Original Article
Blockage of thymic stromal lymphopoietin signaling improves acute lung injury in mice by regulating pulmonary dendritic cells

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Abstract: Objectives: To investigate the effects of blockage of thymic stromal lymphopoietin (TSLP) signaling by TSLP receptor (TSLPR)-immunoglobulin (Ig) on acute lung injury (ALI) induced by lipopolysaccharide (LPS). Methods: C57BL/6 mice received TSLPR-Ig or controlled-Ig before being induced ALI. Lung wet/dry (W/D) weight ratio was recorded. Neutrophil number and albumin concentration of bronchoalveolar lavages fluids (BALF) were determined. Besides, bone marrow dendritic cells (BMDCs) were separated and cultured with medium, TSLP, TSLP plus TSLPR-Ig or TSLP plus controlled-Ig. Protein expression levels of TSLP in lung tissues, phosphorylation extracellular regulated protein kinases (pERK) 1/2, p38, and signal transducers and activators of transcription (STAT) 3 in BMDCs were analyzed using Western blotting. Expression of CD40, CD80 and CD86 on pulmonary DCs and BMDCs was determined using flow cytometry (FCM). Results: The W/D ratio, neutrophil number and albumin concentration were significantly decreased in the TSLPR-Ig group compared with the controlled-Ig and model group. Moreover, there was a noticeable decrease in CD40, CD80 or CD86 expression by TSLPR-Ig on both pulmonary DCs and BMDCs. The protein levels of TSLP, pERK1 and STAT3 were significantly decreased by TSLPR-Ig. However, no significant differences were found in p38 and pERK2. Conclusion: These results suggest that TSLP may be involved in ALI, and blockage of TSLP signaling using TSLPR-Ig improves ALI at least in part by regulation of DCs functions. The underling downstream signaling mediated by TSLP might be associated with activating the ERK1 and STAT3 signaling pathway.

Keywords: Thymic stromal lymphopoietin, thymic stromal lymphopoietin receptor, dendritic cells, acute lung injury, lipopolysaccharide

Introduction

Acute lung injury (ALI) serves as a major cause of acute respiratory failure, leading to significant mortality in critically ill patients [1]. It is characterized by intense pulmonary inflammatory response induced by a variety of inflammatory mediators and effector cells, with neutrophils infiltration into the lung, interstitial edema, epithelial integrity disruption, fibrin leakage into the alveoli [2, 3]. In spite of significant improvements in supportive care, approximately 40% lethality has been reported [4, 5]. Therefore, an improved and a deeper understanding of the molecular mechanisms underlying ALI is of importance.

Airway inflammation is a special kind of immune response, and T lymphocytes play significant roles in regulation of inflammation response.

Recently, DCs has been well reported to be associated with the innate immune responses and initiation of primary immune responses. There are relatively small numbers of dendritic cells (DCs), including myeloid DCs (mDCs) or plasmacytoid DCs (pDCs), in the lung [6]. Indeed, the role of DCs in ALI has been investigated [7-9]. DCs migrate to the lung and direct the T helper (Th) 1, Th2, and Th17 cell response during the development of inflammation [10, 11]. In addition, thymic stromal lymphopoietin (TSLP), an interleukin (IL)-7 like cytokine produced by epithelial cells, has been attracted much attention in Th2 dominant allergic diseases. It serves as an upstream phase of the allergic cascade and could strongly promote the migration of DCs by induction the expression of major histocompatibility complex (MHC) I, II, and some costimulatory molecules (CD40, CD80, and CD86, etc.) [12]. TSLP signaling is

mediated by a heterodimeric receptor complex consists IL-7Rα chain and a unique TSLP receptor binding chain (TSLPR) [13]. It has been reported that TSLP initiates signal transducer and activator of transcription (STAT) 5 phosphorylation [14], mitogen-activated protein kinase (MAPK), and NF-κB, etc. [15]. Zhang *et al.* [16] has suggested that local use of specific inhibition of TSLPR (TSLPR- immunoglobulin (Ig)) prevents airway inflammation induced by allergic disease partly by regulating function of DC. However, little information is available concerning the role of TSLP-TSLPR in ALI. Therefore, we hypothesized that TSLP-TSLPR signaling pathway might be involved in ALI by regulation DCs function.

In order to confirm the hypothesis, we induced the ALI mouse model and separated bone marrow dendritic cells (BMDCs). The TSLP signal was inhibited in vivo and in vitro to explore its functional role in ALI, as well as the underling mechanism.

Materials and methods

Animals and model of ALI

Thirty-two male C57BL/6 mice (4-6 weeks old, Slac Laboratory Animal Co. Ltd., Shanghai, China) weighing 18-22 g were randomly assigned to four groups: control group, model group, TSLPR-Ig group, and controlled-Ig group. Under anesthetized conditions with intraperitoneal injection of 100 μg/g ketamine and 8 μg/g xylazine, 40 μg of lipopolysaccharide (LPS, E. coli, O111: B4 Sigma-Aldrich, St. Louis, MO, USA) dissolved in 40 μL of phosphate-buffered saline (PBS) was slowly injected intra-tracheally during inspiration. The normal control mice only received intratracheal instillation of PBS. The mice in the TSLPR-Ig group and controlled-Ig group were given intratracheal instillation of 40 μg TSLPR-Ig (R & D Systems, Minneapolis, USA) or controlled-Ig (AB-108-C, R & D Systems, Minneapolis, USA) 30 min before receiving LPS. The animal care and use was approved by local Ethics Committee and was complied with the ethical standards.

Samples preparation

The mice were sacrificed 12 h later to assess lung injury with 250 mg/kg ketamine. Bronchoalveolar lavages (BAL) were performed three times by injection of normal saline (0.5 mL, 4°C). BAL fluids (BALF) were centrifuged at

10,000 g for 10 min at 4°C, and then the supernatant was harvested and stored at -20°C. After collection BALF, the lung was excised for further analyses. Neutrophil number and albumin concentration of the BALF were determined. The wet lung was weighed and then was placed in the oven at 90°C for 24 h. After complete dehydration, the dry lung was weighted again. The weight ratio of wet weight and dry weight (W/D) value was recorded.

Analysis of co-stimulatory molecule expression on pulmonary DCs in vivo and BMDCs in vitro

For analysis of co-stimulatory molecule expression on pulmonary DCs, lung tissues were harvested, washed with PBC, and digested with collagenase and DNase I. Then the lung tissues were washed and incubated with RPMI-1640 culture medium supplemented with 0.1% collagenase (Type IV; Sigma, St. Louis, MO, USA) and 0.002% DNase (Sigma, St. Louis, MO, USA). After incubation, the lung tissues were minced. Then the cells were collected, washed and suspended in cold PBS.

For analysis of co-stimulatory molecule expression on BMDCs, mouse BMDCs were firstly prepared according to a previously described method [17]. The femurs and tibias from each mouse were harvested, washed, minced and digested. After centrifugation, the cells were cultured in RPMI1640 medium (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS), 10,000 U/L penicillin (Gibco), 10 g/L streptomycin (Gibco), 50 μL 2-mercaptoethanol (Gibco). Following 8 days of culture with 10 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) and 1 ng/mL IL-4, DCs were collected, purified with anti-CD11c-coated microbeads (Miltenyi-Biotec, Auburn, CA, USA), and then treated with normal medium, TSLP (100 ng/mL), TSLP (100 ng/mL) in combination with controlled-Ig (200 ng/mL, AB-108-C) or TSLP (100 ng/mL) in combination with TSLPR-Ig (200 ng/mL) for 24 h.

After culture, both the cells from pulmonary DCs and BMDCs were harvested, washed, and stained with phycoerythrin (PE)-CD40, CD80 or CD86 mAbs (10 μL/mL, Pharmingen). The fluorescence activated cell sorter (FACS, BD Biosciences) was performed to analyzed cells (at least 1×10^5 cells for pulmonary DCs and 1×10^4 cells for BMDCs). The results were analyzed using CellQuest software (Becton
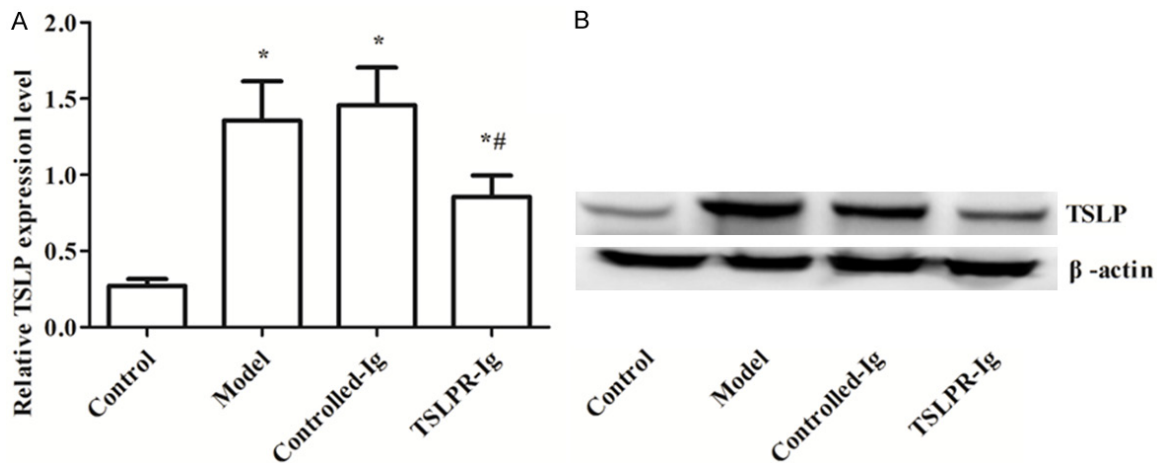
Figure 1. TSLP protein expression in lung. A: Relative TSLP protein expression level; B: Picture of Western blotting; TSLP: thymic stromal lymphopoietin; TSLPR: thymic stromal lymphopoietin receptor; Ig: immunoglobulin. $^*P < 0.05$ compared with control group; $^\#P < 0.05$ compared with model group.

Dickinson, Bedford, MA). The co-stimulatory molecules CD40, CD80 and CD86 were analyzed by flow cytometry (FCM).

Western blotting

For Western blotting analysis, lung tissue and BMDCs were harvested, washed and extracted protein. Protein density of lung tissue was assessed using Bio-Rad DC protein Assay kit (Bio-Rad, Hercules, CA, USA). Proteins (15 µL) were separated by a standard electrophoresis sodium dodecyl sulfate (SDS) polyacrylamide gel and transferred onto nitrocellulose membranes (Sigma). Then the membranes were blocked with 5% defatted milk for 2 h, washed with PBS, and then probed with anti-TSLP (Abcam, Cambridge, MA, USA) antibody, anti-phosphorylation extracellular regulated protein kinases (pERK) 1 antibody (Santa Cruz Biotechnology), anti-pERK2 antibody (Santa Cruz Biotechnology), anti-p38 antibody (Santa Cruz Biotechnology) or anti-signal transducers and activators of transcription (STAT) 3 antibody (Santa Cruz Biotechnology, CA, USA). In addition, β-actin (Santa Cruz Biotechnology, CA, USA) was used as an internal control. After that, the membranes were incubated with HRP-labelled secondary antibody (Santa Cruz Biotechnology, CA, USA). The blots were then developed enhanced chemiluminescence and densitometric analysis.

Statistical analysis

The collected data were expressed as the mean ± standard deviation (SD). Statistical analyses were performed using statistical package for

the social sciences (SPSS) software (version 19.0; SPSS Inc., Chicago, IL). Measurement data were compared by Student's t test (for 2 groups) or analysis of variance (ANOVA, for more than 3 groups). A value of $P < 0.05$ was considered statistically significant.

Results

TSLP protein expression in lung

In order to verify the specific blockage of TSLP activity by TSLPR-Ig, the expression level of TSLP protein in the lung tissues was determined by Western blotting. The results (**Figure 1A** and **1B**) showed that the expression levels of TSLP were significantly increased in the model group and controlled-Ig group compared with the control group ($P < 0.05$). However, the level was significantly decreased in the TSLPR-Ig group compared with model groups, but was significantly higher than the control group ($P < 0.05$).

Analyses of lung injury

Lung wet weight/dry weight (W/D) was firstly recorded to assess lung injury. The results demonstrated that the W/D ratio was significantly higher in the model group and controlled-Ig group than that in the control group ($P < 0.05$), suggesting that the mice in the model group and controlled-Ig group presented with significant pulmonary edema. However, the W/D ratio was significantly decreased in the TSLPR-Ig group compared with the model group, but was still markedly higher than the
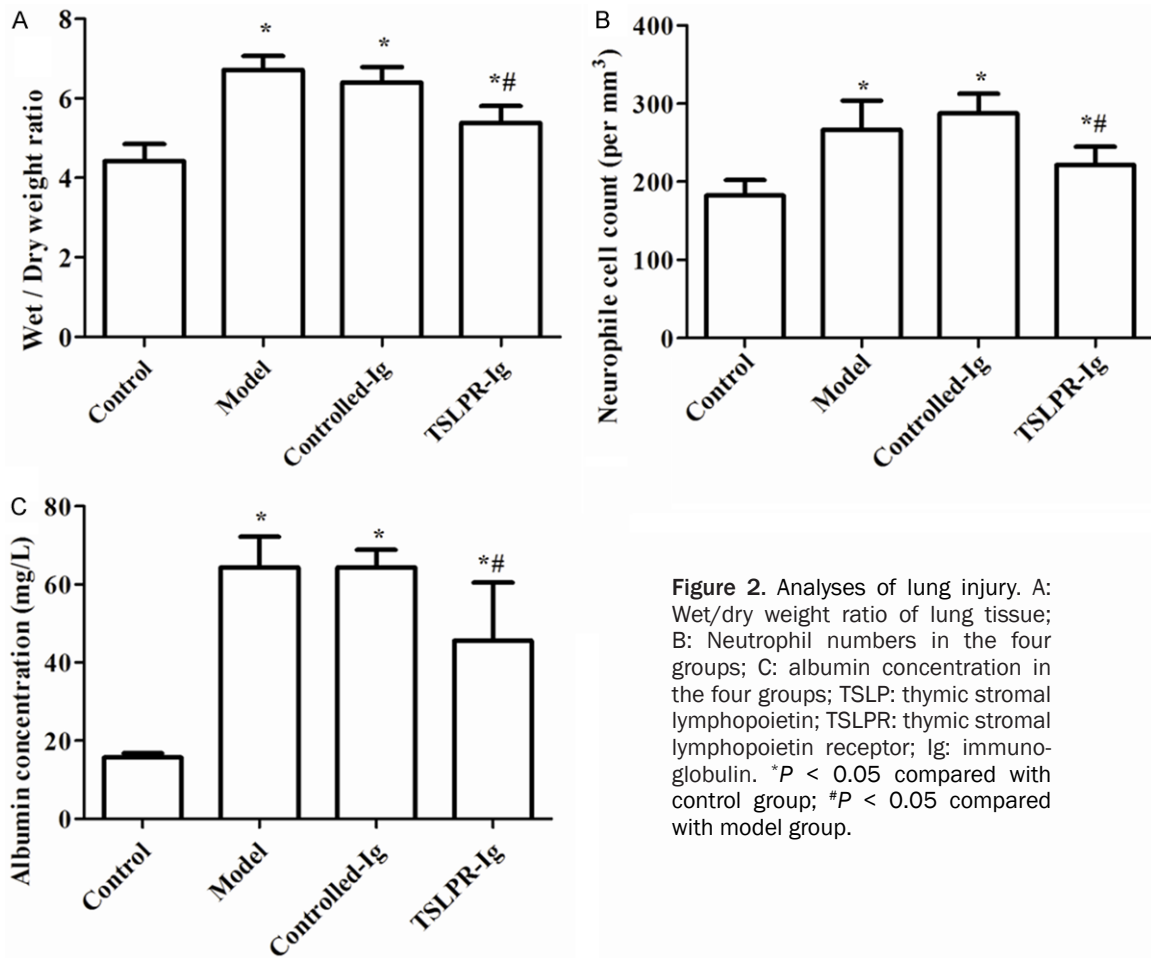
Figure 2. Analyses of lung injury. A: Wet/dry weight ratio of lung tissue; B: Neutrophil numbers in the four groups; C: albumin concentration in the four groups; TSLP: thymic stromal lymphopoietin; TSLPR: thymic stromal lymphopoietin receptor; Ig: immunoglobulin. $^*P < 0.05$ compared with control group; $^\#P < 0.05$ compared with model group.

control group ($P < 0.05$) (**Figure 2A**). The results indicated that specific blockage of TSLP activity by TSLPR-Ig could significantly improve pulmonary edema.

Next, the number of neutrophil and the albumin concentration in the BALF were determined. From the results, we found that both the neutrophil number and albumin concentration were significantly increased in the model group compared with the control group, while were markedly decreased by TSLPR-Ig ($P < 0.05$) (**Figure 2B** and **2C**). These results demonstrated that TSLPR was involved in the development of inflammatory lung injury.

Expression of co-stimulatory molecules on pulmonary DCs and BMDCs

To confirm the effects of TSLPR-Ig might be associated with functional changes in pulmonary DCs, we explored the expression of co-stimulatory molecules. As shown in **Figure 3A**,

there was a noticeable decrease in CD40, CD80 or CD86 expression in the TSLPR-Ig group compared with the controlled Ig group and model group ($P < 0.05$), indicating that TSLPR-Ig treatment could effectively inhibited the maturation of pulmonary DCs.

To determine whether co-stimulatory molecule expression could be influenced by TSLPR-Ig in vitro, BMDCs were treated with normal medium, TSLP, TSLP plus TSLPR-Ig or TSLP plus control IgG for 24 h. FCM was performed to analyze the expression of co-stimulatory molecules by BMDCs. **Figure 3B-D** showed that BMDCs cultured with TSLP in the presence and absence of control Ig significantly up-regulated the expression of CD40, CD80, and CD86 compared with DCs cultured only in normal medium ($P < 0.05$). TSLPR-Ig effectively repressed the CD40, CD80, and CD86 expression induced by TSLP. These results suggested that TSLPR-Ig could effectively suppress co-stimulatory molecule expression in vitro.
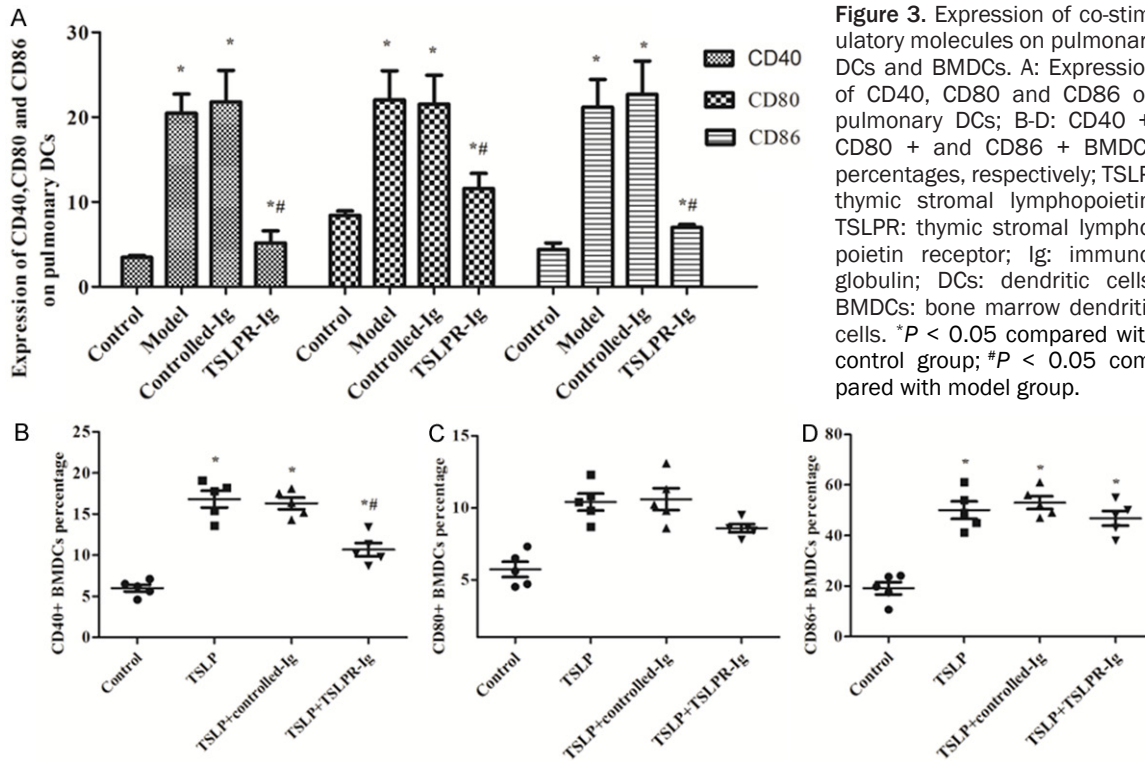
Figure 3. Expression of co-stimulatory molecules on pulmonary DCs and BMDCs. A: Expression of CD40, CD80 and CD86 on pulmonary DCs; B-D: CD40 +, CD80 + and CD86 + BMDCs percentages, respectively; TSLP: thymic stromal lymphopoietin; TSLPR: thymic stromal lymphopoietin receptor; Ig: immunoglobulin; DCs: dendritic cells; BMDCs: bone marrow dendritic cells. *$P < 0.05$ compared with control group; #$P < 0.05$ compared with model group.

Analyses of signaling pathway

To determine the associated underling downstream signaling mediated by TSLP, we detected the proteins expression levels of pERK1/2, p38, and STAT3 in BMDCs (**Figure 4A-E**). The results showed that regulation of TSLP by TSLPR-Ig was associated with pERK1 and STAT3. Both the proteins expression levels of pERK1 and STAT3 were significantly lower in the group treated with TSLPR-Ig than those groups treated with controlled-Ig and TSLP ($P < 0.05$). However, no significant differences were found in the protein expression level of p38 among the four groups. Interestingly, we found that TSLP could effectively activate phosphorylation of ERK2; however, no significant impacts on phosphorylation of ERK2 by TSLPR-Ig were showed.

Discussion

In the present study, the role of TSLP and TSLPR-Ig in ALI was explored in vivo and in vitro. The results suggested that TSLP might be involved in inflammatory response of ALI, and blockage of TSLP signaling by TSLPR-Ig could improve ALI by regulation of DCs through pERK1 and STAT3 signaling pathways.

The pathological mechanism leading to ALI has not been well understood. The associated mechanisms of the pathological changes were complicated, involving with various inflammatory cells such as neutrophils that is regarded as potential cellular mediators of ALI [18-20]. Also, emerging evidence has suggested that lymphocytes and DCs play significant roles in ALI. They have been considered as active players in ALI resolution phase [19]. Morris *et al.* suggested that in addition to neutrophils, lymphocytes infiltrate the lung in a murine model of ALI induced by endotoxin [21]. Also, Nakajima *et al.* showed that BALF lymphocytes, neutrophils, IL-6, tumor necrosis factor (TNF)-α and albumin were increased in ALI induced by endotoxin [22].

DCs including myeloid and plasmacytoid DCs has been reported to reside in the airway epithelium, alveolar septate and around pulmonary vessels lung [23]. These cells contribute to the immune response during infection/inflammation [6]. Indeed, lymphocytes and DCs migrate to the lung to maintain, enhance, and regulate local immune response during the development of inflammation [11, 24]. DCs have been shown the ability of regulating the immune response by bridging innate and adap-
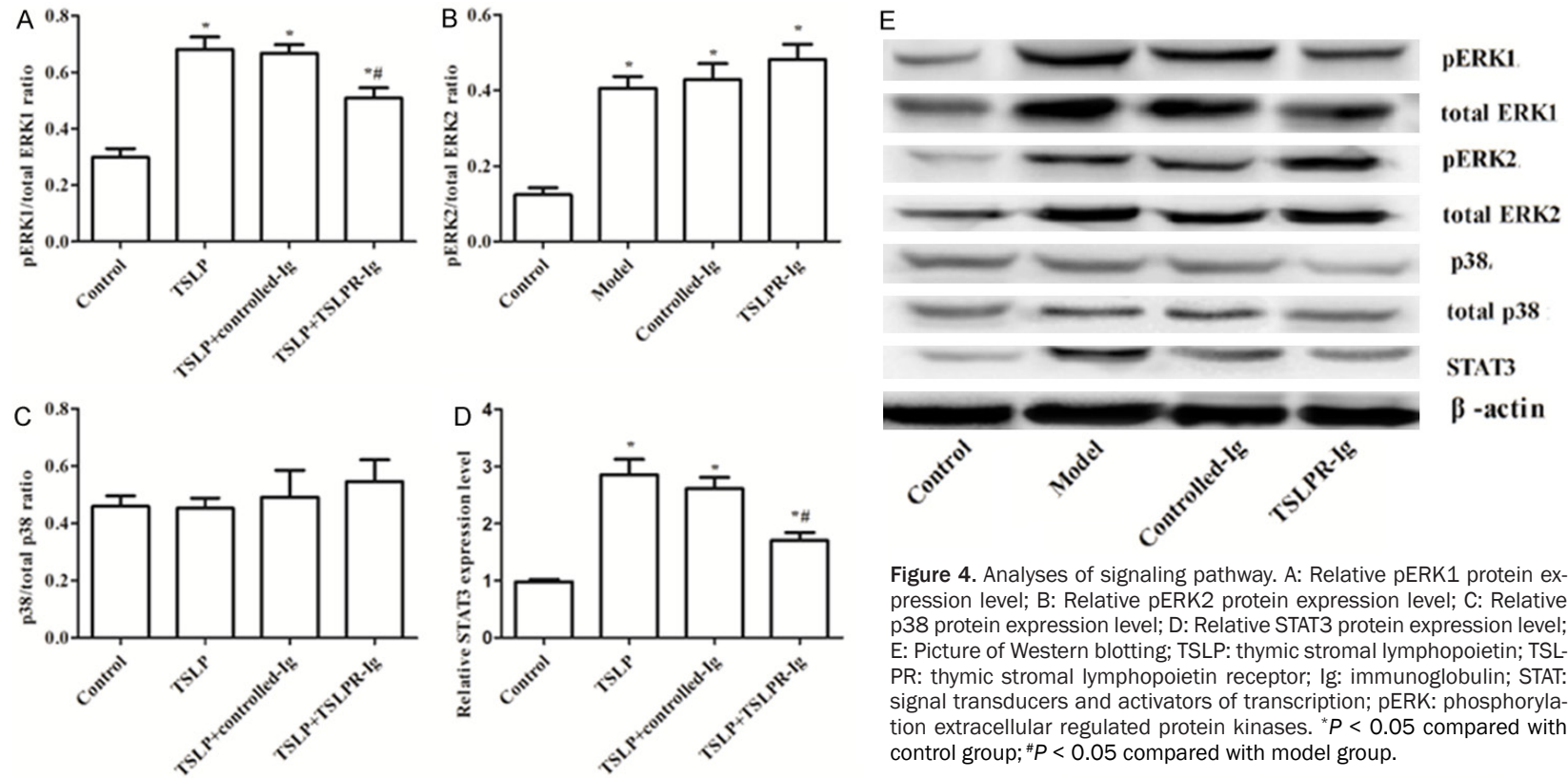
Figure 4. Analyses of signaling pathway. A: Relative pERK1 protein expression level; B: Relative pERK2 protein expression level; C: Relative p38 protein expression level; D: Relative STAT3 protein expression level; E: Picture of Western blotting; TSLP: thymic stromal lymphopoietin; TSLPR: thymic stromal lymphopoietin receptor; Ig: immunoglobulin; STAT: signal transducers and activators of transcription; pERK: phosphorylation extracellular regulated protein kinases. $^*P < 0.05$ compared with control group; $^#P < 0.05$ compared with model group.

tive immunity. Different type of signals that the DCs receive at an immature stage could induce either Th1 or Th2 responses [25]. Also, DCs regulate the function and increase of regulatory T (Treg) cells that closely control overzealous inflammatory T cell responses [6]. Recently, TSLP has been paid attention due to the ability on induction of DC-mediated Th2 response [26, 27]. It activates DCs to stimulate naive T cells and regulate Th2 differentiation by initiating some pro-allergic cytokines, such as IL-4 and IL-13. In addition, TSLP endows CD11c+ DCs with the ability to produce chemokines (C-C motif) ligand (CCL) 17 and CCL22, leading to recruit Th2 cells to the draining lymphatic nodes [28]. TSLPR, the receptor of TSLP, contributes to the importance of TSLP signaling in allergic airway inflammation [29]. TSLP initiates STAT3 and STAT5 phosphorylation, MAPK (pERK1 and p38), and NF-κB, etc. [30, 31]. Researches on TSLP mainly focus on allergic diseases, such as atopic dermatitis lesions and asthmatic airway [32-34]. However, little information is available regarding to the role of TSLP- TSLPR on ALI.

In this study, we explored the effect of blocking TSLP signaling in BMDCs using TSLPR-Ig *in vitro*, and assessed the effect of TSLPR-Ig on the expression of co-stimulatory molecule (CD40, CD80 and CD86) by DCs that prior to treat with TSLP (TSLP-DCs). The results showed that expression of CD40, CD80 and CD86 were all significantly reduced by TSLPR-Ig. Also, the effect of TSLPR-Ig on airway inflammation/injury in ALI model was evaluated. We found that TSLPR-Ig pretreated mice had low levels of TSLP, suggesting that the critical role played by TSLP/TSLPR signaling in airway inflammation of ALI. Additionally, we found that the lung W/D ratio and little infiltration of the BALF by neutrophil and albumin were significantly reduced by TSLPR-Ig. These results indicated a significant lung injury improvement by using TSLPR-Ig, suggesting TSLPR-Ig might be a potentially therapeutic treatment for ALI. To further investigate the mechanisms underlying the protective effects of TSLPR-Ig, we focused on pulmonary DCs that play a major role in the pathogenesis of ALI. The maturation level of DCs is not only the hallmark of DCs activity but also is important for priming naive T cell responses. Therefore, we evaluated the maturation status of pulmonary DCs in the presence

and absence of TSLPR-Ig. The results showed that expression of CD40, CD80 and CD86 on pulmonary DCs were reduced in mice received with TSLPR-Ig compared with DCs treated with controlled-Ig, which demonstrated that TSLPR-Ig inhibited the inflammation response in the mice airways, at least in part, by regulating DC function. Our study was in line with Zhang *et al.* [16] and Shi *et al.* [28]. Both the two researches suggested that local use of TSLPR-Ig protests against airway inflammation of allergic disease partly by regulating functions of DCs, and that blocking TSLP signaling by using TSLPR-Ig might be a novel strategy for treatment of allergic disease.

Furthermore, the underling downstream signaling mediated by TSLP was explored *in vitro* experiment using BMDCs in the presence of TSLPR-Ig or controlled-Ig. We found that TSLP could effectively activate STAT3 and phosphorylation of ERK1 and ERK2 which was consistent with previous reports [35]. It was noteworthy that TSLPR-Ig only inhibited the phosphorylation of ERK1, but had no significant impact on phosphorylation of ERK2.

In conclusion, our study suggests that TSLP might be involved in ALI and blockage of TSLP signaling by TSLPR-Ig could improve ALI in mice, at least in part, by regulating function of pulmonary DCs. The underling downstream signaling mediated by TSLP might be associated with activating the ERK1 and STAT3 signaling pathway. TSLPR-Ig might be a novel therapeutic treatment of ALI. However, further study should be performed to confirm the clinical effect of TSLPR-Ig on ALI.

Acknowledgements

Disclosure of conflict of interest

None.

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