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Original Article

Knockdown of thymic stromal lymphopoietin attenuates acute lung injury in lipopolysaccharide-induced model rats

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Abstract: Acute lung injury (ALI) is a severe syndrome characterized by neutrophil accumulation or edema in pulmonary alveoli, and is accompanied with elevated production of inflammatory cytokines like tumor necrosis factor (TNF) and interleukin 1β (IL-1β). But the regulatory mechanism among key factors remains elusive. This study aims to reveal the role of thymic stromal lymphopoietin (TSLP) in ALI. The ALI model rats were induced by intraperitoneal injection of lipopolysaccharide (LPS), and TSLP was knocked down by intravenous injection of siRNA. *TSLP* mRNA expression was detected by qRT-PCR, and TSLP, TNF and IL-1β concentration in rat serum and bronchoalveolar lavage fluid (BALF) was detected by ELISA. Polymorphonuclear neutrophil (PMN) number and dry/wet weight ratio of the lungs were also compared among different rat groups. Results showed that *TSLP* mRNA was significantly elevated in the lungs of ALI rats ($P < 0.01$). ALI caused increase in PMN number ($P < 0.001$) and pulmonary edema severity ($P < 0.01$), as well as serum TNF and IL-1β concentration ($P < 0.01$) and BALF TNF concentration ($P < 0.001$). Knockdown of TSLP reduced PMN number ($P < 0.01$) and pulmonary edema severity ($P < 0.001$), and inhibited serum TNF and IL-1β levels ($P < 0.01$) and BALF TNF level ($P < 0.01$). No significant BALF IL-1β change was detected. These results indicate that TSLP plays promotive roles in ALI progression and regulates TNF and IL-1β production, and that knockdown of TSLP attenuates ALI symptoms. So TSLP is a potential therapeutic target in ALI treatment.

Keywords: Acute lung injury, thymic stromal lymphopoietin, tumor necrosis factor, interleukin 1β

Introduction

Acute lung injury (ALI), or acute respiratory distress syndrome (ARDS), is a severe syndrome regarding the acute hypoxemic respiratory failure in bilateral pulmonary infiltrates, which is not attributed to left atrial hypertension [1]. Unfortunately, ALI is a disease with high incidence and mortality, with an in-hospital mortality of about 38.5% according to a study in American patients [2]. ALI can be caused by both direct injuries like pneumonia and drowning and indirect injuries including sepsis and transfusions [3]. ALI is usually characterized by diffuse neutrophilic alveolar infiltrate with hemorrhage or the accumulation of a protein-rich pulmonary edema [3]. Polymorphonuclear neutrophil (PMN) is the main effector of ALI, which is recruited and accumulated to pulmonary circulation and alveolar space [4]. Overactivation of PMN leads to pulmonary tissue damage via altering inflammatory responses and oxidative stress [5]. During ALI, a series of signaling pathways like nuclear factor κB and the MAPK signaling are activated in PMN [6-8]. Some cytokines, such as tumor necrosis factor (TNF) and interleukin 1β (IL-1β), are also promoted to provoke inflammation [9, 10]. More mechanisms linked to ALI progression are under research.

Thymic stromal lymphopoietin (TSLP), firstly discovered in a thymic stromal cell line, supports B cell development *in vitro* [11, 12], and is revealed to be a factor associated with allergic inflammatory diseases. It is up-regulated in lungs of mice with antigen-induced asthma and is a necessary factor for the initiation of allergic airway inflammation [13, 14]. Studies also uncover TSLP as a promising therapeutic target in cancer treatment for its vital roles in the pathogenesis of non-allergic diseases such as primary cancer, where TSLP may induce epi-

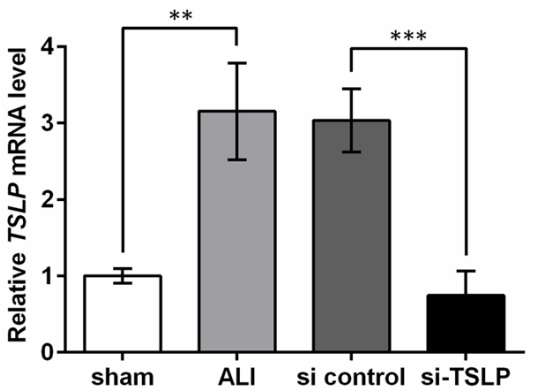

Figure 1. Relative *TSLP* mRNA level. *TSLP* mRNA level was detected by qRT-PCR in the pulmonary lobe of sham (control for ALI model rats), ALI (ALI model rats), si control (the ALI model rats injected with normal saline as controls) and si-TSLP (the ALI model rats injected with siRNA to knockdown TSLP) at 72 h after siRNA injection (n = 10). $^{**}P < 0.01$. $^{***}P < 0.001$. TSLP, thymic stromal lymphopoietin. ALI, acute lung injury.

thelial to mesenchymal transition to promote cancer metastasis [15]. Recently, some researchers found that blocking TSLP receptor (TSLPR) in mice could decrease the expression of phosphorylated extracellular regulated protein kinase 1 and signal transducers and activators of transcription 3 in pulmonary dendritic cells, thus attenuating ALI [16]. So it seems that the TSLP signaling is crucial for ALI progression, but detailed mechanism remains elusive.

This study aims to reveal the role of TSLP in modulating ALI. We constructed a lipopolysaccharide (LPS)-induced ALI rat model and inhibited TSLP using its specific siRNA. TSLP expression changes were compared among these rat groups, and PMN number and dry/wet weight ratio of the rat lungs were examined. We also detected the concentration of TSLP, TNF and IL-1β in rat serum and bronchoalveolar lavage fluid (BALF) by enzyme-linked immunosorbent assay (ELISA). Our findings support the promotive functions of TSLP in ALI progression and suggest TSLP as a potential therapeutic target for ALI treatment.

Materials and methods

Animals

Sprague Dawley rats (220~320 g, SPF grade, Vital River Laboratories, Beijing, China) were randomly divided into four groups: sham, ALI, si control and si-TSLP groups, with ten individuals in each group. The animal experiments were approved by a local animal ethics committee and performed according to the instructions of our institute. Rats were anesthetized with 5% pentobarbital sodium (Sigma-Aldrich, Shanghai, China). The rats in ALI, si control and si-TSLP groups were intraperitoneally injected with LPS (6 mg/kg, Sigma-Aldrich) to induce ALI [17], and the rats in sham group were injected with the same volume of normal saline. For the rats of si-TSLP group, the specific siRNA of TSLP (50 μg, RiboBio, Guangzhou, China) was intravenously injected through the tail at 6 h after LPS injection. The rats in si control group were intravenously injected with the negative control (RiboBio).

Sampling

At 72 h after siRNA injection, the rats of the four groups were anesthetized to expose the trachea. BALF was collected by cannulating the upper trachea and the lavage with 1 mL of phosphate buffered saline for three times. Besides, 2 mL of venous blood was collected and centrifuged to collect the serum. Then the rats were sacrificed for sampling. The lung tissues were collected. The upper right pulmonary lobe was collected and weighed, after which the tissue was dried in an incubator at 60°C for 24 h and weighed again, and the two results of weighting were wet weight and dry weight, respectively. The lower right pulmonary lobe was sampled and immediately fixed in 4% paraformaldehyde. Slides were made and stained with hematoxylin and eosin (HE) for counting PMN number. Another portion of lower right pulmonary lobe was immediately frozen in liquid nitrogen and stored at -80°C for RNA extraction.

ELISA

The collected BALF and serum were centrifuged to remove cell debris. The concentration of TSLP, TNF or IL-1β in BALF and serum was detected using the specific ELISA kits for these factors (Elabscience, Wuhan, China) according to the manufacturer's instructions. The optical density was measured at 450 nm with a microplate reader (Molecular Devices, Silicon Valley, CA). A standard curve was made based on the standards provided in the kit to assess the concentration of tested factors.

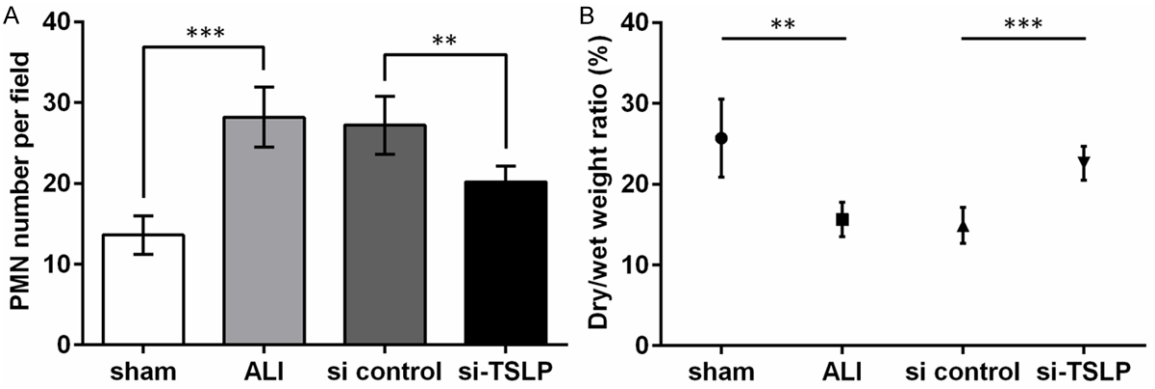

Figure 2. PMN number and dry/wet weight ratio of pulmonary lobe tissues of the four rat groups. A. PMN number per field in the HE staining slides of rat pulmonary lobe tissues (n = 10). PMN, polymorphonuclear neutrophil. B. Dry/wet weight ratio (%) of pulmonary lobe tissues (n = 10). $^{**}P < 0.01$. $^{***}P < 0.001$. ALI, acute lung injury.

qRT-PCR

The pulmonary tissues were lysed in TRIzol (Invitrogen, Carlsbad, CA) for total RNA extraction according the manufacturer's instructions. Protein and DNA contaminations were removed by RNA Purification Kit (TIANGEN, Beijing, China). The complementary DNA (cDNA) was synthesized from 1 μg of total RNA under the catalysis of Prime Script Reverse Transcriptase (TaKaRa, Dalian, China). qRT-PCR was performed on LightCycler 480 (Roche, Basel, Switzerland) with 20 ng cDNA and the specific primers for rat TSLP (Fw: 5'-TAG CAA TCG GCC ACA TTG CT-3' and Rv: 5'-GAA GCG ACG CCA CAA ACC TTG-3') and GAPDH (Fw: 5'-CGC ATT GCC AGA CAT ATC AGC-3' and Rv: 5'-AGG TGA AGC AGG CTC AAT CAA-3'). GAPDH was used as an internal reference. The data were analyzed with $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

All the experiments were repeated for three times and results were represented as the mean ± standard deviation. Data were analyzed by one-way ANOVA followed by Tukey's post-hoc test, and comparison between groups was performed by *t* test in SPSS 19 (IBM, New York, USA). $P < 0.05$ was considered to be statistically significant.

Results

TSLP is up-regulated during ALI

To begin with, we detected *TSLP* mRNA expression in the pulmonary lobe of ALI rats using qRT-PCR (**Figure 1**). Results showed that the ALI rats possessed a significantly higher *TSLP* mRNA level compared to sham group ($P < 0.01$), indicating that TSLP was up-regulated during ALI. After TSLP knockdown via siRNA injection, the *TSLP* mRNA level was markedly reduced compared to si control group ($P < 0.001$), suggesting that the intravenous injection method used in this study could effectively knock down TSLP in ALI rats.

TSLP knockdown attenuates ALI

Since ALI is usually accompanied with pulmonary alveolar edema and overactive PMN, we examined PMN number and lung dry/wet weight ratio after ALI model construction and TSLP knockdown. The PMN number in ALI rats was obviously more than sham group ($P < 0.001$, **Figure 2A**), which was in consistent with the promoted PMN activity during ALI. Besides, the dry/wet weight ratio of pulmonary lobe tissue was decreased in ALI rats ($P < 0.01$, **Figure 2B**), indicating the increased degree of edema. But when TSLP was knocked down, the PMN number decreased ($P < 0.01$) and the dry/wet weight ratio of tissue increased ($P < 0.001$) compared to si control, suggesting that TSLP knockdown was capable of controlling the overactive PMN and edema during ALI. These results implied that TSLP might be a key factor regulating the progression of ALI.

TSLP regulates TNF and IL-1β in serum and BALF

We next detected the concentration of TSLP, TNF and IL-1β in serum and BALF using ELISA.

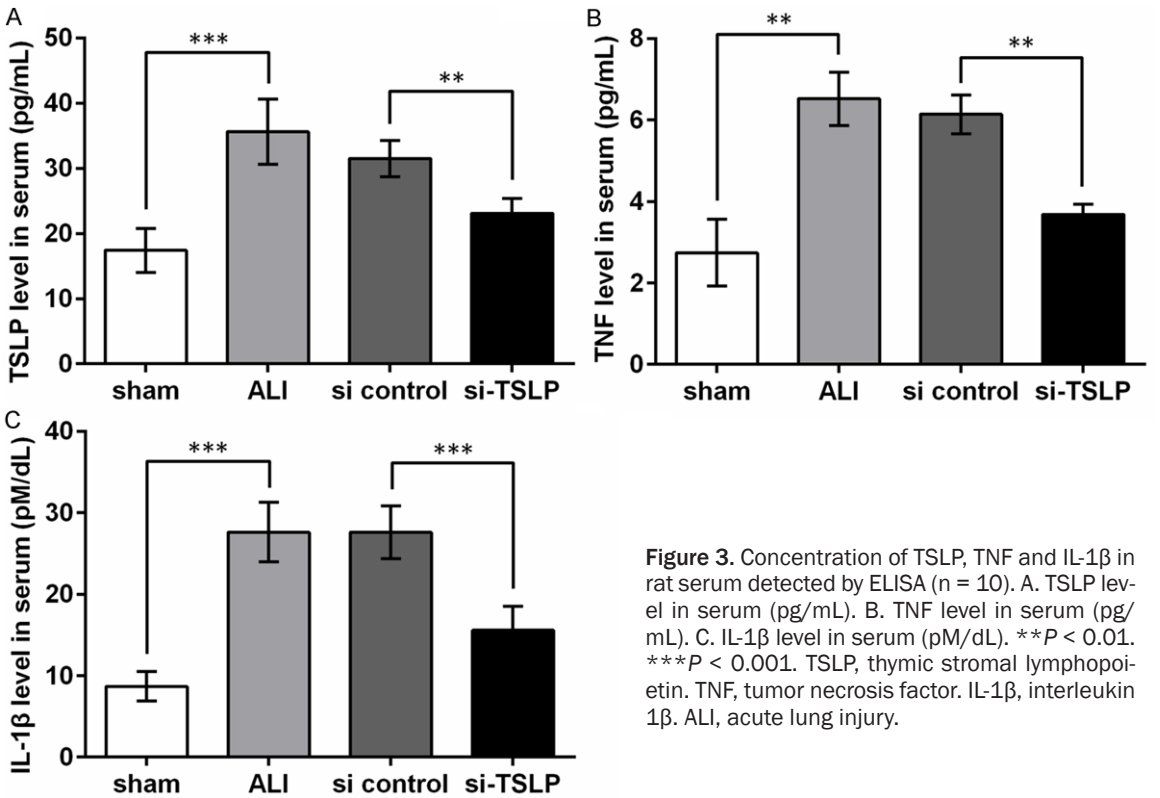

Figure 3. Concentration of TSLP, TNF and IL-1β in rat serum detected by ELISA (n = 10). A. TSLP level in serum (pg/mL). B. TNF level in serum (pg/mL). C. IL-1β level in serum (pM/dL). $^{**}P < 0.01$. $^{***}P < 0.001$. TSLP, thymic stromal lymphopoietin. TNF, tumor necrosis factor. IL-1β, interleukin 1β. ALI, acute lung injury.

In serum of the four rat groups, the concentration of TSLP, TNF and IL-1β was all increased during ALI ($P < 0.01$, **Figure 3A-C**), and TSLP knockdown induced the decrease of all the three factors ($P < 0.01$). Similarly, the concentration of TSLP and TNF in BALF was increased during ALI ($P < 0.001$, **Figure 4A** and **4B**), but was decreased by TSLP knockdown in ALT rats ($P < 0.001$ or $P < 0.01$). The concentration of IL-1β in BALF did not change obviously by ALI or TSLP knockdown ($P > 0.05$, **Figure 4C**). Taken together, the concentration of TSLP in serum and BALF was decreased by TSLP knockdown, which again confirmed the successful knockdown of TSLP; TSLP knockdown in ALI rats could decrease serum TNF and IL-1β levels, as well as TNF level in BALF, indicating that TSLP might regulate the production of TNF or IL-1β.

Discussion

Though TSLPR and the related signaling have been reported to play roles in ALI modulation, the direct involvement of TSLP in ALI has not been studied. In this study, TSLP is found up-regulated in the lung tissue of LPS-induced ALI rat model. The injection of TSLP-specific siRNA successfully reduces TSLP expression. TSLP knockdown leads to decreased PMN number and attenuated pulmonary edema. ELISA results indicate that the concentration of TSLP and TNF in rat serum and BALF, as well as the serum IL-1β level, is increased by ALI and reduced by TSLP knockdown.

TNF and IL-1β are representatives of inflammatory cytokines, which are released by monocytes or macrophages upon ALI and constitute major chemoattractants and activators for neutrophils. TNF plays significant roles in the functioning mechanism of several promising anti-inflammatory mediators during ALI, such as hirudin and asperuloside. Hirudin reduces the production of TNF and matrix metalloproteinase 12, whereby the inflammation and fibrosis caused by lung injury are attenuated [18]. Asperuloside significantly inhibited TNF, IL-1β and IL-6, thus relieving ALI symptoms in a murine model [7]. TNF inhibitors are under investigation as possible strategies for treating asthma [19], sarcoidosis [20] and pulmonary fibrosis [21, 22]. Overexpression of IL-1β in rat lungs induces up-regulation of TNF and the accompanying aggravated inflammatory response and tissue injury, indicating its role

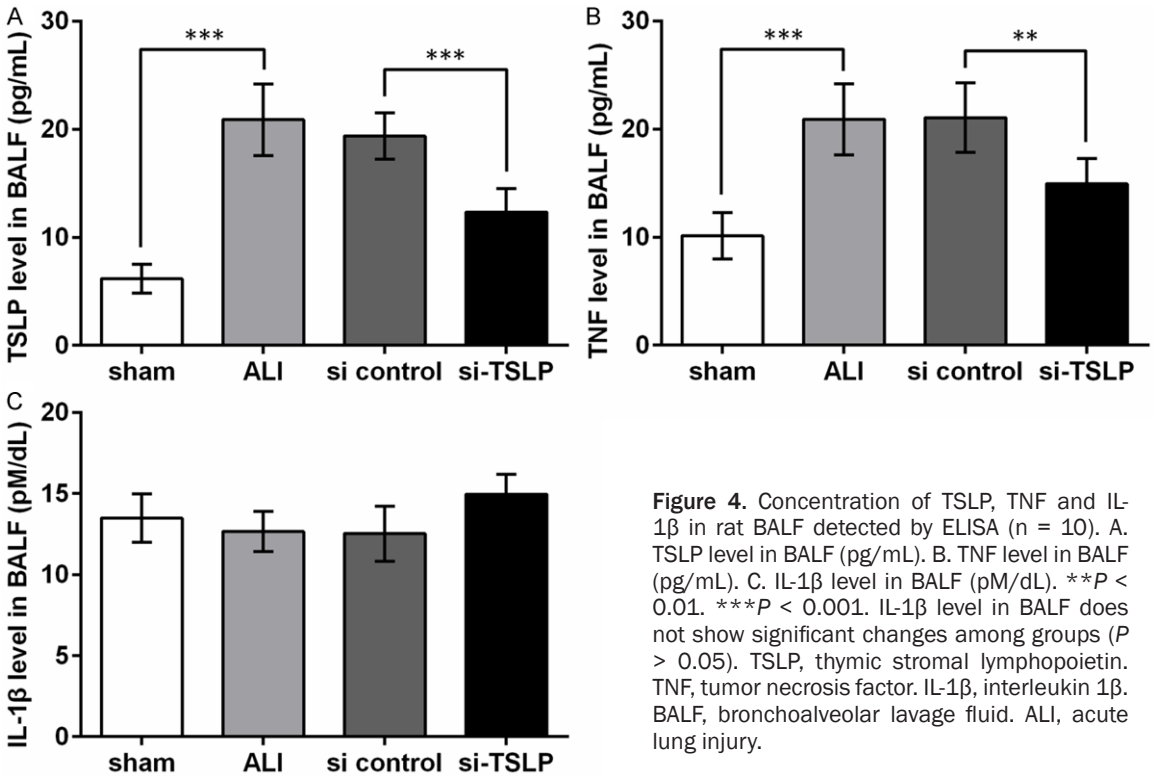

Figure 4. Concentration of TSLP, TNF and IL-1β in rat BALF detected by ELISA (n = 10). A. TSLP level in BALF (pg/mL). B. TNF level in BALF (pg/mL). C. IL-1β level in BALF (pM/dL). $^{**}P < 0.01$. $^{***}P < 0.001$. IL-1β level in BALF does not show significant changes among groups ($P > 0.05$). TSLP, thymic stromal lymphopoietin. TNF, tumor necrosis factor. IL-1β, interleukin 1β. BALF, bronchoalveolar lavage fluid. ALI, acute lung injury.

as an inducer of ALI [23, 24]. In consistent with these former findings, we detected higher TNF and IL-1β concentration in serum or BALF of ALI model rats, which implies the promotive function of the two factors in ALI progression.

The secretion of TNF and IL-1β from macrophages can be induced by LPS [25-27]. Similarly in this study, LPS was used to induce ALI model rats and we found the increase of TNF and IL-1β in rat serum or BALF, which might be caused by LPS function. Also, it is reported that intratracheal administration of TNF or IL-1β induces neutrophil accumulation in pulmonary vessels and alveoli [28, 29]. This study observed increased PMN number along with elevated TNF and IL-1β concentration, consistent with previous research.

When TSLP was knocked down in ALI model rats, we observed reduced PMN number and alleviated pulmonary edema, as well as decreased TNF and IL-1β concentration in serum or BALF. According to previous studies, IL-3/TSLP induces TNF and IL-1β secretion and plays vital roles in the differentiation from peripheral blood $CD34^+$ progenitor cells to eosinophils and basophils [30]. But TNF promotes TSLP expression in nasal polyp fibroblasts [31]. Our findings indicates that TSLP may influence TNF and IL-1β production and secretion to serum or BALF in ALI model rats, but the exact regulatory relationship among these factors remains to be investigated. We failed to detect significant changes in BALF IL-1β level, which needs to be verified and explained in our future studies.

TSLP was found up-regulated in ALI model rats and its knockdown induces alleviation of ALI symptoms. Researchers have uncovered the involvement of TSLP in lung diseases, especially pulmonary fibrosis. For example, TSLP and TSLPR are up-regulated in idiopathic pulmonary fibrosis and TSLP is a master regulator of type 2 immune responses [32]. This study further confirms that TSLP is involved in LPS-induced ALI, and that knockdown of TSLP in ALI model rats reduces PMN number and pulmonary edema in rat lungs. Moreover, TSLP knockdown attenuating ALI symptoms can also be reflected by the influence on TNF and IL-1β concentration changes. So TSLP is a promising target for ALI treatment. Further mechanism studies are necessary to describe the vital position of TSLP in modulating ALI.

In summary, this study uncovers the elevated expression of TSLP in the lungs of ALI model rats. TSLP knockdown reduces PMN number, attenuates pulmonary edema, and inhibits TNF and IL-1β production. So TSLP is a promising therapeutic target in ALI treatment, but its regulatory relationship with key factors in ALI progression needs to be revealed in future studies.

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Disclosure of conflict of interest

None.

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