating and perpetuating the lung injury process (35,36). Many studies have also revealed that levels of the inflammatory cytokines TNF- α , IL-6, IL-1 β and IFN- γ are increased in rats with LPS-induced ALI (22,37,38). UTI has been identified as an innate anti-inflammatory regulator. Clinically, UTI is used for the treatment of acute circulatory disorders, pancreatitis, and for the regulation of hemodynamic stability during surgical stress (22). Furthermore, UTI acted as an anti-inflammatory agent in an infant piglet model by inhibiting inflammatory markers, including IL-10, TNF- α and neuron-specific enolase (39). Consistent with previous reports, the results from the present study also demonstrated that the TNF- α , IL-6, IL-1 β and IFN- γ levels were significantly increased in the BALF from rats with LPS-induced ALI. Conversely, UTI treatment significantly reduced the levels of these inflammatory mediators compared with the LPS group. These results suggested that UTI may have a protective effect on LPS-induced ALI by inhibiting the secretion of the inflammatory cytokines TNF- α , IL-6, IL-1 β and IFN- γ .

Neutrophils arrive quickly at an infection site and are considered the first line of defense against invading microorganisms (40). Neutrophils are predominant inflammatory cells

in ALI and have key roles in the development of most cases of ALI (41). Multiple inflammatory mediators are able to activate neutrophils and macrophages during LPS-induced inflammation, including TNF- α and IL-8 (5,42). Neutrophils are subsequently attracted to the inflammation site by chemotactic factors (43,44), and invade the inflamed endothelial tissue via adhesion molecules, including L-selectin on neutrophils and P-selectin and E-selectin on endothelial cells (45-47). In the present study, total cells, neutrophils and leukocytes were significantly increased in BALF from rats with LPS-induced ALI; however, UTI treatment reduced the amount of these cells in BALF. These data indicated that the anti-inflammatory effect of UTI on LPS-induced ALI may be due to neutrophil and leukocyte repression.

NF- κ B, which is a DNA binding protein, can modulate the transcriptional regulation of numerous genes. A number of cellular stimuli, including LPS, TNF- α , reactive oxygen species and ultraviolet light, are able to activate NF- κ B (48). The activated NF- κ B pathway acts as a trigger that initiates an inflammatory cascade, which results in the upregulation of many pro-inflammatory cytokines (49). In addition, NF- κ B regulates the expression of these cytokines through binding with the consensus sequence of their enhancer/promoter regions (50-52). The main form of NF- κ B is a heterodimer (NF- κ B p50 and NF- κ B p65), which is localized in the cytoplasm and binds to the inhibitory proteins of the I κ B family (28). NF- κ B pathway activation mainly depends on the I κ B kinase (IKK γ) complex, which consists of a regulatory subunit (IKK) and catalytic subunits (IKK α and IKK β), and on I κ B α as its downstream substrate. In unstimulated cells, the heterodimer NF- κ B p50 and NF- κ B p65 binds to I κ B α . Phosphorylated IKK α and IKK β promote the phosphorylation of I κ B α following stimulation. Subsequently, the ubiquitin proteasome pathway degrades phosphorylated I κ B α , which causes NF- κ B p50/NF- κ B p65 dimer phosphorylation and translocation, eventually leading to gene transcription (53,54). A previous study demonstrated that NF- κ B is activated via MAPKs, including SAPK/JNK, p38 MAPK and ERK1/2. MAPKs are considered to be evolutionarily conserved and respond to stress by activating signal transduction during a phosphorylation cascade from cytoplasmic to nuclear targets (55,56).

To explore the underlying mechanisms of the inhibitory effect of UTI on cytokine production, the influence of UTI on the NF- κ B and MAPK signaling pathways was evaluated in the present study. Increasing evidence indicates that the expression of pro-inflammatory mediators is tightly modulated at the transcriptional level via the MAPK (ERK, JNK and p38) and NF- κ B signaling pathways (57-59). A previous study demonstrated that activation of JNK and p38 results in sepsis-induced organ injury, whereas JNK and p38 repression improves survival in septic mice (60). The results from the present study demonstrated that LPS administration resulted in p-ERK1/2, p-JNK and p-p38 MAPK upregulation in lung tissues. However, UTI treatment significantly downregulated the phosphorylation of ERK1/2, JNK and p38 MAPK in lung tissues from rats with LPS-induced ALI. These data indicated that UTI may have anti-inflammatory properties in LPS-induced ALI via downregulation of the MAPK signaling pathways. NF- κ B is considered a key factor in the

inflammatory response and is involved in the development of ALI (61). Activated NF- κ B is able to promote the expression of numerous inflammatory cytokines, including TNF- α , IL-1 and IL-6, which causes inflammation (60). It has been suggested that NF- κ B activation could be regulated by the phosphorylation of ERK, JNK and p38 MAPK (62). In particular, the p38 MAPK pathway has an essential role in the expression of multiple pro-inflammatory cytokines via activation of NF- κ B (63). The anti-inflammatory effect of UTI was therefore investigated, notably on the NF- κ B signaling pathway in rats with LPS-induced ALI. The results demonstrated that UTI treatment may suppress the NF- κ B signaling pathway by reducing the level of NF- κ B p65 and blocking I κ B- α degradation in rats with LPS-induced ALI.

In conclusion, the results from the present study suggested that UTI may exert anti-inflammatory effects in LPS-induced ALI by alleviating pathological changes, decreasing lung vascular leakage and pulmonary edema, and reducing lung inflammatory responses. In addition, UTI affected the phosphorylation of ERK1/2, JNK, p38 MAPK and NF- κ B p65, which suggested that the inhibitory effect of UTI on pro-inflammatory cytokine release may be mediated by the MAPK and NF- κ B signaling pathways. These findings indicated that UTI may be used as a potential therapeutic agent in the treatment of ALI.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MJ, HH, SC, YiL, YuL, SP, YZ, LX and DZ performed the experiments, contributed to data analysis and wrote the manuscript. MJ, HH, SC and YiL analyzed the data. ZL designed the study, contributed to data analysis and experimental materials. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by Zhongshan Hospital, Fudan University Ethics Committees.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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