

# CysLT1R expression on ILC2s and effects of CysLT1R antagonist on ILC2 activity in patients with allergic rhinitis

To the Editor,

Group 2 innate lymphoid cells (ILC2s) produce the canonical type 2 cytokines interleukin (IL)-5 and IL-13 in response to epithelial cytokines IL-25, IL-33, and thymic stromal lymphopoietin (TSLP).<sup>1</sup> ILC2s

have been to be involved in the pathogenesis of asthma, allergic rhinitis (AR) and atopic dermatitis (AD).<sup>2</sup> We have reported the higher ILC2 levels in patients with asthma and patients with AR.<sup>3,4</sup> Recently, the cysteinyl leukotriene (CysLT) D4 (LTD4) and LTE4 were reported

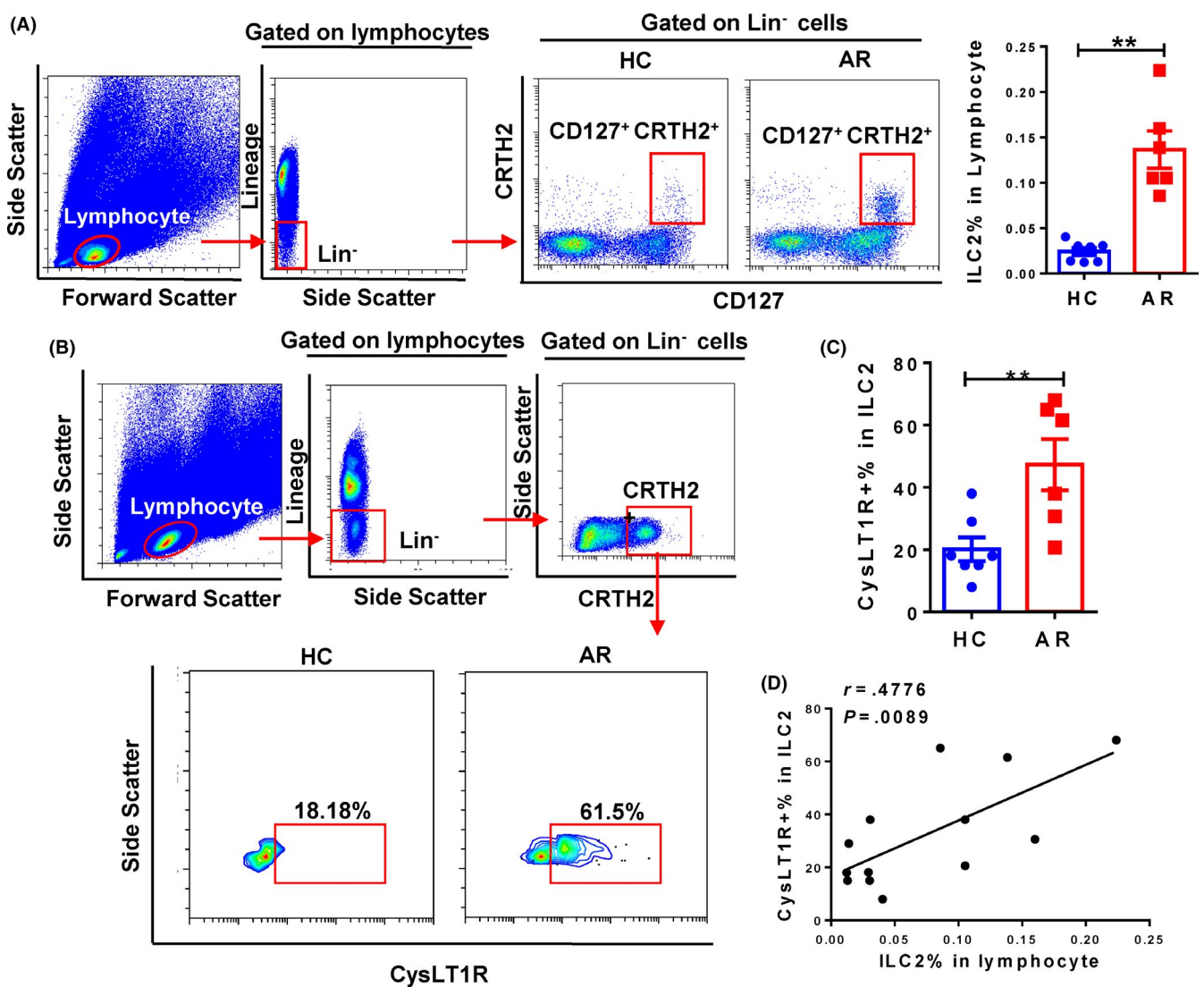


**FIGURE 1** The levels of ILC2s and the expression of CysLT1R on ILC2s in patients with allergic rhinitis. (A) ILC2s were identified as Lin<sup>-</sup>CRTH2<sup>+</sup>CD127<sup>+</sup> lymphocytes. Representative cytograms and statistical analysis of ILC2s in HC (n = 7) and AR (n = 6) groups as determined by flow cytometry. (B) Representative gating strategy of Lin<sup>-</sup>CRTH2<sup>+</sup>ILC2s and representative cytograms of CysLT1R<sup>+</sup>ILC2s; (C) Statistical analysis of CysLT1R<sup>+</sup>ILC2s in HC (n = 7) and AR (n = 6) groups as determined by flow cytometry; (D) ILC2 levels were correlated with CysLT1R<sup>+</sup>ILC2 levels (Spearman's method). Data are shown as mean ± SEM. \*\*P < .01. AR: allergic rhinitis; HC: healthy control; ILC2s: Group 2 innate lymphoid cells

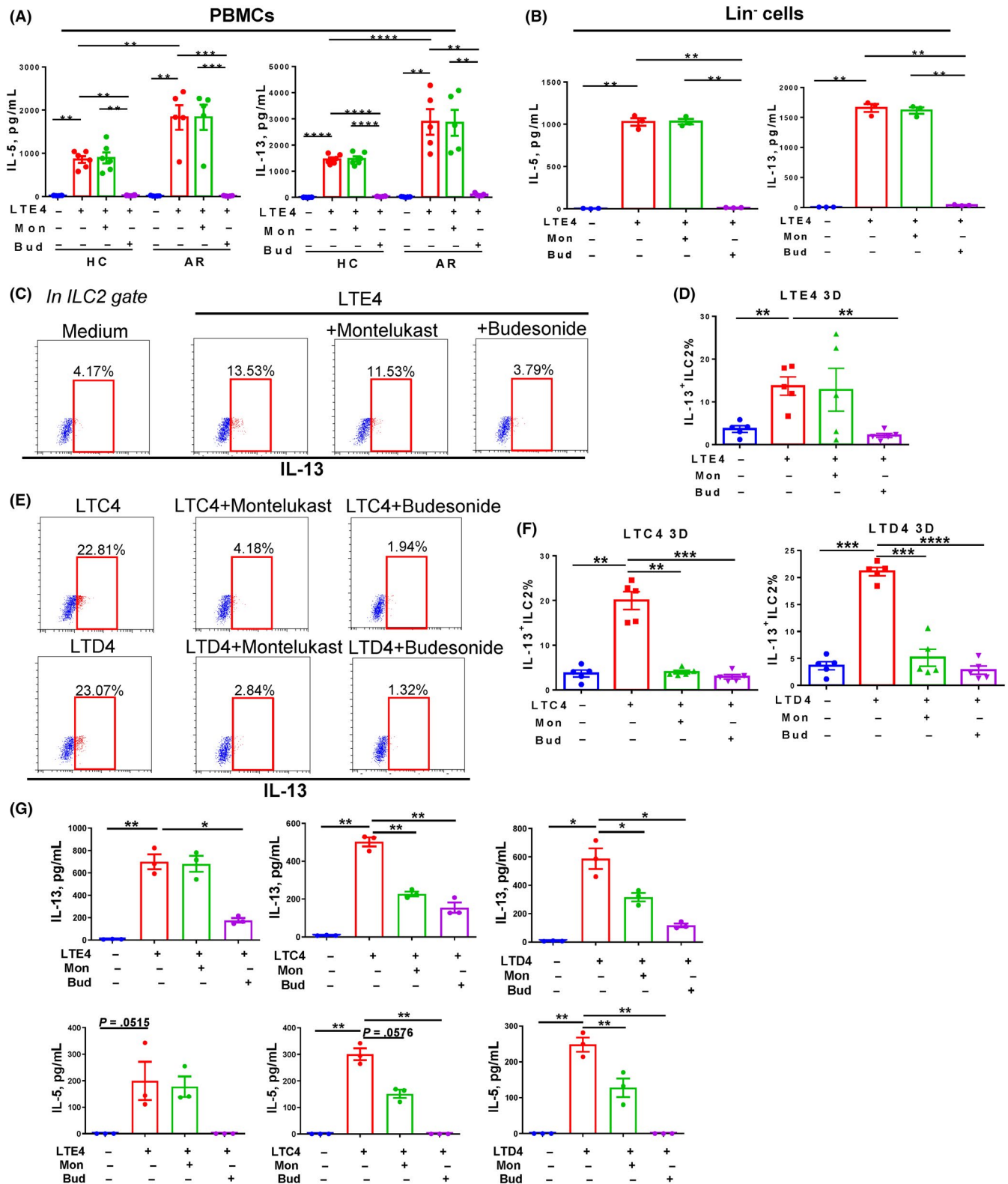


**FIGURE 2** The effects of montelukast on ILC2s under LT stimulation. PBMCs from patients with AR or healthy controls (A), or Lin<sup>-</sup> cells from human buffy coat (B) or PBMCs from patients with AR (C-F) or ILC2s from human buffy coat (G) were stimulated with LTE4, LTC4 or LTD4. IL-5 and IL-13 levels in PBMCs from HC (n = 6) and AR (n = 5) groups (A), in Lin<sup>-</sup> cells (n = 3) (B) under LTE4 stimulation; Representative cytograms (C, E) and statistical analysis (n = 5) (D, F) of IL-13<sup>+</sup> ILC2s in ILC2 gate. (G) IL-5 and IL-13 levels under different stimulations (n = 3). Data are shown as mean ± SEM. \*P < .05, \*\*P < .01, \*\*\*P < .001 and \*\*\*\*P < .0001. AR: allergic rhinitis; HC: healthy control; Lin<sup>-</sup> cells: Lineage negative cells; LT: Leukotriene; PBMC: Peripheral blood mononuclear cell

to induce Th2 cytokine production from ILC2s.<sup>5,6</sup> LTE4 is the stable and dominant CysLT in the pathogenesis of allergic conditions. Salimi et al<sup>6</sup> recently reported that CysLT receptor 1 (CysLT1R) is expressed on circulating ILC2s in patients with AD, that LTE4 promoted IL-5, IL-13 production in vitro, and that the effects could be blocked by montelukast, a CysLT1R antagonist. It suggested that the therapy directed at blocking CysLT-CysLT1R binding might have the potential to inhibit ILC2 function in allergic diseases. The expression of CysLT1R, the roles of LT, and its antagonist on ILC2s in patients with AR are still unclear.

This study enrolled 19 patients with AR, 10 patients with AD, and 13 healthy control subjects (HC) (Table S1). The CysLT1R expression on circulating ILC2s was examined by flow cytometry. Peripheral blood mononuclear cells (PBMCs) from patients were stimulated with LTE4, LTC4, or LTD4, and treated with montelukast. The levels of soluble Th2 cytokines and intracellular cytokines were measured. Detailed methods are given in the Data S1. We found higher prevalence of ILC2s (Lin<sup>-</sup>CRTH2<sup>+</sup>CD127<sup>+</sup> cells, Figure 1A) and the higher expression of CysLT1R in ILC2s (Figure 1B-C) from patients with AR compared to healthy controls. More importantly, ILC2 levels showed a positive correlation with CysLT1R<sup>+</sup> ILC2s (Figure 1D).

LTE4 strongly upregulated the levels of IL-5 and IL-13 on PBMCs both in patients with AR and healthy subjects but with more effects from patients (Figure 2A). However, we did not observe any effects of montelukast on the high levels of IL-5 and IL-13. Additionally, as our previous study,<sup>3</sup> the treatment of budesonide significantly reversed the high levels of IL-5 and IL-13 production to almost basal levels (Figure 2A). Similar results were found for Lin<sup>-</sup> cells (Figure 2B). Moreover, we found no effects of montelukast on high IL-13 production of Lin<sup>-</sup> cells in response to LTE4 either when different dosages of montelukast, or when drug formulations of the leukotriene inhibitor obtained from different companies were tested, or when montelukast was pre-administrated (Figure S1). The treatment of LTE4 upregulated IL-13<sup>+</sup> ILC2 percentage from AR patients at 3 days, and budesonide but not montelukast significantly abolished the enhancing effects (Figure 2C-D). However, both montelukast and budesonide significantly reversed the increased response of IL-13<sup>+</sup> ILC2s to LTC4 or LTD4 at 3 days (Figure 2E-F). Additionally, montelukast decreased the high levels of IL-13<sup>+</sup> ILC2s in response to LTD4 but not LTE4 and LTC4 at 4 hours after the treatment (Figure S2). Using freshly isolated ILC2s, montelukast was found to significantly decrease the production of IL-13 and IL-5 with the administration of LTC4 and LTD4 but not LTE4 (Figure 2G).

Treatment with LTE4, IL-25, IL-33, and TSLP as in the Salimi et al<sup>6</sup> study showed higher levels of IL-13<sup>+</sup> ILC2s (Figure S3). However, we found that montelukast had no effects on it. For sorted ILC2s, similar finding was observed under the treatment of LTE4 alone or combined stimulation (Figure S3). Here we found that p-STAT3, p-STAT5, and p-STAT6 in ILC2s under the above stimulation kept high levels after treated with montelukast (Figure S3).

Salimi et al<sup>6</sup> reported that CysLT1R is expressed in ILC2s of patients with AD and that the effect of LTE4 on increasing IL-5, IL-13 production in vitro could be inhibited by montelukast. Using PBMCs

from patients with AD, we found that LTE4 stimulation upregulated IL-5, IL-9, and IL-13 levels and the treatment of montelukast significantly reversed their high levels (Figure S4). Both patients with AR and AD had high levels of CysLT1R in ILC2s but with higher levels in patients with AD compared to patients with AR (Figure S4).

In this study, we found the evidence of high CysLT1R expression on human circulating ILC2s in patients with AR and with AD which is consistent with Salimi et al's<sup>6</sup> work in patients with AD and patients with AR.<sup>7</sup> We observed that LTE4, LTC4, and LTD4 significantly activated ILC2 function from patients with AR or patients with AD, suggesting that LT-mediated ILC2 activation could be a critical contributor to allergic inflammation. We found that montelukast blocked Th2 cytokine production of PBMCs from patients with AD but not AR and healthy subjects under the stimulation of LTE4. However, montelukast reversed the production of Th2 cytokines in ILC2s in response to LTC4 and LTD4 in patients with AR. It suggests that the activation of ILC2s to LTC4, LTD4 but not LTE4 in AR is related to the increased expression of CysLT1R in AR, at least in our study. A recent study in Japan reported that montelukast significantly decreased IL-5 and IL-13 production on sorted ILC2s from AR which were cultured for several weeks before the experiments.<sup>7</sup> The difference may be because of different experimental condition, different nations or different allergic situation of the patients, or just a phenomenon under certain experimental conditions.

Previous studies have shown that both CysLT1R and CysLT2R have low affinity for LTE4, and the P2Y12 and GPR99 have been proposed as receptors with a preference of LTE4.<sup>8</sup> However, Salimi et al<sup>6</sup> reported that both P2Y12 and GPR99 are not expressed in ILC2s, and the activity of LTE4 in human ILC2s was blocked completely by the CysLT1R antagonist montelukast. The current reports suggest the possibility of involvement of another unknown LTE4 receptor. Montelukast treatment inhibited the IL-5 production induced by LTC4 and LTD4, but not LTE4 in *Alternaria*-challenged mice,<sup>5</sup> which was consistent with our study. It may because LTC4 might directly bind to CysLT1R on ILC2s or undergo conversion in vivo to LTD4 resulting in higher affinity binding to CysLT1R.<sup>9</sup> These difference might be consistent with involvement of that novel LTE4 receptor, which may exert different responses in different diseases to montelukast under the stimulation of LTE4.<sup>6</sup> The expression of this possible receptor may have similar patterns of expression as CysLT1R in healthy subjects, and patients with AR and AD in our study, resulting in the different effects of montelukast in response to LTE4.

The inhibitory effects of montelukast in response to LTE4 stimulation were not observed for patients with AR in under certain experimental conditions. The detailed mechanism and clinical implication about the above negative findings should be further studied in the future. Of course, montelukast therapy may still be beneficial for patients with AR because of the presence of LTC4 and LTD4.

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## CONFLICTS OF INTEREST

The authors declared no conflict of interest.

Zi-Li Qin<sup>1</sup>  
Ya-Qi Peng<sup>1</sup>  
Shu-Bin Fang<sup>1</sup>  
Ai-Yun Jiang<sup>1</sup>  
Cheng-Lin Li<sup>1</sup>  
Xing-Liang Fan<sup>1</sup>  
Dong Chen<sup>1</sup>  
Wen-Na Chen<sup>2</sup>  
Joseph.A. Bellanti<sup>3</sup>  
Song Guo Zheng<sup>4</sup>   
Qing-Ling Fu<sup>1</sup> 

<sup>1</sup>Department of Otorhinolaryngology, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, China

<sup>2</sup>Department of Dermatology, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, China

<sup>3</sup>Department of Pediatrics and Microbiology-Immunology, Georgetown University Medical Center, Washington, DC, USA

<sup>4</sup>Department of Internal Medicine, Ohio State University College of Medicine and Wexner Medical Centre, Columbus, OH, USA

## Correspondence

Qing-Ling Fu, Department of Otorhinolaryngology, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, Guangdong, China.  
Email: fuqingl@mail.sysu.edu.cn

## ORCID

Song Guo Zheng  <https://orcid.org/0000-0002-5611-4774>  
Qing-Ling Fu  <https://orcid.org/0000-0002-5969-628X>

Zi-Li Qin and Ya-Qi Peng contributed equally to this study.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

## **Supporting information**

### **METHODS**

#### **1 | Study population**

The study included 19 patients with allergic rhinitis (AR), 10 patients with atopic dermatitis (AD) and 13 healthy controls (Table S1), who were recruited from The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, China. The study protocol was approved by the Ethics Committee of the hospital (No. 2015-48). The inclusion criterion for allergic rhinitis patients were included as established according to the Allergic Rhinitis and its Impact on Asthma (ARIA): (1) history of nasal symptoms by nose itching, obstruction, sneezing and rhinorrhea; (2) positive skin prick test (SPT) result to *Dermatophagoides pteronyssinus* (Derp1) (ALK-Abello', Hoersholm, Denmark); (3) positive for specific immunoglobulin E (IgE) to Derp1 (Phadia, Uppsala, Sweden). The inclusion criterion for patients with AD were included as established according to The Hanifin and Rajka criteria<sup>1</sup>. The patients had not received antihistamines, intranasal steroid treatments, oral steroids or other medications (e.g. antibiotics or antileukotrienes) at least 4 weeks prior to this study. Subjects were excluded if had any acute viral infections, chronic obstructive pulmonary disease, cardiovascular disease, immunodeficiency, cystic fibrosis or pregnancy within at least 1 month before the study. Healthy subjects had no history of allergy, asthma or sinus disease, and their allergen SPT was negative. The healthy subjects' specific IgE was under 0.35 kU/L.

#### **2 | Blood sample collection**

The peripheral blood was sampled into heparin anticoagulant tubes and was layered over the lymphocyte separation medium (MP Biomedicals, Santa Ana, CA, USA) by density gradient centrifugation. Then the peripheral blood mononuclear cells (PBMCs) were collected from the interface between lymphocyte separation medium and plasma.

#### **3 | Lineage negative (Lin<sup>-</sup>) cell and ILC2 sorting**

Because there were only a few group 2 innate lymphoid cells (ILC2s) in PBMCs in the blood, here Lin<sup>-</sup> cells and ILC2s were sorted from the buffy coats of healthy volunteers provided by Guangzhou Blood Center (n = 15) using human ILC2 isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Healthy human blood buffy coats were from ‘anonymous donors’ and exemption of the written informed consent was approved by the Ethics Committee of The First Affiliated Hospital, Sun Yat-sen University, China. PBMCs were stained with Biotin-Antibody Cocktail and Anti-Biotin MicroBeads. About 10% PBMCs were firstly separated as Lin<sup>-</sup> cells after magnetic separation using two LS Columns. The isolated Lin<sup>-</sup> cells were further stained with CRTH2-PE and Anti-PE MicroBeads. After positive selection using two MS Columns, CRTH2<sup>+</sup> ILC2s were obtained.

#### **4 | The stimulation and treatment of PBMCs, Lin- cells and ILC2s**

PBMCs ( $1 \times 10^6$  cells/ml in 24-well plates with 1 ml medium/well), Lin<sup>-</sup> cells ( $1 \times 10^6$  cells/ml in 24-well plates with 1 ml medium/well) and ILC2s ( $1 \times 10^5$  cells/ml in 96-well plates with 200  $\mu$ l medium/well) isolated from patients with allergic rhinitis, patients with atopic dermatitis or healthy human blood buffy coats were cultured, and stimulated with the cysteinyl leukotriene (CysLT) C4 (LTC<sub>4</sub>), LTD<sub>4</sub> or LTE<sub>4</sub> (all for 50 nM, Enzo, NY, USA ), or IL-25 (50 ng/ml) (R & D system, MN, USA), IL-33 (50 ng/ml) (R & D system, MN, USA) , IL-2 (20 U/ml) (R & D system, MN, USA) and TSLP (50 ng/ml) (R & D system, MN, USA), with or without montelukast (1  $\mu$ M) (Cayman Chemical, Ann Arbor, USA ) or budesonide ( $10^{-8}$  M) (Cayman Chemical, Ann Arbor, USA) for 4 h or 3 day, then the cytokine levels in the supernatant were evaluated using enzyme-linked immunosorbent assay (ELISA), and the possible mechanisms about STATs involved in were evaluated using western blotting. The intracellular IL-13 and the levels of CysLT<sub>1</sub>R in ILC2s were evaluated using flow cytometry.

We also examined the dose-dependent effects of montelukast on ILC2 function. PBMCs from healthy human blood buffy coats were stimulated with LTE<sub>4</sub> (50 nM)

and treated with montelukast from two different companies (Cayman Chemical, Ann Arbor, USA, or Sigma-Aldrich, St. Louis, USA) from 10 pM, 100 pM, 1 nM, 10 nM, 100 nM to 1  $\mu$ M for 3 days. The supernatant was collected for the examination of IL-13 using ELISA. Additionally, the PBMCs were also pre-treated with montelukast for 0.5 h prior to stimulation with LTE4.

## **5 | Flow cytometry**

PBMCs were stained for 30 min at 4°C immediately with the following antibodies for ILC2s: FITC-conjugated lineage cocktail (consisting of CD2, CD3, CD14, CD16, CD19, CD56, CD235a) (eBioscience, San Diego, CA); PE-conjugated CRTH2 or PE-CF594-conjugated CRTH2 (BD Bioscience, NJ); PE-Cy7-conjugated CD127 (eBioscience, San Diego, CA); PBMCs were stained for the ILC2 surface markers as the above description. After fixation and permeabilization, PBMCs were stained for 30 min at room temperature with anti-CysLT1R rabbit antibody (1:100, ABCAM, Cambridge, UK) and donkey F(ab')<sub>2</sub> anti-rabbit IgG H & L (PE) preadsorbed (1:200, ABCAM, Cambridge, UK). Cells were analyzed using Beckman CytoFlex S flow cytometer machine (Beckman Coulter, Hercules, CA, USA). The data were analyzed with CytoFlex S-equipped CytExpert software (Beckman Coulter, Hercules, CA, USA), and ILC2s were identified as Lin<sup>-</sup> CD127<sup>+</sup> CRTH2<sup>+</sup> lymphocytes. As previous reports, CysLT1R<sup>+</sup> ILC2s were gated in Lin<sup>-</sup> CRTH2<sup>+</sup> lymphocytes.<sup>2-4</sup>

For the intracellular IL-13 examination, a combination of phorbol myristate acetate (PMA, 50 ng/mL, Sigma-Aldrich, St. Louis, USA), ionomycin (1  $\mu$ g/mL, Sigma-Aldrich, St. Louis, USA) and monensin (1:1000, Invitrogen, Waltham, MA, USA) was administered 5 h before cell collection. PBMCs were stained for the ILC2 surface markers as the above description. After fixation and permeabilization, PBMCs were stained for 30 min at room temperature with V450-conjugated IL-13 antibody (BD Bioscience, NJ). IL-13<sup>+</sup> ILC2s were gated in Lin<sup>-</sup> CRTH2<sup>+</sup> CD127<sup>+</sup> ILC2s.

## **6 | Enzyme-linked immunosorbent assay**

The IL-5 and IL-13 levels in the cell culture supernatant of PBMCs from HC and AR, Lin<sup>-</sup> and ILC2s were measured using an ELISA kit (RayBiotech Inc, Norcross, GA). The detection limit for IL-13 was 0.15 pg/ml and the detection limit for IL-5 was 5 pg/ml. Sometimes, the supernatant was diluted for the examination of IL-5 and IL-13 because their concentration was very high and exceeded the limit of the kits. The cell supernatant from PBMCs from patients with AD was analyzed using IL-13 ELISA kit (Invitrogen, Waltham, MA, USA), IL-5 ELISA kit (Invitrogen, Waltham, MA, USA) and IL-9 (NeoBioscience, Shenzhen, Guangdong, China), according to the manufacturer's instruction. The analytical sensitivity for IL-13 was 4 pg/mL, IL-5 was 4.7 pg/mL and IL-9 was 24 pg/mL. All values below the detectable limit were set as 0 for convenient analysis. The ELISA kits' protocols are recommended by the manufacturer.

## **7 | Western blotting**

As our previous study,<sup>5</sup> equal amounts of protein from isolated ILC2s with different treatments were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Roche Diagnostics, Indianapolis, IN). After blocking with 10% nonfat milk, the membranes were incubated with rabbit anti p-STAT3 mAb (Tyr705, 1:500), rabbit anti p-STAT5 (Tyr694, 1:500), rabbit anti p-STAT6 (Tyr641, 1:500), and mouse anti  $\beta$ -actin (1:1000, all from Cell Signaling, MA). After washing, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibody and then visualized using Enhanced Chemiluminescence Plus (Millipore Corporation, Billerica, MA, USA).

## **8 | Statistical analyses**

GraphPad Prism 6.0 software (GraphPad Software, La Jolla, CA, USA) was used for data analysis. The data were presented as mean  $\pm$  SEM. Differences between two groups were analyzed using paired or unpaired t test for the data with normal distribution or using Mann-Whitney test for the data with abnormal distribution. Three or more groups were compared using one-way ANOVA with Bonferroni post

hoc test or Kruskal-Wallis test for repeated measurements. Correlations were analyzed with Spearman rank test. P value less than .05 was considered statistically significant.

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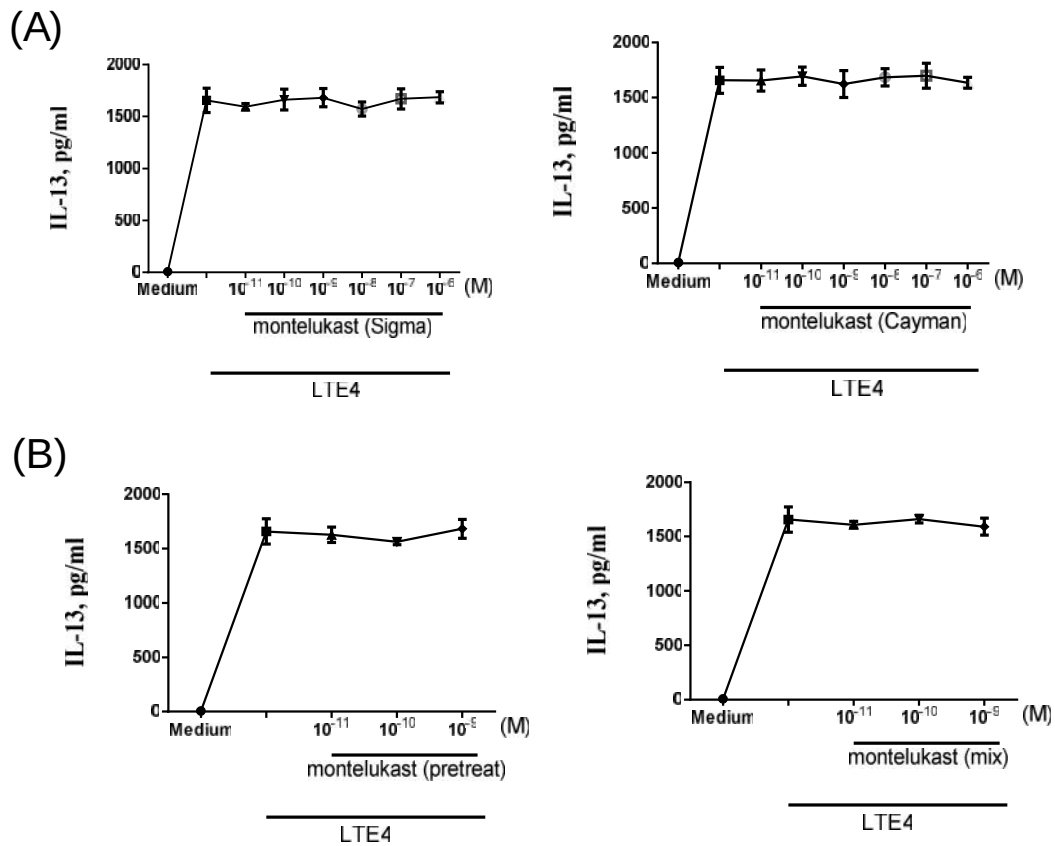
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**Table S1** Demographics and characteristics of the in this study

| <b>Characteristic</b>        | Healthy control<br>(n = 13) | Allergic rhinitis<br>(n = 19) | Atopic dermatitis<br>(n = 10) |
|------------------------------|-----------------------------|-------------------------------|-------------------------------|
| <b>Age (y), mean±SD</b>      | 30.9±7.1                    | 33.1±5.7                      | 15.2±7.2                      |
| <b>Sex, female/male</b>      | 6/7                         | 10/9                          | 7/3                           |
| <b>Total IgE positive</b>    | 0/13                        | 19/19                         | 10/10                         |
| <b>Specific IgE positive</b> | 0/13                        | 19/19                         | 10/10                         |
| <b>/subjects tested</b>      |                             |                               |                               |
| <b>Dust mite (Derp1) , %</b> | 0                           | 94.74%                        | 100%                          |
| <b>House dust , %</b>        | 0                           | 15.79%                        | 20%                           |
| <b>Cat/dog dandruff , %</b>  | 0                           | 10.53%                        | 0                             |
| <b>Artemisia , %</b>         | 0                           | 0                             | 0                             |
| <b>Shrimp&amp;Crab , %</b>   | 0                           | 0                             | 0                             |

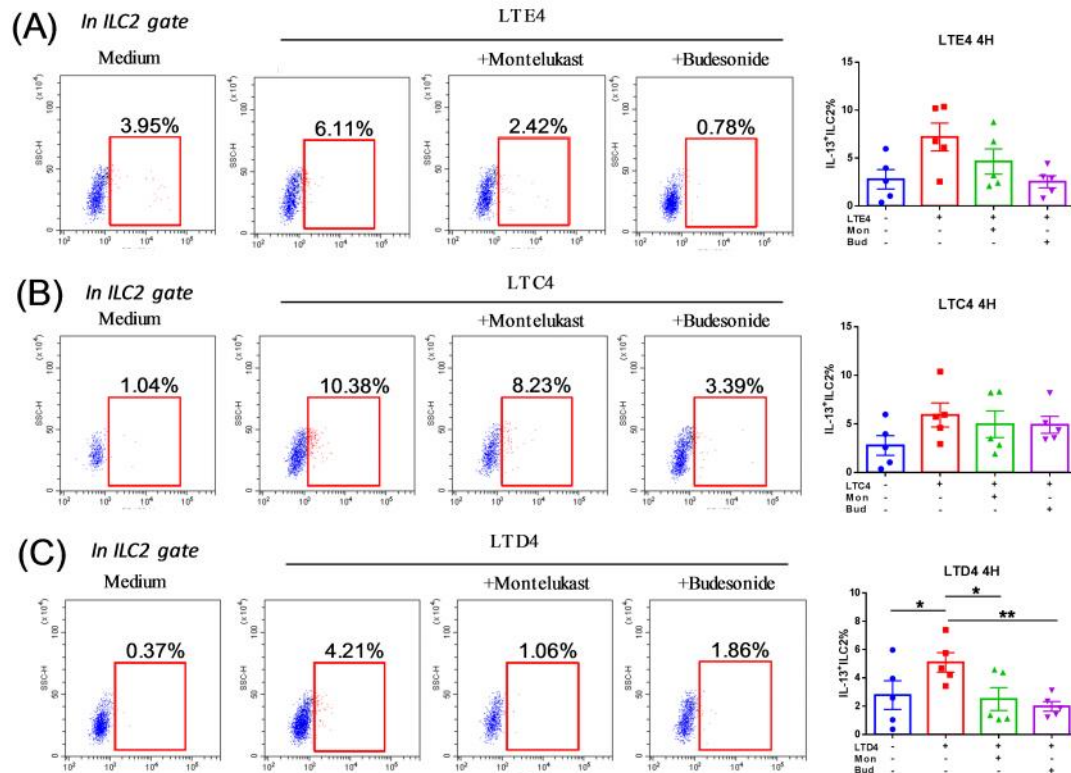
## SUPPLEMENTARY FIGURES

FIGURE S1



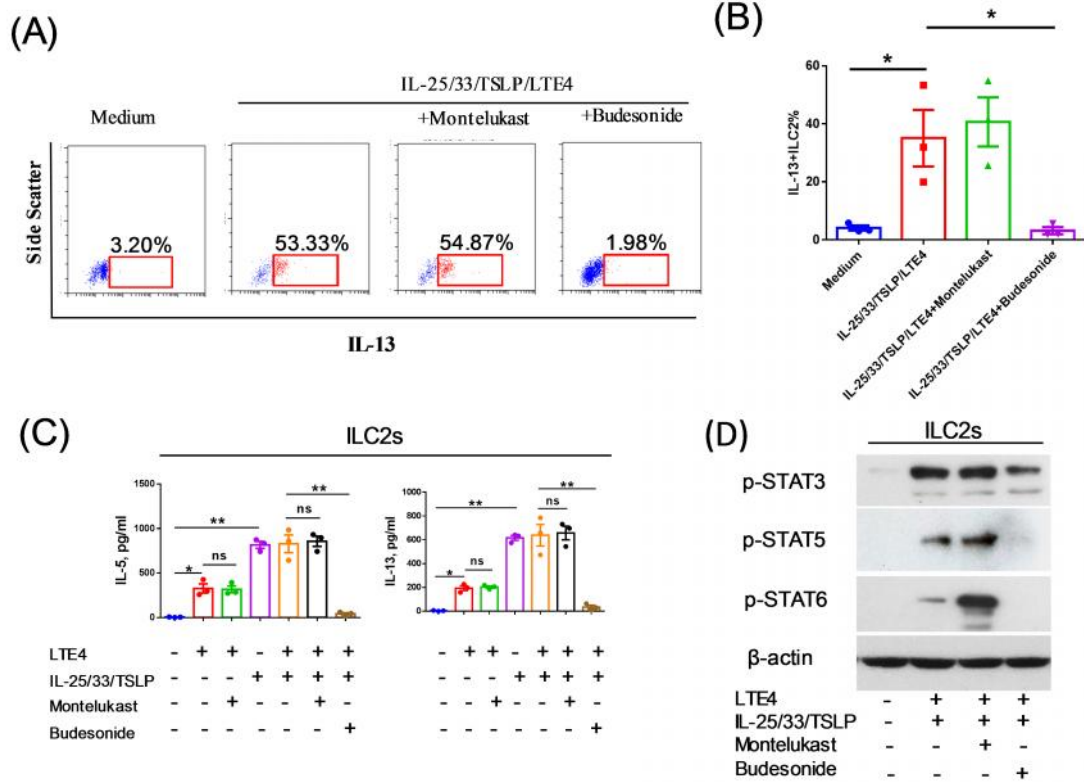
**Montelukast treatment had no effects on IL-13 levels in response to leukotrienes E4.** Lin<sup>-</sup> cells isolated from healthy human buffy coats were stimulated with LTE4 for 3 days. (A) The cells were treated with montelukast with different concentration from Sigma or Cayman. Different sources of montelukast did not significantly decreased the IL-13 levels (n = 3). (B) The cells were pretreated or treated (Mix) with montelukast with different concentration from Cayman at the same time of LTE4 stimulation (n = 3). Data are shown as mean  $\pm$  SEM. LTE4: Leukotriene E4.

**FIGURE S2**



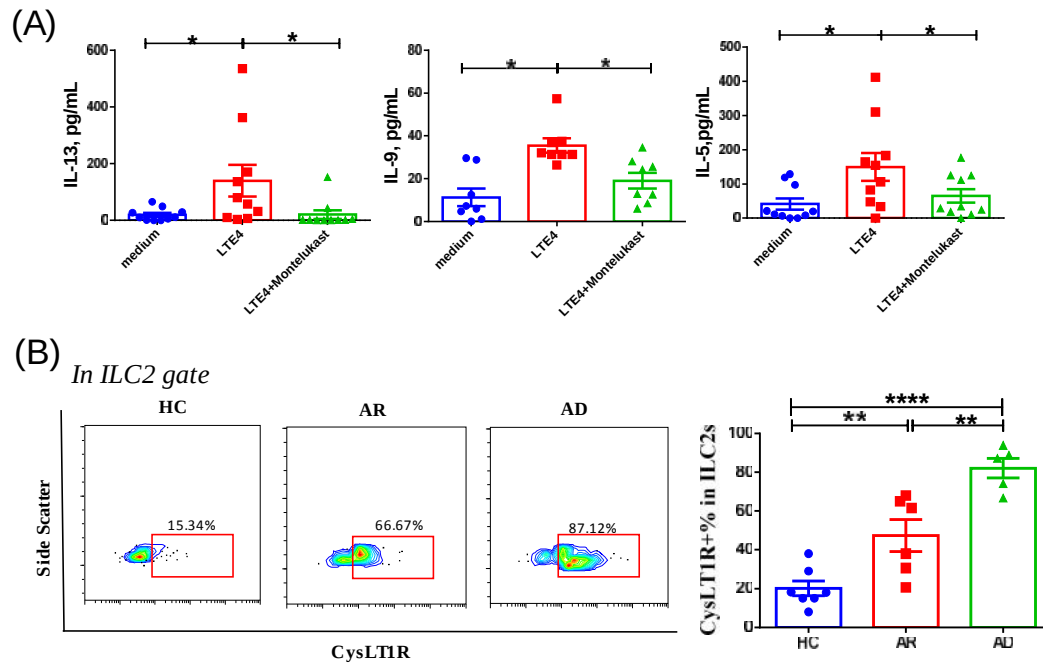
**The effects of montelukast on PBMCs from patients with allergic rhinitis under LT stimulation after 4 hours.** PBMCs from patients with AR were stimulated with LTE4 (A), LTC4 (B) or LTD4 (C), and treated with montelukast or budesonide for 4 h (n = 5). Data are shown as mean  $\pm$  SEM. \*  $P < .05$  and \*\*  $P < .01$ . LT: Leukotriene; PBMC: Peripheral blood mononuclear cell.

**FIGURE S3**



**The effects of montelukast on ILC2 function under LTE4 stimulation.** PBMCs from patients with allergic rhinitis (A, B), ILC2s from healthy human buffy coat (C, D) were stimulated with LTE4 or combined with IL-25, IL-33 plus TSLP, and treated with montelukast or budesonide. (A) Representative cytograms of IL-13<sup>+</sup>ILC2s. (B) Statistical analysis of IL-13<sup>+</sup>ILC2s (n = 3); (C) Statistical analysis of IL-5 and IL-13 levels in ILC2 supernatants under different stimulations (n = 3). (D) The phosphorylated STAT-3, STAT-5 and STAT-6 levels in ILC2s under different treatments as determined by western blot analysis (n = 3). Data are shown as mean ± SEM. \*  $P < .05$  and \*\*  $P < .01$ . ILC2: group 2 innate lymphoid cells; LTE4: Leukotriene E4; ns: not significant; PBMC: Peripheral blood mononuclear cell; TSLP: thymic stromal lymphopoietin.

**FIGURE S4**



**The effects of montelukast on ILC2 function and the expression of CysLT1R on ILC2s from patients with AD.** (A) PBMCs from patients with AD were stimulated with LTE4 and treated with montelukast. IL-5 (n = 10), IL-9 (n = 8) and IL-13 (n = 10) levels were determined in the supernatant; (B) Representative gating strategy and statistical analysis of CysLT1R<sup>+</sup> ILC2s in HC (n = 7) and AR (n = 6) and AD (n = 5) groups as determined by flow cytometry; Data are shown as mean ± SEM. \*  $P < .05$ , \*\*  $P < .01$  and \*\*\*\*  $P < .0001$ . AD: atopic dermatitis; AR: allergic rhinitis; HC: healthy control; ILC2: group 2 innate lymphoid cells; LTE4: Leukotrienes E4.