



# A mouse allergic asthma model induced by shrimp tropomyosin

Lei Fang<sup>a,b,1</sup>, Fangchao Zhou<sup>a,b,1</sup>, Fan Wu<sup>a,b,1</sup>, Ying Yan<sup>a</sup>, Zhenpeng He<sup>a</sup>, Xiaoling Yuan<sup>c</sup>,  
Xiumei Zhang<sup>d</sup>, Tong Zhang<sup>d</sup>, Duonan Yu<sup>a,b,d,\*</sup>

<sup>a</sup> Institute of Translational Medicine, Medical College, Yangzhou University, Yangzhou 225009, China

<sup>b</sup> Jiangsu Key Laboratory of Experimental & Translational Non-coding RNA Research, Yangzhou University Medical College, Yangzhou 225009, China

<sup>c</sup> Yangzhou Maternal & Child Care Service Center, Yangzhou University, Yangzhou, Jiangsu 225002, China

<sup>d</sup> Xinghua People's Hospital, Yangzhou University, Xinghua, Jiangsu 225700, China

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## ABSTRACT

Allergic asthma remains an important worldwide health issue. Animal models are valuable for understanding the pathophysiological mechanisms of asthma and the development of effective therapeutics. This study aims to develop an alternative murine model induced by shrimp tropomyosin (ST) instead of ovalbumin (OVA). To investigate responses to short-term exposure to antigens, mice were sensitized with intraperitoneal injections of ST or ST plus aluminum adjuvant on days 0, 7, 14 followed by an intranasal challenge with ST for seven consecutive days. We reveal that sensitization with ST alone or ST plus aluminum induces significant levels of serum total IgE and ST-specific IgE in mice. Challenge results show that ST causes severe eosinophilic airway inflammation. Histology analysis of the lung tissues demonstrates airway inflammation and mucus hypersecretion within the bronchi in mice exposed to ST. Analysis of the cell composition in bronchoalveolar lavage fluid (BALF) shows a significant increase in eosinophil count in ST alone and ST plus aluminum groups. We also detect increased CD4<sup>+</sup> T lymphocytes in lung tissues and production of helper T cell type 2-associated cytokines (IL-4 and IL-5) in BALF. In addition, airway hyperresponsiveness to methacholine in ST alone and ST plus aluminum groups is much higher than that in control groups. For the chronic model, mice were sensitized by ST or ST plus aluminum adjuvant for 3 weeks and challenged with ST for 6 weeks. We find severe structural changes in animals upon prolonged exposure to ST, including goblet cell hyperplasia, collagen deposition, and smooth muscle thickening. In conclusion, ST-induced asthma is a simple murine model for studying pathogenesis of asthma and evaluating new therapeutic drugs.

## 1. Introduction

Allergic asthma is the most prevalent respiratory disease affecting 235 million people worldwide [1] and causing 250,000 deaths each year [2]. Asthma is diagnosed based on airflow limitation and respiratory symptoms such as wheezing, shortness of breath, and chest tightness [3]. The pathological characteristics are airway inflammation, mucus overproduction, hyperreactivity (AHR) and airway remodeling [4].

Murine models have been used extensively for investigating the mechanisms of sensitization and immune responses in human patients, as well as therapeutic strategies to reduce allergic asthma [5,6]. These models could mimic clinical critical features and phenotypes of human asthma, including AHR, increased eosinophils in bronchoalveolar lavage

fluid (BALF) and lung tissue, and elevated Th2 cytokine levels [7].

Allergic asthma, one of the most common forms of type I allergy, is mediated by immunoglobulin E (IgE) to various allergens. The antigens such as ovalbumin (OVA) are commonly used in asthma models [8]. The OVA-induced asthma requires the use of both OVA and Th2-adjuvant aluminum hydroxide to sensitize mice. In fact, allergen exposure without adjuvant represents the natural sensitization process in humans or animals. However, the adjuvant-free OVA protocol failed to induce a high IgE level even with multiple allergen exposures. Moreover, not all asthma features can be induced by OVA in the presence of adjuvants. Th2 cytokine levels, especially IL-4, are not high enough in OVA-sensitized C57BL/6 mice [9]. In addition, prolonged exposure to OVA usually induces tolerance in the animals, resulting in reduced airway

\* Corresponding author at: Jiangsu Key Laboratory of Experimental & Translational Non-coding RNA Research, Yangzhou University Medical College, 136 Jiangyangzhong Road, Yangzhou, Jiangsu Province 225009, China.

E-mail address: [dnyu@yzu.edu.cn](mailto:dnyu@yzu.edu.cn) (D. Yu).

<sup>1</sup> These authors contributed equally to this work.

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inflammation [10,11].

Components from shrimp are also known as common human allergens [12], often presenting severe reactions including respiratory symptoms and anaphylaxis [13]. Tropomyosin is a potent allergen in shrimp [14] with cross-reactivity with asthma-related inhalant allergens arising from mites and cockroach [15]. Thus we think shrimp might be a more reliable source of allergen for asthma models. In this study, we found that intraperitoneal (i.p.) administration of shrimp tropomyosin (ST) purified from *Metapenaeus ensis* induces more intense immunological and histopathological changes than OVA with or without adjuvants. Moreover, ST recapitulates many asthma features in the absence of adjuvants, indicating that ST-induced asthma represents a simple mouse model useful for basic and therapeutic studies.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Mouse IgE, IL-4 and IL-5 ELISA kits were obtained from Biolegend Co. (San Diego, California, USA). O-phenylenediamine and OVA were purchased from Sigma (St Louis, MO). Rat anti-mouse IgE antibody conjugated with HRP was purchased from Abcam Co. (Cambridge, UK). PLUS-Agarose was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Methacholine Chloride was purchased from TCI Co. (Shanghai, China). 3, 3', 5, 5'-Tetramethylbenzidine (TMB) was purchased from Sangon Biotech Co. (Shanghai, China). Periodic Acid Schiff (PAS) staining kit was from Wanleibio Co. (Shenyang, China). Anti-mouse  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) antibody and immunohistochemistry assay kit were from Boster (Wuhan, China). HiScript III RT SuperMix for qPCR and ChamQ Universal SYBR qPCR Master Mix were from Vazyme Biotech Co., Ltd. (Nanjing, Jiangsu, China). All other reagents were of analytical grade. Primers for RT-PCR were synthesized in Sangon Biotech Co. (Shanghai, China) and the sequences were listed in Supplementary table 1.

### 2.2. Shrimp tropomyosin (ST) preparation

ST was extracted from shrimp (*Metapenaeus ensis*). Fresh shrimp (about 100 g) was minced, homogenized in PBS, and 300 mL acetone was added. The mixture was centrifuged and the precipitate was washed with acetone. Shrimp acetone powders were prepared by drying the precipitate for 24 h. Extraction and purification of ST were conducted by isoelectric precipitation as previously described [16]. In brief, the acetone powders were dispersed in a buffer (1 M KCl, 0.5 mM dithiothreitol, pH 7) for 24 h and centrifuged at 5000g for 15 min. After centrifugation, the precipitate was dissolved in an extraction buffer, adjusted to pH 7 and stirred for 30 min at room temperature. This isoelectric precipitation step was repeated twice. The final precipitate was dissolved in 0.5 mM dithiothreitol (DTT, pH 7) and dialyzed against PBS. After saturation by 55% ammonium sulphate, the precipitate was dissolved in 0.5 mM DTT, then dialyzed and lyophilized. The purity was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) based on the presence of a prominent band with a molecular weight of approximately 36 kDa.

### 2.3. Mice and ST sensitization/intranasal challenge

C57BL/6 mice (female, 6–8 weeks, 16–18 g) were purchased from the Comparative Medicine Centre of Yangzhou University (Yangzhou, China) and kept in standard laboratory temperature and humidity with a 12-h light/dark cycle. The experimental protocol was shown in Fig. 1A and sFig. 1A. Mice were divided randomly into four groups: (1) two control groups: saline group (Saline) and aluminum hydroxide (alum) adjuvant group (Alum); (2) two model groups: ST group (ST) and ST plus alum adjuvant group (ST+alum). Mice were immunized once per week (days 0, 7, and 14) by an i.p. injection of 20  $\mu$ g of ST alone or ST emulsified in 2.5 mg alum with a total volume of 0.1 mL. For short-term exposure to ST, mice were challenged intranasally (i.n.) with 50  $\mu$ g of ST in 50  $\mu$ L of saline daily for 7 days. For long-term exposure to ST, mice were challenged i.n. with ST twice a week for 6 weeks. Control mice

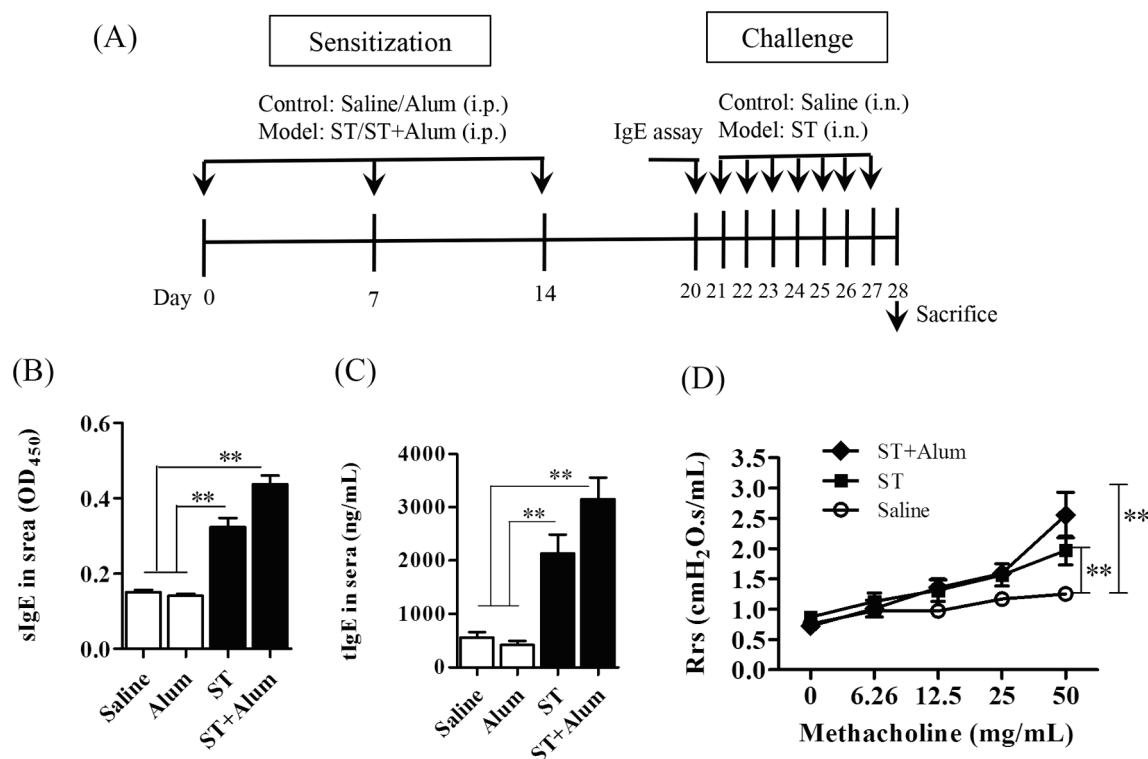


**Fig. 1.** The IgE levels in sera of ST-sensitized mice. (A) Sensitization and challenge protocol for ST-induced asthma models in C57BL/6J mice. (B) The sIgE levels in sera ( $n = 10$ ). (C) The tIgE levels in sera. ( $n = 10$ ). (D) The lung resistance ( $n = 6$ ). Data are reported as means  $\pm$  SE. \*\* $P < 0.01$  by one-way ANOVA.

were sensitized by an equal volume of saline (0.9% NaCl) or alum adjuvant and challenged by saline using the same protocol.

Three independent experiments were conducted. In each experiment, 6 mice were used for AHR analysis and BALF collection and 10 mice were used to measure the serum levels of tIgE and sIgE. In addition, in each experiment, 6 mice were used to analyze eosinophil peroxidase (EPO) activities and histopathology, 3 mice were used to examine CD45<sup>+</sup>CD4<sup>+</sup> T cells in lung tissues, and 6 mice were used to investigate airway remodeling. All experiments were approved by the Animal Ethics Committee of Medical College of Yangzhou University (Approval No. 20180912, 20181121, 20190301).

#### 2.4. ELISA for tIgE and sIgE in sera

On day 20, before the i.n. challenge, a small volume of blood was collected from the tails of the immunized mice for measuring total IgE (tIgE) and ST-specific IgE (sIgE) levels in sera. tIgE was assayed using ELISA kits according to the manufacturer's instructions. Levels of sIgE were measured by ELISA as described previously with modifications [17]. Briefly, IgG in serum samples were removed by Protein G PLUS-Agarose. Microplate wells coated with 10 µg/mL of ST were incubated for 1 h at 37 °C with 100 µL diluted serum samples (1:100 dilutions in PBS). Plates were then washed, and 100 µL of rat anti-mouse IgE antibody conjugated with HRP (1:5000) was added to each well. The plates were incubated for another 1 h at 37 °C. The reaction was developed by incubation with TMB for 5 min at 37 °C, stopped with the addition of 2 M H<sub>2</sub>SO<sub>4</sub>, and the reaction product was measured by Multiskan FC (Thermo Fisher, USA) at 450 nm.

#### 2.5. AHR

On day 28, mice were anesthetized by an i.p. injection of pentobarbital sodium (50 mg/kg) and the tracheas were cannulated. Airway resistance (cm H<sub>2</sub>O. s/mL) was measured using the Flexivent system (SCIREQ, Montreal, Quebec, Canada) when mice were exposed to methacholine at the concentrations of 12.5, 25 and 50 mg/mL.

#### 2.6. Collection of bronchoalveolar lavage fluid (BALF)

On day 28, mice were sacrificed and BALF was collected as we previously described [18]. Mouse tracheas were cannulated and lavaged twice with 0.4 mL ice-cold PBS and 0.7 mL BALF was collected. The total numbers of inflammatory cells were measured using a hemocytometer. BALF was centrifuged at 500g for 5 min at 4 °C, and supernatants were stored at -80 °C for cytokine assay. The cell pellet was resuspended in PBS. Smears of cells on slides were prepared using a cytospin machine. The eosinophil count in BALF was determined by cytospin preparations and H&E staining.

#### 2.7. ELISA for IL-4 and IL-5 in BALF

IL-4 and IL-5 in BALF were assayed using ELISA kits according to the manufacturer's instructions.

#### 2.8. Eosinophil peroxidase (EPO) activity assays

At the termination of the experiment, mice were sacrificed and lungs were excised. The left lung lobes were weighed and homogenized in 0.5% hexadecyltrimethylammonium bromide (pH 6.0) using an automatic tissue homogenizer. The homogenate was centrifuged at 3000 rpm for 10 min at 4 °C. Supernatants were used for EPO activity assay as previously described [19]. The supernatant (50 µL) was added to 100 µL of reaction mixture (0.1% Triton X-100, 1 mM o-phenylenediamine, and 500 mM hydrogen peroxide in 50 mM Tris-HCl buffer, pH 8.0) in a microplate and incubated for 30 min at room temperature. The reaction was stopped by adding 2 M H<sub>2</sub>SO<sub>4</sub> (50 µL), and absorbance at 490 nm

was measured.

#### 2.9. Histology and morphometry

The right lung lobes were fixed in 10% neutral-buffered formalin solution, embedded in paraffin and cut into 4 µm thick sections. Serial tissue sections were stained with hematoxylin and eosin (H&E) to examine inflammatory cell infiltration. The degree of peribronchial and perivascular inflammation was evaluated according to a 0–5 scoring system as previously described (0, no cells; 1, a few cells; 2, a layer of cells; 3, a ring of cells with 2–4 layer of cells; 4, focal inflammatory cells clustered around bronchi or vessels; 5, intense inflammatory infiltration) [20]. The airway goblet cell hyperplasia (mucus hypersecretion) was quantified with PAS staining and scored as previously described (0, no goblet cells; 1, goblet cells occupying < 25% of the epithelium; 2, 25–50% of the epithelium; 3, 50–75% of the epithelium; 4, >75% of the epithelium) [21]. At least 3–5 bronchioles were analyzed in each tissue slide and average scores were calculated.

For the airway remodeling, Masson's trichrome method for observing elastic and collagen fibers was used. The immunohistochemistry assay was performed using antibodies against α-SMA at a 1:500 dilution according to the manufacturer's instructions. Positive expression was quantified in airways using ImageJ software. Data are expressed as percentage of α-SMA expression per total tissue area [22].

#### 2.10. CD4<sup>+</sup> T cells in lung tissues

Lung tissue suspension cells were prepared as previously described [23]. The cells were stained with FITC-labeled anti-CD45 and PE-labeled anti-CD4 mAbs. The percentage of CD45<sup>+</sup>CD4<sup>+</sup> cells was analyzed by flow cytometry on a Calibur flow cytometer (Becton Dickinson, California, USA).

#### 2.11. Statistical analysis

Prism 5.0 (GraphPad Software, San Diego, CA, USA) was used for statistical analysis. The data were expressed as mean ± SE and analyzed by a one-way ANOVA followed by Tukey's test. For lung inflammation scores and PAS scores, Kruskal-Wallis test and Dunn's test were used. Statistical difference was set at  $p < 0.05$ .  $p < 0.01$  indicates significantly different.

### 3. Results

#### 3.1. tIgE and sIgE levels in sera

IgE is an important mediator of allergic reactions. To assess IgE levels after ST sensitization, the IgE levels in sera before allergy challenge were determined. Mice in the ST group and ST plus alum group produced much high levels of tIgE and sIgE in sera compared to the unsensitized mice (Fig. 1B/C).

#### 3.2. Asthma symptoms and AHR

AHR, a hallmark of bronchial asthma, is caused by airway smooth muscle contraction. Asthma symptoms, including scratching around the nose, forelimb crimpation and difficult breathing, were observed after ST challenge in all sensitized mice. None of the mice from the control group showed symptoms of asthma during the course of challenging. AHR was provoked by inhalation of methacholine aerosol and assessed by the changes of airway resistance. Airway resistance was higher in ST-challenged mice than that in control mice (Fig. 1D), indicating lung function disorder in ST-induced asthma mice.

### 3.3. Histopathological changes in lung tissues

Pathological analysis detected a marked inflammatory reaction in the lung characterized by the infiltration of inflammatory cells, especially in the peribronchial and perivessel areas in ST-induced animals compared with control mice (Fig. 2A/B). The majority of infiltrated inflammatory cells were eosinophils. Normal bronchial epithelium layers were observed in mice from saline and alum groups (Fig. 2A/B). In contrast, PAS-positive goblet cells in the bronchiolar epithelium were evident in the ST and ST plus alum groups (Fig. 2C/D). Moreover, a structural remodeling of airway walls including collagen deposition and smooth muscle thickening in animals long exposed to ST (up to 6 weeks) was observed (SFig. 1B-E), indicating a pathological alteration of lung airway by ST.

### 3.4. Infiltration of inflammatory cells in BALF

To assess airway inflammation induced by ST, we examined the total numbers and classification of inflammatory cells in BALF in ST-induced mice. As shown in Fig. 3A and B, ST-challenged mice had a 16.6-fold higher total cell count in BALF than in control mice. The characteristics of cells were observed on cytospin slides. Of note, a significant higher eosinophils count was observed in ST-challenged mice than in control mice (Fig. 3C).

### 3.5. EPO activity in lung tissues

The activity of EPO, an enzyme located mainly in the primary granules of eosinophil, reflects the degree of eosinophil infiltration during the pathogenesis of allergic airway inflammation. To confirm the eosinophil accumulation, EPO activity in lung tissue was measured. As shown in Fig. 3D, EPO activity was significantly increased upon ST

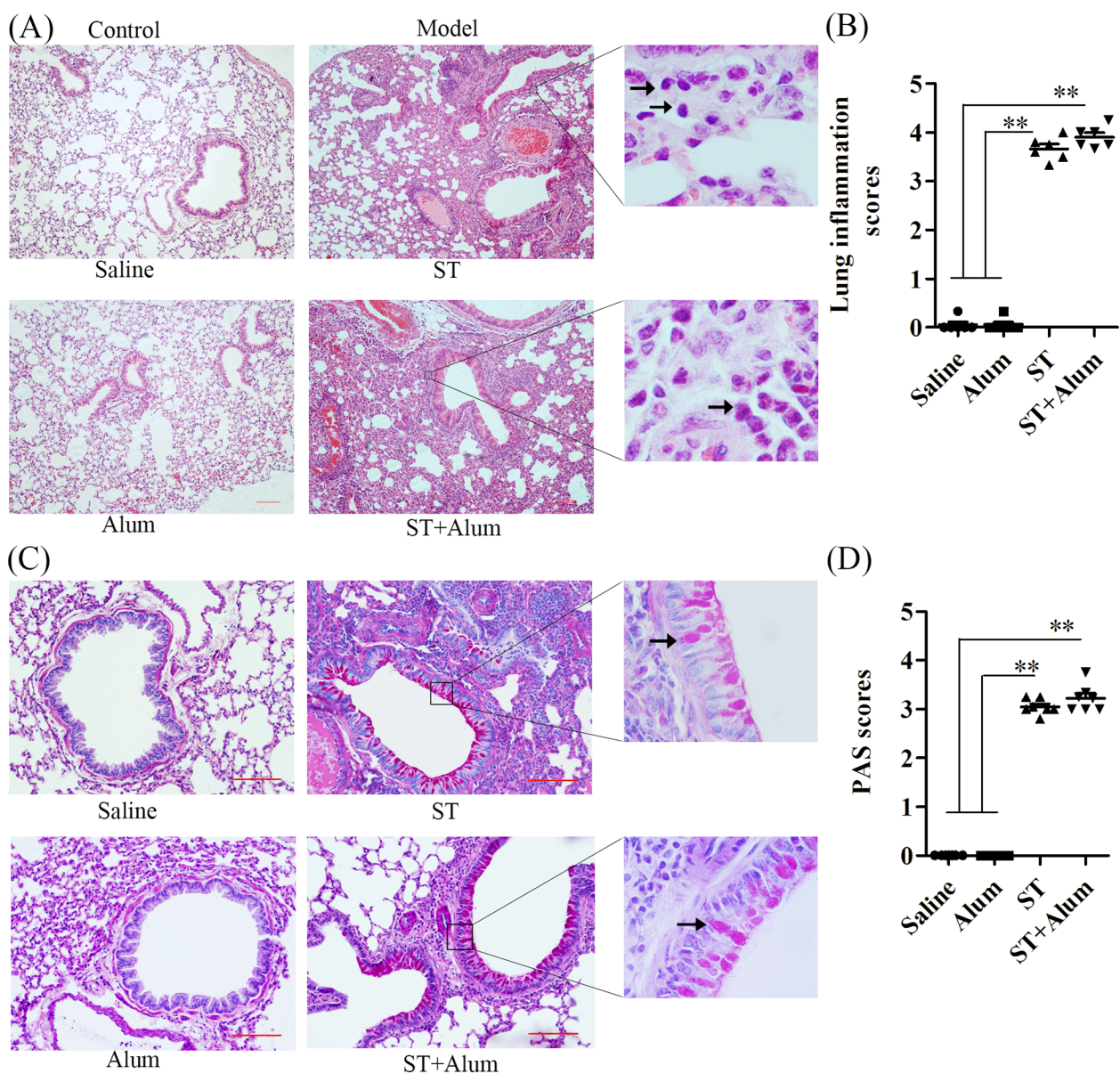


**Fig. 2.** The histopathological changes in lung tissues in ST-sensitized mice. (A) H&E staining showing lung inflammation (100x, scale bar = 200 μm). The magnified images (1000x) represent the small squares outlined in ST or ST+alum groups. The arrows point to infiltrated eosinophils. (B) Lung inflammation scores in different groups. (C) PAS staining showing goblet cell hyperplasia (200x, scale bar = 100 μm). The magnified images (400x) represent the squares in ST or ST+alum groups. The arrows point to goblet cells. (D) PAS scores. Data represent means ± SE (n = 6). \*\*P < 0.01 by Kruskal-Wallis test.

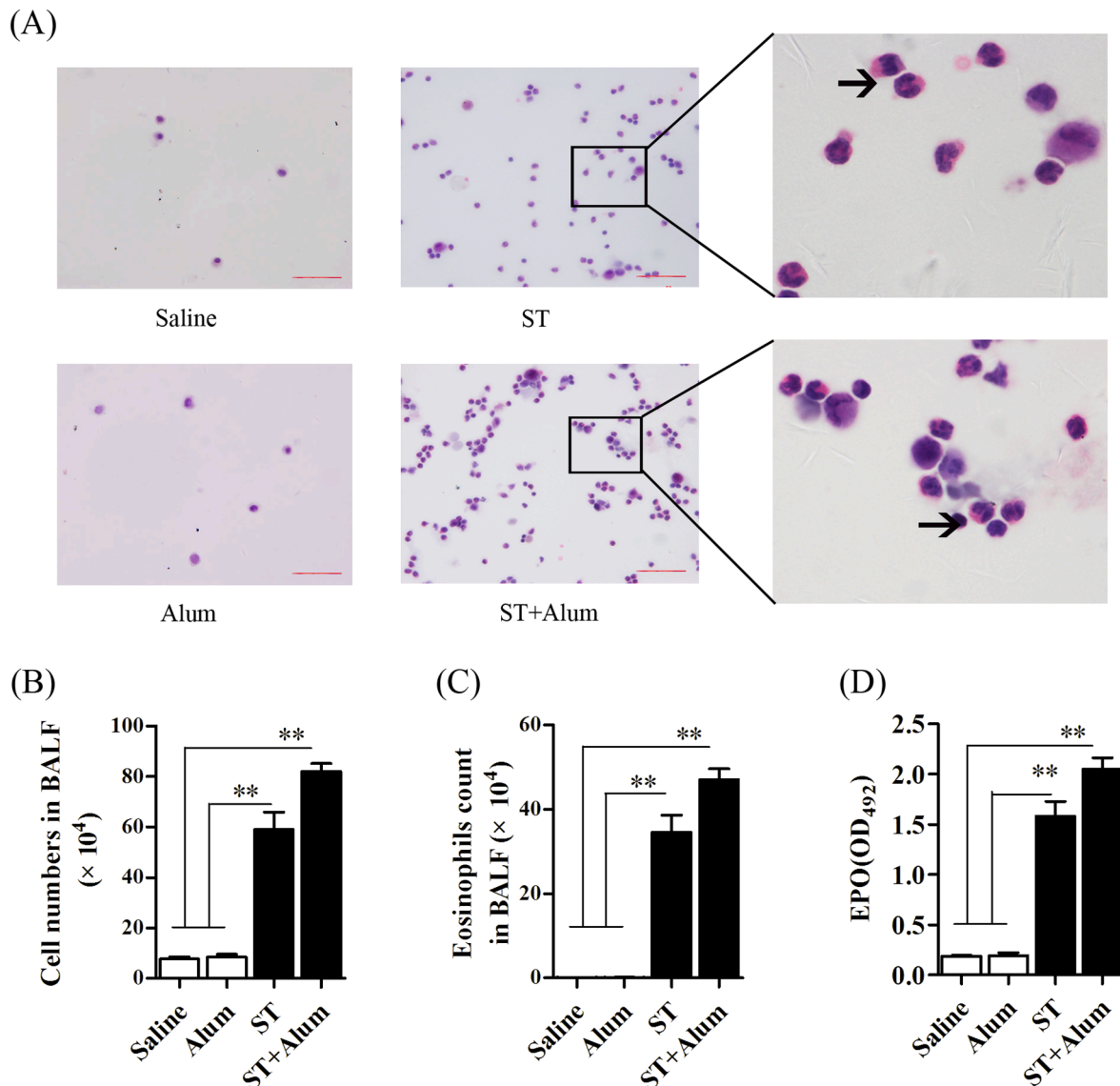


**Fig. 3.** The airway inflammation in ST-sensitized mice. (A) Leukocyte recruitment in BALF from mouse lungs (400×). The high magnification images (1000×) represent the squares in ST or ST+alum groups. The arrows point to eosinophils. (B) Total cell numbers in BALF. (C) Eosinophil counts in BALF. (D) Lung EPO activity. Data represent means  $\pm$  SE ( $n = 6$ ). \*\* $P < 0.01$  by one-way ANOVA.

stimulation.

### 3.6. IL-4 and IL-5 production in BALF and CD4<sup>+</sup> T cells in lung tissues

Th2-mediated immune responses are associated with bronchial AHR, inflammatory cell infiltration, and airway remodeling. We detected Th2 cytokine production in BALF. As shown in Fig. 4A/B, the levels of IL-4 and IL-5 produced in BALF were significantly higher in ST-sensitized mice than that in control groups. Immunization with ST or ST plus alum also resulted in significantly increased numbers of CD45<sup>+</sup>CD4<sup>+</sup> T cells in lung tissues (Fig. 4C/D). All these results suggest a polarization toward a Th2 cell-mediated immune response in ST-sensitized mice.

## 4. Discussion

In this study, we successfully established an asthma model induced by ST instead of OVA. This is an alternative asthma model featuring enhanced airway response to methacholine, eosinophil infiltration, mucus hypersecretion in airways, airway remodeling, and increased production of Th2 cytokines in BALF and high levels of serum IgE.

At present, stimulation with OVA is a classic approach to induce allergic asthma. To mimic the pathogenesis of human asthma, protocols for development of animal models usually include sensitization and challenge phases. However, mouse response to OVA is less sensitive. First, in the sensitization phase, mice usually require multiple systemic OVA immunizations (via i.p. or subcutaneous route) with adjuvants. Lack of repeated systemic OVA sensitization does not allow mice to generate the main features of allergic asthma after allergy challenge [24,25]. One of the most commonly used protocols is animal sensitization using two i.p. injections with OVA plus an adjuvant after 7–14 days followed by the allergy challenge one week later [5]. However, our study demonstrates that sensitization using two i.p. injections with a mixture of OVA and aluminum hydroxide results in low IgE levels in mice (SFig. 2A). Our study also reveals that expression of several genes (IL-4, IL-5, IL-13, IL-33 and eotaxin) in the lung tissues are not high enough after OVA challenge (SFig. 2B–G). Moreover, we do not observe significant eosinophilic inflammation in OVA without adjuvants (SFig. 3). We speculate that the low asthma activity by OVA might be associated with the development of tolerance [26] induced by long-term exposure of mice to OVA allergen-containing mouse diet.

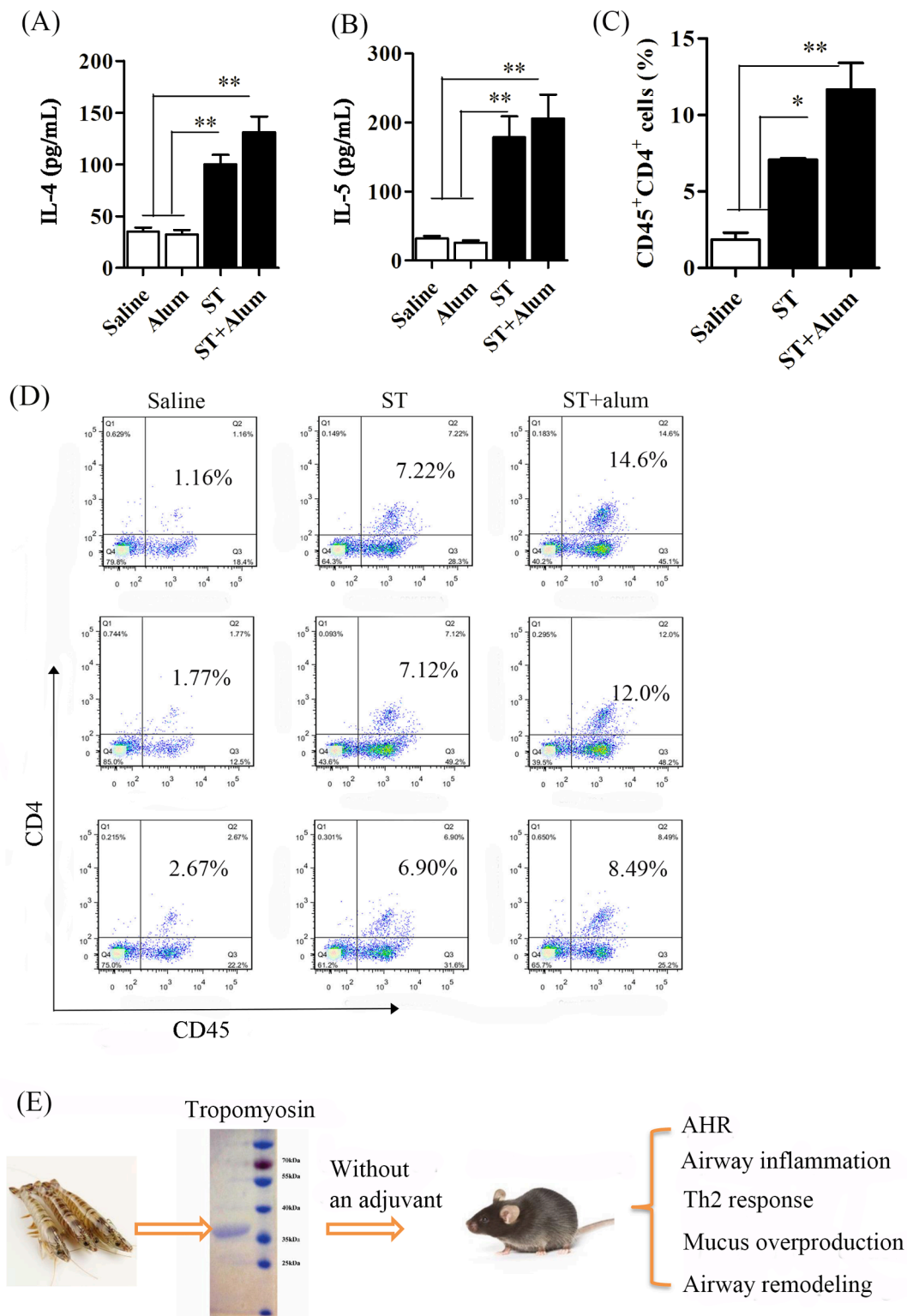


**Fig. 4.** The Th2 response in ST-induced asthma mice. (A) IL-4 levels in BALF ( $n = 6$ ). (B) IL-5 levels in BALF ( $n = 6$ ). (C) Percentage of CD45<sup>+</sup>CD4<sup>+</sup> double positive cells in lung tissues measured by flow cytometry ( $n = 3$ ). (D) Raw data for CD45<sup>+</sup>CD4<sup>+</sup> double positive cells in lung tissues measured by flow cytometry ( $n = 3$ ). (E) Graphical abstract. Data present the means  $\pm$  SE.  $^{**}P < 0.01$  by one-way ANOVA.

Like eggs, shrimp can cause human allergy [27]. ST is derived from shrimp and can be produced in large quantities, making it less expensive for experimentation. In the presence of an adjuvant, ST dramatically elevates the tIgE levels in mouse sera (SFig. 2A), indicating a more sensitive response compared with that from OVA. We have previously developed a systemic hypersensitivity mouse model induced by i.p.

injections with ST plus alum [28]. Alum, a widely used adjuvant, is known to preferentially prime Th2 response. However, alum is not a physiological substance, which causes distress in experimental animals and impedes the test of adjuvant-containing drugs [29]. In this study, we demonstrate that without alum the sensibility of mouse response to ST is still very high. In fact, mice sensitized by three i.p. injections of ST alone

produce higher levels of IgE (Sfig. 2A).

Eosinophilic inflammation of the airways is a common feature of allergic asthma. Loss of asthma control and asthma exacerbations are associated with increased sputum eosinophil count [30]. Compared to the aerosolized route, the advantage of i.n. and intratracheal routes in the challenge phase is that allergens are instilled directly inside the airways, and thus drive a more intense allergic inflammation [31]. In this study, ST treatment induces airway inflammation in mice, characterized by increased cell numbers in BALF. Strikingly, the numbers of lavaged eosinophils are dramatically increased. Lungs from ST-challenged mice contain large numbers of perivascular and peribronchial eosinophils, along with high levels of EPO activity. These mice also show an increase in AHR by approximately two-fold under naive conditions after challenge by ST, while non-immunized mice do not exhibit pulmonary inflammation and AHR. Airway remodeling is the consequence of ongoing inflammation and repair, and there is increasing evidence that eosinophils are important in the development of this pathological process [32]. In this study, we find that mice exposed to ST for up to six weeks develop chronic inflammations with airway remodeling, characterized by various structural changes including collagen deposition, airway smooth muscle hypertrophy and goblet cell hyperplasia.

Eosinophilic asthma is driven by a response from Th2 CD4<sup>+</sup> T cells. Allergen-induced airway eosinophilia is abrogated in the absence of conventional CD4<sup>+</sup> T cells [7]. Th2-associated cytokines such as IL-4, IL-5 and IL-13 are usually found in BALF and are responsible for inflammatory reactions. IL-4 is a cytokine that promotes Th2 cell differentiation and drives B-lymphocytes to conduct class-switch and differentiate into IgE-producing plasma cells [33]. IL-4 is required for mice to manifest AHR. AHR does not occur following IL-4 receptor blockades [34]. IL-5 is responsible for proliferation, maturation, activation, and survival of eosinophils. In asthma, eosinophils are recruited into mucosal airways [35]. Using IL-5 to neutralize monoclonal antibodies is a valuable therapeutic option for patients with severe eosinophilic asthma [36]. Our study demonstrates that upon intranasal challenge by ST, CD4<sup>+</sup> T cells are increased in lung tissues and the levels of Th2 cytokines in BALF including both IL-4 and IL-5 are elevated, further confirming that a ST-induced asthma mouse model mimics the pathophysiology of human asthma disease.

In conclusion, ST induces more allergic response than OVA in mice. Moreover, ST-induced allergy without adjuvants exhibits several hallmarks of human asthma including high IgE production, AHR, eosinophilic inflammation and Th2 response. Furthermore, repeated i.n. challenges using ST without managing adjuvants leads to airway remodeling. Our findings also demonstrate that a local administration of ST through i.n. instillation generates enough challenge for asthma development, and thus a ST-induced murine asthma model mimics the natural exposure of humans to allergens via the nose and trachea. We conclude that ST-induced asthma with a nasal challenge is a simple and alternate murine model for studying the pathogenesis of asthma and drug development of asthma therapeutics.

#### CRedit authorship contribution statement

**Lei Fang:** Writing - original draft, Investigation, Methodology, Data curation. **Fangchao Zhou:** Investigation, Methodology, Data curation. **Fan Wu:** Writing - original draft, Methodology. **Ying Yan:** Validation, Formal analysis. **Zhenpeng He:** Investigation. **Xiaoling Yuan:** Investigation. **Xiumei Zhang:** Investigation. **Tong Zhang:** Formal analysis. **Duonan Yu:** Conceptualization.

#### Declaration of Competing Interest

The authors claim no conflict of interests.

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#### Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.intimp.2020.107289>.

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## Supplementary information

Table 1 Primers used for RT-PCR analysis.

| Target gene | Primer sequence                                          |
|-------------|----------------------------------------------------------|
| IL-4        | F: CATCGGCATTTTGAACGAG<br>R: CGAGCTCACTCTCTGTGGTG        |
| IL-5        | F: ACATTGACCGCCAAAAAGAG<br>R: CACCATGGAGCAGCTCAG         |
| IL-13       | F: ACCCAGAGGATATTGCATGG<br>R: TGGGCTACTTCGATTTTGGT       |
| IL-33       | F: CAAAACAAAACATAACAGATTGGTCA<br>R: GACACATTGAGCATCCAAGG |
| Eotaxin     | F: AGAGCTCCACAGCGCTTCT<br>R: GCAGGAAGTTGGGATGGA          |