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Triptolide ameliorates lipopolysaccharide-induced acute lung injury in rats

Jianling Gao, Ying Zhan, Jun Chen, Lina Wang and Jianping Yang*

Abstract

Background: Acute lung injury (ALI) is a serious clinical syndrome with a high rate of mortality. In this study, the effects of triptolide on lipopolysaccharide (LPS)-induced ALI in rats were investigated.

Methods: Sixty-five male Sprague Dawley rats (approved by ethics committee of the First Affiliated Hospital of Soochow University) were randomly divided into five groups. The control group was injected with 2.5 mL saline/kg body weight via the tail vein and intraperitoneally with 1% dimethyl sulfoxide (DMSO) ($n = 5$). The L group was administered with 0.2% LPS dissolved in saline (5 mg/kg) to induce ALI via the tail vein ($n = 15$). The TP1, TP2, and TP3 groups were treated as rats in the L group and then intraperitoneally injected with 25, 50, and 100 μg triptolide/kg body weight, respectively (15 rats per group). Blood samples from the left heart artery were taken for blood gas analysis at 1 hour before injection and at 1, 3, 6, and 12 hours after saline and DMSO administration in the control group, LPS injection in the L group, and triptolide injection in the TP1, TP2, and TP3 groups. Lung wet-to-dry weight (W/D) ratio, diffuse alveolar damage (DAD) score, TNF- α levels, and mRNA and protein expression of toll-like receptor 4 (TLR4) were analyzed.

Results: Compared with the control group, the arterial partial pressure of oxygen (PaO_2) declined ($P < 0.05$), the W/D ratio and DAD score increased ($P < 0.05$), and TNF- α levels in serum and bronchoalveolar lavage fluid (BALF) and mRNA and protein expression of TLR4 were significantly increased in the L group ($P < 0.05$). Compared with the L group, PaO_2 significantly increased in the TP2 and TP3 groups ($P < 0.05$), while the W/D ratio and DAD score were significantly decreased in the TP2 and TP3 groups ($P < 0.05$). TNF- α levels and mRNA and protein expression of TLR4 were significantly decreased in the TP2 and TP3 groups compared with the L group ($P < 0.05$).

Conclusions: Triptolide can ameliorate LPS-induced ALI by reducing the release of the inflammatory mediator TNF- α and inhibiting TLR4 expression.

Keywords: Triptolide, Lipopolysaccharide, Acute lung injury, Immunosuppressive agents, Toll-like receptor 4

Background

Acute lung injury (ALI) or its more severe form, adult acute respiratory distress syndrome (ARDS), is the pulmonary manifestation of an acute systemic inflammatory process and an important cause of mortality in the human population [1]. When ARDS occurs in the context of multisystem organ failure, mortality can reach 60% and can result in significant pulmonary impairment in more than 50% of survivors [2-4]. Therefore, there is an urgent need to improve treatment and prevention strategies to minimize the mortality of ALI.

Triptolide, a diterpenetriepoxide, is extracted from *Tripterygium wilfordii*, which is a traditional Chinese medicine and has multiple pharmacological activities including immunosuppressive, anti-inflammatory, and anti-tumor activities [5-7]. Triptolide exhibits anti-inflammatory activities in cultured lung cells and in rat models with acute chlorine-induced lung injury [8]. However, there have been no reports to date on triptolide activity for lipopolysaccharide (LPS)-induced ALI.

LPS is a component of the cell wall in gram-negative bacteria. In a clinical situation, ARDS is usually induced by LPS shock, which is very difficult to treat and is associated with high mortality [1]. ARDS is a major complication in patients with leukemia, AIDS, transplantation,

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and steroid treatment. LPS inducing in an animal model has been developed as a means to establish an ALI model and test potential therapies [9-11]. In lung injury, arterial partial pressure of oxygen (PaO₂), lung wet-to-dry weight (W/D) ratio, and diffuse alveolar damage (DAD) can be used as indicators of lung injury according to previous reports [12,13]. LPS activates monocytes and macrophages to produce cytokines such as TNF- α , IL-1, and IL-6 that serve as endogenous inflammatory mediators [14,15]. TNF- α is one of the cytokines that can induce pulmonary permeability and edema [16].

The sensing of LPS by innate immune cells is vital for host defenses against gram-negative bacteria. Toll-like receptor 4 (TLR4) can recognize LPS and activate innate immunity, meanwhile transmit signals to antigen-presenting cells and activate acquired immunity [17]. Wu *et al.* [18] found that TLR4 played a critical role in LPS-induced ALI and transfection of adenovirus Ad-siTLR4 could effectively downregulate TLR4 expression *in vitro* and *in vivo*, accompanied by alleviation of LPS-induced lung injury. A recent study demonstrated that plasminogen activator inhibitor type-1 (PAI-1) increased the expression of TLR4 to promote lung injury [19]. Thus a therapeutic strategy against these abnormalities and targets could be effective for ARDS treatment.

This study aimed to investigate whether triptolide can ameliorate LPS-induced ALI and to explore the possible mechanisms responsible for the effects of triptolide on LPS-induced ALI. PaO₂, lung W/D ratios, and DAD scores were determined to assess whether the ALI rat model is well established and the function of triptolide. TNF- α levels in serum and bronchoalveolar lavage fluid (BALF) and mRNA and protein expression of TLR4 were analyzed to explore the possible mechanisms.

Methods

Animal models

All protocols were performed in accordance with guidelines of the Ethics Committee of the First Affiliated Hospital of Soochow University, Jiangsu, China. Sixty-five male Sprague Dawley rats weighing 200 to 250 g (purchased from Shanghai Slack Laboratory Animal Limited Liability Company, Shanghai, China) were used in the study. The animals were randomly divided into five groups: control group (n = 5), L group (n = 15), TP1 group (n = 15), TP2 group (n = 15), and TP3 group (n = 15). The control group was injected via the tail vein with saline (2.5 mL/kg body weight) and intraperitoneally with 1% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St Louis, MO, USA). The L group was administered via the tail vein with 0.2% LPS (*Escherichia coli* serotype O111: B4, Sigma-Aldrich) and dissolved in saline (5 mg/kg) to induce ALI. The TP1, TP2, and TP3 groups were treated as rats in the L group and then intraperitoneally injected with 25, 50, and

100 μ g triptolide (dissolved in 1% DMSO, a gift from Nanjing Institute of Skin Disease Prevention and Control, Nanjing, China)/kg body weight, respectively.

Collection of blood and bronchoalveolar and measurement of TNF- α in serum and BALF

Rats were anesthetized with chloral hydrate (300 mg/kg) and then blood (0.3 mL) was collected from the left heart artery for blood gas analysis by an artery blood gas analyzer (Nova Biomedical, Waltham, MA, USA) at 1 hour before injection and at 1, 3, 6, and 12 hours after saline and DMSO administration in the control group, LPS injection in the L group, and triptolide injection in the TP1, TP2, and TP3 groups. Blood was anticoagulated by heparin and centrifuged at 2,000 rpm for 20 minutes at 4°C. Supernatant was then collected and stored at -20°C.

After blood collection at 12 hours, as mentioned above, rats were killed with an overdose of pentobarbital. The right bronchus was clamped and a 24G trocar (BD, Franklin Lakes, USA) was placed in the cannula through the tracheal ring gap. The bronchoalveolar was washed with 25 mL/kg saline three times at 4°C. BALF obtained from rats was pooled and centrifuged at 1,200 rpm for 10 minutes at 4°C. The supernatant was then taken and stored at -20°C. TNF- α levels in the serum and BALF were determined by an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA).

Collection of lung tissue and measurement of lung W/D

After rats were executed, the inferior lobe of the right lung was taken and washed with saline at 4°C. The tissue was plated in ice and cut into pieces. Next, 100 mg of tissue was placed in centrifuge tubes containing RNAlater and incubated at room temperature for 1 hour, then 4°C overnight, and stored at -20°C until RNA extraction and PCR. The remaining inferior lobe of the right lung was cut into small pieces and placed in freezing tubes equipped with glycerol. It was then frozen with liquid nitrogen for 10 minutes and stored at -70°C until histological examination and western blot analysis.

The lung W/D ratio was calculated as previously described [20]. The middle lobe of the right lung was excised and rinsed with saline at 4°C, blotted with filter paper to absorb the surface water, and weighed to obtain the wet weight (W). The lung was then placed in an oven at 80°C for 24 hours to obtain the dry weight (D). Finally, the lung W/D ratio was calculated.

Histological examination

Lung tissues were fixed in 4% paraformaldehyde (PFA), embedded in paraffin, and cut into 5 μ m thick sections. Tissues were stained with hematoxylin and eosin (H&E) and observed with a Nikon Eclipse E800 microscope (Nikon, Tokyo, Japan). ALI was scored (as described

previously) [21] according to the following DAD criteria: 1) effusion of neutrophils to the alveolar space and the interval: 0 (no effusion), 1 (doubtful effusion), 2 (scattered effusion), and 3 (more or special mess distributed' effusion)); 2) broadening of the alveolar septum: 0 (no broadening), 1 (a little broadening), 2 (clear broadening), 3 (loss of the normal alveolar structure); 3) bleeding in the alveolar space: 0 (no red blood cells), 1 (a few red blood cells), 2 (more red blood cells), and 3 (almost filled with red blood cells); and 4) effusion of the fibrin in the alveolar space: 0 (no fibrin), 1 (a few fibrin), 2 (more fibrin), and 3 (almost filled with fibrin). Scores were added up and expressed as mean \pm SD (standard deviation).

Extraction of RNA and quantitative PCR analysis

Total RNA was extracted from the lung tissue using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). RNA purification was conducted with an RNeasy kit (Takara, Shiga, Japan) and residual DNA was removed by DNase I (Takara). PCR primers were designed using Primer Premier version 5.0 software (Premier Biosoft, Palo Alto, CA, USA) and sequences were as follows (Invitrogen): TLR4: forward: 5'- CCTTTCCTGCCTGAGACC-3', reverse: 5'-TTCTTGGTTGAATAAGGGATGT-3', product length: 123 bp; β -actin: forward: 5'-CCCATCTATGAGGGTTACGC-3', reverse: 5'-TTTAATGTCACGCACGATTTC-3', product length: 150 bp. β -actin was used as an internal standard. Quantitative PCR reaction conditions were as follows: initial denaturation at 95°C for 2 minutes followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing at 60°C for 15 seconds, and extension at 72°C for 20 seconds. The comparative C_T method (also known as the $2^{-\Delta\Delta C}$ method) was used to calculate the relative expression of TLR4 [22].

Western blot analysis

The inferior lobe of the right lung was stored with glycerol at -70°C and taken for western blot analysis. The total protein of the lung tissue was extracted and the concentration was determined using a BCA Protein Quantification Kit (Abcam, Hongkong, China). Next, 50 μ g of protein was loaded, separated by 10% SDS-PAGE, and transferred to polyvinylidene difluoride (PVDF) membranes. After blocking with 5% nonfat dry milk at room temperature for 1 hour and 4°C overnight, the membranes were blotted with the primary antibodies, including TLR4 and GAPDH antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), for 2 hours at 37°C. Membranes were washed with Tris-buffered saline containing 0.2 to 0.4% Tween-20 (TBST) three times and then incubated with horseradish peroxidase (HRP) conjugated rabbit-anti-mouse secondary antibodies (Shanghai Boyao Biotechnology, Shanghai, China)

for 1 hour at room temperature. The optical density was detected by a gel image analysis system (Bio Image System, Inc, Aquarias, Singapore).

Statistical analysis

Data were analyzed using the SAS statistical package (version 8; SAS Institute, Cary, NC, USA) and presented as means \pm standard deviation. One-way analysis of variance (ANOVA) was used for comparison among groups. Data was considered statistically significant when $P < 0.05$.

Results

Effect of triptolide on LPS-induced lung PaO₂

Table 1 demonstrates that PaO₂ in the L group declined by 44.04% after injection of LPS compared with before injection. PaO₂ in the L group decreased significantly after approximately 3 to 12 hours of LPS injection

Table 1 Effect of triptolide on partial pressure of oxygen (PaO₂), partial pressure of carbon dioxide (PaCO₂), and pH in arterial blood

Group	Time (hours)	PaO ₂ (mmHg)	PaCO ₂ (mmHg)	pH
Control group (n = 5)	1	104.4 \pm 2.3	35.6 \pm 2.2	7.3 \pm 0.1
	1	101.4 \pm 2.7	36.4 \pm 2.3	7.4 \pm 0.0
	3	103.9 \pm 3.0	35.5 \pm 2.2	7.4 \pm 0.0
	6	102.1 \pm 3.8	34.6 \pm 3.2	7.3 \pm 0.1
L group (n = 15)	12	103.8 \pm 3.0	35.9 \pm 3.7	7.4 \pm 0.1
	1	102.1 \pm 2.4	35.5 \pm 3.1	7.3 \pm 0.0
	1	94.7 \pm 6.7	36.2 \pm 4.4	7.3 \pm 0.1
	3	72.5 \pm 8.2 ^a	35.5 \pm 4.7	7.2 \pm 0.2
TP1 group (n = 15)	6	64.2 \pm 6.8 ^a	36.6 \pm 5.8	7.3 \pm 0.2
	12	57.2 \pm 5.5 ^a	36.9 \pm 4.2	7.2 \pm 0.3
	1	103.1 \pm 2.4	35.6 \pm 1.4	7.4 \pm 0.1
	1	93.5 \pm 6.7	35.5 \pm 1.9	7.3 \pm 0.1
TP2 group (n = 15)	3	72.5 \pm 7.6 ^a	36.3 \pm 2.4	7.3 \pm 0.1
	6	59.2 \pm 6.8 ^a	36.9 \pm 4.7	7.3 \pm 0.2
	12	53.4 \pm 8.2 ^a	36.5 \pm 5.0	7.3 \pm 0.2
	1	105.1 \pm 2.4	35.5 \pm 1.6	7.3 \pm 0.0
TP3 group (n = 15)	1	94.8 \pm 6.4	35.3 \pm 1.6	7.4 \pm 0.1
	3	79.9 \pm 7.2 ^a	35.8 \pm 2.2	7.3 \pm 0.1
	6	82.2 \pm 6.8 ^{ab}	36.2 \pm 3.8	7.3 \pm 0.2
	12	77.4 \pm 7.4 ^{ab}	36.4 \pm 4.2	7.3 \pm 0.2
TP3 group (n = 15)	1	102.1 \pm 2.4	35.4 \pm 1.7	7.4 \pm 0.0
	1	93.8 \pm 5.7	35.3 \pm 1.4	7.3 \pm 0.1
	3	83.8 \pm 6.8 ^a	35.9 \pm 2.5	7.3 \pm 0.2
	6	79.2 \pm 6.8 ^{ab}	35.6 \pm 3.4	7.3 \pm 0.2
	12	74.4 \pm 5.6 ^{ab}	36.0 \pm 3.6	7.3 \pm 0.2

Data presented as mean \pm standard deviation. ^a $P < 0.05$, compared with control group; ^b $P < 0.05$, compared with L group.

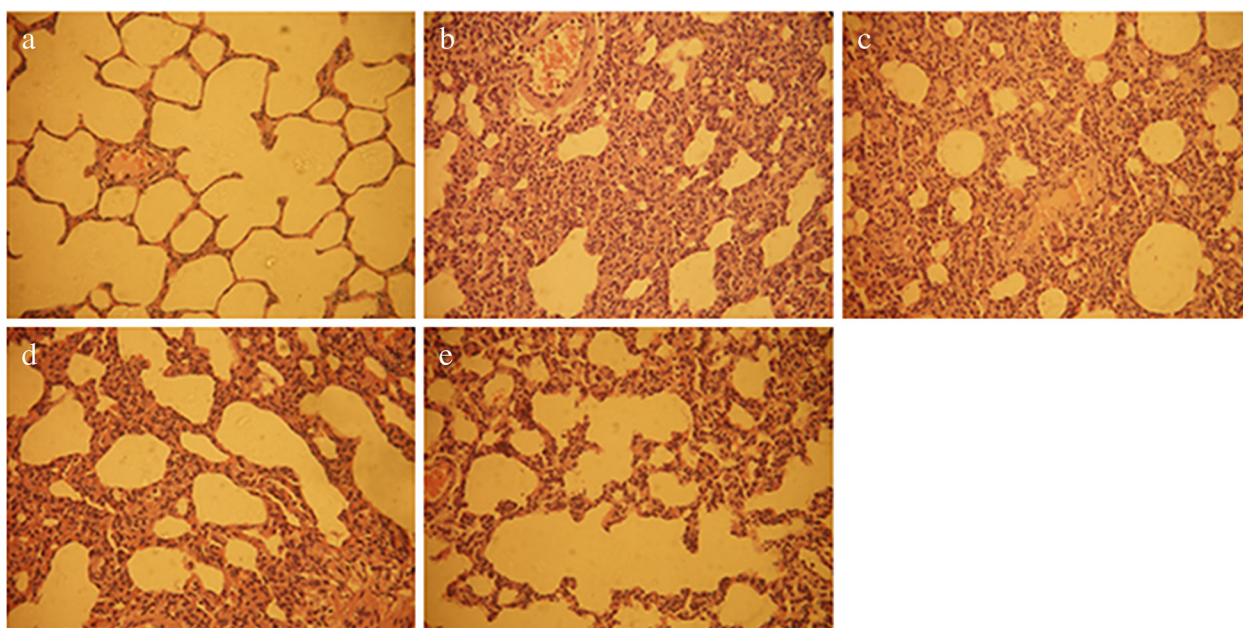


Figure 1 Lung pathologies. (a) Control group, (b) L group, (c) TP1 group, (d) TP2 group, and (e) TP3 group with hematoxylin-eosin (H&E) staining ($\times 400$). Injection of LPS caused infiltration of inflammatory cells into the lung interstitial and alveolar spaces, alveolar wall thickening, and intra-alveolar exudation. In TP1, TP2, and TP3 groups, triptolide attenuated these histological changes. LPS, lipopolysaccharide.

compared with the control group ($P < 0.05$). PaO_2 in the TP2 and TP3 groups were relatively higher at 6 and 12 hours compared with the L group ($P < 0.05$). PaCO_2 and pH values had no statistical significance among these groups ($P > 0.05$).

Effect of triptolide on LPS-induced lung pathological changes

H&E staining results showed that the alveolar structure of the control group was complete and had no abnormal changes such as effusion of inflammatory cells and broadening of the pulmonary interstitial space (Figure 1a). After injection of LPS, there was significant alveolar interval widening, white blood cell exudation and gathering, pulmonary capillary expansion and congestion, alveolar cavity effusion, and even transparent membrane formation could be observed (Figure 1b). Pathological changes such as alveolar septum and effusion of inflammatory cells could be seen in groups TP1, TP2, and TP3 under microscope. But pathological changes of groups TP2 and TP3 were lighter than group TP1 (Figure 1c,d,e).

Effect of triptolide on DAD score and W/D ratio

Pulmonary edema is a hallmark of ALI, and the standard assessment of edema is to measure the amount of water in the lungs. Table 2 shows the DAD score and W/D ratio for the control group, L group, and TP1, TP2, and TP3 groups. The DAD score and W/D ratio in the L group was 3.8 and 1.7 times greater than the control

group, respectively ($P < 0.05$). In the TP1 group, the DAD score and W/D ratio were not significantly different compared with those of the L group, and lower in the TP2 and TP3 groups than in the L group ($P < 0.05$).

Effect of triptolide on TNF- α levels in serum and BALF

As shown in Figure 2, TNF- α in serum and BALF were increased by 1.8 and 1.7 times, respectively, in the L group compared with the control group ($P < 0.05$) and also higher in the TP1 group compared with the control group ($P < 0.05$). However, there was no statistical significance between the TP1 group and L group ($P > 0.05$). TNF- α in serum and BALF were significantly decreased in the TP2 and TP3 groups compared with the L group ($P < 0.05$). It was observed that TNF- α levels decreased with the increase of triptolide dose. The results showed

Table 2 Effect of triptolide on the diffuse alveolar damage (DAD) score and wet-to-dry weight ratio (W/D)

Group	DAD score	W/D ratio
Control group (n = 5)	2.55 \pm 0.45	3.49 \pm 0.18
L group (n = 15)	9.84 \pm 1.50 ^a	5.84 \pm 0.32 ^a
TP1 group (n = 15)	9.22 \pm 1.35 ^a	5.39 \pm 0.24 ^a
TP2 group (n = 15)	6.35 \pm 1.05 ^{ab}	4.42 \pm 0.26 ^{ab}
TP3 group (n = 15)	5.40 \pm 0.65 ^{ab}	4.23 \pm 0.22 ^{ab}

Data are presented as mean \pm standard deviation. ^a $P < 0.05$, compared with control group; ^b $P < 0.05$, compared with L group.

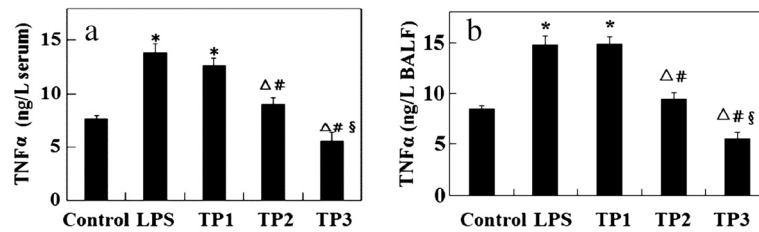


Figure 2 Effect of triptolide on TNF- α levels. (a) Serum and (b) BALF. Data presented as mean \pm standard deviation. * $P < 0.05$, compared with control group; ^Δ $P < 0.05$, compared with L group. Pairwise comparison among TP1, TP2, and TP3 groups: [#] $P < 0.05$, compared with TP1 group; [§] $P < 0.05$, compared with TP2 group. BALF, bronchoalveolar lavage fluid.

that triptolide inhibited TNF- α expression in a dose-dependent manner ($P < 0.05$).

Effect of triptolide on expression level of TLR4

As shown in Figure 3, mRNA and protein expression levels of TLR4 were upregulated in the L group compared with the control group ($P < 0.05$). However, there was no statistical significance between the TP1 group and L group ($P > 0.05$). Their levels were significantly downregulated in the TP2 and TP3 groups compared with the L group ($P < 0.05$). The mRNA and protein expression levels decreased with the increase of triptolide dose. Triptolide affected expression levels of TLR4 in a dose-dependent manner ($P < 0.05$).

Discussion

ALI can be induced by exposure of the lung to LPS [23] and in this study we used LPS-induced rats to assess the function of triptolide. The results showed that triptolide can increase the PaO₂ level, ameliorate LPS-induced histological changes, and attenuate the increased DAD value and lung W/D ratio after LPS injection. Triptolide also decreased the levels of TNF- α in serum and BALF

and reduced mRNA and protein expression levels of TLR4.

In the present study, a rat model of ALI was established by tail vein injection of LPS, which decreased PaO₂ and increased DAD values and lung W/D ratios. Rojas *et al.* [12] observed that endotoxin (LPS) injection led to an increase of W/D ratio and changes of histology which could be characterized as the onset of ALI. DAD is a well-recognized histological pattern associated with an acute clinical presentation [24]. Our results indicated that triptolide intervention can improve PaO₂, decrease W/D ratio and pulmonary edema, and alleviate lung injury in ALI rats. Laffey *et al.* [25] observed that hypercapnic acidosis attenuated the increased PaO₂ in LPS-induced ALI. Itoh *et al.* [26] demonstrated that adrenomedullin attenuated the increase of lung W/D ratio and histological changes to ameliorate ALI [26]. Taken together, the studies indicate that triptolide ameliorates LPS-induced ALI in rats.

LPS has been reported to induce production of several cytokines. TNF- α is a proinflammatory cytokine and participates in several important processes involved in inflammatory response. Triptolide is the most potent inhibitor of lung inflammation in a library of 446

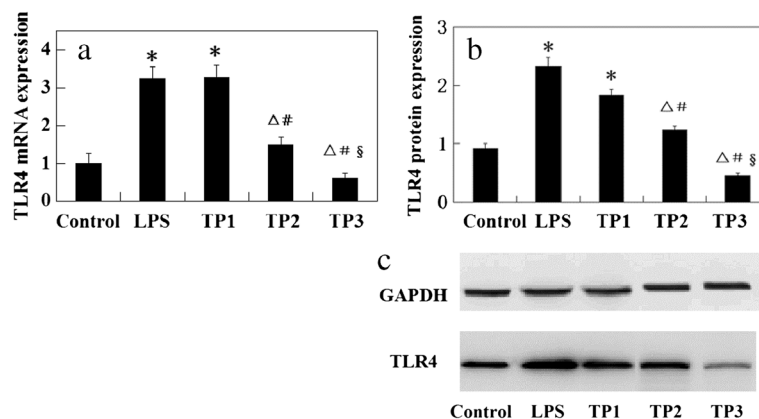


Figure 3 Effect of triptolide on expression level of TLR4. (a) mRNA and (b) protein expression of TLR4 in rat lung tissue. * $P < 0.05$, compared with control group; ^Δ $P < 0.05$, compared with L group. Pairwise comparison among TP1, TP2, and TP3 groups: [#] $P < 0.05$, compared with TP1 group; [§] $P < 0.05$, compared with TP2 group.

compounds in the National Institutes of Health (NIH) Clinical Collection [8]. In our study, TNF- α markedly increased in serum and BALF after LPS injection and triptolide decreased the TNF- α level. The results can be supported by earlier *in vitro* findings that the triptolide suppresses the TNF- α /TNFR2 signal pathway in IL-10-deficient mice [27]. This study showed that triptolide reduced LPS-stimulated lung inflammation through inhibition of TNF- α production.

TLR4 plays a critical role in LPS-induced ALI [18]. Baumgarten *et al.* [28] found that the pulmonary gene expression of proinflammatory cytokines such as TNF, IL-1 β , and IL-6 as well as their corresponding receptors TNFRp75 and IL-1RII were regulated by LPS via TLR4. In our study, mRNA and protein expression levels of TLR4 increased after injection of LPS in rats and triptolide infusion attenuated the increase, which corresponded with TNF- α level changes in serum and BALF. A previous study showed that triptolide suppressed TLR expression in IL-1 β -treated human intervertebral disc cells [29]. Triptolide ameliorated Crohn's colitis associated with inhibition of the TLR/NF- κ B signaling pathway [30]. The results demonstrated that triptolide can ameliorate LPS-induced ALI via TLR4. However, details of the regulation of TLR4 in LPS-induced ALI by triptolide requires further study.

Conclusions

In summary, triptolide can ameliorate LPS-induced ALI by reducing the release of the inflammatory mediator TNF- α and inhibiting TLR4 expression. These results demonstrate that triptolide could provide potential therapy for LPS-induced ALI.

Abbreviations

ALI: Acute lung injury; ANOVA: Analysis of variance; ARDS: Acute respiratory distress syndrome; BALF: Bronchoalveolar lavage fluid; DAD: Diffuse alveolar damage; DMSO: Dimethyl sulfoxide; ELISA: Enzyme-linked immunosorbent assay; H&E: Hematoxylin and eosin; HRP: Horseradish peroxidase; IL: Interleukin; LPS: Lipopolysaccharide; NIH: National institutes of health; PaCO₂: Partial pressure of carbon dioxide; PAI-1: Plasminogen activator inhibitor type-1; PaO₂: Arterial partial pressure of oxygen; PFA: Paraformaldehyde; PVDF: Polyvinylidene difluoride; TBST: Tris-buffered saline and tween 20; TLR4: Toll-like receptor 4; TNF: Tumor necrosis factor; W/D: Wet-to-dry weight.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JLG and YZ participated in the design of the study, and performed the statistical analysis. JC carried out the study and together with LNW collected important background information. JPY drafted the manuscript. All authors read and approved the final manuscript.

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