

## Original Article

# Decreased Rac1 expression leads to upregulation of ILC2-related cytokines and aggravation of airway inflammation in allergic asthma mice

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**Abstract:** Allergic asthma is an inflammatory disease of the airways in which epithelial cells and group 2 innate lymphoid cells (ILC2s) are increasingly implicated. Recent findings suggest that airway epithelial undergo apoptosis after encountering environmental allergens then apoptotic cells are efficiently phagocytosed by airway epithelial cells via a Rac1-dependent manner and secrete anti-inflammatory cytokines. However, the expression level of Rac1 in asthma and its relationship with ILC2 polarization remains unclear. In this study, Rac1 and the ILC2s related cytokines in lung tissue, PBMC, BALF from the mice were measured, and the potential correlation between them was also analyzed. Our data showed that the expression levels of IL-25, IL-33, IL-5 and IL-13 mRNA were significantly increased while Rac1 level was decreased in asthmatic mice, and there was a negative correlation between the expression level of Rac1 and ILC2s related cytokines. After the Rac1 inhibitor EHT1864 was administrated, the expression levels of the ILC2s related cytokines were further increased, meanwhile the inflammatory was aggravated in asthmatic mice. These results suggested that the decreased Rac1 may contribute to the polarization of ILC2 and promote the development of asthma inflammation.

**Keywords:** Asthma, ILC2s, Rac1, inhibitor, mice

## Introduction

Asthma is a common respiratory tract disease in human, which afflicts more than 300 million people in the world including probably 6.2 million children under 18 years old [1]. Its pathogenesis and how to treat has attracted widespread attention. The important characteristics of asthma are the imbalance differentiation of T helper cell subset (Th1/Th2) and aberrant Th2 lymphocyte immune response. When exposed to allergens, bronchial epithelial cells can produce the inflammatory cytokines IL-25 and IL-33 [2], which contribute to the Group 2 ILCs polarization and up-regulate the secretion of IL-5 and IL-13, and then lead to Th1/Th2 imbalance involve in pathological process of asthma [3-6].

Rac1 (Ras-related C3 botulinum toxin substrate 1) is a member of the small guanosine triphos-

phatase Rho family of proteins which also includes Rho and Cdc42. Rac1 has been shown to play important roles in a wide variety of cellular processes. Changes in Rac1 activation may be triggered by a variety of extracellular signals including matrix adhesion, growth factors, cytokines, and endocrine hormones, and by intracellular signals including cytosolic free calcium and lipid raft trafficking [7, 8]. Rac1 is powerful regulators in a wide range of pathways inducing actin cytoskeleton reorganization, cell adhesion and migration, phagocytosis and apoptosis [9-11]. Accumulating evidences indicate that Rac1 is overexpressed in tumorigenic cells, including gastric cancer, breast, testicular, and lung carcinomas tissues compared to the corresponding normal tissue, it regulates a diverse range of functions containing tumorigenesis, angiogenesis and metastasis in cancer cells [11-13]. All the research achievements

**Table 1.** The sequences and length of primers

| Gene           | Sequence (5'-3')            | Length/bp |
|----------------|-----------------------------|-----------|
| Rac1           | Fwd: GTAAACCTGCTGCTCATCA    | 163       |
|                | Rev: GGACGCAATCTGCATAATCTTC |           |
| IL-33          | Fwd: TCCCAACAGAAGACCAAGAA   | 162       |
|                | Rev: CCGTGGATAGGCAGAGAAGT   |           |
| IL-25          | Fwd: CTCGTCCTCCACTTTACCACA  | 126       |
|                | Rev: CACACACACAAGCCAAGGA    |           |
| IL-13          | Fwd: GGAATCATCAGACTATAAGATT | 176       |
|                | Rev: GTGTGCTCCATTTCTTCTA    |           |
| IL-5           | Fwd: AGGATGCTTCTGCACTTGAG   | 144       |
|                | Rev: CCTCATCGTCTCATTGCTTG   |           |
| $\beta$ -actin | Fwd: ATGGAAATGGGGAAGATGGTC  | 349       |
|                | Rev: GCGGGGAGGGTGTGAAC      |           |

are enough to show that Rac1 can serve as a promising therapeutic target for these cancers. Recent studies have found that Rac1 is conducive to control asthma through regulation of apoptotic epithelial cells phagocytosis and anti-inflammatory cytokines secretion after encountering environmental allergens [14], but its mechanism of action and its relationship with ILC2s remains unclear.

To date, most efforts have focused on Rac1 inhibitors that indirectly block Rho GTPase function. One of the small molecule inhibitor is EHT1864, which binds to Rac1 with high affinity, compared to GDP/GTP, and attenuates Rac1 in an inert/inactive state by a novel mechanism involving guanine nucleotide displacement (GDP/GTP) [15, 16]. EHT1864 can specifically inhibit all known Rac isoforms (Rac1>Rac2>Rac3) [17]. The inhibitor have been tested for a variety of disorder in vivo, that prevented spread of breast cancers and also reduced proliferation at the invading edge. It is thought to be beneficial cancer treatment [18]. EHT1864 was used in this study with a purpose to prove the role of Rac1 in asthma mice, and hope to find a new immunotherapy approaches for asthma based on the Rac1 associated metabolites and cytokines.

## Materials and methods

### Animals and asthma model induction

Female Balb/c mice (4-6 weeks old), weighing  $18 \pm 2$  g, were purchased from the laboratory animal center of Yangzhou University (Yangzhou, China). Mice were housed in plastic cages

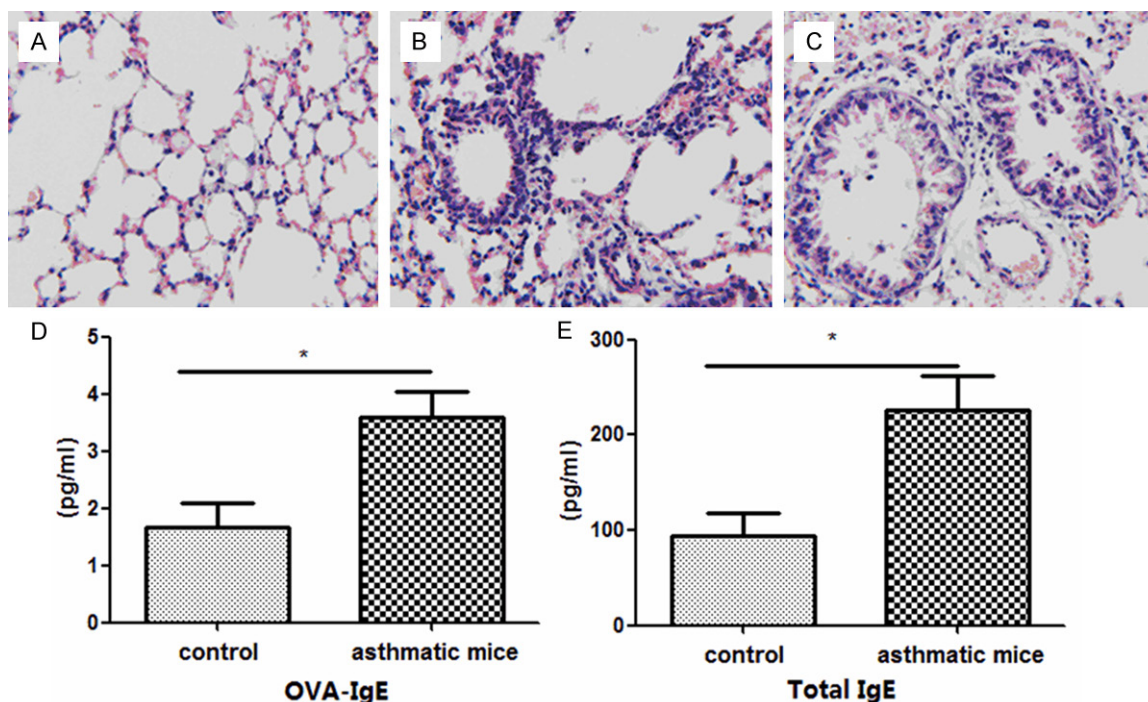
with sterilized wood-chip bedding, bred in animal rooms kept at a temperature of  $23^\circ\text{C} \pm 2^\circ\text{C}$  and a relative humidity of  $55\% \pm 10\%$  with a 12-hour light-dark cycle. They had free access to tap water and normal diet. The mouse model of allergic airway inflammation was established as described by Ying et al [19] with slight modification. Briefly, the mice were sensitized by intraperitoneal injection of 50  $\mu\text{g}$  OVA protein (Sigma, St. Louis, MO, USA) and 2 mg of 10% aluminum hydroxide gel (Thermo, Rockford, IL, USA) in 1 ml PBS saline. A second sensitization was given 10 days after the initial sensitization. On day 22 after initial sensitization, The ultrasonic atomizer (model 100, Yadu, Shanghai, China) was used for inhalation administration of 2% OVA ( 5 mg OVA in 5 ml PBS) to challenge mice for 40 minutes each day from day 22 to 26. Control mice were both sensitized and challenged with PBS. The Rac1 inhibitor EHT1864 (40 mg/kg) was obtained from Selleckchem (Selleck, TX, USA) and was given by intraperitoneal injection on day 10, 19, 20, 21. In addition, the inhibition group were sensitized and challenged as the asthmatic mice. On day 27, all mice were sacrificed and samples were collected for further analysis.

### Collection of specimens

**Bronchoalveolar lavage:** The mice tracheas were exposed for endotracheal intubation, each mouse was slowly injection of 1 ml PBE, the recovery rate was 50%~90%, the recovery liquid was bronchoalveolar lavage (BAL) and centrifuge by  $500 \times g$  for 5 min. Then 1 mL Trizol (Invitrogen, CA, USA) was added in the precipitation to extract RNA for fluorescence quantitative PCR and the supernatant for ELISA.

**Blood samples:** Peripheral blood samples were collected from healthy and asthmatic mice. The collection tubes contained 0.2 mL EDTA-K2 anticoagulant. The supernatant was used to detect cytokines by ELISA. The precipitate was used to separate peripheral blood mononuclear cells (PBMCs) by standard Ficoll-Hypaque density centrifugation (Tianjin Hao Yang Company, China), and 1 mL Trizol was added to crack PBMCs and then frozen at  $-80^\circ\text{C}$  for extracting total RNA.

**Lung tissue:** The left lung tissue was freshly obtained and Trizol was used to extract RNA. The right lung tissue was fixed in 10% formalin and embedded in paraffin for H&E (hematoxylin and eosin) and Immunofluorescence staining.



**Figure 1.** Pathological changes in asthma mice. A: The results of H&E staining in lung tissue from control mice ( $\times 200$ ); B and C: H&E staining in lung tissue from asthma mice ( $\times 200$ ). D: ELISA analysis for OVA-IgE in plasma from control and asthma mice; E: ELISA analysis for total IgE in plasma from control and asthma mice. Data shown were represented as mean  $\pm$  SD, and all samples were measured in triplicate. \* $P < 0.05$ .

#### RNA extraction, primer design and quantitative real-time PCR

Total RNA was extracted by guanidinium thiocyanate phenol chloroform method. Total RNA (500 ng) was reverse transcribed using Prime-Script Reverse Transcriptase kit (TaKaRa, Ohtsu, Japan) as the manufacturer's instructions. The following PCR amplification was assayed by quantitative real-time PCR performed with a SYBR Premix ExTaq (TaKaRa, Ohtsu, Japan) according to the manufacturer's instructions. On the basis of Genbank sequences, the primers used in this study were designed by Premier 5.0 software and synthesized by Shanghai Invitrogen. All sequences of primers are shown in **Table 1**. Quantitative Real-time PCR (qRT-PCR) was conducted in a SYBR<sup>®</sup> Premix Ex Taq<sup>™</sup> (TaKaRa, Ohtsu, Japan) according to the manufacturer's instructions. Fold changes in the expression of each objective gene relative to  $\beta$ -actin were calculated based on the threshold cycle (Ct). All samples were performed in triplicate.

#### H&E and immunofluorescence assay

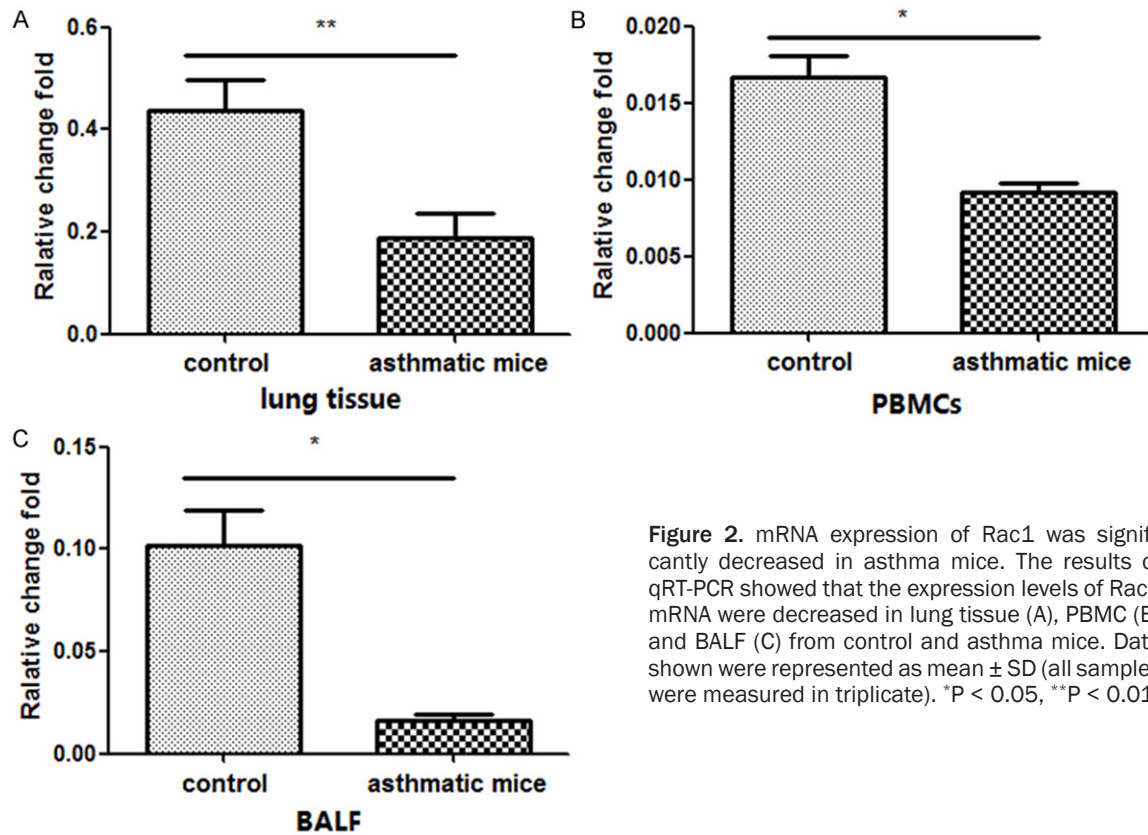
The lung tissue of asthma mice was freshly obtained and fixed in 4% paraformaldehyde

and embedded in paraffin. The blocks were cut into 4  $\mu$ m slices, heated 3 h in a 37°C incubator, and then dewaxed and stained with H&E. One slice was chosen from each mouse and was analyzed under a microscope.

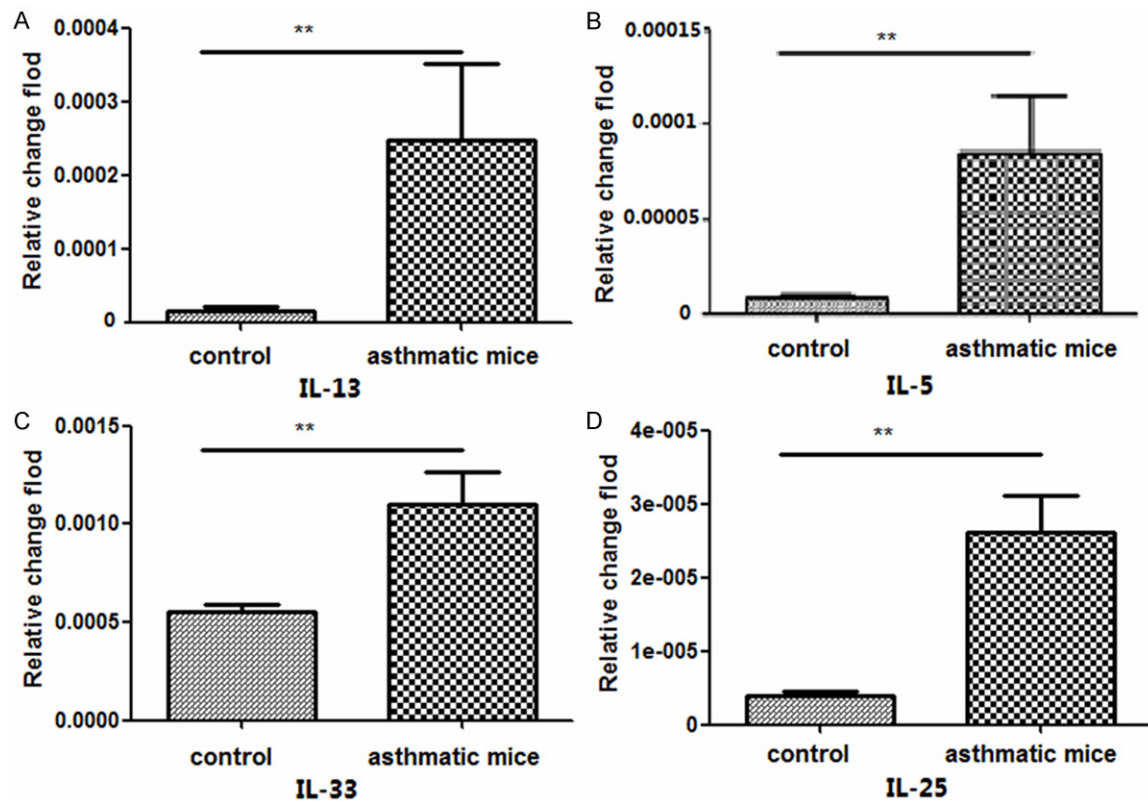
Immunofluorescence staining on paraffin embedded mouse lung tissue was performed as described previously [20]. Briefly, after dewaxing and antigen unmasking, the slides were blocked in 1% (weight/volume) BSA for 60 minutes, and then the primary antibodies rabbit-anti-mouse Rac1 (1:1000, Santa Cruz, Texas, USA) was applied for 2 h at room temperature. After washing the labeled second antibodies goat-anti-rabbit IgG (1:5000, Invitrogen, CA, USA) was added for 90 min at room temperature. Finally, the slides were dyed in Hoechst 33342 for 10 min. All the sections were cover-slipped with Vecta shield mounting medium, viewed with an Olympus fluorescence microscope, and analyzed using Image J software.

#### ELISA

The serum IL-13, IL-5, IL-33 was measured by ELISA kit (eBioscience, CA, USA) following the manufacturer's protocols. All samples were

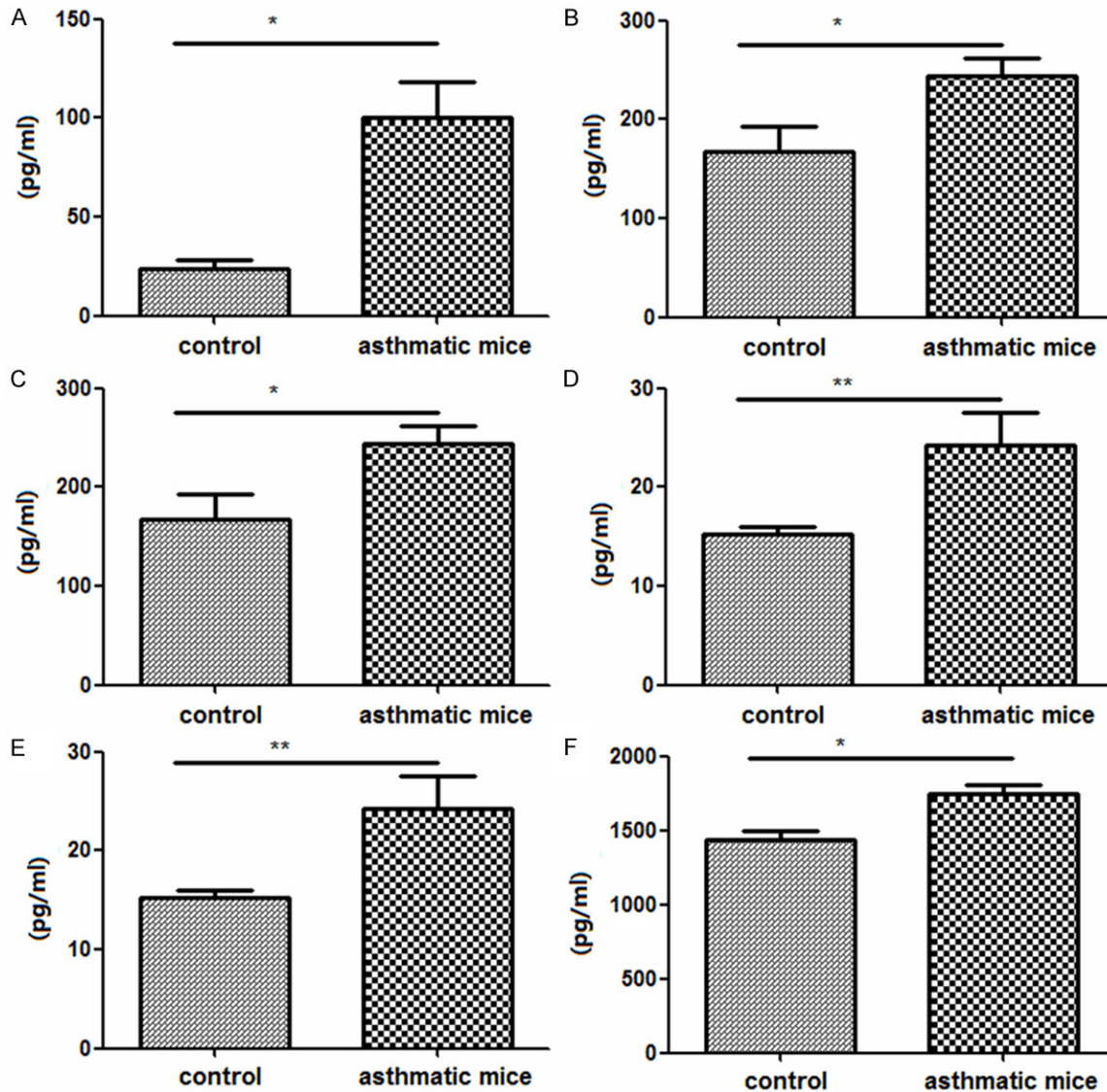


**Figure 2.** mRNA expression of Rac1 was significantly decreased in asthma mice. The results of qRT-PCR showed that the expression levels of Rac1 mRNA were decreased in lung tissue (A), PBMC (B) and BALF (C) from control and asthma mice. Data shown were represented as mean  $\pm$  SD (all samples were measured in triplicate). \*P < 0.05, \*\*P < 0.01.





**Figure 3.** mRNA expression of ILC2s associated cytokines were significantly increased in asthma mice. The mRNA expression levels of ILC2s associated cytokines in lung tissue were detected by qRT-PCR. The data indicated that the IL-13 (A), IL-5 (B), IL-33 (C) and IL-25 (D) mRNA levels were obviously increased in lung tissue from control and asthma mice. Data shown were represented as mean  $\pm$  SD, and all samples were measured in triplicate. \*\*P < 0.01.



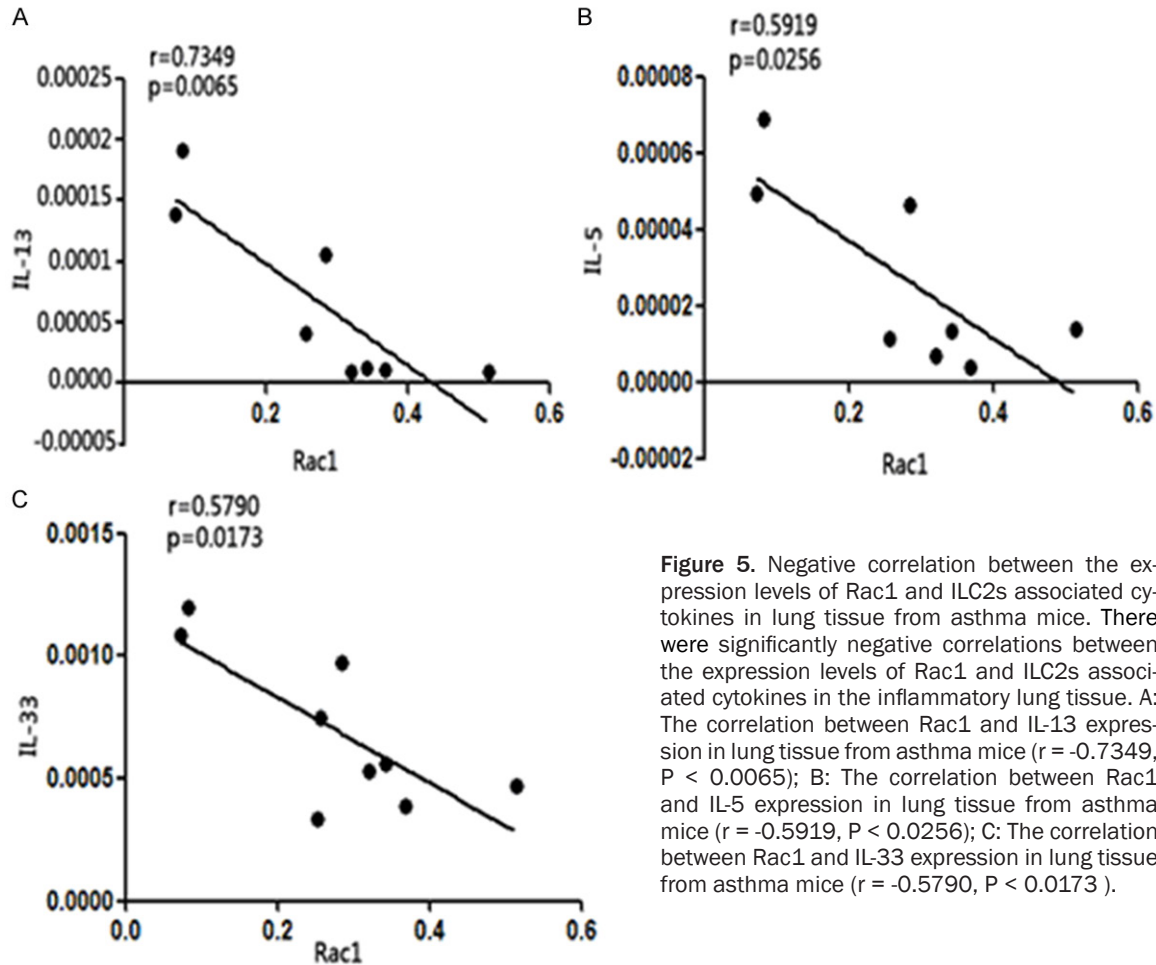
**Figure 4.** Enhanced protein levels of ILC2s related cytokines in some specimens from asthma mice. The protein levels of ILC2s related cytokines in BALF and plasma were detected by ELISA. The three cytokines were increased in BALF from asthma mice (A-C), (A) IL-13, (B) IL-5, (C) IL-33; and they were also increased in plasma from asthma mice (D-F), (D) IL-13, (E) IL-5, (F) IL-33. Data shown were represented as mean  $\pm$  SD, all samples were measured in triplicate. \*P < 0.05, \*\*P < 0.01.

measured in triplicate, and the mean concentration was calculated from the standard curve.

#### Statistical analysis

All statistical analysis was performed using GraphPad Prism Version 5.0 software (San

Diego, CA, United States). Data were expressed as the mean  $\pm$  SD in figures. Comparisons between groups were performed using the Unpaired Student's t-test. Pearson's correlation was used to test correlation between two continuous variables. P<0.05 was considered to be statistically significant.



**Figure 5.** Negative correlation between the expression levels of Rac1 and ILC2s associated cytokines in lung tissue from asthma mice. There were significantly negative correlations between the expression levels of Rac1 and ILC2s associated cytokines in the inflammatory lung tissue. A: The correlation between Rac1 and IL-13 expression in lung tissue from asthma mice ( $r = -0.7349$ ,  $P < 0.0065$ ); B: The correlation between Rac1 and IL-5 expression in lung tissue from asthma mice ( $r = -0.5919$ ,  $P < 0.0256$ ); C: The correlation between Rac1 and IL-33 expression in lung tissue from asthma mice ( $r = -0.5790$ ,  $P < 0.0173$ ).

## Results

### Lung tissue pathology

The results of H&E staining revealed that more eosinophil infiltration was found between the alveolar and the blood vessels in OVA sensitized mice, and existed the airway wall thickening, mucous membrane wrinkle wall decreases, and goblet cells hypertrophy in airway, showed an obvious allergic inflammation performance. The inflammation was significantly enhanced by EHT1864 treatment. In addition, the total IgE and OVA-IgE in asthma mice were both increased than that in control mice (**Figure 1**).

### Decreased expression levels of Rac1 in PBMC, lung tissue and alveolar lavage fluid from asthmatic mice

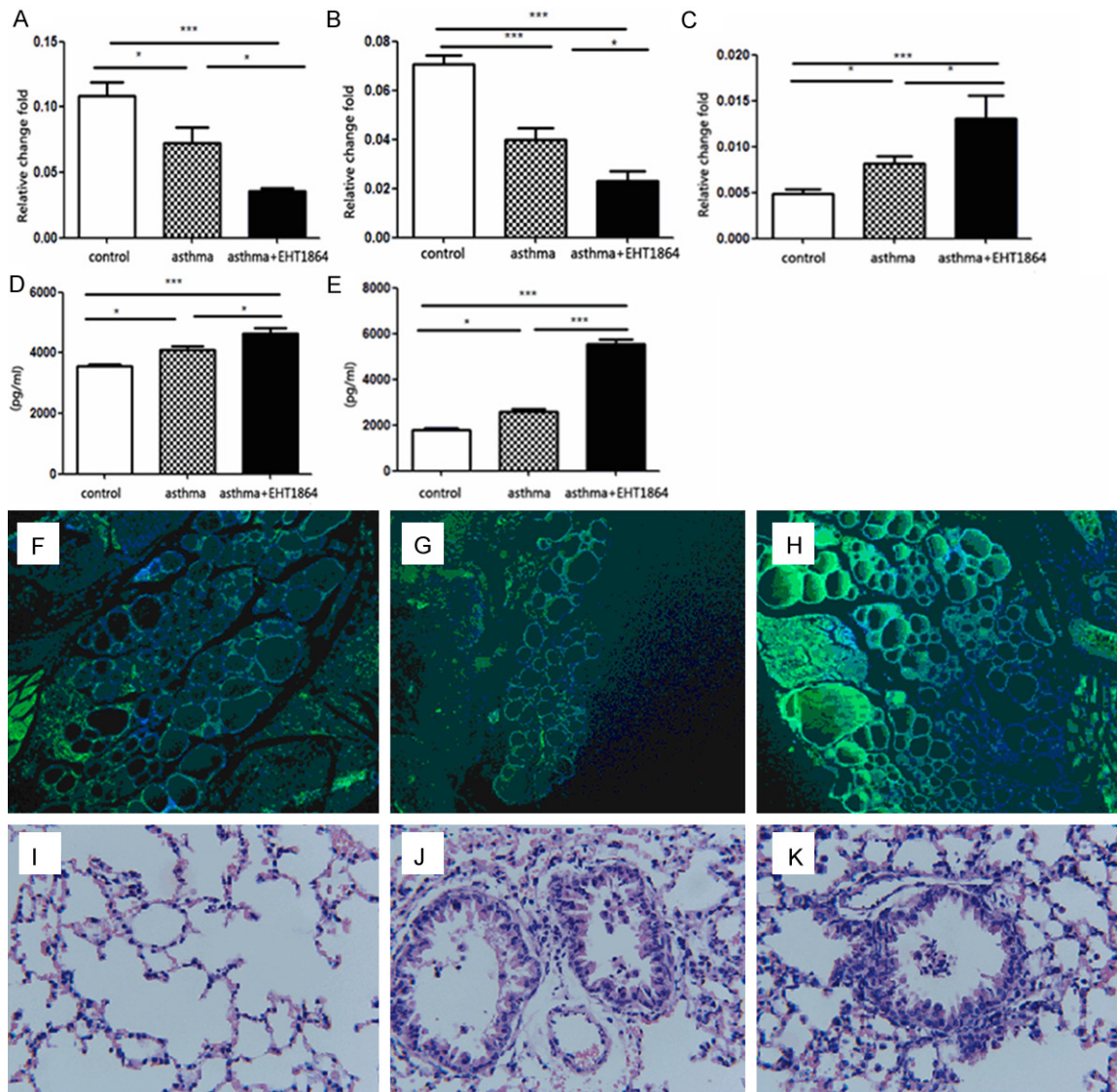
In order to understand the expression level of Rac1 in asthma, the lung tissue, peripheral blood mononuclear cells and alveolar lavage

fluid from healthy and asthmatic mice were respectively used to detect the Rac1 mRNA by qRT-PCR method. As shown in **Figure 2**, the expression levels of Rac1 mRNA in all specimens from asthmatic mice were significantly decreased compared with control mice.

### Increased ILC2s associated cytokines in asthmatic mice

As we know, both IL-25 and IL-33 can stimulate ILC2s, which produce characteristic cytokines including IL-5 and IL-13 and are critical for maintaining Th2 cells polarization involved in asthmatic inflammation. In this experiment, the qRT-PCR was used to analyze the mRNA levels of IL-33, IL-25, IL-13 and IL-5 in lung tissue from healthy and asthma mice. The results were judged according to the relative changes of purpose gene and internal genes  $\beta$ -actin mRNA expression levels. As shown in **Figure 3**, the expression levels of IL-25, IL-5, IL-13 and IL-33 in asthmatic mice were significantly higher than

## Rac1 influences ILC2 cytokines production in asthma mice



**Figure 6.** EHT1864 influences the expression of Rac1 and upregulates ILC2 associated cytokines. In order to further prove the role of Rac1 in the occurrence of asthma, the mice were treated by EHT1864 before allergen provoking, it resulted in more enhanced expression levels of ILC2s related cytokines along with decreased expression level of Rac1 in asthmatic mice. (A) The expression level of Rac1 mRNA in the lung tissue; (B) The expression level of Rac1 mRNA in PBMC; (C) The expression level of IL-33 mRNA in the lung tissue; (D) IL-33 protein levels in BALF detected by ELISA; (E) IL-33 protein levels in plasma detected by ELISA. The results of immunofluorescence assay showed that the Rac1 expression in the bronchial from control group (F), asthma group (G) and asthma+EHT1864 group (H). In addition, the results of H&E staining showed that the inflammatory was aggravated in asthma mice and asthma+EHT1864 group. (I) The pathological changes ( $\times 200$ ) in lung tissue from control group; (J) The pathological changes ( $\times 200$ ) in lung tissue from asthmatic group; (K) The pathological changes ( $\times 200$ ) in lung tissue from EHT1864 inhibited group. Data shown are represented as mean  $\pm$  SD (all samples were measured in triplicate). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

that in control mice. In addition, the expression levels of characteristic cytokines in plasma and BALF from asthma mice were also detected by ELISA, the results as exhibited in **Figure 4**, the protein levels of IL-33, IL-13 and IL-5 were significantly increased compared with control mice.

### *Correlation between the expression levels of Rac1 and ILC2s associated cytokines*

In order to understand the expression level of Rac1 in asthma and its relationship with ILC2 polarization, The correlation between the expression levels of Rac1 and ILC2s associated



cytokines IL-5, IL-13 or IL-33 in asthma mice was analyzed respectively. The results were showed in **Figure 5**, there was a significantly negative correlation between the mRNA levels of Rac1 and ILC2s associated cytokines in asthma mice.

*EHT1864 inhibits the expression of Rac1, which mediates the up-regulation of ILC2s related cytokines*

In this experiment, EHT1864 was used to further prove the role of Rac1 in the occurrence of asthma. The mice were treated by EHT1864 before allergen provoking, and then the expression levels of Rac1 and ILC2s associated cytokines were measured. Our results suggested that after the Rac1 inhibitor treatment, the expression levels of Rac1 were significantly decreased in the lung tissue and PBMCs, and the bronchial immunofluorescence showed a similar result. Furthermore, the expression of IL-33 mRNA in lung tissue and protein levels in BALF and plasma were significantly increased (**Figure 6**).

### Discussion

Allergic asthma is a respiratory disease that influences human health, the incidence has a tendency to rise, but its mechanism is not fully clear. ILC2 is a new type of immune cell found in recent years, it is no-T and no-B lymphocytes that can be stimulated by IL-25 and IL-33 because of the receptors (T1/ST2, IL-17RB) [21], the administration of IL-33 or IL-25 into mice induces airway high pressure reaction, and goblet cell hyperplasia accompanied with increased eosinophil infiltration and type 2 cytokines such as IL-4, IL-5, IL-9 and IL-13 independent of acquired immunity [22, 23]. Rac1 is a small GTPase in the members of Ras superfamily and is key regulator in the signaling pathways that control a variety of cellular and biological functions such as cytoskeletal organization, cell division, differentiation, and nuclear assembly [24]. In the tumor cell proliferation and apoptosis, cell invasion and metastasis has been paid attention by people.

The pathological mechanism of asthma is closely related to Th2 cells polarization or immune imbalance. Th2 cells highly express transcription factor GATA3, which is required for the Th2 cytokines produced by ILC2s. Thus the

activity of ILC2s is an important factor to maintain the polarization of Th2 cells. IL-33 and IL-25 were previously implicated in the initiation and regulation of innate immune responses associated with ILC2s-mediated inflammatory diseases, and the function of IL-33 is more forcible which has been confirmed by Mjösberg [25]. Furthermore, an influenza-induced airway hyper-reactivity model also confirmed the idea of IL-33 is more potent in the induction of ILC2s [26]. Li et al also confirmed the important role of IL-33-IL-13 axis in a murine biliary injury model [27]. Recent study has confirmed that IL-33 was a primary influence on the innate immune axis that drives IL-13 dependent lung disease both in mouse model and in human [28]. Recent study demonstrated that administration of endotoxin-low ovalbumin or HDM to mice lacking Rac1 in bronchial epithelial cells leads to increased development of Th2 immunity and the inflammation features of asthma, there was an increase in IL-33 [29]. Thus we speculate that the expression of Rac1 may be inhibited in asthma, perhaps it also promotes the Th2 polarization by influencing the activity of ILC2s.

In the current study, the Rac1 and the ILC2s related cytokines in lung tissue, PBMC, serum and BALF from the mice were measured, and the potential correlation between them was also analyzed. Our data showed that the IL-5, IL-13 and IL-33 mRNA and protein expression levels in the asthma mice were higher than that in control group, while the expression level of Rac1 is decreased obviously. The correlation analysis showed that there was a significant negative correlation between the expression of Rac1 and ILC2s related cytokines (IL-5, IL-13 or IL-33). In order to further prove the role of Rac1 in the occurrence of asthma, the mice were treated by EHT1864 before allergen provoking, it resulted in more enhanced expression levels of ILC2s related cytokines along with decreased expression level of Rac1, and the inflammatory was aggravated in asthmatic mice. For these reason, it is arguable that the Rac1 can affect the pathogenesis of asthma. If the Rac1 agonist is used in asthma patient, it may effectively improve the inflammatory state. Further studies on the role of Rac1 in asthma, is beneficial to understand the immune mechanism of asthma and can help us to find new targets for the prevention and treatment of asthma.



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## Disclosure of conflict of interest

None.

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