

Increased expression of type 2 innate lymphoid cells in pediatric patients with allergic rhinitis

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Abstract. Type 2 innate lymphoid cells (ILC2s) are a newly identified group of innate immune cells. ILC2s promote features of allergic airway diseases through the secretion of Th2 type cytokines, including interleukin (IL)-4, IL-5 and IL-13. It remains unknown whether ILC2s aggregate in the peripheral blood. The present study examined the ILC2 levels in pediatric patients with allergic rhinitis (AR), and the correlation with the severity of clinical symptoms. Flow cytometry detected the ILC2s frequency in the peripheral blood of 12 healthy controls (HCs), 12 patients with AR sensitized only to house dust mites (HDM-AR), and 18 AR patients monosensitized to other antigens including weeds, animal danders and *Blattella germanica*, but not including HDM (non-HDM-AR). Clinical symptoms of AR were expressed according to the Total 5 Symptom Score (T5SS). The percentages of ILC2s in the peripheral blood were increased significantly in patients with HDM-AR and non-HDM-AR, compared with that in the HCs. A subgroup analysis of patients with AR indicated that the proportion of ILC2s was significantly increased in HDM-AR in comparison with that in non-HDM AR. Furthermore, there was a notable correlation between ILC2 levels and T5SS scores. ILC2s frequencies in PBMC were increased significantly in pediatric

patients with AR, irrespective of the type of allergen. HDM may trigger more severe allergic reactions and an increase in the number of ILC2s. These discoveries indicate the unique function of ILC2 in AR and provide a potential therapeutic target.

Introduction

Allergic rhinitis (AR) is one of the most common diseases affecting quality of life and work and school attendance (1). AR is characterized by the infiltration and activation of immune cells in the nasal mucosa, which then produce T helper type 2 (Th2) cytokines and proinflammatory molecules (2,3). It is well-known that CD4⁺ Th2 cells serve an essential role in allergic airway disease pathogenesis through producing the Th2 type cytokines interleukin (IL)-4, IL-5, and IL-13 (4). Following the identification of type 2 innate lymphoid cells (ILC2s) in 2010, this traditional understanding of AR has been challenged (5).

Innate immunity comprises the first line of response against invading pathogens and environmental insults. ILC2s are a newly identified group of innate immune cells. ILC2s have lymphocyte characteristics, but do not express T cell, B cell, natural killer cell or other granulocyte-monocyte lineage markers (6). They produce IL-5 and IL-13 in response to the epithelium-derived cytokines IL-25, IL-33, and thymic stromal lymphopoietin (7). It remains unclear whether ILC2s aggregate in the peripheral blood in AR. Bartemes *et al* (8) suggested that ILC2s levels were increased in asthma, but did not change in AR. Doherty *et al* (9) demonstrated that cat-sensitized AR adults challenged locally by cat allergens exhibited a significantly increased percentage of ILC2s in the peripheral blood at 4 h after challenge. Subsequently, Fan *et al* (10) suggested that patients with AR sensitized to house dust mite (HDM) or mugwort allergens have different levels of ILC2 expression, which represent a critical source of type 2 cytokines in HDM-AR (10). The present study determined whether ILC2 levels were increased in systemic peripheral blood and their association with clinical manifestations in pediatric patients with AR.

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Patients and methods

Clinical specimens. Patients with HDM-AR (n=12), non-HDM-AR (n=18) and healthy controls (HCs) (n=12) were recruited from the Children's Hospital of Chongqing Medical University from November in 2017 to February in 2018. AR was diagnosed according to the criteria of the Initiative on Allergic Rhinitis and its Impact on Asthma (11). Patients with AR presented with a characteristic history of watery nasal discharge, nasal obstruction, sneezing, itching in the nose, were positive for IgE specific to antigens such as HDM, weeds (mugwort and ragweed), animal danders (cat and dog) and *Blattella germanica* (Pharmacia CAP System, Pharmacia Diagnostics). The severities of clinical symptoms were further assessed using the Total 5 Symptom Score (T5SS) (12). According to the clinical symptoms of itching in the nose, nasal obstruction, rhinorrhea, sneezing and itching in the eyes, the severity of symptoms was assessed by a 0-3 point system [0, no symptoms; 1, mild (symptoms exist, but not annoying); 2, moderate (symptoms annoying, but easy to tolerate); 3, serious (symptoms annoying and intolerable)].

Prior to inclusion in the present study, all medications such as corticosteroids, antihistamines or leukotriene receptor antagonists were prohibited for at least 4 weeks. Patients with infectious, vasomotor, hormonal, drug-induced and occupational rhinitis, or any complications, were excluded. HCs had to satisfy the conditions of no history of allergic diseases, and a negative skin prick test and absence of IgE specific to antigens (total IgE levels <100 kU/l). Patient characteristics are summarized in Table I. The present study was approved by and performed in accordance with the local regulations of the Ethical Committee of Chongqing Medical University (ethics approval no. 038/2014) and the Declaration of Helsinki. Informed consent was acquired from all subjects' legal guardians prior to enrolment in the study. All participants showed no adverse reactions.

Separation of peripheral blood mononuclear cells (PBMCs). A total of 5 ml blood was collected from each subject into a heparin anticoagulant tube. Peripheral blood was managed by Ficoll-Hypaque density gradient centrifugation at 800 x g for 30 min at 4°C. The PBMCs were collected from the cell layer between the lymphocyte separation medium and the plasma.

Sorting ILC2s with flow cytometry. To examine the role of ILC2 in AR, ILC2s were sorted from PBMCs using the lineage cocktail method. Firstly, PBMC cells were stained with fluorescein isothiocyanate (FITC)-conjugated antibodies against lineage markers [cluster of differentiation (CD)3, B-lymphocyte antigen CD19, hematopoietic progenitor cell antigen CD34, natural killer cells antigen CD94, IL-3 receptor, T cell receptor (TCR) $\alpha\beta$, C-type lectin domain family 4 member C and high-affinity IgE receptor) and phycoerythrin (PE)-conjugated lineage markers (T-cell surface glycoprotein CD1a, integrin α -X, monocyte differentiation antigen CD14, TCR $\gamma\delta$ and neural cell adhesion molecule), which aimed to exclude T cells, monocytes, macrophages, B cells, mast cells, basophils, dendritic cells and hematopoietic progenitor cells. Lineage-negative (Lin⁻) cells were collected from PBMCs by depleting lineage-positive (Lin⁺)

Table I. Demographics and characteristics of participants.

Characteristics	Patient groups		
	Healthy controls	HDM-AR	non-HDM-AR
Patients (n)	12	12	18
Age, years	6.9±1.1	7.2±1.0	7.0±0.9
Sex (male/female)	7/5	4/8	9/9
Specific IgE, kU/l			
HDM	<0.35	47±3.8	0
Weeds	<0.35	0	1.8±0.2
Animal danders	<0.35	0	3.4±0.4
Cockroach	<0.35	0	5.9±0.8
T5SS	0	9.5±1.69	7.56±1.92
Allergen positive/subjects tested	0/12	12/12	18/18

Data are presented as mean ± standard deviation. AR, allergic rhinitis; HDM, house dust mite; IgE, immunoglobulin E; T5SS, Total 5 Symptom Score.

cells, which express the aforementioned markers. Secondly, lineage-negative (Lin⁻) cells were further sorted following staining with Alexa Fluor 647-conjugated prostaglandin D2 receptor (CRTH2/CD294) and phycoerythrin-Cy7-conjugated interleukin-7 receptor subunit α (CD127; both from BD Biosciences). ILC2s were defined by their Lin⁻CRTH2⁺CD127⁺ expression (Fig. 1). In each analysis, at least 20,000 events were collected in order to conduct an analysis of the data. Flow cytometry was conducted on a FACScan flow cytometer (BD Biosciences). The number of ILC2s was expressed as a percentage of PBMCs. Data were analyzed with CellQuest software (CellQuest Pro 5.2.1; BD Biosciences).

Statistical analysis. All data are presented as the mean ± standard deviation. Analysis of variance followed by Tukey's post hoc test was used to compare ILC2 data among the HDM-AR, non-HDM-AR and HCs groups. The association between ILC2 levels and T5SS scores was examined using Spearman's correlation analysis. SPSS v.19.0 statistical software (IBM Corp.) was used for statistical analysis, and GraphPad Prism software (v.5.00; GraphPad Software, Inc.) was used to generate the figures. P<0.05 was considered to indicate a statistically significant difference.

Results

Clinical characteristics of the patients. In the HDM-AR group (n=12), the patients were AR were only allergic to HDM. In the non-HDM-AR group (n=18), 6 patients were monosensitized to weeds, 6 patients were monosensitized to animal danders, and 6 patients were monosensitized to cockroach. In the HCs group (n=12), all subjects had no symptoms of AR or other allergic disease. Their T5SS scores were not detectable. All the subjects with AR expressed high specific IgE in the plasma and had a high T5SS. As indicated in Table I, there were no significant differences

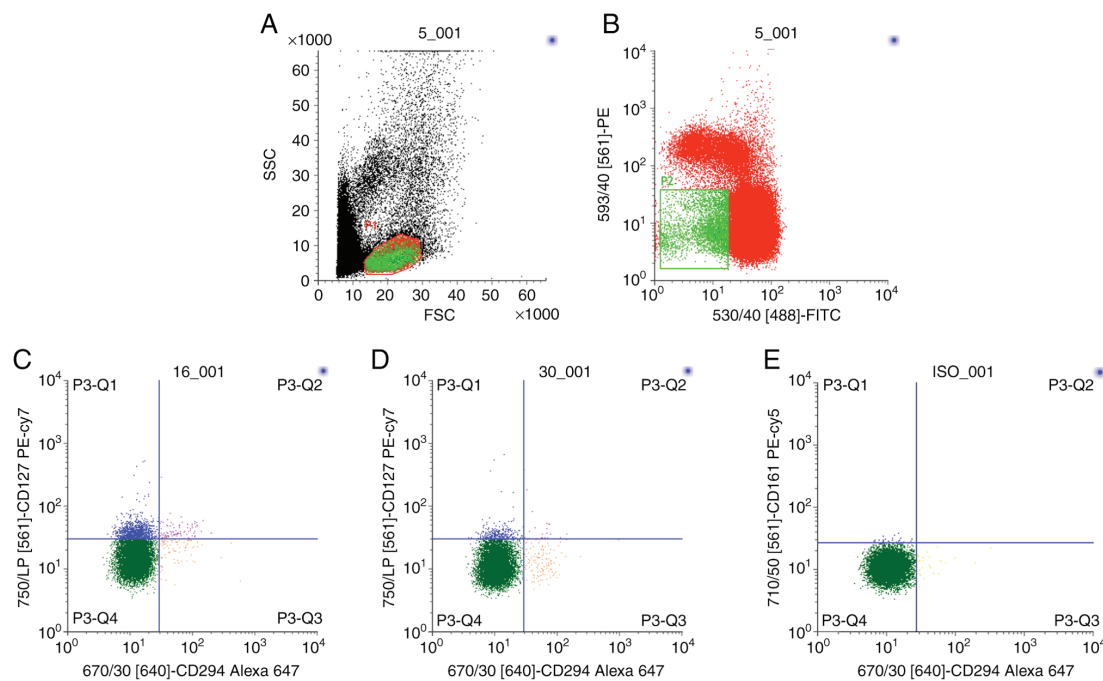


Figure 1. Sequential gating to determine ILC2s frequencies in lymphocyte fractions of HCs (n=12), patients with HDM-AR (n=12) and non-HDM-AR (n=18). (A) Lymphocytes were isolated from whole peripheral blood mononuclear cells, and (B) Lin⁻ cells were gated and further assessed for co-expression of CD127 and CD294 (CRTH2). ILC2s were identified as Lin⁻CRTH2⁺CD127⁺ lymphocytes. (C) ILC2s frequencies in the HDM-AR and non-HDM-AR groups. (D) Expression of ILC2s frequencies in the HC group. (E) Isotype control staining. The numbers in the figures indicate the percentages of ILC2s in Lin⁻ cells. ILC2s, type 2 innate lymphoid cells; HCs, healthy controls; HDM, house dust mite; AR, allergic rhinitis; Lin⁻, lineage-negative; SSC, side scatter; FSC, forward scatter; CD127, interleukin-7 receptor subunit α ; CD294, prostaglandin D2 receptor; PE, phycoerythrin; FITC, fluorescein isothiocyanate.

in age or the sex ratio among the three groups. However, significant differences appeared in the specific IgE levels and T5SS between the HDM-AR, non-HDM-AR and HCs groups.

Differences in ILC2s frequencies between HDM-AR, non-HDM-AR and HC subjects. PBMCs were resuspended into single cell suspension for flow cytometry. Lin⁻ cells that co-expressed CRTH2 and CD127 were defined as ILC2s (Fig. 1). HCs exhibited rare ILC2s expression on PBMCs ($0.01 \pm 0.008\%$; Fig. 2). However, in the HDM-AR ($0.08 \pm 0.018\%$; Fig. 2) and non-HDM-AR groups ($0.06 \pm 0.013\%$; Fig. 2), ILC2s percentages were significantly increased when compared to the HC group ($P < 0.01$). Furthermore, subgroup analysis revealed that subjects with HDM-AR exhibited a higher level of ILC2s compared with those with non-HDM-AR (Fig. 2; $P < 0.01$). In addition, there were no significant differences among the three non-HDM-AR patient subgroups ($P > 0.05$, data not shown).

Circulating ILC2 level is positively correlated with clinical parameters. The correlation between ILC2 levels and clinical parameters was further analyzed. There was a positive linear correlation between the proportion of ILC2s in PBMCs in the HDM-AR group ($R^2 = 0.91$; $P < 0.01$) and the T5SS score (Fig. 3A). Similarly, a positive correlation between the percentages of circulating ILC2s and the T5SS score was observed in patients with non-HDM-AR (Fig. 3B; $R^2 = 0.86$; $P < 0.01$).

Discussion

In the present study, it was demonstrated that blood ILC2s levels were significantly increased in pediatric patients with

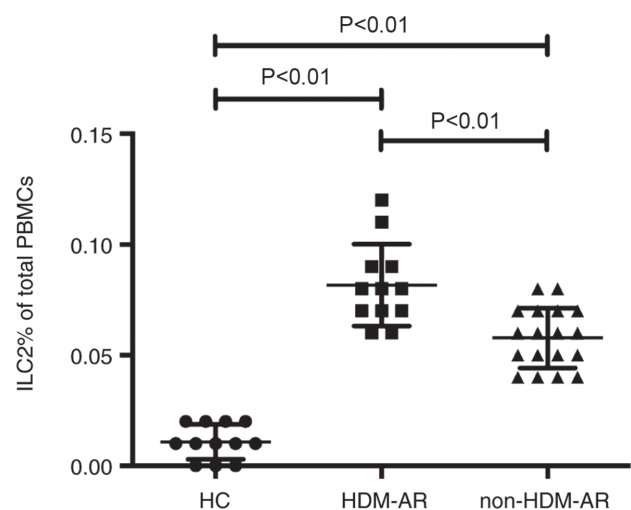


Figure 2. ILC2 levels in peripheral blood mononuclear cells are significantly increased in HDM-AR (n=12) and non-HDM-AR (n=18) groups compared with the HCs (n=12). A significant difference also was identified between patients with HDM-AR and non-HDM-AR. Error bars represent the standard deviation. ILC2, type 2 innate lymphoid cells; HDM, house dust mite; AR, allergic rhinitis; HCs, healthy controls.

AR compared with those in the HCs. Furthermore, there was a positive correlation between ILC2 levels and T5SS. These data suggest that ILC2s may serve a significant role in AR.

Allergic rhinitis is caused by IgE-mediated and type 2 inflammatory responses to inhaled allergens, which results in nasal symptoms that include watery nasal discharge, nasal obstruction, sneezing and itching in the nose (13,14). The

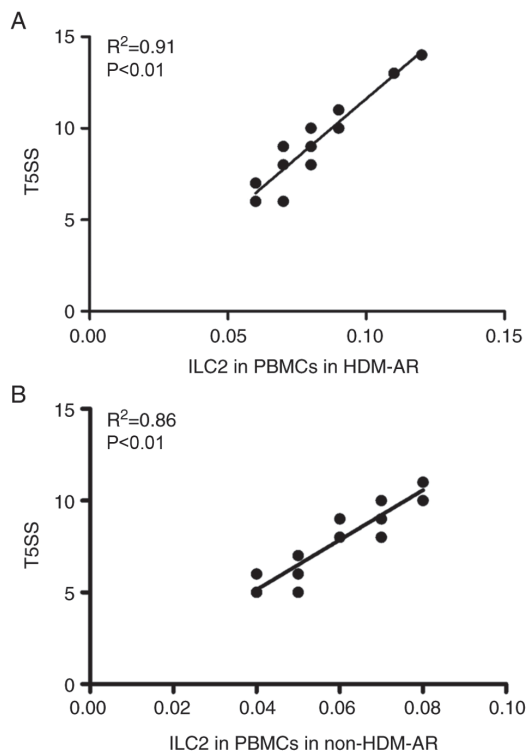


Figure 3. Patients' symptom severity (T5SS) was correlated with ILC2 levels. (A) Positive Spearman's correlation between ILC2s level in PBMCs and T5SS in the HDM-AR group. (B) Positive Spearman's correlation between ILC2s level in PBMCs and T5SS in the non-HDM-AR group. T5SS, Total 5 Symptom Score; ILC2, type 2 innate lymphoid cells; PBMCs, peripheral blood mononuclear cells; HDM, house dust mite; AR, allergic rhinitis.

pathogenesis of AR is not fully clear. ILC2s are a newly recognized subset of the innate lymphoid cell family and have been gaining attention in AR research. To the best of our knowledge, the first study to verify the association between ILC2s and AR indicated that the percentage of ILC2s in the peripheral blood of patients with cat-sensitized AR increased significantly after nasal cat allergen challenge, relative to the control challenge (9). Subsequently, Lao *et al* (15) identified that ILC2s levels were elevated in patients with grass pollen-sensitized AR during the pollen season compared with the control group, and that the ILC2 levels were decreased following subcutaneous immunotherapy. A recent study identified that ILC2s levels were significantly increased in patients with AR who were monosensitized to HDM compared with the HCs (16). In addition, elevated ILC2s levels were detected in patients with other allergic airway diseases, including asthma, chronic rhinosinusitis and aspirin exacerbated respiratory disease (17-23). Consistent with these results, the present study identified that blood ILC2s levels were significantly increased in pediatric patients with AR compared with the HCs. ILC2s respond to IL-25, IL-33 and leukotrienes to promote features of allergic airway diseases via the production of Th2 type cytokines IL-4, IL-5 and IL-13 (24-28).

However, a different previous study demonstrated that there were neither enhanced type 2 responses nor increased ILC2 levels in the peripheral blood in patients with AR outside of the allergy season (8). Fan *et al* (10) demonstrated that the ILC2s level of patients with monosensitized mugwort-AR and HCs were similar, while the percentage of ILC2s in patients

with HDM-AR was significantly increased compared with those in the other two groups (10). The results of the present study were inconsistent with these aforementioned data. The present study identified that pediatric patients with AR may have significantly increased levels of blood ILC2s compared with the HCs, irrespective of the type of allergen. In addition, a subgroup analysis of patients with AR indicated that the proportion of ILC2s in HDM-AR was significantly increased compared with that in non-HDM AR. Another previous study indicated that the frequencies of ILC2s were elevated in seasonal Timothy grass (*Phleum pratense*)-sensitized AR subjects, 66.7% of whom were polysensitized to HDM allergen (14). Miao *et al* (29) has suggested that during and outside mugwort pollen season, an increased level of circulating ILC2s was detected in patients with asthma monosensitized to mugwort or HDM compared with the HCs (29). These data suggest that there is different immunogenicity between dust mites and other allergens such as mugwort pollen. Mugwort is one of the most common pollen allergens in China (30,31). Allergic immune responses to the major mugwort pollen allergen Art, which belongs to the pectate lyase allergen family, are characterized by IgE binding and T-cell proliferation (32-34). By contrast, house dust mites (HDM) are a perennial allergen present globally. HDM and their fecal particles contain several trypsin/chymotrypsin-like enzymes that may directly lead to tissue damage and increase the passage of allergens through the epithelial barrier. These effects may additionally enhance allergic reactions through different pathways, including an increase in the release of IL-33 by epithelial cells (35,36). It has been confirmed that IL-33 has a strong stimulating effect on the activation and migration of ILC2s *in vitro*, and promotes the aggregation of ILC2s in the airway during the initiation phase of Th2 immunity induced by HDM (37). Wang *et al* (38) has demonstrated that *Dermatophagoides farinae*-31, a type of HDM, upregulated the levels of IL-33 in epithelial cells via the activation of Toll-like receptor 2, which induced eosinophil-like airway allergy and increased the number of lung-resident ILC2s (38).

The present study also observed a correlation between peripheral ILC2s levels and disease severity in patients with AR. The results demonstrated that the high expression of peripheral ILC2s was positively correlated with T5SS scores, representing the severity of the clinical symptoms. These results were consistent with previous studies: Compared with mild atopic asthma and the HCs group, the number of activated ILC2s in peripheral blood and sputum of patients with severe asthma was increased significantly (39). In addition, recent studies have demonstrated that peripheral ILC2s are significantly increased in patients with AR sensitized to HDM, and that there is a positive correlation between ILC2 levels and VAS scores (16). These data further demonstrate the close association between ILC2s and allergic inflammation.

In summary, the present study demonstrated that blood ILC2s levels were significantly increased in pediatric patients with AR, irrespective of the type of allergen. In addition, the proportion of ILC2s in HDM-AR was significantly increased compared with in non-HDM AR. Furthermore, there was a positive correlation between ILC2 levels and the severity of the clinical symptoms. These data indicate that ILC2s may serve an important role in the pathogenesis

of AR, and therefore may represent a potential therapeutic target for AR.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

RS and XT made substantial contributions to the experimental design, analysis of experimental results and selection of subjects. YY and ZG were responsible for the collection of clinical specimens and separation of PBMCs. QH and PW were responsible for the flow cytometry to determine the expression levels of ILC2s. All the authors have read and approved the final version of this manuscript.

Ethics approval and consent to participate

The present study was approved by and performed in accordance with the local regulations of the Ethical Committee of Chongqing Medical University (ethics approval no. 038/2014) and the Declaration of Helsinki. Informed consent was acquired from all subjects' legal guardians prior to enrolment in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors confirm that they have no competing interests.

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