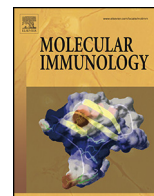




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## SP600125 promotes resolution of allergic airway inflammation via TLR9 in an OVA-induced murine acute asthma model

Hui-Mei Wu, Lei Fang, Qi-Ying Shen, Rong-Yu Liu\*

Anhui Geriatric Institute, Department of Pulmonary, The First Affiliated Hospital of Anhui Medical University, Jixi Road 218, Hefei, Anhui 230022, PR China

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### ABSTRACT

**Background:** c-Jun N-terminal kinase (JNK) relays extracellular stimuli through phosphorylation cascades that lead to various cell responses. In the present study, we aimed to investigate the effect of the JNK inhibitor SP600125 on the resolution of airway inflammation, and the underlying mechanism using a murine acute asthma model.

**Methods:** Female C57BL/6 mice were sensitized with saline or ovalbumin (OVA) on day 0, and challenged with OVA on day 14–20. Meanwhile, some of the mice were treated with SP600125 (30 mg/kg) intraperitoneally 2 h before each challenge. The airway inflammation was evaluated by counting the numbers of various types of inflammatory cells in bronchoalveolar lavage fluid (BALF), histopathology, cytokines production and mucus secretion in individual mouse. In addition, we analyzed the protein levels of phosphorylated JNK and TLR9 in the lung tissues.

**Results:** SP600125 markedly reduced the invasion of inflammatory cells into the peribronchial regions, and decreased the numbers of eosinophils, monocytes, neutrophils and lymphocytes in BALF. SP600125 also reduced the level of plasma OVA-specific IgE, lowered the production of pro-inflammatory cytokines in BALF and alleviated mucus secretion. Meanwhile, SP600125 inhibited OVA-induced, increased expression of p-JNK and TLR9 in the lung tissues.

**Conclusions:** Collectively, our data demonstrated that SP600125 promoted resolution of allergic airway inflammation via TLR9 in an OVA-induced murine acute asthma model. The JNK-TLR9 pathway may be a new therapeutic target in the treatment for the allergic asthma.

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### 1. Introduction

The hallmarks of asthma are inflammation, airway hyperresponsiveness and remodeling (Nath et al., 2005). Despite significant progress in the therapy and understanding of the pathophysiology, asthma control is still very poor (Beeh et al., 2013). JNK is a conserved enzyme that relays external stimuli through phosphorylation cascades to generate various cell responses (Bennett, 2006). Even though its effect on chronic inflammation has been evaluated in several models, its effect on allergic airway inflammation and the underlying mechanism remains to be described.

Allergic inflammation plays an important role in asthma development, and the pathogenesis is dominantly promoted by T helper 2 (Th2) cells (Beeh et al., 2013). Upon sustained activation, Th2 cells produce pro-inflammatory cytokines, which lead to an imbalance between Th1 and Th2 immune responses and activate intracellular

signaling pathway (Eynott et al., 2004; Larche et al., 2003), particularly the mitogen-activated protein kinase (MAPK) pathway (Roux and Blenis, 2004). MAPK pathways include three subgroups: JNK, extracellular signal-regulated kinase (ERK) and p38 (Bennett, 2006; Duan and Wong, 2006). Among them, activation of JNK leads to the phosphorylation of activator protein-1 (AP-1) (Morton et al., 2003), which regulates the production of many cytokines central to airway allergic inflammation (Nath et al., 2005; Van Seuning et al., 2000). Therefore, JNK may play a pro-inflammatory role. Indeed, there is much evidence that indicates a pro-inflammatory role of JNK in the pathogenesis of airway inflammation. Inhibitor of JNK is able to suppress TGF- $\beta$ -stimulated ASM cell migration, suggesting a prominent role for JNK in mediating airway remodeling (Xie et al., 2005). Chung and co-workers have demonstrated that SP600125, an inhibitor of JNK, inhibits airway inflammation and remodeling in both rat and mouse models of chronic airway inflammation (Nath et al., 2005; Zhai et al., 2004; Eynott et al., 2003). Recently, it has been also reported that the absence of JNK protects against pulmonary remodeling induced by house dust mite (van der Velden et al., 2014).

\* Corresponding author. Tel.: +86 551 62922809; fax: +86 551 65120742.  
E-mail address: [rongyuliu@163.com](mailto:rongyuliu@163.com) (R.-Y. Liu).

On the other hand, several studies have revealed that Toll-like receptors (TLR) are prominent factors which mediate the observed pro-asthmatic or anti-asthmatic effects (Duechs et al., 2011). This may be due to that TLRs act as the host's first line of defense against invading pathogens and aeroallergens, making them be involved in the pathophysiology of allergic airway diseases such as asthma (Duechs et al., 2011; Aryan et al., 2014). Until now, TLR9 has been reported to be abundantly expressed in cells including eosinophils, mast cells, plasmacytoid DCs and epithelial cells, and has been widely studied in asthma (Adner et al., 2013; Ramaprabhakar and Hogaboam, 2010; Volpi et al., 2013).

In the present study, we were prompted to reveal the effect of SP600125, an inhibitor of JNK, on the resolution of allergic airway inflammation in an OVA-induced murine acute asthma model, and to assess whether TLR9 is involved in this process.

## 2. Materials and methods

### 2.1. Animal

C57BL/6 mice (Female, 6–7 weeks-old, weighing 20–25 g; Shanghai Laboratory Animal Center) were housed under a 12-hr light/dark cycle at an ambient temperature of  $24 \pm 1^\circ\text{C}$  in a specific pathogen free animal facility. All animal experiments were approved by the Committee on the Ethics of Animal Care and Use of University of Science and Technology of China.

### 2.2. Experiment protocols and treatment

Mice were assigned randomly and evenly into four groups ( $n = 7$  each): the control group treated with saline (Control group); asthmatic group injected and challenged with OVA (OVA group); control mice treated with 30 mg/kg of SP600125 (Con + SP600125 group); asthmatic mice treated with 30 mg/kg of SP600125 (OVA + SP600125 group).

The protocol for the murine acute asthma model used in this study has been reported previously (Locke et al., 2007), and was illustrated in Fig. 1. In brief, all experimental mice were injected intraperitoneally (i.p.) with 10  $\mu\text{g}$  OVA (Sigma, St. Louis, MO, USA) mixed with 1 mg potassium aluminum sulfate (Sangon Biotech, Shanghai, China) in 0.5 ml of saline on day 0, meanwhile, the mice in the control group received 0.5 ml of saline. Fourteen days later, the OVA-sensitized mice were challenged with 1% aerosolized OVA, and the saline-sensitized mice were challenged with nebulized saline for 30 min per day from day 14 to day 20. Two hours before each challenge, the mice in the Con + SP600125 group and OVA + SP600125 group were injected intraperitoneally with 30 mg/kg SP600125. Twenty-four hours after the last challenge, the left lung of each mouse was subjected to bronchoalveolar lavage, and then all the mice were sacrificed for further assays. Part of the right lung of each mouse was used for histological examination, and

the rest of the right lung was frozen at  $-80^\circ\text{C}$  for western blotting analysis.

### 2.3. Bronchoalveolar lavage

One day after the last challenge, mice were injected intraperitoneally with 60 mg/kg of pentobarbital. The left lung was then lavaged with 0.5 ml of cold PBS thrice. The collected BALF were centrifuged at  $700 \times g$  at  $4^\circ\text{C}$  for 5 min, and the pellets were obtained for differential cell counting. The supernatants were stored at  $-80^\circ\text{C}$  for ELISA analysis.

### 2.4. Differential cell count

The cell pellets harvested from BALF were resuspended in 200  $\mu\text{L}$  PBS and the total cell numbers were counted. Giemsa was used for the cell staining and then the numbers of eosinophils, neutrophils, lymphocytes, and monocytes were counted in a blinded manner. More than 200 cells were counted for each mouse.

### 2.5. Enzyme-linked immunosorbent assay (ELISA)

The production of tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), IL-10 in individual BALF samples and blood OVA-specific IgE in each mouse were measured by ELISA using specific kits (Cusabio, Wuhan, China) according to the protocols from the manufacturer. The detection limit for TNF- $\alpha$  and IL-10 was 3.9 pg/ml and 0.8 pg/ml, respectively.

### 2.6. Histological examination

The lung tissue specimens of each mouse were fixed in 4% paraformaldehyde at  $4^\circ\text{C}$  for 3 days and then embedded in paraffin. Then the inflammation and mucus productions were analyzed by hematoxylin/eosin (H&E) or periodic acid-Schiff (PAS) staining, respectively. Histopathologic analysis of peribronchial inflammatory in H&E stained lung slices from seven mice was performed using a semi-quantitative scoring system as previously described (Xirakia et al., 2010; Koltsida et al., 2013).

### 2.7. Western blotting analysis

The lung tissue from each mouse was homogenized, and the total protein concentration was determined. Total proteins (25  $\mu\text{g}$ /lane) of each sample were separated on 12% SDS-PAGE gels and were then transferred to PVDF membranes (Millipore, Billerica, MA, USA). Membranes were blocked in 5% non-fat milk and probed with antibodies against p-JNK (Signaling Technology Inc., Beverly, MA, USA), JNK (Signaling Technology Inc.), TLR9 (Abcam, Boston, MA, USA) and actin (Sigma-Aldrich, St. Louis, MO, USA). Intensity of protein bands was determined by using Image J 1.38x software (NIH, Bethesda, MD, USA).

### 2.8. Statistical analysis

Statistical analysis was performed by using SPSS 19. Data were expressed as means  $\pm$  SEM. The differences among the groups were analyzed by using one-way ANOVAs followed by post hoc Bonferroni tests. Significance was set to  $p < 0.05$ .

## 3. Results

### 3.1. SP600125 ameliorates pathologic features of OVA-induced allergic mice

Histological staining showed that there were more inflammatory cells invading the peribronchial regions in the lung of

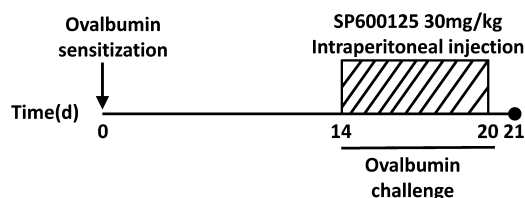


**Fig. 1.** Protocol of induction of allergic airway inflammation. Individual mice were sensitized by intraperitoneal injection with OVA or saline. Two weeks later, the mice were treated with SP600125 (30 mg/kg) for 2 h and then challenged with aerosolized OVA or saline. Twenty-four hours later, the mice were sacrificed for further experiments.

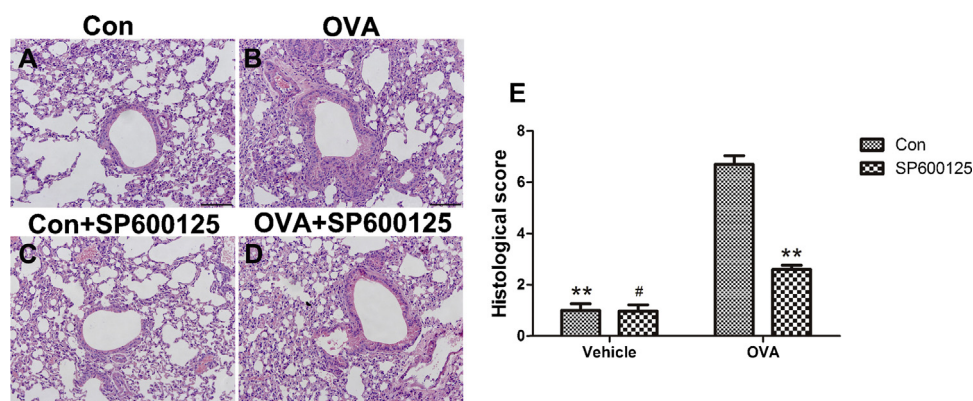


**Fig. 2.** Effect of SP600125 on pathologic features of OVA-induced allergic mice. (A–D) Representative H&E-stained sections of lungs from control group (A), OVA group (B), control + SP600125 group (C), OVA + SP600125 group (D), magnification of  $\times 200$ . (E) Histological scoring was expressed as means  $\pm$  SEM of 7 mice per group from three independent experiments. 0, normal; 1, few cells; 2, a ring of inflammatory cells one cell layer deep; 3, a ring of inflammatory cells two to four cells deep; 4, a ring of inflammatory cells of more than four cells deep. \*\* $p < 0.01$  versus the OVA group; # $p < 0.05$  versus the OVA + SP600125 group.

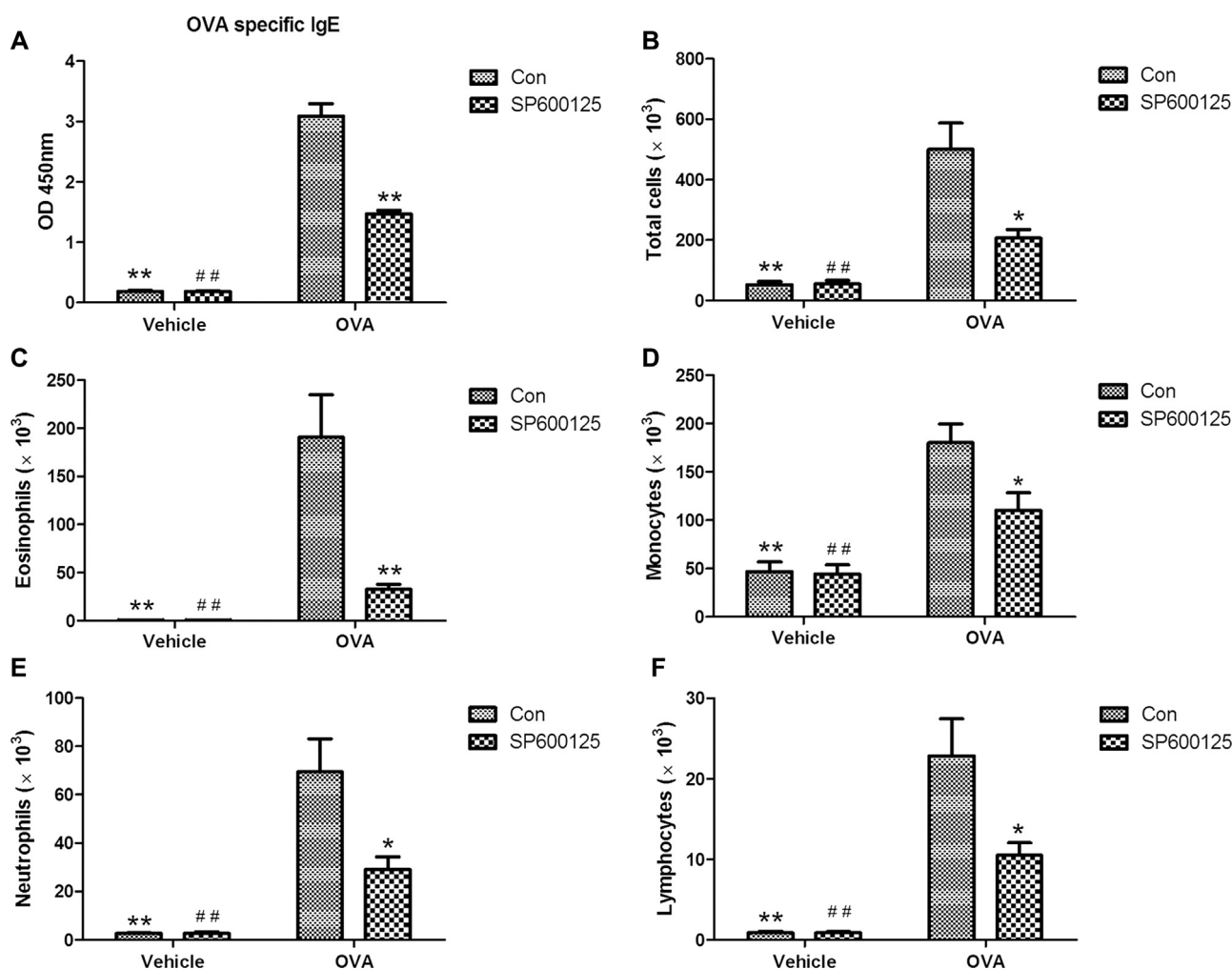


**Fig. 3.** Effect of SP600125 on IgE levels in serum and differential cell counts in BALF. (A) Changes of the serum levels of OVA-specific IgE. (B–F) Changes of the numbers of total cells, eosinophils, monocytes, neutrophils and lymphocytes in BALF. Data were expressed as means  $\pm$  SEM of 7 mice per group. \* $p < 0.05$ , \*\* $p < 0.01$  versus the OVA group; # $p < 0.05$ , ## $p < 0.01$  versus the OVA + SP600125 group.

the OVA group mice (Fig. 2B) compared with that of the control group mice (Fig. 2A). The OVA group mice administrated with SP600125 showed marked reduction of inflammatory cells in the peribronchial regions (Fig. 2D). The peribronchial inflammation

was scored, and the histological score in the OVA group mice lungs was considerably increased compared with that of the control group mice (Fig. 2E), while it was markedly reduced in the lungs from the OVA + SP600125 group mice (Fig. 2E).

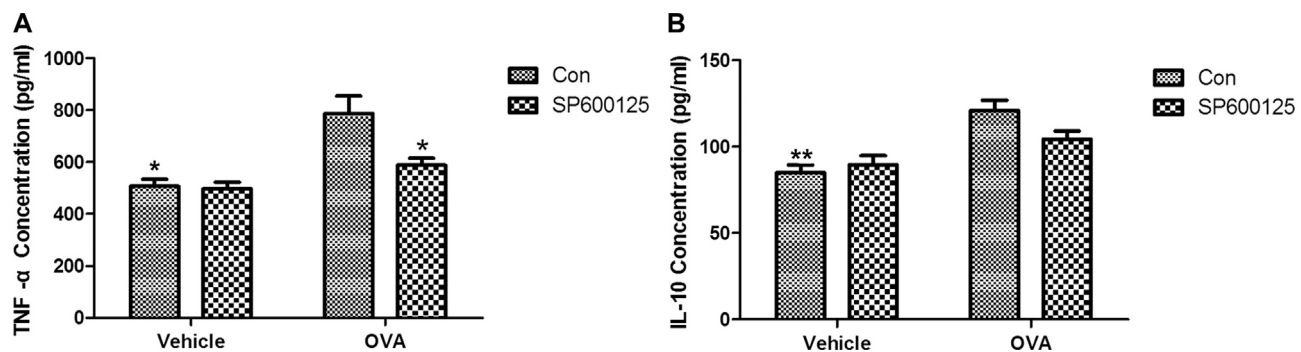


**Fig. 4.** Effect of SP600125 on allergen-induced production of cytokines in BALF. The levels of TNF- $\alpha$  and IL-10 in BALF were determined by ELISA. (A) The level of TNF- $\alpha$ . (B) The level of IL-10. Data were expressed as the means  $\pm$  SEM of 7 mice per group from three separate experiments. \* $p$  < 0.05, \*\* $p$  < 0.01 versus the OVA group.

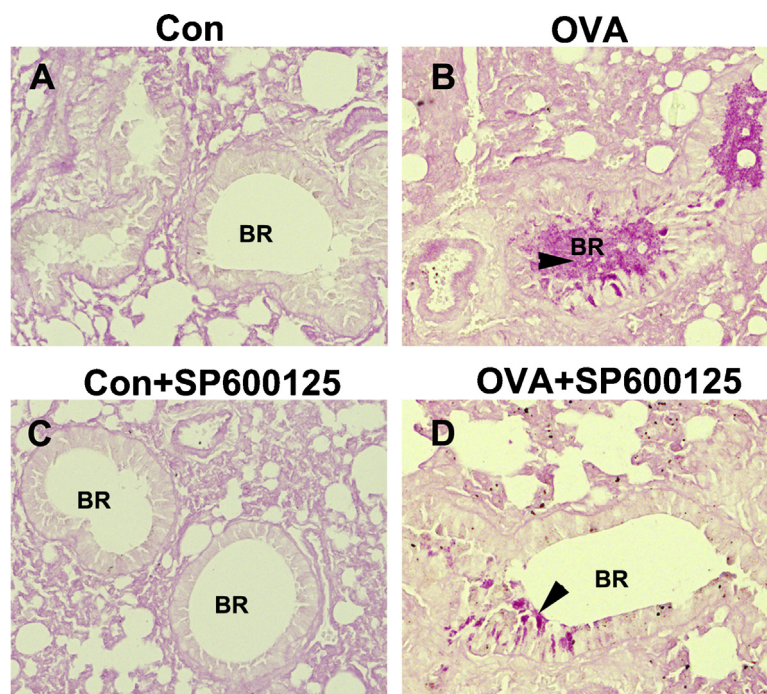


**Fig. 5.** Effect of SP600125 on allergen-induced mucus production in the lung. Representative periodic acid-Schiff (PAS) staining of lung sections from mice challenged with saline (A), OVA (B), or saline after SP600125 treatment (C), OVA after SP600125 treatment (D). Goblet cells stain magenta in color, magnification of  $\times 200$ .

### 3.2. SP600125 reduces the plasma level of OVA-specific IgE in allergic mice

Allergic asthma is a class of diseases characterized by elevated plasma level of IgE (Camateros et al., 2007). The plasma IgE level in the OVA group mice was significantly higher than that in the control group mice. SP600125 administration markedly reduced the OVA-induced IgE level, although it remained higher than those in the control mice (Fig. 3A).

### 3.3. SP600125 prevents the invasion of inflammatory cells into BALF

The numbers of total cells, eosinophils, monocytes, neutrophils and lymphocytes in BALF of allergic mice were significantly increased compared with that of the control mice. However, administration of SP600125 significantly reduced the increase of these cells in BALF of allergic mice (Fig. 3B–F).

### 3.4. SP600125 affects the production of inflammatory cytokines in allergic mice

ELISA analysis showed that the production of TNF- $\alpha$  and IL-10 in BALF of the allergic mice was significantly increased compared with that of the control mice. Treatment of SP600125 markedly reduced the OVA-induced increase of TNF- $\alpha$ , comparable to that in the control mice (Fig. 4A). However, SP600125 pretreatment had no effect on the OVA-induced production of IL-10 (Fig. 4B).

### 3.5. SP600125 alleviated mucus secretion in allergic mice

Mucus secretion and goblet cell hyperplasia were assessed by PAS staining as described in Materials and Methods. OVA challenge led to a considerable increase in airway intensity of PAS staining, which was inhibited by administration of SP600125 (Fig. 5A–D).

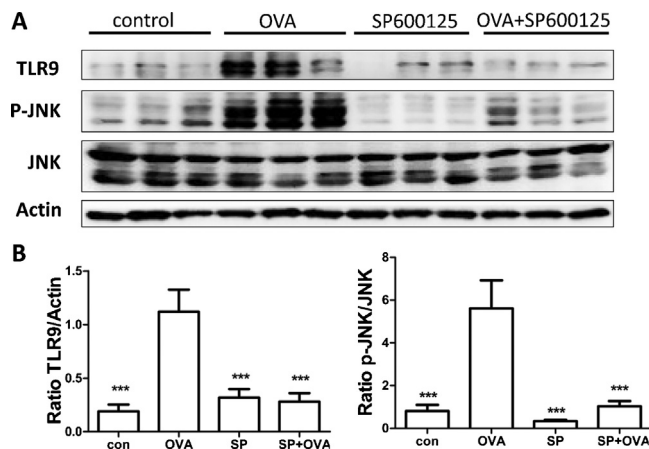


**Fig. 6.** Effect of SP600125 on allergen-induced expressions of p-JNK and TLR9 in the lung tissues. The expression of p-JNK and TLR9 in the lung of the different groups of mice was characterized by western blot assays. (A) Western blot analysis of the expression of p-JNK, JNK, TLR9 and actin in the lung tissues. (B) The relative level of p-JNK and TLR9 in the lung. Data were expressed as the means  $\pm$  SEM of 7 mice per group from three separate experiments. \*\*\* $p$  < 0.001 versus the OVA group.

### 3.6. SP600125 inhibits OVA-induced TLR9 and p-JNK expression in the lung of allergic mice

There was an obviously increased expression of phosphorylated-JNK and TLR9 in the lung tissues from OVA group mice as shown by western blot (Fig. 6A and B). Pretreatment with SP600125 abolished the increased expression of p-JNK, accompanied by the decreased expression of TLR9 (Fig. 6A and B).

## 4. Discussion

In the present study, we demonstrated that pretreatment of SP600125, a JNK inhibitor, markedly reduced the infiltration of inflammatory cells into the submucosal regions, and decreased the numbers of eosinophils, monocytes, neutrophils and lymphocytes in BALF. Moreover, SP600125 reduced the level of plasma OVA-specific IgE, lowered the production of pro-inflammatory cytokines and alleviated airway goblet cell hyperplasia. Interestingly, SP600125 inhibited OVA-induced, increased activity of phosphorylated JNK and counteracted the accompanying increased expression of TLR9 in the lung tissues.

MAPK signaling cascades mediate numerous cell functions via phosphorylating target proteins (Nath et al., 2005) and their involvement in the inflammatory responses is one of the best studied functions (Hommes et al., 2003). JNK, being a major subgroup of MAPKs signal transduction pathway, has been demonstrated to activate AP-1, which controls cellular responses associated with the pathogenesis of asthma (Caramori et al., 2008). In addition, JNK activity has been shown to be enhanced in severe glucocorticoid-resistant asthma (Lane et al., 1998). Thus, JNK inhibitors as potential therapeutics for asthma have attracted more and more attention. Chung et al. have reported that JNK plays a pro-inflammatory role in the pathogenesis of chronic asthma, and JNK inhibitor SP600125 significantly mitigates mucus hypersecretion, airway inflammation, bronchial hyperresponsiveness and airway smooth muscle (ASM) proliferation (Nath et al., 2005; Zhai et al., 2004; Eynott et al., 2003). In accordance with these findings, our present study showed that in a murine acute asthma model, SP600125 markedly reduced the invasion of inflammatory cells into the submucosal regions, and decreased the numbers of eosinophils, monocytes, neutrophils and lymphocytes in BALF. Our results support the role of JNK in cellular trafficking of inflammatory cells. Since it has been demonstrated that SP600125 was able to reduce the expression of intracellular

adhesion molecule-1 (ICAM-1) (Lee et al., 2015), cell migration may be reduced through a mechanism where SP600125 inhibits the endothelial cell adhesion.

Allergic asthma is a class of diseases featured by elevated plasma IgE levels (Camateros et al., 2007). The switch from an IgG- to an IgE-producing B cell is a tightly regulated process (Geha et al., 2003), as the level of IgE in the plasma is much less than that of IgG (Kim et al., 2010). MAPKs, especially JNK, play important roles in IgE switching (Jabara and Geha, 2005). Although inhibition of JNK is insufficient to affect OVA-specific serum IgE production in rat model of allergic asthma (Eynott et al., 2004), our present study showed that SP600125 pretreatment counteracted the OVA-increased plasma IgE levels in a murine acute asthma model. These different results may be caused by the different treatment protocol and species.

There is a close relationship between the IgE-mediated immune response and the elevated Th2 cytokines in allergic asthma (Camateros et al., 2007). We observed a significant reduction of TNF- $\alpha$ , a pro-inflammatory cytokine, in the OVA-challenged mice pretreated with SP600125. It has been reported that there is a JNK binding element in the TNF- $\alpha$  gene promoter, and that SP600125 can inhibit the expression of TNF- $\alpha$  (Bennett et al., 2001). On the other hand, we demonstrated that pretreatment with SP600125 was insufficient to alter the level of IL-10, an anti-inflammatory cytokine, in OVA-challenged mice. Therefore, it was obvious that the ratio of IL-10 to TNF- $\alpha$  was increased in OVA-challenged mice pretreated with SP600125. The imbalance of anti-inflammatory and pro-inflammatory cytokine responses may explain the observed effects of SP600125 in our model.

JNK activation is important for cell proliferation (Nath et al., 2005), and its inhibitor SP600125 inhibits chronic allergen-induced airway smooth muscle and epithelial cell proliferation in a rat model (Eynott et al., 2003). Our data showed that exposure to OVA for a short period was sufficient to elicit early changes of airway remodeling, as demonstrated by mucus hypersecretion, while SP600125 pretreatment led to the inhibition of allergen-induced mucus hypersecretion.

It was quite clear that JNK inhibitor SP600125 promoted resolution of allergic airway inflammation from our observations, but what was the mechanism? Several studies have suggested a mechanistic correlation between JNK signaling pathway and TLR9 (Tsai et al., 2012; Holley et al., 2012; Mitchell and Olive, 2010), and the levels of TLR9 are indeed higher in obese asthmatic patients (Sanchez-Zauco et al., 2014). TLRs are abundant on various innate immune cells involved in asthma pathogenesis, including eosinophils, mast cells, plasmacytoid DCs and epithelial cells (Sanchez-Zauco et al., 2014). When pathogens or allergens entering the airway, they may be recognized by the expressed TLRs of these cells, which triggers the activation of TLRs downstream signaling to induce defense action via initiating cytokines secretion (Lin et al., 2010). Our results demonstrated that SP600125 inhibited OVA-induced, increased activity of p-JNK and counteracted the accompanying increased expression of TLR9 in the lung tissues, suggesting that TLR9 may be the downstream signal of JNK in an OVA-induced murine acute asthma model. To our knowledge, this is the first study to propose the involvement of JNK-TLR9 in the pathogenesis of allergic asthma.

Collectively, our data demonstrated that SP600125 may promote resolution of allergic airway inflammation via TLR9 in an OVA-induced murine acute asthma model. The JNK-TLR9 pathway therefore may be a new therapeutic target in the treatment for the allergic asthma.

### Conflict of interest statement

The authors have no conflict of interest to declare.

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