

# Effect of triptolide on the regulation of ATP-binding cassette transporter A1 expression in lipopolysaccharide-induced acute lung injury of rats

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**Abstract.** The aim of this study was to investigate the effect of triptolide on ATP-binding cassette transporter A1 (ABCA1) expression in lipopolysaccharide (LPS)-induced acute lung injury (ALI) in rats. Thirty male Sprague Dawley rats weighing 200-250 g were randomly divided into six groups: Normal (N, n=5), Control (C, n=5), LPS (L, n=5), Triptolide 25  $\mu$ g (TP<sub>1</sub>, n=5), Triptolide 50  $\mu$ g (TP<sub>2</sub>, n=5) and Triptolide 100  $\mu$ g (TP<sub>3</sub>, n=5). The N group was not administered anything; the C group was administered 5 ml/kg normal saline intravenously and 7.5 ml/kg 1% dimethylsulfoxide (DMSO) intraperitoneally; the L group was administered 5 mg/kg 0.1% LPS and 1% DMSO; and the TP<sub>1</sub>, TP<sub>2</sub> and TP<sub>3</sub> groups were separately injected with 0.1% LPS and 25, 50 or 100  $\mu$ g/kg triptolide, respectively. All groups had the same liquid-injection volume. Arterial blood gases, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and ABCA1 expression and general pathology were examined following the treatments. It was found that increasing the triptolide dose in the TP<sub>1-3</sub> groups resulted in an increase in the expression of ABCA1 mRNA and protein. As compared with the L group, the ABCA1 expression showed a significant increase in TP<sub>2</sub> and TP<sub>3</sub> groups ( $P<0.05$ ). In addition, the expression level of TNF- $\alpha$  was significantly increased in the L and TP<sub>1</sub> groups, as compared with that in the N or C groups ( $P<0.05$ ). Conversely, a marked decrease in TNF- $\alpha$  expression was detected in the TP<sub>2</sub> and TP<sub>3</sub> groups, as compared with the L or TP<sub>1</sub> groups ( $P<0.05$ ). In conclusion, this study found that triptolide could promote the expression of ABCA1 mRNA and protein and inhibit other inflammatory factors during LPS-induced ALI in rats. Regulating the expression of ABCA1 may be one of the protective mechanisms of triptolide. Furthermore, triptolide-induced increases in ABCA1 expression occurred in a dose-dependent manner between 25 and 100  $\mu$ g/kg.

## Introduction

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are common and severe conditions, with high mortality rates. ALI/ARDS most seriously affects those with existing medical conditions, affecting both the quality of life and chance of survival in patients (1). The pathogenesis of the condition has not been fully elucidated; however, it is known that the pathophysiological basis involves a loss of control of the regulatory network consisting of cells, cytokines and inflammatory mediators. This leads to the damage of target cells, including pulmonary capillary endothelial and alveolar epithelial cells (2-5). Previous studies (6-8) have shown that ATP-binding cassette transporter A1 (ABCA1) has an essential effect on the regulation of the inflammatory response. ABCA1-mediated cholesterol efflux can reduce the inflammatory signaling induced by lipopolysaccharide (LPS) in cell membrane. The metabolic balance between cholesterol and phospholipids has an important effect on the inflammatory response. Phospholipids are key components of surface-active substances, and a decrease in phospholipid content can aggravate lung collapse (9). Bates *et al* (10) indicated that ABCA1-knockout mice manifest low levels of high-density lipoprotein and exhibit lung morphological abnormalities. Lipid analysis of the alveolar lavage fluid suggested that the quantity and metabolism of phospholipids in the lung tissues were abnormal. Considering the high distribution of ABCA1 in the lungs under physiological conditions, it is speculated that ABCA1 may be involved in the pathogenesis of ALI/ARDS. Triptolide is a diterpene lactone epoxide compound with high biochemical activity. The compound is isolated from *Celastraceae tripterygium* (*Tripterygium wilfordii* Hook. f.) and has been shown to have extensive pharmacological effects and a potent immunomodulatory capacity (11,12).

It has yet to be elucidated whether TP can recover the balance of the systemic inflammatory response and the compensatory anti-inflammatory response systems, relieve the pathological changes of ALI/ARDS and improve prognosis by regulating the transcription and protein expression of ABCA1 and inhibiting or reducing the release of inflammatory cells, transmitters and cytokines. The aim of the present study was to generate a rat model of ALI mediated by LPS, in order to investigate the *in vivo* changes in the transcription and protein expression of ABCA1 and the protective effects of triptolide on LPS-mediated rat ALI.

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## Materials and methods

**Materials.** LPS (*E. coli* O111:B4; lot no. L2630) and 1% dimethylsulfoxide (DMSO, lot no. D5879) were purchased from Sigma (St. Louis, MO, USA). Triptolide (dissolved with 1% DMSO; lot no. 20090321) was obtained from the Nanjing Skin Disease Prevention Institute (Nanjing, China), and a blood gas analyzer was purchased from Nova Biomedical (Waltham, MA, USA). A tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) ELISA kit (article no. HY12849E) was obtained from R&D Systems (Minneapolis, MN, USA). ABCA1 monoclonal antibody (article no. p3490RB) was purchased from R&D Systems (Minneapolis, MN, USA), GAPDH antibody (article no. sz-293072) was obtained from Santa Cruz Biotechnology, Inc., (Santa Cruz, CA, USA) and rabbit anti-mouse immunoglobulin G (IgG) (article no. KTB4027) was purchased from Shanghai Boyao Biological Technology Co., Ltd., (Shanghai, China). A LightCycler 480 fluorescent quantitative polymerase chain reaction (qPCR) instrument was obtained from Roche Diagnostics (Indianapolis, IN, USA), TRIzol<sup>TM</sup> RNA extraction solution (article no. 15596018) was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA), a bicinchoninic acid (BCA) protein quantification kit (article no. SK3021-500) was ordered from Shanghai Sangon (Shanghai, China) and SDS (article no. H10236) was obtained from Sigma.

**Laboratory animals and groups.** Thirty male Sprague Dawley rats, weighing 200–250 g, were provided by Shanghai Laboratory Animal Center, CAS (Shanghai, China). The rats were randomly distributed into six groups as follows: Normal (N group, n=5), Control (C group, n=5), LPS (L group, n=5) and three triptolide dosage groups (TP<sub>1-3</sub> groups, n=5/group). Rats in the L and TP<sub>1-3</sub> groups were injected with 5 mg/kg LPS (dissolved with normal saline) via the vena caudalis, as previously described (13), and the TP<sub>1-3</sub> groups were additionally administered an intraperitoneal injection of triptolide at 25, 50 or 100  $\mu$ g/kg, respectively. Rats in the N group were not treated. Rats in the C group were injected with normal saline via the vena caudalis and were intraperitoneally injected with 1% DMSO. The present study was approved by the Animal Care and Use Committee of Soochow University (Suzhou, China).

**Arterial blood gas analysis.** Arterial blood samples (0.3 ml) were collected 1 h before treatment administration and 1, 3, 6 and 12 h after treatment for blood gas analysis. Data of the arterial oxygen tension (PaO<sub>2</sub>) were collected.

**Collection and storage of samples.** Rats were anesthetized with 4% chloral hydrate at a dose of 400 mg/kg, 12 h after treatment administration. A thoracotomy was then performed and the trachea and left and right bronchi were exposed and isolated. Animals were then sacrificed by exsanguination, taking blood from the left heart 12 h after administration. The left bronchus was clipped using a vascular clamp. An intravenous 24G Y type trocar (BD Biosciences, San Jose, CA, USA) was placed in casing along the tracheal ring gap, below the middle section of the trachea. Normal saline at 4°C was used for bronchoalveolar lavage, repeated three times, at a total dose of 25 ml/kg. The bronchoalveolar lavage fluid (BALF) was obtained and,

following centrifugation at 241 x g for 10 min at 4°C, the supernatant was stored at -20°C. The blood samples were anticoagulated with heparin and then centrifuged at 671 x g for 20 min at 4°C. The supernatant was then stored at -20°C. The concentration of TNF- $\alpha$  in the serum and BALF was measured by double-antibody sandwich ELISA (R&D Systems).

The rats were sacrificed and fresh lung tissues were collected from the inferior lobe of the right lung. The tissues were washed with normal saline at 4°C and then placed on ice. Ophthalmic scissors soaked in diethylpyrocarbonate-treated water were used to collect ~100 mg tissue, which was then transferred to a centrifuge tube containing a five-fold volume of RNeasy<sup>®</sup> (article no. 15596018; Invitrogen Life Technologies). The tube was stored at room temperature for 1 h and then incubated at 4°C overnight, followed by storage at -20°C. The remaining left lung inferior lobe tissue was cut into small pieces, which were placed in cryovials containing glycerol. The cryovials were frozen in liquid nitrogen for 10 min and then stored at -70°C.

**Pathology of lung tissue and wet/dry ratio (W/D).** The connective tissue was removed from the middle lobe of the right lung. The tissue was washed with normal saline at 4°C and soaked in paraformaldehyde solution (Invitrogen Biotechnology Co., Ltd., Shanghai, China) for 24 h, followed by conventional dehydration and embedding to create a wax block for microtome cutting. Continuous 4- $\mu$ m slices were prepared and stained with a hematoxylin and eosin staining kit according to the manufacturer's instructions. The pathology status of the lung tissue was observed under an optical microscope and graded in accordance with the criteria for the evaluation of the diffuse alveolar damage (DAD) score (14,15) (Table I). The W/D of the middle lobe of the right lung of the rats was calculated.

**Detection of ABCA1 mRNA with reverse transcription-fluorescent qPCR.** Right lung inferior lobe tissue (100 mg), was collected and stored at -20°C prior to extraction of total RNA by the TRIzol<sup>TM</sup> method. PCR primers were designed with Primer 5.0 software (Primer, Inc., Ottawa, ON, Canada), and were synthesized by Invitrogen Biotechnology Co., Ltd. (Shanghai, China). The sequences were as follows: ABCA1 upstream primer, 5'-CCCCAATCCCCAACACTCC-3' and downstream primer, 5'-GCTACACTGGCAGGAAGG-3', product size 133 bp;  $\beta$ -actin upstream primer, 5'-CCCATC TATGAGGGTTACGC-3' and downstream primer, 5'-TTT AATGTCACGCACGATTTC-3', product size 150 bp. The reaction conditions for the PCR were as follows: Predenaturation at 95°C for 2 min; denaturation at 95°C for 10 sec, annealing at 60°C for 15 sec and elongation at 72°C for 20 sec (40 cycles in total). The fluorescence detection point was set at 72°C. The cycle threshold (CT) value for each amplicon was measured using a LightCycler 480 fluorescent quantitative PCR instrument (Roche Diagnostics). The  $\Delta\Delta$ CT method was used for relative quantification of the target gene, with the following formula:  $\Delta\Delta$ CT=2-[(CT1-CT2)-(CT3-CT4)]. CT1 and CT2 were the average CT values of the target and housekeeping gene in the control group, respectively. CT3 and CT4 were the average CT values of the corresponding genes in the experimental group respectively.

**Analysis of ABCA1 protein expression by western blotting.** Right lung inferior lobe tissue (400 mg) stored at -70°C was

Table I. Evaluation criteria of diffuse alveolar damage.

Pathological damage	Diffuse alveolar damage score			
	0	1	2	3
Widened alveolar septum	Not widened	Slightly widened	Obviously widened	Abnormal alveolar structure
Hemorrhage in alveolar space	No erythrocytes	Few erythrocytes	Many erythrocytes	Alveolar space filled with erythrocytes
Effusion of fibrin in alveolar space	No effusion	Slight effusion	Obvious effusion	Alveolar space filled with fibrin
Effusion of neutrophils in alveolar space and alveolar septum	No effusion	Effusion of neutrophils	Scattered effusion	Obvious effusion or focal distribution

used for the extraction of total proteins from the lung tissue subsequent to lysis. The concentration of the extracted proteins was measured using a BCA protein quantification kit. A total of 50  $\mu$ g protein solution was dissolved in 2X SDS sample loading buffer. The samples were then boiled at 100°C for 5 min, followed by separation by 10% SDS-PAGE. Following electrophoresis, the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane. Blocking solution was made with 10 g skimmed milk powder added to 200 ml phosphate-buffered saline. The mixture was filtered to prepare 5% sealing solution. The PVDF membrane was incubated in sealing solution and agitated in a shaker for 2 h, followed by further incubation at 4°C overnight. Monoclonal ABCA1 and GAPDH antibodies were added and incubated on the membranes at 37°C for 2 h before washing with Tris-buffered saline with Tween® 20, three times. Horseradish peroxidase-labeled rabbit anti-mouse IgG was added to the membranes for 1 h. Following the western blotting, the membranes were developed and scanned and the optical density of the bands was measured using a gel image analysis system. The ratio of ABCA1 to GAPDH optical density was calculated. The band intensity of each group was compared with that of GAPDH, and the ratio represented ABCA1 protein expression.

**Statistical analysis.** The data were processed with Statistical Analysis System (SAS) version 8 software (SAS Institute, Inc., Cary, NC, USA). Measurement data are expressed as the mean  $\pm$  standard deviation. One-way analysis of variance was used for comparisons between groups.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Pathological changes and DAD score of lung tissue, and W/D of the right lung middle lobe.** As shown in Fig. 1, the lung tissue structure of rats in the N group was normal. The lungs of rats in the C group had a normal structure and no notable widening of the alveolar septum. The alveolar septum of rats in the L group was widened significantly, and alveolar trapping and effusion in the alveolar space were observed. Similarly, the alveolar septum of rats in the TP<sub>1</sub> group was widened, and alveolar trapping and effusion in the alveolar

Table II. Comparison of pathological score and W/D of rats in all groups.

Group	DAD score	W/D
N	2.68 $\pm$ 0.36	4.38 $\pm$ 0.12
C	2.55 $\pm$ 0.45	4.29 $\pm$ 0.13
L	9.68 $\pm$ 1.32 <sup>a</sup>	6.43 $\pm$ 0.71 <sup>a</sup>
TP <sub>1</sub>	9.46 $\pm$ 1.35 <sup>a</sup>	6.92 $\pm$ 0.68 <sup>a</sup>
TP <sub>2</sub>	5.53 $\pm$ 0.86 <sup>a,b</sup>	5.18 $\pm$ 0.52 <sup>b</sup>
TP <sub>3</sub>	5.58 $\pm$ 0.83 <sup>a,b</sup>	4.99 $\pm$ 0.73 <sup>b</sup>

<sup>a</sup> $P < 0.05$  compared with the N and C groups; <sup>b</sup> $P < 0.05$  compared with the L group. n=5 for all groups. DAD, diffuse alveolar damage; W/D, wet/dry ratio; N, normal group; C, control group; L, LPS-treated group; TP<sub>1-3</sub>, triptolide-treated group at 25, 50 or 100  $\mu$ g/kg, respectively.

space were observed. As compared with the N and C groups, the differences in the DAD score of pathological changes and the W/D of the middle lobe of the right lung of the TP<sub>1</sub> group were statistically significant ( $P < 0.05$ ). As compared with the L group, the differences were not statistically significant ( $P > 0.05$ ). Observations by optical microscopy indicated that the alveolar structure of the TP<sub>2</sub> group was normal, with the presence of interstitial edema. Angiotelectasis, congestion and inflammatory cell infiltration were observed. In the TP<sub>2</sub> group, the DAD score, representing pathological change, was higher than that in the N and C groups, but lower than that in the L group. The difference in the DAD score between the TP<sub>2</sub> and the L group was statistically significant ( $P < 0.05$ ) (Table II). In the TP<sub>3</sub> group, the alveolar septum was slightly widened, and partial alveolar trapping, interstitial edema, partial angiotelectasis, congestion and inflammatory cell infiltration were observed. As compared with the L group, the differences in the DAD score and W/D of the middle lobe of the right lung in the TP<sub>3</sub> group were statistically significant ( $P < 0.05$ ). However, as compared with the TP<sub>2</sub> group, the differences were not statistically significant ( $P > 0.05$ ) (Table II).

**Arterial blood gas analysis.** The differences in PaO<sub>2</sub> at 3, 6 and 12 h between the L and TP<sub>1</sub> groups and the N and C groups were statistically significant ( $P < 0.05$ ). In addition, the differences in PaO<sub>2</sub> at 3, 6 and 12 h between the TP<sub>2</sub> and TP<sub>3</sub> groups



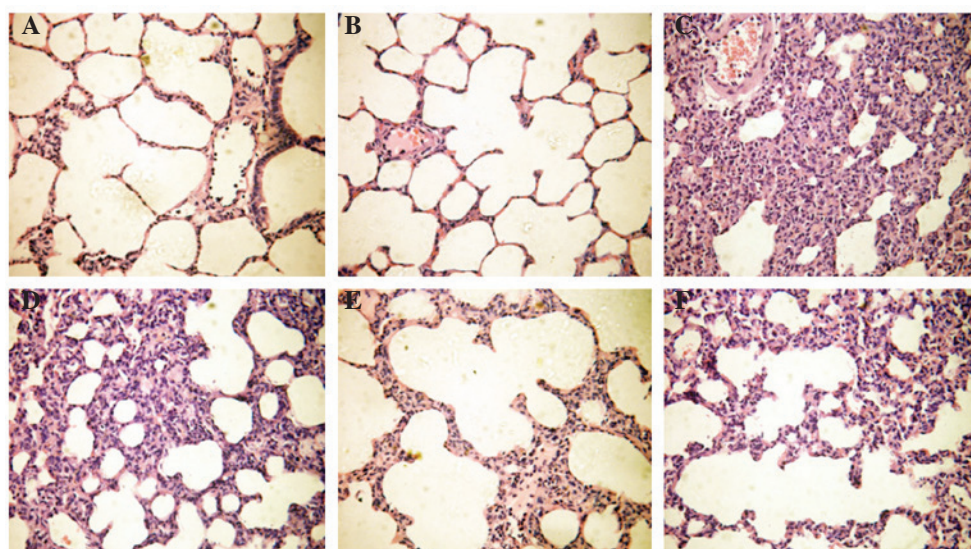


Figure 1. Pathological changes in the lungs of rats in all groups (hematoxylin and eosin staining; magnification, x400). (A) The lung tissue structure of rats in the N group was normal. (B) The alveolar septum of rats in the C group was not notably widened and the structure was basically normal. (C) A notably widened alveolar septum, alveolar trapping and effusion in the alveolar space were observed in the L group. (D) A widened alveolar septum, alveolar trapping and effusion in the alveolar space were observed in the TP<sub>1</sub> group. (E) Under the optical microscope, the alveolar structure in the TP<sub>2</sub> group was basically normal, although interstitial edema, partial angiotectasis, congestion and inflammatory cell infiltration were observed. (F) A slightly widened alveolar septum, edema, partial alveolar trapping, angiotectasis, congestion and inflammatory cell infiltration were observed in the TP<sub>3</sub> group. N, normal group; C, control group; L, LPS-treated group; TP<sub>1-3</sub>, triptolide treated group at 25, 50 or 100  $\mu\text{g/kg}$ , respectively.

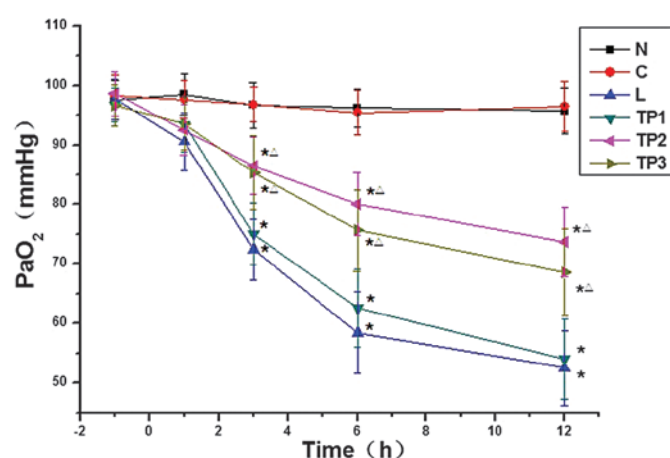


Figure 2. Arterial blood gas analysis of rats in all groups. \* $P < 0.05$  compared with the N and C groups at the same time-point.  $\Delta P < 0.05$  compared with the L group at the same time-point. N, normal group; C, control group; L, LPS-treated group; TP<sub>1-3</sub>, triptolide-treated group at 25, 50 or 100  $\mu\text{g/kg}$ , respectively; PaO<sub>2</sub>, arterial oxygen tension.

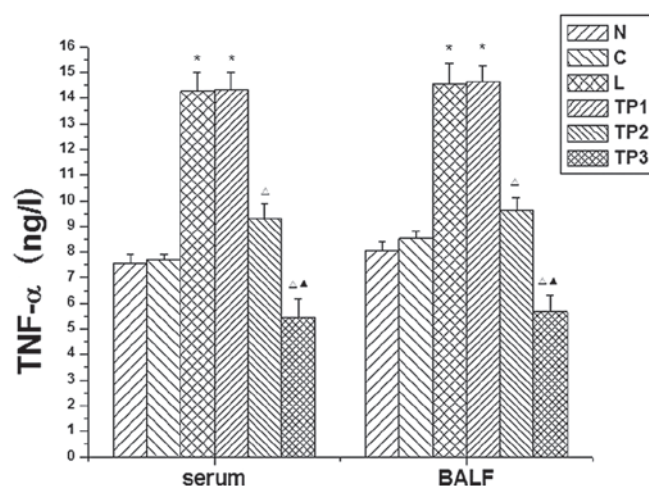


Figure 3. Changes in TNF- $\alpha$  expression in the serum and BALF of rats in all groups. \* $P < 0.05$  compared with the N and C groups.  $\Delta P < 0.05$ , compared with the N and C group. N, normal group; C, control group; L, LPS-treated group; TP<sub>1-3</sub>, triptolide-treated group at 25, 50 or 100  $\mu\text{g/kg}$ , respectively; BALF, bronchoalveolar lavage fluid; TNF- $\alpha$ , tumor necrosis factor  $\alpha$ .

and the N and C groups were statistically significant ( $P < 0.05$ ). The PaO<sub>2</sub> in the TP<sub>2</sub> and TP<sub>3</sub> groups was relatively high as compared with that in the L and TP<sub>1</sub> groups, and the differences were statistically significant ( $P < 0.05$ ) (Fig. 2).

**Changes in TNF- $\alpha$  expression in serum and BALF.** The expression levels of TNF- $\alpha$  in the serum and BALF were increased at 12 h in the L and TP<sub>1</sub> groups. As compared with the N and C groups, the differences were statistically significant ( $P < 0.05$ ). The TNF- $\alpha$  expression in the serum and BALF at 12 h in the TP<sub>2</sub> and TP<sub>3</sub> groups was decreased as compared with that in the L and TP<sub>1</sub> groups. These differences were statistically significant ( $P < 0.05$ ). As compared with the N and

C groups, the expression of TNF- $\alpha$  in the serum and BALF in the TP<sub>3</sub> group was significantly decreased ( $P < 0.05$ ) (Fig. 3).

**Analysis of ABCA1 expression in lung tissue.** The mRNA and protein expression levels of ABCA1 in the L group were significantly decreased as compared with those in the N and C groups ( $P < 0.05$ ). With the increasing doses of triptolide, the mRNA and protein expression of ABCA1 in the TP<sub>2</sub> and TP<sub>3</sub> groups showed an increasing trend. As compared with the L group, the corresponding expression of ABCA1 in the TP<sub>2</sub> group was significantly increased ( $P < 0.05$ ). As compared with the TP<sub>2</sub> group, the mRNA and protein expres-

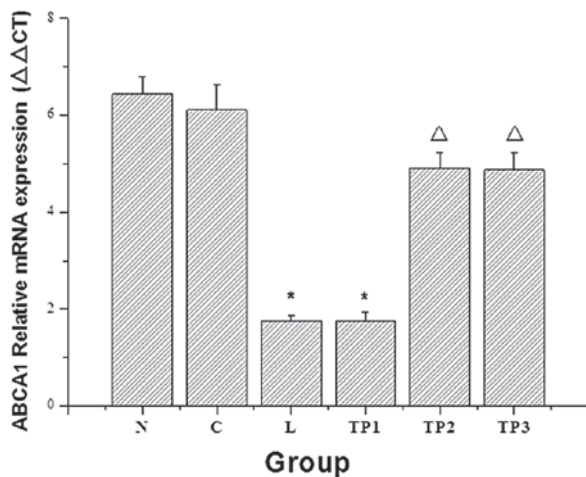


Figure 4. Changes in ABCA1 mRNA expression in the lung tissue of rats in all groups. \* $P < 0.05$  compared with the N and C groups.  $^{\Delta}P < 0.05$  compared with the L group. N, normal group; C, control group; L, LPS-treated group; TP1-3, triptolide treated group at 25, 50 or 100  $\mu\text{g/kg}$ , respectively; ABCA1, ATP-binding cassette transporter A1.

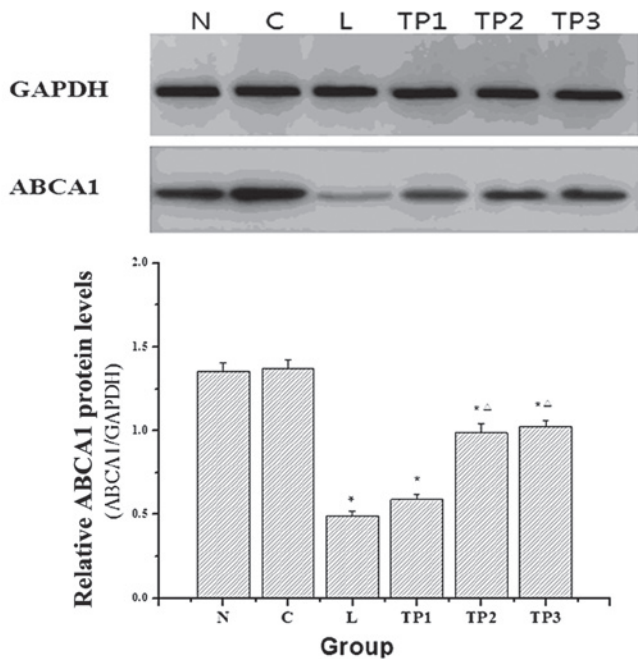


Figure 5. ABCA1 protein expression in lung tissue. \* $P < 0.05$  compared with the N and C groups.  $^{\Delta}P < 0.05$  compared with the L group. N, normal group; C, control group; L, LPS-treated group; TP1-3, triptolide-treated group at 25, 50 or 100  $\mu\text{g/kg}$  respectively; ABCA1, ATP-binding cassette transporter A1.

sion levels of ABCA1 in the TP<sub>3</sub> group were not significantly different ( $P > 0.05$ ) (Figs. 4 and 5).

## Discussion

The inflammatory response and the changes in the expression of active substances on the lung surface play an important role in the pathogenesis of LPS-mediated ALI. The regulation of signaling factors at the molecular level, in addition to the termination of the cytokine chain reaction (16-20), may be areas in which targeted therapies could be designed. ABCA1 is a transmembrane protein that is most highly expressed in the

lung. In the lung, the protein expression of ABCA1 is closely associated with the inflammatory response (6,7,10,11), and ABCA1-mediated cholesterol transport activity is correlated with the anti-inflammatory activity of ABCA1. Adjustments to the lipid composition in the lipid raft region may be the potential mechanism underlying the ABCA1-mediated inflammatory regulation. LPS stimulates alveolar macrophages in ABCA1-knockout rats, which leads to an increase in the expression of pro-inflammatory cytokine, as well as the activation of the nuclear factor  $\kappa$ -light-chain-enhancer of activated B cells-mitogen-activated protein kinase pathway (21,22).

In this study, rats were injected with endotoxin (LPS) through the caudal vein. Twelve hours after the LPS challenge, the PaO<sub>2</sub> of each group was analyzed. The PaO<sub>2</sub> of each of the LPS-treated groups was observed to be significantly decreased 3, 6 and 12 h after injection compared with that of the N and C groups ( $P < 0.05$ ). Additionally, the BALF and TNF- $\alpha$  serum expression levels, the pathological changes in the rat lung tissue and the W/D were concordant with the criteria of an animal model of ALI as compared with the C group. Compared with the C and N groups, ABCA1 gene and protein expression in the L group was respectively reduced, and these differences were statistically significant ( $P < 0.05$ ). These data preliminarily suggest that ABCA1 is involved in the formation and development of ALI/ARDS.

The results of this study showed that ABCA1 mRNA and protein expression levels in the TP<sub>2</sub> and TP<sub>3</sub> groups increased with the increasing dose of triptolide. Compared with the L group, the ABCA1 mRNA and protein expression levels were significantly different ( $P < 0.05$ ). When 50  $\mu\text{g/kg}$  was exceeded, there was no notable change. The analysis of PaO<sub>2</sub> at 3, 6 and 12 h after injection indicated that PaO<sub>2</sub> was significantly higher in the TP<sub>2</sub> and TP<sub>3</sub> groups as compared with that in the L group ( $P < 0.05$ ). The W/D in the TP<sub>2</sub> and TP<sub>3</sub> groups was lower than that in observed in the L group ( $P < 0.05$ ). The degree of pathological change in the lung was low in the TP<sub>2</sub> and TP<sub>3</sub> groups and, compared with the L group, the differences in pathological score were statistically significant ( $P < 0.05$ ). TNF- $\alpha$  expression in the serum and BALF in the TP<sub>2</sub> and TP<sub>3</sub> groups was significantly decreased 12 h after injection, as compared with the L group ( $P < 0.05$ ).

The results of the present study suggest that triptolide is capable of promoting the expression of ABCA1, reducing the secretion of inflammatory factors and relieving the lung pathological injury associated with ALI. Therefore, it is speculated that the regulation of ABCA1 gene and protein expression by triptolide may be a potential strategy for lung protection. Within a range of 25-100  $\mu\text{g/kg}$  triptolide, the regulation of ABCA1 gene and protein expression was dose-dependent, however, 100  $\mu\text{g/kg}$  was considered to be the threshold dose for dose-dependent effects.

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