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Original Article

Lumican overexpression exacerbates LPS induced lung injury in mice

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Abstract: To investigate the role of lumican in mice with endotoxin-acute lung injury (ALI). Lumican transgenic mice and wild type mice were injected with lipopolysaccha (LPS, 10 mg/kg) to establish the ALI model. Mice were sacrificed at 24 h and lung tissue samples and serum were collected. Lung wet-to-dry weight (W/D) ratio was measured to determine pulmonary edema. ELISA was used to measure the concentrations of cytokines in serum such as proinflammatory cytokines including tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), IL-4 and IL-10 in LPS-stimulated mice. The expressions of TLR4 and NFκB p65 proteins in lung tissues were examined using western blot analysis. Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) was performed to monitor apoptosis of lung tissue. Light microscopy and electron microscopy were used to observe the structure changes of lung tissues. After LPS administration, the values of lung wet-to-dry weight (W/D) in the lumican transgenic group were higher than those in the control group. Compared with those in the control mice, the expression levels of lung TLR4 and NFκB, and the cytokines of TNFα, IL-6, IL-4 and IL-10 in serum were upregulated in lumican transgenic mice. Apoptosis was detected mainly on pulmonary vascular endothelial cell and pneumocytes. There was significant difference in the optical density of apoptotic body between the control mice and the lumican transgenic mice. Results of light and electron microscopy exhibited more severe alveolar hemorrhage, pulmonary interstitial edema, visible inflammatory cell infiltration and edema of connective tissue in lung interstitium in the lumican transgenic mice than in the control mice. We found that LPS may cause excessive apoptosis in the pneumocytes via the TLR4 signal transduction pathway and ALI. Lumican may play a role in this process and exacerbates ALI.

Keywords: Lipopolysaccharide, acute lung injury, apoptosis

Introduction

Lumican is one of the major extracellular proteins in interstitial extracellular matrix (ECM) of the skin, corneal stroma, sclera, aorta, muscle, lung, kidney, bone, cartilage and intervertebral discs. It is a member of the small leucine-rich proteoglycans family, with a core protein of 30-50 kDa comprising a signal peptide, a negatively charged N-terminal domain [1], a highly conserved leucine-rich internal domain and a carboxyl-terminal domain. Both the protein core and the glycan chains of lumican can interact with various cellular effectors [2], including cytokines, growth factors, and cell surface receptors to modulate cell adhesion, proliferation, and migration [3]. The primary function of lumican is for the rigidity of collagen fibers [4]. Additionally, lumican plays roles in cellular migration and cell differentiation, and has paracrine function [5]; therefore, lumican is classified as a matrikine. Lumican is also involved in cancer cell proliferation and metastasis [6]. Recently, it has been reported that lumican-deficient (Lum-/-) mice is defective in immunological response through the Fas-Fas ligand pathway [7]. Lumican have also been shown to regulate host response to pathogen-associated molecular patterns [8]. Thus, the Lum-/- mice are hyporesponsive to bacterial lipopolysaccharide (LPS) endotoxins, and Lum-/- macrophages in cell culture produce lower levels of pro-inflammatory cytokines in response to LPS [9]. Lumican facilitates innate immune response by binding LPS and transfers the LPS signal to toll-like receptor 4. However, the detail immunological role of lumican is unclear.

Acute lung injury (ALI) is a life-threatening illness characterized by wide spread capillary

leakage, low lung compliance, severe resistant hypoxemia, severe difficulty breathing, increased rate of breathing, and even multiple organ failure [10]. Pathophysiology of ALI shows as alveolar-capillary barrier damage with noncardiogenic pulmonary edema, excessive invasion of inflammatory cells, and release of inflammatory mediators [11, 12]. Lipopolysaccharide (LPS) is a main component of the gram-negative bacteria cell wall, which could activate Toll like receptor (TLR) 4 and trigger an inflammatory response, including recruit polymorphonuclear leukocytes (PMNs) into the lung with increasing in capillary permeability [13, 14]. The production of inflammatory mediators plays an important role in the pathophysiology of inflammation in lung injury. In this study, we determined the role of lumican in LPS-induced systemic inflammation and lung injury using a mouse endotoxemia model. We found that lumican exacerbated LPS-induced inflammation and lung injury in murine sepsis models. The potential mechanism may be partly through the adjustment of the TLR4-NFκB pathway.

Materials and methods

Mouse husbandry

C57 mice were cross-bred with EF1-Lum transgenic mice (DBF1) overexpressing lumican under the control of the EF1 promoter. After genotyping, the transgenic EF1-Lum mice from one line were used in this study and compared with age-matched and strain-matched C57 mice. Eight to ten-week old male C57BL/6 mice and lumican transgenic mice weighed 20-25 g were obtained from the Experimental Animal Center of China Medical University (Shenyang, China). Mice were housed in rooms maintained at 26°C and 12-hour light/dark cycle for at least one week to acclimate them to the surroundings with free access to water and standard mouse chow. The study was approved by the ethics committee of the China Medical University (Shenyang, China) and conformed to the guide for the care and use of laboratory animals published by the National Institutes of Health (Bethesda, MA, USA).

Experimental protocols

Mice were randomly divided into four groups: wild type control (WT CTR) group, LPS (WT LPS) group, lumican transgenic control (TG CTR) group and LPS (TG LPS) group. We used an endotoxemia mouse model with intraperitoneal injection of a dose of LPS (*Escherichia coli* serotype 055: B5; Sigma, St. Louis, MO, USA). Three different LPS doses were tested (10, 15 and 20 mg/kg body weight), and the lowest dose was selected because it was sufficient to induce septic shock in wild type mice. Each mouse in the LPS group was administered 10 mg/kg body weight LPS. In the control group, an equal volume of sterile saline (Beijing Tiantan Biological Products Co., Ltd, Beijing, China) was administered. Blood samples (0.5 ml) were collected from the inferior vena cava of the mice while the mice were under anesthesia with isofurane (2%; Abbott Laboratories Co., Shanghai, China), at 24 h following LPS injection. The blood samples were immediately centrifuged at 1,000 x g for 20 min and the serum was stored at -80°C. Following the collection of blood, the mice were sacrificed by cervical dislocation and the lungs were excised. Each of these experimental groups included ten mice.

Assessment of lung edema

To assess the lung edema, the wet-to-dry weight (W/D) ratio was calculated as described earlier [15]. The right lung was excised and its inferior lobe was blotted dry and weighed to obtain the “wet” weight. Then it was placed in an oven at 70°C for 3 days to obtain the “dry” weight.

Measurement of serum cytokines

Serum was collected for the measurement of cytokines by standard sandwich enzyme linked-immunosorbent assay (ELISA). Mouse ELISA kits for TNFα, IL-6, IL-4 and IL-10 were obtained from R&D Systems (Minneapolis, MN, USA). Recombinant cytokines were used as standard controls. Experimental samples, negative controls, and diluted standard markers were added into each well. Absorbance was measured using a Biotek ELx808 absorbance reader (BioTek Instruments, Inc., Winooski, VT, USA) at 540 nm. Total protein concentration was measured with the Bradford assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

Determination of the expression of TLR4 and NFκB by western blot analysis

50 mg lung tissue samples were lysed with lysis buffer (Sigma-Aldrich), and another 50 mg were lysed with CelLytic™ NuCLEAR™ Extr-

Table 1. Lung wet-to-dry weight (W/D) ratio in each group ($x \pm s$, $n = 10$)

Group	W/D
WT CTR	4.12 ± 0.13
WT LPS	6.29 ± 0.24#
TG CTR	4.57 ± 0.16
TG LPS	7.53 ± 0.26#,*

#$P < 0.05$ *vs* WT CTR group; *$P < 0.05$ *vs* WT LPS group. Values are expressed as the mean ± standard error (n = 10). WT, wild-type; TG, transgenic mice; CTR, contro l group; LPS, LPS treatment group; W/D,wet to dry ratio.

action Kit (Sigma-Aldrich) for nuclear protein extraction. Protein concentrations were determined using protein assay kit (Bio-Rad, Hercules, CA, USA). Equal quantity of protein (50 μg) from tissue homogenates was separated using 10% SDS-PAGE and was subsequently transferred onto nitrocellulose membranes (Bio-Rad Laboratories). Following blocking of the membrane with 5% non-fat milk in Tris-buffered saline (Sigma-Aldrich) at room temperature for 1 h, the membrane was incubated with mouse monoclonal primary antibodies against TLR4 (1:1,000; cat. no. 2246) and NFκB p65 (1:1,000; cat. no. 8242) purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA) at 4°C for 12 h. The membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology, Inc.) for 1 h and signals were observed using an enhanced chemiluminescence kit (Amersham Pharmacia, GE Healthcare, Amersham, UK). The membranes were subsequently reprobed with a mouse monoclonal antibody against actin (1:1,000; cat. no. 12262) and histone H4 (1:1,000; cat. no. 13919) purchased from Cell Signaling Technology, Inc. at room temperature for 2 h as indicators for equal loading of the samples. Western blotting data were quantified by densitometric analysis using Image J version 1.38x (NIH Image software, Bethesda, MA, USA). Values are expressed as relative differences following normalization against the expression levels of actin and histone H4.

Determination of lung cell apoptosis by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)

Apoptotic cells were detected using a TUNEL assay kit (Promega Corporation, Madison, WI, USA). Fresh lung sections were fixed in 10% buffered formalin and embedded in paraffin, and 4-μm slices were stained using the in situ Cell Death Detection kit (Promega Corporation) according to the manufacturer's instructions. Three tissue sections from each sample were randomly selected and 10 microscopic fields per section were assessed by two independent observers. In each field, the nuclei were quantified and the percentage of TUNEL-positive nuclei was determined.

Observation of pathological changes by light microscopy and electron microscopy

After 24 h of LPS injection, animals were sacrificed by cervical dislocation, followed by immediate organ collection for histological analysis. Fresh left lung sections were fixed in 10% buffered formalin (Sigma-Aldrich) and embedded in paraffin; 4 μm sections were stained with hematoxylin and eosin (Sigma-Aldrich). Samples were assessed using an Olympus CX22 light microscope (Olympus, Tokyo, Japan). For electron microscopy, lungs were perfusion fixed with 1.25% glutaraldehyde (Sigma-Aldrich) in 0.1 M phosphate buffer (pH 7.4) and were subsequently cut in sagittal and horizontal cross sections for image analysis. Sections (1 μm) were dried overnight at 45°C on gelatin-coated slides (Sigma-Aldrich), stained at 60°C for 2 h in Giemsa (Sigma-Aldrich), cooled to room temperature, dehydrated, cleaned in xylene and mounted in permount (Sigma-Aldrich). A JEOL 1011 transmission electron microscope with a Hamamatsu Orca-HR Digital Camera (JEOL, Inc., Peabody, MA, USA) and the Advanced Microscopy Techniques Corp. AMT16000B image capture system (Advanced Microscopy Techniques Corp., Danvers, MA, USA) were used. A pathologist analyzed the samples and determined the levels of injury in a blinded manner.

Statistical analysis

Data were analyzed with SPSS 13.0 (SPSS, Inc., Chicago, IL, USA), differences were compared by ANOVA followed by Bonferroni correction for post hoc t-test where appropriate. And the significance level was set at $p < 0.05$.

Results

Lung edema

In this investigation, we examined the lung edema (as shown in **Table 1**) after LPS treat-

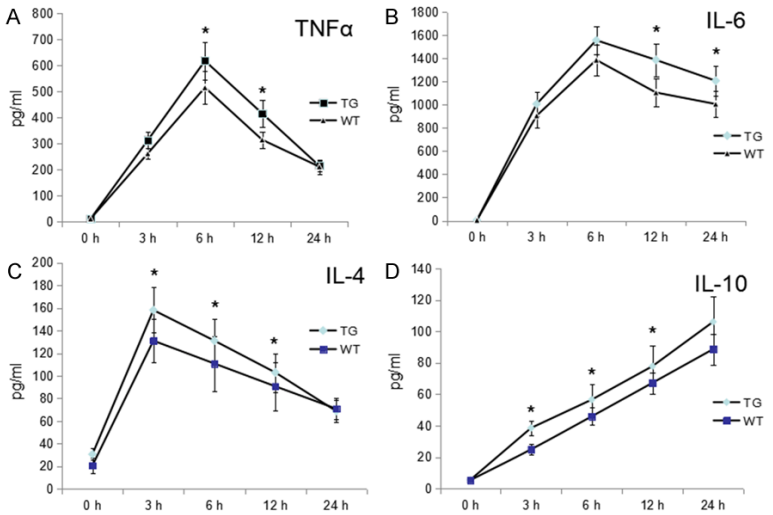

Figure 1. Release of inflammatory cytokines into serum. Following treatment with lipopolysaccharides for 3, 6, 12 and 24 h, blood were collected and serum were separated and selected cytokines, including (A) TNFα, (B) IL-6, (C) IL-4 and (D) IL-10, were measured by standard sandwich enzyme linked-immunosorbent assay (ELISA). *P < 0.05, vs WT LPS. WT, wild-type; TG, transgenic mice; IL, interleukin; TNF, tumor necrosis factor.

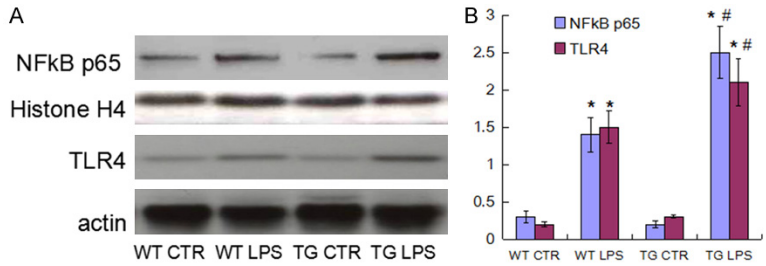

Figure 2. Expression of TLR4 and NFκB following treatment with LPS. A: Western blot bands of expression levels of TLR4 and nuclear NFκB following treatment with LPS. B: Densitometric values normalized by actin and histone H4. Results are expressed as the mean ± standard error (n = 6 for each group). *P < 0.05, vs WT CTR; #P < 0.05, vs WT LPS. WT, wild type; TG, transgenic mice; CTR, control; LPS, lipopolysaccharide; NF, nuclear factor; TLR, Toll-like receptor.

ment. LPS injection led to significant increase in wet-to-dry weight (W/D) ratio compared with WT and TG mice in the control group (p < 0.05), but the increase was attenuated in the WT group (P < 0.05). The results were in accordance with histological changes confirmed as mentioned below.

Release of inflammatory cytokines into serum

LPS induced increases of TNF-α, IL-6, IL-4 and IL-10 compared with the control group (P < 0.05); however, TNF-α, IL-6, IL-4 and IL-10 in the TG LPS group were significantly higher than those in the WT LPS group (P < 0.05) (**Figure 1**).

TLR4 and NFκB expression after LPS treatment

As shown in **Figure 2**, LPS promoted the increases in nuclear NFκB p65 and TLR4 expressions compared with those in the control mice in both strain of mice, but the expressions of NFκB p65 and TLR4 were reduced in the WT LPS group compared with TG LPS group (**Figure 2**).

Apoptosis in lung tissues after LPS treatment

Apoptosis was detected mainly on pneumocytes. There was significant difference in the optical density of apoptotic body between the control mice and the lumican transgenic mice after LPS treatment. LPS induced the increase of TUNEL staining in WT and TG group compared with the control group (P < 0.05), however, the increase was significant lower than that in the TG LPS group (P < 0.05) (**Figure 3**).

Observation of pathological changes by light microscopy and electron microscopy

Lung sections were stained with hematoxylin and eosin, and sections were evaluated for the severity (score: 0-3) of lung bleeding, pulmonary interstitial exudation and inflammatory cell infiltration in the alveolar cavity by an experienced pathologist who was blinded to the treatment each animal had received. The results are shown in **Figure 4**. In the control group, no obvious abnormality was observed under the light microscope, the lung tissue was clear, and there was no inflammatory cell infiltration in the alveolar cavity. And no bleeding, no obvious pulmonary interstitial exudation; While in the LPS group, part of alveolar wall fracture and part of wider, emphysema, pulmonary capillary dilation, partial alveolar hemorrhage, pulmonary interstitial edema, visible inflammatory cell in-

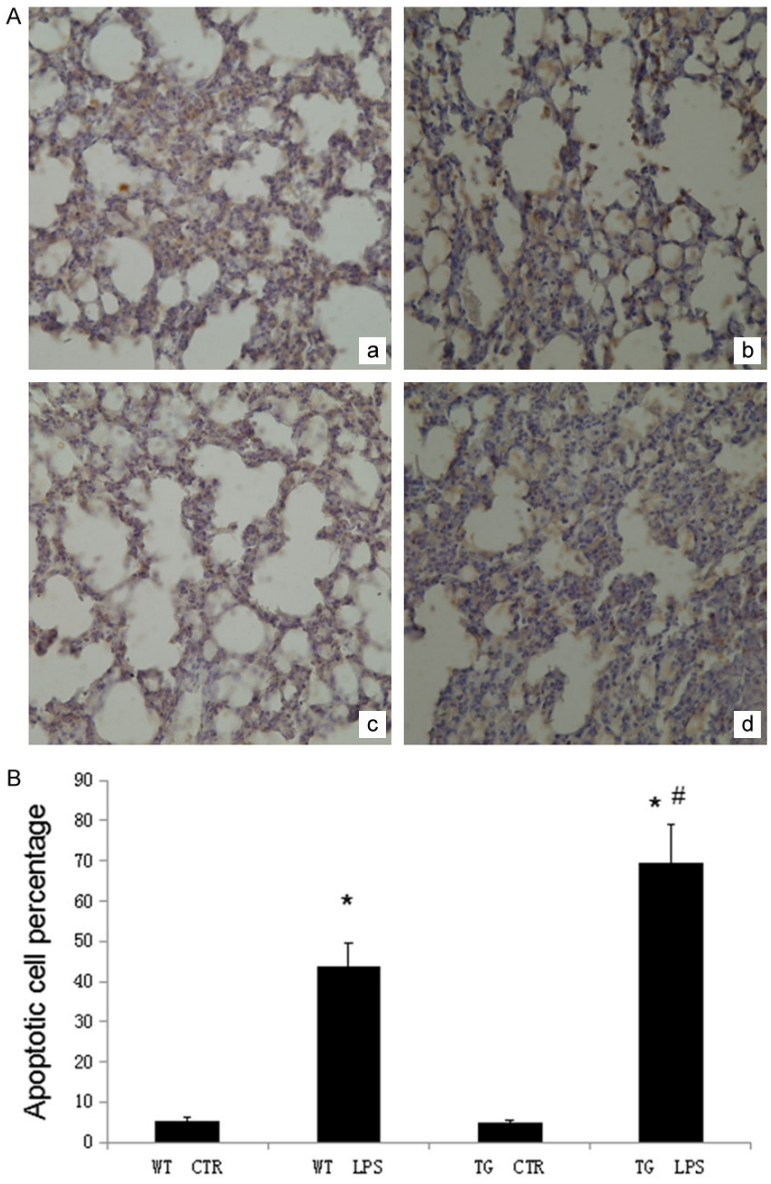

Figure 3. Apoptosis in lung tissues after LPS. TUNEL staining showed the tissue apoptosis after LPS treatment. Percentage of positive apoptosis nuclei and total nuclei are expressed as mean ± SEM (n = 6 for each group). WT: wild type mice; TG: transgenic mice; CTR: control group; LPS: LPS treatment group. A. A picture of TUNEL staining (400×), a: WT CTR group, b: WT LPS group, c: TG CTR group; d: TG LPS group. B. The percentage of apoptotic cells. *$P < 0.05$, WT LPS vs WT CTR; #$P < 0.05$, TG LPS vs WT LPS.

filtration and edema of connective tissue in lung interstitium were observed (**Figure 4A** and **4B**). Transmission electron microscopy of lung tissues from mice that received LPS demonstrated significant ultrastructural changes compared with the controls, in the LPS group, the microvilli of the type II pneumocytes reduced and cells appeared swollen, and some contained extensive vacuoles. (**Figure 4C**). None of the pathologic findings was observed in the lungs of the control mouse; while results of light and electron microscopy exhibited more severe lung tissue injury in the lumican transgenic mice than in the control mice.

Discussion

Proteoglycans (PG) are widely distributed in the stromal tissues of mammalians and considered to be closely related to the extracellular matrix (ECM) and growth factors [13]. Proteoglycans belong to the small leucine-rich proteoglycan (SLRP) family, and are characterized by the presence of multiple adjacent leucine rich regions, which may possess between 20 and 29 amino acid residues that may be repeated up to 30 times [1]. Members of small leucine rich proteoglycan include keratocan, mimecan, decorin, biglycan fibromodulin, epiphycan, osteoadherin, and lumican [14]. SLRPs, including lumican, have important roles in cell migration, cell proliferation, tissue repair and tumor growth, in addition to their extracellular matrix functions in tissue hydration and collagen fibrillogenesis [1]. Lumican was first isolated from chick cornea, and then has also been reported to be localized in the skin dermis, lung, bone, cartilage and heart of adult mice [2]. In adult cornea, lumican is present as keratan sulfate proteoglycans [15]; however, in non-corneal tissues, lumican exists as low- or non-sulfated glycoprotein [16]. Gene-targeting studies indicate that lumican plays an important role in determining the structural phenotype of the mature collagen fibril in various tissues. In addition, we believe that lumican sequesters in the pericellular matrix and interacts with cell surface pro-

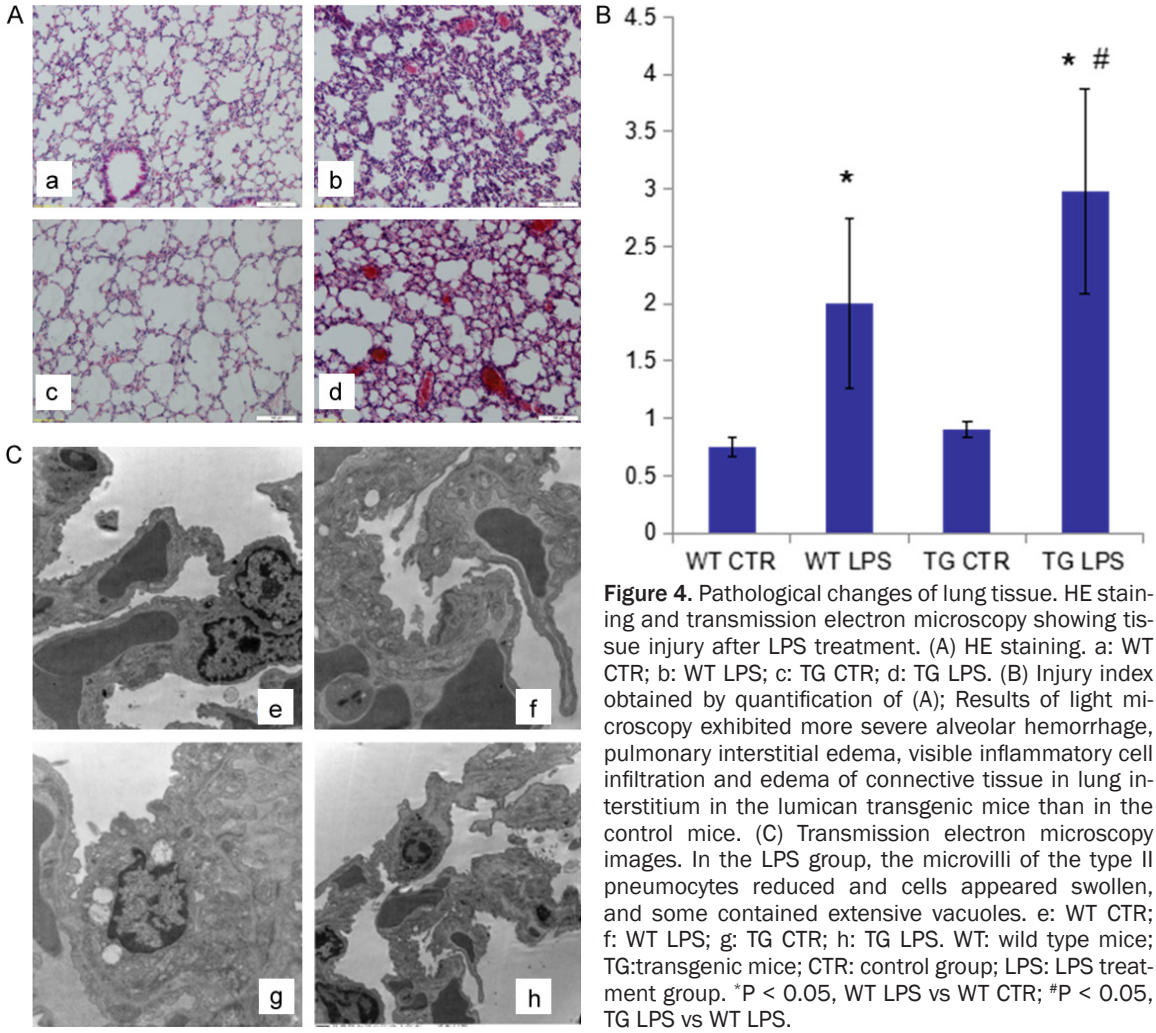

Figure 4. Pathological changes of lung tissue. HE staining and transmission electron microscopy showing tissue injury after LPS treatment. (A) HE staining. a: WT CTR; b: WT LPS; c: TG CTR; d: TG LPS. (B) Injury index obtained by quantification of (A); Results of light microscopy exhibited more severe alveolar hemorrhage, pulmonary interstitial edema, visible inflammatory cell infiltration and edema of connective tissue in lung interstitium in the lumican transgenic mice than in the control mice. (C) Transmission electron microscopy images. In the LPS group, the microvilli of the type II pneumocytes reduced and cells appeared swollen, and some contained extensive vacuoles. e: WT CTR; f: WT LPS; g: TG CTR; h: TG LPS. WT: wild type mice; TG:transgenic mice; CTR: control group; LPS: LPS treatment group. $^{*}P < 0.05$, WT LPS vs WT CTR; $^{\#}P < 0.05$, TG LPS vs WT LPS.

teins for specific cellular functions. Lumican-deficient mice suffer from increased skin fragility and corneal opacities due to abnormal fibril assembly and altered interfibrillar spacing, indicating lateral fusion of collagen fibrils [17]. By contrast, lumican mediates Fas-FasL-induced apoptosis by inducing Fas in mouse embryonic fibroblasts [18]. Lumican, by binding and signaling via FasL, enhances the synthesis and secretion of proinflammatory cytokines and the recruitment of macrophages and neutrophils [7]. Here we show that bacterial lipopolysaccharides are sensed by the Toll-like receptor 4 signaling pathway and regulated by lumican. Lipopolysaccharide (LPS) endotoxins from the cell wall of Gram-negative bacteria are recognized by the TLR4 signaling pathway [19]. LPS sensing begins with its binding to the LPS-binding protein in the blood. The LPS recognition complex also requires soluble MD-2 protein, heat shock proteins, and additional factors that remain to be identified [20-22]. The Toll-like receptor 4 signaling pathway involves the phosphorylation of IkB kinase, nuclear translocation, and the activation of NF-kB. The NF-kB transcription factor up-regulates pro-inflammatory cytokines such as tumor necrosis factor α (TNFα), IL-6, and other co-stimulatory molecules, as well as microbicidal activities [23, 24]. TNFα is the cytokine prototype and is often used to assess host innate immune response. These mediators help to clear infections. However, unrestricted systemic overproduction of pro-inflammatory cytokines and proteins can lead to severe sepsis, multiple organ failure, and death. Host response to infection and bacterial endotoxins is being investigated at multiple levels to define events that lead to sepsis and septic shock. Understanding the molecular events from pathogen recognition to inflamma-

tion mediators is becoming important in the treatment of sepsis and in identifying patients at risk.

Bacterial infection causes shock, acute respiratory failure, multiple organ dysfunction syndrome, and disseminated intravascular coagulation. Despite improved understanding of the epidemiology, pathophysiology, and genetic predisposition to sepsis, morbidity and mortality associated with severe sepsis and septic shock remain high throughout the world [25, 26]. Respiratory failure is one of the most common and life-threatening diseases during septic shock.

Our study of an ECM protein lumican introduces a new modulator of host response to inflammation and sepsis. Lumican modulates host sensing of bacterial lipopolysaccharides (LPS) by toll-like receptor (TLR) 4 in our mouse model of LPS-induced systemic inflammation. We examined serum levels of TNF-α, IL-6, IL-4 and IL-10. These cytokines are normally strongly induced by LPS and are secreted during the early phase of the inflammatory response, thus playing an important role in organ dysfunction [27]. TNFα is a primary mediator of inflammation and its release leads to activation of other cytokines such as IL-6, which is associated with cellular damage [28, 29]. On the other hand, IL-6 may be a more consistent predictor of sepsis and appears to correlate better with sepsis severity and mortality [30]. We found that Lum+/+ mice are hyper-responsive to LPS-induced septic shock, with further induction of pro-inflammatory cytokines such as TNFα and interleukins 6 and anti-inflammatory cytokines such as interleukins 4 and 10 in the serum. Our model of mouse treated with LPS successfully induced multiple organ dysfunction syndrome, including acute respiratory failure symbolized with increased wet-to-dry weight (W/D) ratio levels, increased TUNEL positive staining, especially in epithelial cells and histological damage in the lung, accompanied with TLR4 pathway activation. Lum+/+ mice manifested more severe injury compared with wild type mice.

In conclusion, we found that LPS may cause excessive apoptosis in the pneumocytes via the TLR4 signal transduction pathway and ALI. Lumican may play a role in this process and exacerbates ALI.

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Disclosure of conflict of interest

None.

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