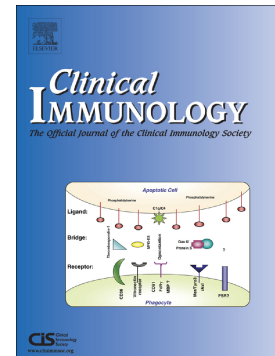


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Increased innate type 2 immune response in house dust mite-allergic patients with allergic rhinitis

Running title: Increased ILC2s response in HDM-AR patients

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Abstract

Group 2 innate lymphoid cells (ILC2s) are essential in initiating and driving allergic immune responses. However, there were inconsistent findings of the ILC2 levels in allergic rhinitis (AR) patients. This study investigated the ILC2 levels in the peripheral blood of house dust mite (HDM)-allergic AR patients and their ability to secrete type 2 cytokines. The levels of ILC2s with phenotypic ILC2 characteristics were increased in the HDM-AR patients. The AR patients' symptom score and IL-13 levels were positively associated with the ILC2s in HDM-AR patients. The epithelial cytokine stimulation induced dramatic production of IL-5 and IL-13 in PBMCs of AR patients. We successfully sorted ILC2s from AR patients and identified their ability of type 2 cytokines production. The number of ILC2s increased in the HDM-AR patients and ILC2s produced the amount of T_H2 cytokines in the presence of epithelial cytokines, which suggested the important role of ILC2 in AR patients.

Highlights

- Increased ILC2 levels were found in AR patients who are positive to HDM antigen, and there was positive correlation between ILC2 levels and VAS scores/ T_H2 cytokines in plasma.
- ILC2s from AR patients produced more T_H2 cytokines, such as IL-5 and IL-13, after the epithelial cytokine stimulation.
- ILC2s may be involved in the HDM-AR, which could be a potential therapeutic target for AR.

Key words: allergic rhinitis; innate immunity; group 2 innate lymphoid cells; T_H2 cytokines

Abbreviations

AR: allergic rhinitis;

ARIA: Allergic Rhinitis and its Impact on Asthma;

Der p 1: Dermatophagoides pteronyssinus;

ELISA: enzyme-linked immunosorbent assay;

FITC: fluorescein isothiocyanate;

HDM-AR: house dust mite-allergic allergic rhinitis;

IgE: immunoglobulin E;

ILC2s: Group 2 innate lymphoid cells;

T_H2: T helper type 2;

TSLP: thymic stromal lymphopoietin;

VAS: visual analog scales.

1. Introduction

Group 2 innate lymphoid cells (ILC2s) are newly discovered cells first reported by several research groups by different names, such as monocytes, natural helper cells, and innate type 2 helper cells [1-3]. ILC2s are morphologically similar to but smaller than, lymphocytes and lack T-cell, B-cell, natural-killer cell or other cell lineage markers. ILC2s vigorously produce T helper type 2 (T_H2) cytokines (particularly for IL-5 & IL-13) under the stimulation of IL-33, IL-25 or thymic stromal lymphopoietin (TSLP) [4]. The discovery of ILC2s puts forward a new challenge to the traditional opinions that T_H2 cells play the dominant role in T_H2 -skewed allergic diseases. ILC2s were found to mediate allergic airway inflammation under the stimulation of IL-33 independent of adaptive immunity in mice [5]. Similarly, the levels of ILC2s were reported to be elevated in allergic asthma patients in several reports [6-8].

However, there were inconsistent findings of ILC2 levels in patients with allergic rhinitis (AR), which is a type of T_H2 -skewed airway inflammation similar to asthma. Until now, there are only a few studies investigating ILC2s in allergic rhinitis in humans [9, 10]. Bartemes KR et al. [11] reported that ILC2 levels were elevated in peripheral blood mononuclear cells (PBMCs) in allergic asthma, but not AR, in American patients compared with healthy controls. However, Lao-Araya M et al. [12] found elevated levels of ILC2s in seasonal AR patients' peripheral blood during the grass pollen season, but not outside of this season. Moreover, Doherty TA et al. [13] found that exposure to cat antigens in sensitized allergic rhinitis subjects rapidly induced increases in peripheral blood ILC2 levels. These results [12, 13] highlight the

effects of natural allergen exposure in increasing the ILC2 number in peripheral blood.

Therefore, we hypothesize that allergen exposure may be one of the important factors affecting ILC2 levels in AR patients. In this study, we investigated the ILC2 levels in the house dust mite -allergic AR patients and their function under the stimulation of epithelial cytokines.

2. Material and methods

2.1 Study subjects

A total of 27 patients with persistent AR and 20 healthy controls 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 (Approval No. 2015-48). The diagnosis of persistent AR was established according to the criteria of the Initiative on Allergic Rhinitis and its Impact on Asthma (ARIA) [14]. Inclusion criteria for AR patients were as follows: (1) Clinical history of persistent allergic rhinitis evidenced by nose itching, sneezing, and clear rhinorrhea; (2) No history or symptoms of chronic rhinosinusitis or asthma; (3) First visit patient and not using glucocorticoids or anti-histamines within the past 4 weeks; (4) Each AR patient had a positive skin prick test (SPT) result to *Dermatophagoides pteronyssinus* (Der p 1) (ALK-Abello', Hoersholm, Denmark) and was positive for specific immunoglobulin E (IgE) to Der p 1 (Pharmacia CAP System, Pharmacia Diagnostics, Uppsala, Sweden) (Table 1). Clinical symptoms were further evaluated using self-reported visual analog

scales (VAS) [15, 16]. Healthy subjects should have no history of allergy, asthma, or sinus disease. Besides, their allergen SPT should also be negative. In addition, the total/sIgE were detected with Unicap system (Pharmacia CAP System, Pharmacia Diagnostics, Uppsala, Sweden) according to the manufacturer's instructions. And the healthy subjects' sIgE should be under 0.35 kU/L. Exclusion criteria for AR and healthy control groups were as follows: (1) systemic or nasal glucocorticoids and anti-histamines within the past 4 weeks; (2) smoking within the past 12 months or a history of more than 10 packs/y; (3) immunodeficiency or cystic fibrosis diagnosis; (4) immunotherapy for any allergens; or (5) pregnancy.

2.2 Isolation and stimulation of PBMCs

The peripheral blood was sampled into heparin anticoagulant tube and was layered over the lymphocyte separation medium (MP Biomedicals, LLC) by density gradient centrifugation. Then the PBMCs were collected from the interface between lymphocyte separation medium and plasma. PBMCs were cultured in 96-well round-bottom plates (1×10^6 cells/mL) at 37°C, 5% CO₂ for 3 days with cytokines (IL-25 (10 ng/mL) and IL-33 (10 ng/mL), eBioscience, San Diego, USA) or Der p 1 (25 µg/ml) in the presence of IL-2 (50 ng/mL). The IL-5 and IL-13 levels in plasma and cell-culture supernatant were measured using an enzyme-linked immunosorbent assay (ELISA) kit. IL-5 or IL-13 positive ILC2 levels were measured using flow cytometry. In addition, CD3⁺ T cells were also depleted from the PBMCs (CD3-depleted PBMCs) using CD3 MicroBeads (Miltenyi Biotec, Shanghai, China), which was to verify the role of CD3⁺ T cells in the elevated PBMC responses.

2.3 ILC2 sorting and stimulation

In order to further investigate the function of ILC2 in AR patients, ILC2s were sorted from PBMCs. AR patients' lineage-negative (Lin^-) cells were enriched from PBMCs by depleting lineage-positive (Lin^+) cells using fluorescein isothiocyanate (FITC)-conjugated antibodies to CD2, CD3, CD14, CD16, CD19, CD56, CD235a and FcεRI, along with the EasySep FITC selection kit (StemCell Technologies, Vancouver, British Columbia, Canada) according to manufacturer's instructions. This process left approximately 50% pure Lin^- cells. These Lin^- enriched cells were stained with phycoerythrin (PE)-conjugated CRTH2 and phycoerythrin-Cy7-conjugated CD127 (BD Bioscience, NJ), then sorted using an FACS Aria (BD Biosciences, NJ) into 3 populations on the basis of CRTH2 and CD127 expression, resulting in $\text{Lin}^- \text{CD127}^+ \text{CRTH2}^+$, $\text{Lin}^- \text{CD127}^+ \text{CRTH2}^-$ and $\text{Lin}^- \text{CD127}^- \text{CRTH2}^-$ populations. The $\text{Lin}^- \text{CD127}^+ \text{CRTH2}^+$ (ILC2s) cells were cultured at 2.5×10^5 cells/mL in 96-well round-bottom plates in RPMI-1640 containing 10% FBS (Gibco, Carlsbad, Calif) and 1% penicillin/streptomycin for 3 days, the culturing condition was 37°C and 5% CO₂ humidified. ILC2s were stimulated using medium alone, in a combination of IL-25 (10 ng/mL), IL-33 (10 ng/mL) and IL-2 (50 ng/mL) or Der p 1. Cell-free supernatants were analysed for IL-5 and IL-13 using ELISA. Furthermore, the stimulated ILC2s were spun onto a microscope slide using cytocentrifugation at 800 rpm for 3 min. Diff-quick staining was then used to visualize the ILC2s on the slide.

2.4 Flow cytometry for ILC2

PBMCs from AR patients were stained with a FITC lineage cocktail (CD2, CD3,

CD14, CD16, CD19, CD56, CD235a, eBioscience, San Diego, CA) and FcεRI (eBioscience), which allowed for the exclusion of B, T, natural killer, and natural killer T cells as well as mast cells and basophils. Cells were further stained with PE-conjugated CRTH2 (BD Bioscience, NJ) and PE-Cy7 conjugated CD127 (BD Bioscience, NJ). Flow cytometry was performed using the Beckman flow cytometer machine (Beckman Coulter, Hercules, CA, USA) and ILC2s were identified as Lin⁻CRTH2⁺CD127⁺ lymphocytes.

In addition, IL-5 and IL-13 positive ILC2 levels were also evaluated using flow cytometry. IL-5 and IL-13 were detected by intracellular cytokine staining using Cytofix (BD Bioscience, NJ) as per the manufacturer's instructions. Briefly, after stimulated with epithelial cytokines cocktail or Der p 1, brefeldin A (3 µg/ml) and monensin (1/2000 final dilution; both from eBiosciences, San Diego, CA) were added into the PBMCs to block cytokine secretion. Then, the cells were incubated with fluorochrome-conjugated anti-IL-5 and anti-IL13 antibodies or appropriate negative control and were subjected to flow cytometric analysis.

2.5 Enzyme-linked immunosorbent assay

The IL-5 and IL-13 levels in plasma and cell-culture supernatant were measured using an ELISA kit (RayBiotech Inc, Norcross, GA) read with a microplate reader (Biotek). The detection limit for IL-13 was 0.15 pg/mL and the detection limit for IL-5 was 5 pg/mL. All values below the detectable limit were set as 0 for convenient analysis. The ELISA kits' protocols are recommended by the manufacturer.

2.6 Statistical analyses

Data are presented as the Mean $\pm$ SEM. All ILC2 data and T_H2-cytokine (IL-5 and IL-13) levels were analysed with unpaired t-tests. Linear correlation analysis was performed between ILC2 levels and VAS scores or IL-13 levels (Spearman's method). A *P* value less than 0.05 represented statistical significance.

3. Results

3.1 Functional ILC2 increase in peripheral blood of AR patients

There were 27 AR patients and 20 healthy controls recruited in this study. As shown in Table 1, there was no significant difference in age, gender, the smoking status between these two groups. None of the participants were either diagnosed with asthma or had medications including glucocorticosteroids, antihistamines, decongestant and chromones, etc. The AR patients were all allergic to HDM and 100% positive for plasma specific IgE, visual analog scale (VAS) for AR patients was around 54.6 mm. While the healthy controls were negative for plasma specific IgE, and their VAS level was not detectable (Table 1).

Single cell suspensions of PBMCs were stained to identify the ILC2 subset. When sorted by flow cytometry, Lin⁻ lymphocytes that co-expressed the prostaglandin D2 receptor (CRTH2) and IL-7 receptor (CD127) (Lin⁻CRTH2⁺CD127⁺ cells) were considered to be ILC2s (Fig 1A). The ILC2s percentages in PBMCs in healthy controls were very low (Fig 1B, 0.02% $\pm$ 0.002%). However, the percentages of ILC2s in PBMCs in the AR patients (0.08% $\pm$ 0.01%) were significantly higher compared to those in healthy controls (Fig 1B, *P* < 0.0001). Similarly, the percentages

of ILC2s in Lin⁺ cells in AR subjects were higher compared to those in healthy controls (Fig 1B, $P < 0.0001$). More importantly, for AR patients, there was a positive linear correlation between the proportion of ILC2s in PBMCs ($R^2=0.39$, $P=0.0004$) or in Lin⁺ lymphocytes ($R^2=0.29$, $P=0.0037$) and VAS score (Fig 1C).

3.2 IL-13 levels are associated with ILC2s in AR patients

IL-13 and IL-5 have been identified as the major cytokines produced by ILC2s and play major roles in allergic inflammation [4]. We observed a similar upregulation of IL-13 levels in the plasma in AR patients compared to those in healthy controls (Fig 2A, $P < 0.001$). Moreover, we found a strong correlation between the percentages of ILC2s in PBMCs ($R^2=0.7$, $P < 0.0001$)/Lin⁺ lymphocytes ($R^2=0.41$, $P < 0.0001$) and IL-13 levels in AR patients (Fig 2B). The IL-5 levels were undetectable in both groups, so data is not shown.

3.3 IL-33/IL-25/IL-2 and Der p 1 antigen stimulate the production of type 2 cytokines in PBMCs and in ILC2s from HDM-allergic AR patients

The IL-13 levels in the culture medium were relatively low for both AR patients and healthy controls, but with higher levels for AR patients. We found that there were an increase of IL-13 and IL-5 levels in healthy controls after the stimulation of cytokines (combination of IL-33, IL-25, and IL-2) or Der p 1. However, higher levels of IL-13 and IL-5 were observed in AR patients after both types of stimulation. There was significant difference for IL-13 and IL-5 between AR patients and healthy controls after stimulation. In addition, there were higher levels of IL-5 and IL-13 with the stimulation of IL-33/IL-25/IL-2 compared to that of Der p 1 stimulation (Fig 3A, $P <$

0.01). Considering the functional redundancy between ILCs and T_H2 , CD3-depleted PBMCs were applied to test whether CD3⁺ cells are required for the elevated PBMC responses *in vitro*. CD3⁺ T cells were magnetically depleted using CD3 MicroBeads. Both PBMCs and CD3-depleted PBMCs showed significantly increased T_H2 cytokines (IL-5 and IL-13), and the CD3-depleted PBMCs even showed a higher ability of IL-13 expression compared to that of PBMCs (Supplementary Fig 1, $P < 0.0001$).

We further investigated the levels of IL-5 and IL-13 positive ILC2s from AR patients under stimulation. The identification of ILC2s in PBMCs was shown in Fig 3B, and the percentage of IL-5 and IL-13 double positive ILC2s was determined using flow cytometry. More IL-5 and IL-13 double positive ILC2s were found in the presence of IL-33/IL-25/IL-2 compared to that of control (Fig 3C&D, $P < 0.001$). Der p 1 stimulation also increased the percentage of IL-5 and IL-13 double positive ILC2s, but not as high as that in IL-33/IL-25/IL-2 stimulated group (Fig 3D). Taken together, these findings suggested that human PBMCs in AR patients produce dramatic high level of type 2 cytokines (IL-5 and IL-13) after the stimulation with the IL-33/IL-25/IL-2 cocktail and Der p 1 antigen. And the IL-33/IL-25/IL-2 cocktail is stronger than the Der p 1.

3.4 Sorted ILC2s produced T_H2 cytokines after the stimulation

In order to further investigate the function of ILC2s, we freshly sorted ILC2s from AR patients' PBMCs using flow cytometry. We followed the protocol used by Kathleen RB et al. [11]. The sorted ILC2s were stained with diff-quick, they exhibited a small

cell size with 4 μm diameter and big nuclei with less cytoplasm (Fig 4A). The sorted ILC2s were cultured with IL-33/IL-25/IL-2 cytokines cocktail. Cytokines stimulation dramatically increased IL-5 and IL-13 concentrations in the culture medium, whereas IL-5 and IL-13 were barely detected from the ILC2s without stimulation (Fig 4B). Taken together, these findings suggested that human ILC2s are able to produce type 2 cytokines IL-5 and IL-13 when stimulated with the IL-33/IL-25/IL-2 cocktail.

4. Discussion

The connections between innate type 2 immune responses and allergic diseases were previously described with relatively small sample size, which precluded the researchers from performing detailed analyses [11]. In this study, we verified that ILC2 levels were significantly increased in AR patients who were positive to HDM antigen, and there was a positive correlation between ILC2 levels and VAS scores/ T_H2 cytokines in plasma. The sorted $\text{Lin}^- \text{CD127}^+ \text{CRTH2}^+$ cells from HDM-AR patients produced more T_H2 cytokines than those from healthy subjects after the epithelial cytokine stimulation. These findings suggested the pivotal actors of ILC2s in HDM-AR, which could be a potential therapeutic target for such allergic disease.

Our data demonstrated an elevation of peripheral ILC2s in HDM-allergic AR patients. More importantly, peripheral ILC2 levels had a strong positive correlation with the severity of the clinical symptoms, and also with their functional cytokine IL-13 levels in the plasma. Our findings were consistent with previous studies about the increased peripheral ILC2s in the grass pollen season [12] or after the challenge of

cat antigen in AR patients [13]. However, D.C. Fan *et. al* [17] found there was no significant difference of the ILC2s level between monosensitized mugwort-AR patients (but negative to HDM) and healthy controls. The stronger generation of ILC2s stimulated by HDM might due to the different mechanisms, by which HDM stimulate inflammatory responses, from pollen allergens. Dust mites are a type of common, persistent, and perennial allergen that is present almost everywhere in the world. HDM-mediated direct nonspecific damage and allergic reactions in the respiratory epithelium are induced by trypsin/chymotrypsin-like enzymatic activities associated with mite allergen, such action may potentially lead to greater epithelial cell-derived IL-33 release and generation of systemic ILC2s [17-19]. Our results were inconsistent with the findings of Bartemes KR *et al.* [11] concerning equal ILC2 levels between American AR subjects and healthy controls. However, we noticed that only 33% of patients were sensitive to dust mites and 50-81% subjects were sensitive to seasonal allergens, such as ragweed or trees, in their study. Moreover, the authors did not report on whether the patients were enrolled during allergy season or during the off-season. Therefore, one of the advantages of our study is that we enrolled 27 AR patients and 20 healthy controls, more samples than the studies by Bartemes KR [11] (16 AR subjects), Lao-Araya M [12] (12 AR subjects) and Doherty TA [13] (7 AR subjects). All of the findings in this study indicate that mite allergen exposure has an important effect on the increase of peripheral ILC2 number for AR subjects, even though ILC2s are a type of innate lymphocyte.

In our study, ILC2s from HDM-AR patients secreted type 2 cytokines (IL-5 and

IL-13) in the presence of epithelial cytokines cocktail. IL-5 induces survival and degranulation of eosinophil, and IL-13 causes airway hyper-responsiveness (AHR) and airway remodelling by induction of matrix metalloproteinase. Although the abundance of ILC2s is relatively low in PBMCs, their type 2 cytokines are able to augment type 2 cytokines from T_H2 cells. Such action might reflect the pivotal actors of ILC2s in allergic diseases. A recent study showed that after repetitive antigen exposure, there were three feedback and feed-forward circuits involving epithelial cells, ILC2s and T_H2 cells for the mechanisms to sustain asthma, in which IL-13 produced by both ILC2s and T_H2 cells further induced IL-33 production and then induced more ILC2s [20]. Furthermore, $CD4^+$ T-cell-derived IL-2 promotes ILC2 proliferation and enhances T_H2 cytokine production of ILC2s [21]. Therefore, early activation of ILC2 may contribute to the later development of a $CD4^+$ T_H2 response, whereas T cell production of IL-2 may reinforce this response in a feedback loop by further activating ILC2 [21]. It is clear that the discrepancy in ILC2 levels between different groups may also be the result of the different pathological states of patients, such as differing inflammatory profiles, allergens, races and environments. The possible detailed relationships between ILCs and T cells in a specific situation should be further investigated.

We further identified that PBMCs from AR patients produced dramatic IL-13 and IL-5 after the stimulation of IL-33/IL-25/IL-2. Der p 1 antigen also stimulated PBMCs from HDM-allergic AR patients to produce some but much lower IL-13 and IL-5 compared to those stimulated by above epithelial cytokines. It suggested that

IL-33/IL-25 had much stronger effects on PBMCs in AR patients to induce the production of T_H2 cytokines. Demonstrating that different allergen could induce different allergic effects. These findings were consistent with the previous study, which found that ILC2 percentage was significantly elevated in house dust mite-allergic AR (HDM-AR) patients compared to mugwort-AR patients, the T_H2 cytokines release was also different according to the source of allergens [17]. Considering the functional redundancy between ILCs and T_H2 , CD3-depleted PBMCs were applied to test whether $CD3^+$ cells are required for the elevated PBMC responses *in vitro*. The CD3-depleted PBMCs showed similar elevated responses as that of PBMCs in the presence of epithelial cytokines, which demonstrated that $CD3^+$ T cells are not necessary for the enhanced type 2 immune response. Using flow cytometry, we next found that there were high levels of IL-5 and IL-13 positive ILC2s after IL-33/IL-25/IL-2 stimulation, suggesting ILC2s were one production source of type 2 cytokines. Furthermore, we sorted ILC2s from PBMCs of AR patients and successfully identified the morphology of ILC2s with small size, big nuclei and a thin rim of cytoplasm, similar to previous studies [22]. We further identified that the sorted ILC2s produced IL-5 and IL-13 after the stimulation of IL-33/IL-25/IL-2. In this study, there's a positive correlation between IL-13 levels and ILC2 levels in HDM-AR patients, it is possible that circulating ILC2s might have a relationship with the symptom levels of AR patients. Furthermore, ILC2s may also contain antigen specific memory cells in the blood. Although ILC2s lack antigen-specific receptors and therefore memory ILC2s do not have antigen specificity, recent studies have

shown that ILC2s can record previous activations and respond more vigorously on reactivation [23], IL-33-experienced ILC2s differ from naïve and effector ILC2s in gene expression profiles, such findings further supporting the notion that ILC2s could acquire memory-like properties and enhance allergic inflammation [24]. The severity of AR may be reflected by the circulating antigen specific ILC2s. So far, there have been several studies demonstrated that decreased ILC2s at the sinonasal mucosal barrier may relieve ongoing local inflammation [17, 25, 26]. Of course, further investigation with more extensive research should be performed to further confirm the role of ILC2s, and identify the potential mechanisms in AR patients.

In this study, we found the increased levels of ILC2 in AR patients were positive to HDM antigen, and there was a positive correlation between ILC2s levels and VAS scores/ T_H2 cytokines in serum. Furthermore, we identified that ILC2s from AR patients produced more IL-5 and IL-13 after the epithelial cytokine stimulation. Our data indicate the important role of ILC2s in AR patients especially positive for antigens. ILC2s may be involved in the HDM-AR, which could be a potential therapeutic target for AR.

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Disclosure of potential conflict of interest

The authors have declared that they have no conflict of interest.

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Figure legends

Figure 1. Increased levels of ILC2s in PBMCs of HDM-AR patients.

A, Lymphocytes were identified from whole PBMCs, and Lin⁻ cells were gated and further assessed for co-expression of CD127 and CCR2. ILC2s were identified as Lin⁻CCR2⁺CD127⁺ lymphocytes. B, ILC2 levels in PBMCs or in Lin⁻ lymphocytes were significantly elevated in the AR group (n=27) compared with those in the healthy controls (n=20) (unpaired t-test for two-group comparisons). C, HDM-AR patients' symptom severity (visual analog scale [VAS]) was correlated with ILC2 levels (Spearman's method). FSC, Forward scatter; HDM-AR, house dust mite positive allergic rhinitis; HC, healthy control; Lin⁻, lineage-negative; PBMCs, peripheral blood mononuclear cells; SSC, side scatter.

Figure 2. IL-13 levels in plasma were positively correlated with ILC2 levels in

HDM-AR group. A, IL-13 levels in the plasma were significantly elevated in HDM-AR (n=27) compared with those in the healthy controls (n=20) (unpaired t-test for two-group comparisons). B, IL-13 levels were positively correlated with ILC2 levels (Spearman's method). AR, allergic rhinitis; HC, healthy control; HDM-AR, house dust mite-allergic allergic rhinitis.

Figure 3. Induction of type 2 cytokine positive ILC2s with the stimulation of epithelial cytokines cocktails and Der p 1 antigen in PBMCs from AR patients.

PBMCs from healthy controls and AR patients were cultured with Der p 1 or

cytokines of IL-33 and IL-25 in the presence of IL-2 for 3 days. IL-5 and IL-13 levels in the supernatants were elevated by the stimulation (A); ILC2s in the PBMCs were further identified (B), and the percentage of IL-5 and IL-13 double positive cells in ILC2s were determined using flow cytometry (C, D). Data are the mean $\pm$ SEM of 5 subjects. * $P < .05$; ** $P < .01$, *** $P < .001$. AR, house dust mite positive allergic rhinitis; HC, healthy control.

Figure 4. Sorted ILC2s from AR patients produced T_H2 cytokines after stimulation. ILC2s were separated from the PBMCs in HDM-AR group using flow cytometry and cultured with cytokines of IL-33 and IL-25 in the presence of IL-2 for 3 days. Diff-quick staining showing the morphology of the sorted ILC2s (Scale bar is 4 μ m) (A); (B) The IL-5 and IL-13 levels in ILC2s. Data are the mean $\pm$ SEM of 5 subjects. * $P < .05$; ** $P < .01$; *** $P < .001$.

Supplementary figure legends

Supplementary Figure 1. CD3-depleted PBMCs from AR patients were cultured with IL-33 and IL-25 in the presence of IL-2. Levels of IL-5 and IL-13 in the supernatants were evaluated to test the role of CD3⁺ cells for the elevated PBMC responses *in vitro*.

Table 1. Demographics and characteristics of the study subjects in this study

Characteristic	HC (n=20)	AR (n=27)	P value
Age (y), mean $\pm$ SD	27.7 $\pm$ 1.4	30 $\pm$ 7.4	0.6308
Sex, female/male	8/12	16/11	0.3884
SPT, positive/subjects tested	0/20	27/27	
Dust mite (%) (Der p 1)	0	100%	< 0.0001
House dust(%)	0	18.5%	< 0.0001
Trees (%)	0	3.7%	< 0.0001
Artemisia (%)	0	3.7%	< 0.0001
Shrimp & Crab (%)	0	7.4%	< 0.0001
Total IgE concentration(kU/L), mean $\pm$ SD	89 $\pm$ 6.1	94 $\pm$ 5.4	< 0.01
sIgE concentration(kU/L), mean $\pm$ SD	< 0.35	78 $\pm$ 4.2	< 0.0001
Dust mite (Der p 1)	< 0.35	61 $\pm$ 6.9	< 0.0001
House dust	< 0.35	45 $\pm$ 7.4	< 0.0001
Trees	< 0.35	23 $\pm$ 6.7	< 0.0001
Artemisia	< 0.35	17 $\pm$ 8.1	< 0.0001
Shrimp&Crab	< 0.35	44 $\pm$ 3.7	< 0.0001
VAS level, mean $\pm$ SD	0	54.6 $\pm$ 19.6	< 0.0001

SPT, skin prick test; tIgE, total IgE; sIgE, specific IgE

Figures

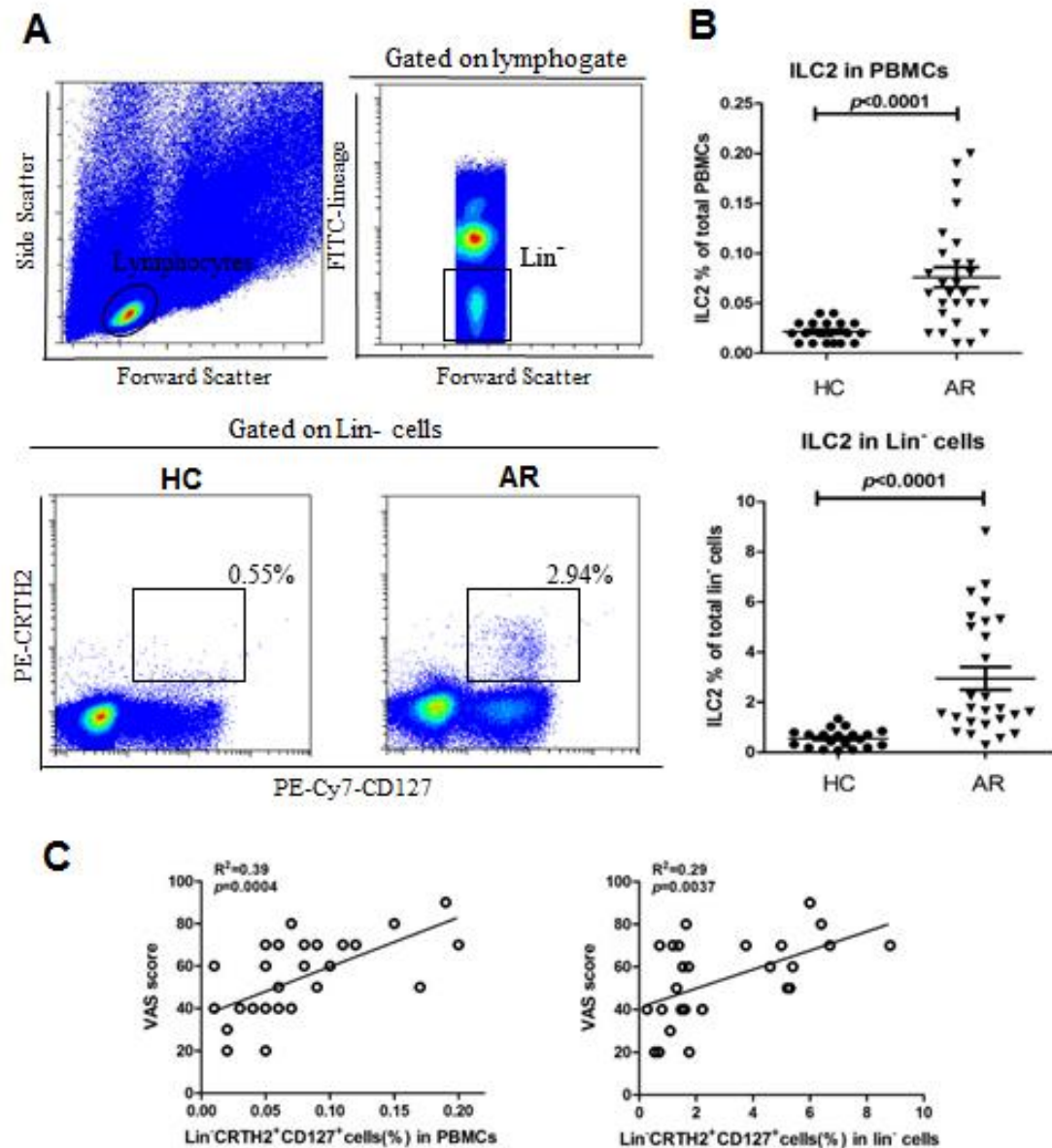


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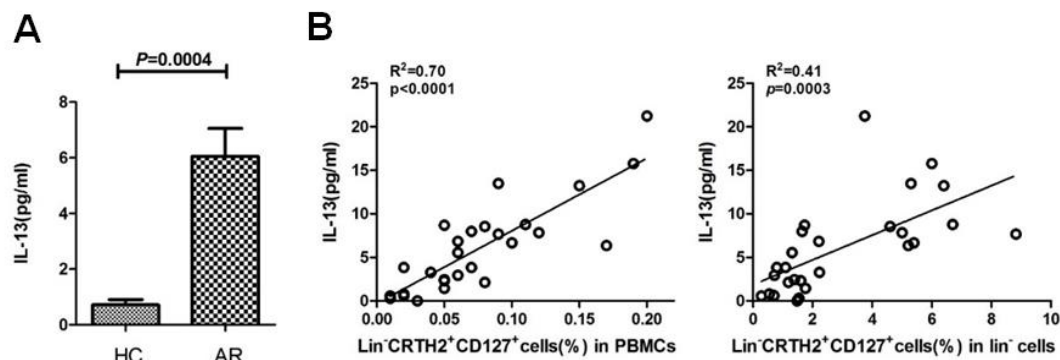


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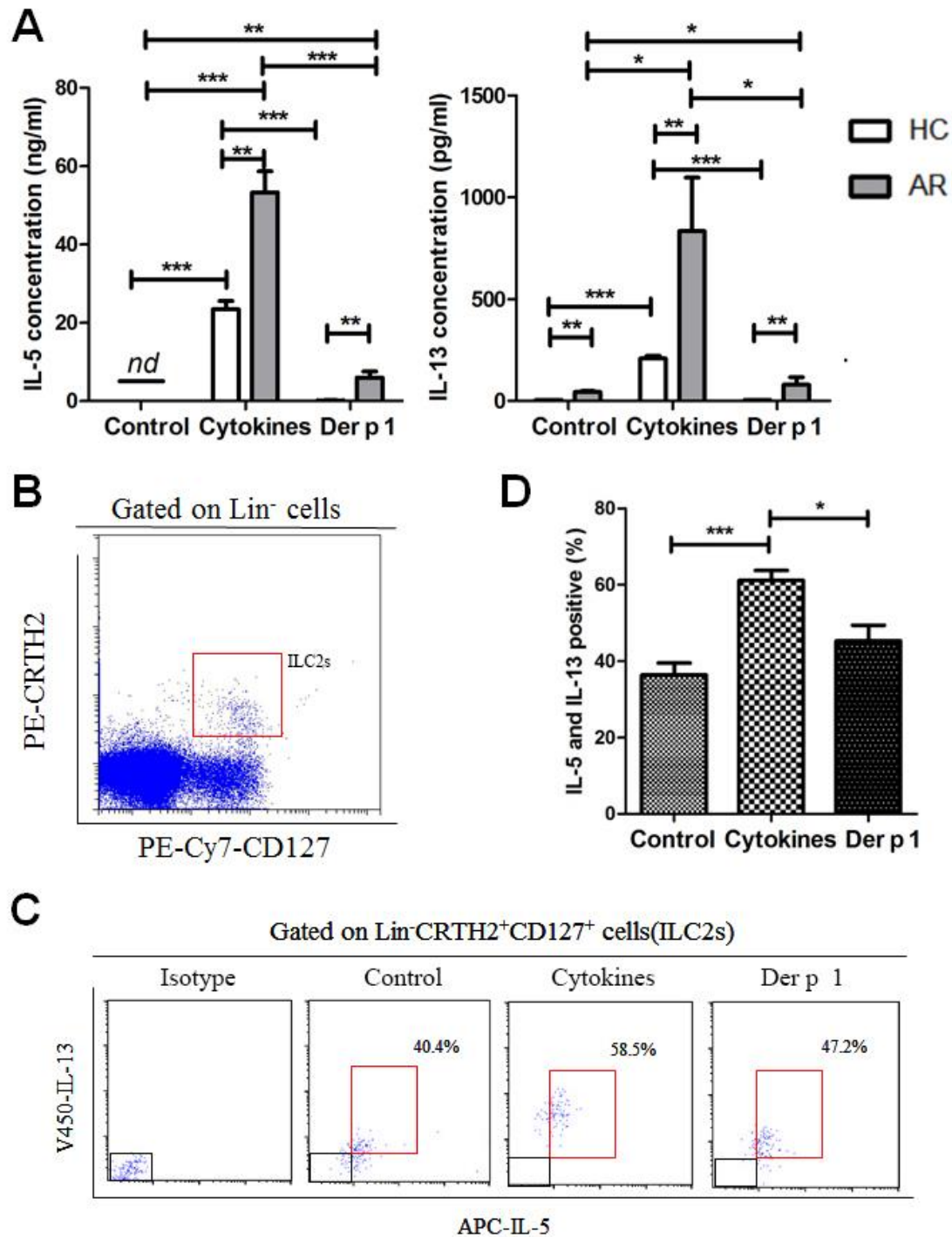


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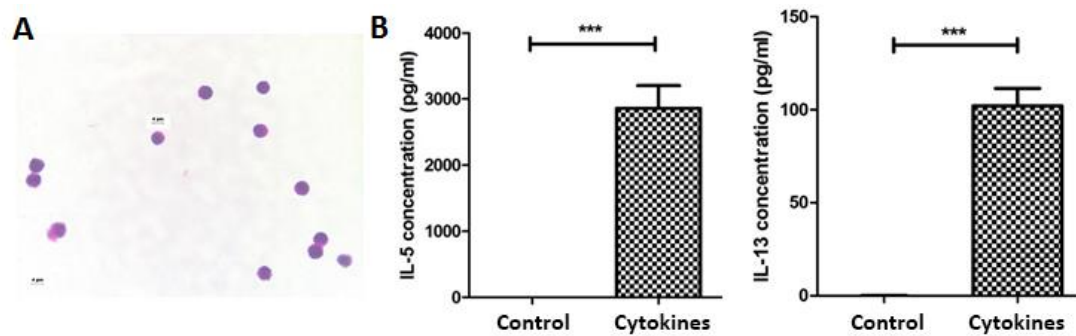
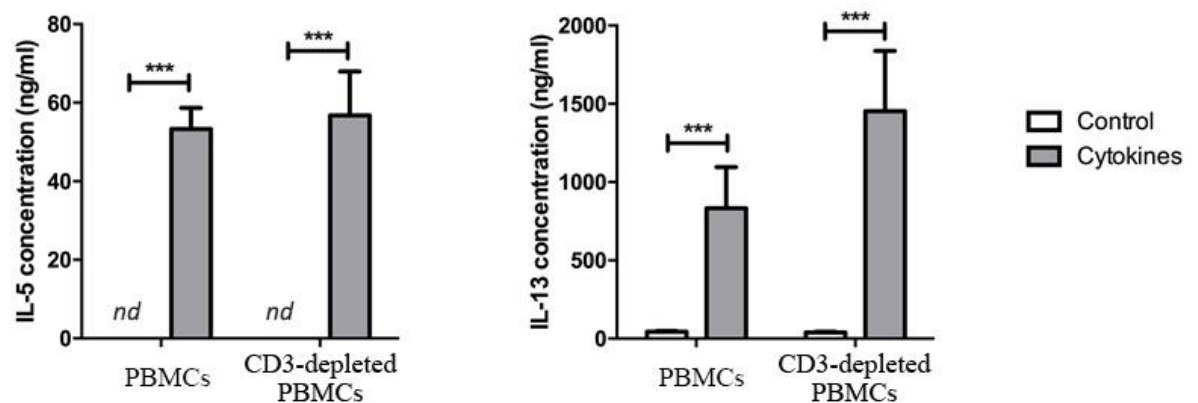


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Supplementary Figure



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