

Increased Group 2 Innate Lymphoid Cells Are Correlated with Eosinophilic Granulocytes in Patients with Allergic Airway Inflammation

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Keywords

Group 2 innate lymphoid cells · Allergic airway disease · Eosinophils · Asthma control

Abstract

The T helper 2 (Th2)-type response was considered the hypostasis of allergic airway diseases, including asthma and allergic rhinitis (AR). However, more recent studies have suggested that allergic airway inflammation also depends on innate immunity and is closely related to group 2 innate lymphoid cells (ILC2s). This study evaluated the ILC2 levels of asthma subjects, patients with asthma and AR, and healthy individuals, regarding how to investigate the relationship between clinical data and ILC2 levels. It was found that asthma patients and asthma with AR patients had higher ILC2 levels compared to healthy subjects. ILC2s were positively correlated with the percentage of eosinophils in patients with asthma and AR, but not with pulmonary function. ILC2 levels were higher in mild asthma subjects than in patients with severe asthma. This study provides a new interpretation of the pathogenesis of allergic airway inflammation and may provide a new direction for the diagnosis and assessment of allergic airway diseases.

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Introduction

Allergic airway diseases, including asthma and allergic rhinitis (AR), have become a global problem that not only affects patients' physical wellbeing, thus lowering their quality of life, but also increases economic burdens [1, 2]. Traditionally, the T helper 2 (Th2)-type response of CD4⁺ T cells was considered the hypostasis of these allergic airway diseases [3]. However, more recent studies have suggested that allergic airway inflammation also depends on innate immunity. Recent studies have shown that group 2 innate lymphoid cells (ILC2s) contribute to allergen-induced airway hyperresponsiveness and Th2-type responses [4]. Most of the research focusing on ILC2s have come from mouse studies, and only limited information is available for human patients with allergic airway inflammatory diseases [5–9]. Human ILC2s are characterized by a lack of T and B cells, or natural killer T cell surface markers, but do express CCR2⁺ and CD127 (IL-7Ra), etc. [5]. Human ILC2s produce large amounts

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of Th2 cytokines, including interleukin (IL)-5 and IL-13 after being induced by IL-25, IL-33, and/or thymic stromal lymphopoietin [6–8]. The secretion of IL-5 by ILC2s leads to the recruitment and activation of eosinophils. ILC2s secrete type 2 cytokines in response to eosinophils in order to orchestrate type 2 immunity and its associated pathologies [10].

So far, it has been found that innate type 2 immune responses are increased in asthma subjects [6]. AR patients sensitized to house dust mite (HDM) [11] or mugwort allergen have distinct ILC2 frequencies [9]. However, the level of ILC2s in the population with asthma and AR has not been considered. There is a critical network of feedback and feed-forward interactions between epithelial cells and ILC2s, which is involved in maintaining chronic asthma in these patients [8]. Indeed, ILC2s play an important role in the human clinical immunopathology of allergic diseases [6–9, 12–17]. For instance, there are high levels of ILC2s in human eosinophilic chronic rhinosinusitis with nasal polyps [12, 15]. ILC2s are raised in severe asthma patients who have persistent airway eosinophilia [17]. However, detailed clinical data analyses have not been sufficiently employed. The relationship between ILC2s and the clinical characteristics of asthma remains obscure, as well as whether ILC2s have a relationship with the severity of diseases.

Here, a prospective study was performed to evaluate the ILC2 levels of subjects with asthma, patients with asthma and AR, and healthy individuals, and to clarify the relationship between the severity of disease and the ILC2 levels. Detailed clinical data, including the fraction of exhaled nitric oxide (FeNO), blood routine indexes, and pulmonary function, were also collected and compared. An asthma control test (ACT) was used to measure the state of the disease.

Materials and Methods

Study Subjects

Patients with asthma and those with asthma and AR were recruited from The First Affiliated Hospital, Sun Yat-Sen University, China. Written informed consent was obtained from all subjects. The study protocol was approved by the Ethics Committee of The First Affiliated Hospital, Sun Yat-Sen University (approval No. 2015-48).

Patients newly diagnosed with asthma were recruited according to the Global Strategy for Asthma Management and Prevention (GINA) [1]. The specific inclusion criteria were a history of respiratory symptoms and a positive bronchial provocation test or bronchial dilation test. In addition, asthma with AR patients were also recruited according to the Allergic Rhinitis and its Impact on Asthma (ARIA) [2] criteria, which includes a history of nasal

symptoms and positive specific immunoglobulin E (SIgE). Individuals with immunodeficiency or cystic fibrosis, who were pregnant, or had used systemic glucocorticoids in the past 4 weeks were excluded.

Clinical Data

As shown in Table 1, 75 volunteers, including 15 healthy controls, 30 asthma patients, and 30 asthma and AR patients were evaluated for pulmonary function and underwent a routine blood and serum SIgE test. FeNO as a biomarker for eosinophilic airway inflammation was measured using a chemiluminescence analyzer. Volunteers in the 2 patient groups also completed the ACT and Asthma Control Questionnaire 7 (ACQ-7) evaluation.

Sample Collection and Flow Cytometry Analysis for ILC2s

Peripheral blood mononuclear cells (PBMCs) were isolated from 75 participants using centrifugation through a Ficoll-sodium metrizoate solution (MP Biomedicals LLC). PBMCs were surface labelled for 30 min at 4 °C immediately with the following antibodies for ILC2s: fluorescein isothiocyanate-conjugated lineage cocktail (consisting of CD2, CD3, CD14, CD16, CD19, CD56, CD235a, and FcεR1; eBioscience, San Diego, CA, USA); phycoerythrin-conjugated CRTH2 (BD Bioscience, Franklin Lakes, NJ, USA), and phycoerythrin-Cy7-conjugated CD127 (BD Bioscience). Cells were resuspended in PBS and analyzed within 6 h after blood collection using a Beckman flow cytometer machine (Beckman Coulter, Hercules, CA, USA). ILC2s were identified as lineage FcεR1–CRTH2+CD127+ lymphocytes.

Statistical Analyses

SPSS10.0 software was used for the data analysis. The data are presented as the mean ± SEM. Statistical analyses were performed using nonparametric tests (Kruskal-Wallis test) and Pearson correlation analysis. A *p* value <0.05 was considered statistically significant.

Results

Baseline Characteristics of the Experimental Groups

The detailed clinical baseline characteristics of all participants are summarized in Table 1. There was no significant difference in age, gender, or smoking status among the 3 groups, and no significant difference in the ACT and ACQ-7 scores between the 2 patient groups. However, SIgE levels, eosinophils percentages, and FeNO levels among the 3 groups were significantly different, even between the asthma and asthma with AR groups. In addition, we found that only 23.3% of asthma patients were SIgE positive compared with 100% of the asthma with AR patients (Table 1).

The FeNO levels (ppb) and eosinophils percentages were compared among the 3 groups (Fig. 1). FeNO has been introduced as one of the most practical tools for the diagnosis and management of bronchial asthma [18].

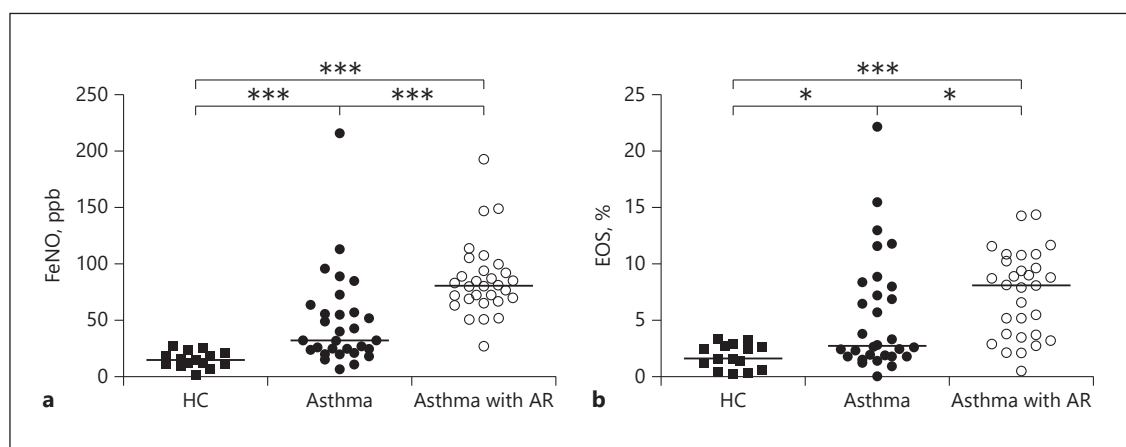


Fig. 1. The relation between FeNO levels (ppb; **a**) and eosinophil (EOS) percentages (**b**) in asthma patients ($n = 30$), asthma with AR patients ($n = 30$), and healthy controls ($n = 15$). Each symbol represents 1 patient; horizontal bars indicate the median for each group. Nonparametric test: * $p < 0.05$, *** $p < 0.001$.

Table 1. Summarized baseline characteristics of all volunteers involved in the study

Characteristic	Controls ($n = 15$)	Asthma ($n = 30$)	Asthma with AR ($n = 30$)	p value
Age, years	34.3 $\pm$ 2.2	38.8 $\pm$ 2.6	36.6 $\pm$ 2.6	0.55
Female/Male	10/5	21/9	21/9	0.97
Ever smoked, %	0	6.7	3.3	0.55
Specific IgE positive/subjects tested, %	0	23.3	100	<0.0001
Blood eosinophils, %	1.8 $\pm$ 0.3	5.4 $\pm$ 0.9	7.4 $\pm$ 0.7	<0.0001
FEV ₁ , % predicted before bronchodilation	107.7 $\pm$ 1.6	86.4 $\pm$ 3.7	80.8 $\pm$ 3.3	<0.0001
FEV ₁ /FVC ratio, %	91.9 $\pm$ 1.8	77.7 $\pm$ 2.1	71.4 $\pm$ 1.9	<0.0001
FeNO, ppb	15.5 $\pm$ 1.8	48.3 $\pm$ 7.6	86.0 $\pm$ 6.0	<0.0001
ACT	–	16.6 $\pm$ 0.7	16.4 $\pm$ 0.8	0.99
ACQ-7	–	1.5 $\pm$ 0.2	1.4 $\pm$ 0.1	0.78

ACT, asthma control test; ACQ-7, asthma control questionnaire 7; FeNO, fractional exhaled nitric oxide; FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity.

Both asthma patients (48.27 ± 7.59 ppb) and asthma with AR patients (86.00 ± 5.95 ppb) showed significantly higher levels of FeNO than the healthy control group (15.47 ± 1.82 ppb, $p < 0.0001$; Fig. 1a). FeNO levels in the asthma with AR group were even higher compared with the asthma group ($p < 0.0001$; Fig. 1a).

Eosinophil percentages also showed higher levels in the patient groups. Significantly lower eosinophil percentages in the healthy control group ($1.81 \pm 0.28\%$) were detected compared with those in the asthma group (5.41 ± 0.94 , $p < 0.05$) and asthma with AR group (7.35 ± 0.69 ,

$p < 0.001$). Also, the eosinophil percentage was higher in the asthma and AR patients compared with the asthma-only patients ($p < 0.05$; Fig. 1b).

Increased ILC2s in Asthma Patients

The gating strategy is shown in Figure 2a and online supplementary Figure S1 (for all online suppl. material, see www.karger.com/doi/10.1159/000488050). The cluster that coexpressed CD127 and CRTH2 in the lineage-negative population from PBMCs was the target ILC2s. The percentage of ILC2s in PBMCs was signifi-

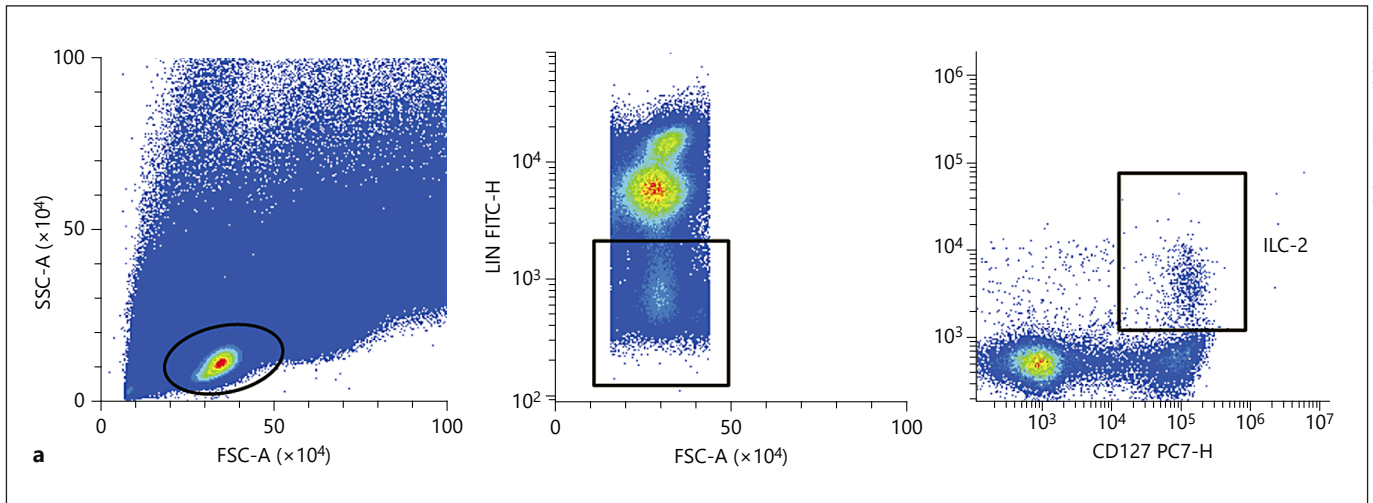
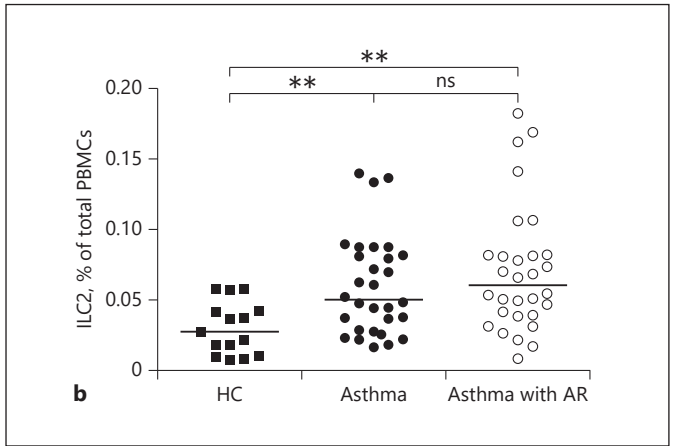


Fig. 2. ILC2s were increased in asthma patients. **a** ILC2s were identified as lineage FcεR1–CRTH2+CD127+ lymphocytes. Lymphocyte-sized human PBMCs were flow sorted. Lineage-negative (CD2–, CD3–, CD14–, CD16–, CD19–, CD56–, CD235a–) FcεR1–cells were gated and further assessed for coexpression of CD127 and CRTH2. **b** ILC2 levels in PBMCs of the blood of asthma patients ($n = 30$), asthma with AR patients ($n = 30$), and healthy controls ($n = 15$). Each symbol represents 1 patient; horizontal bars indicate the median for each group. Nonparametric test: ** $p < 0.01$.



cantly increased in the asthma patient group ($0.060 \pm 0.096\%$) compared with the healthy control group ($0.030 \pm 0.005\%$, $p < 0.01$; Fig. 2b), which was consistent with previous discoveries [6]. Bartemes et al. [6] reported that ILC2 levels were not elevated in a group of American AR patients compared with healthy controls. We recently reported that the percentage of ILC2s in PBMCs in the AR group sensitized to HDM was significantly higher compared with a healthy control group [11]. However, the level of ILC2s in patients with asthma and AR remained unclear. In this study, the percentage of ILC2s in PBMCs was significantly increased in the asthma with AR group ($0.071 \pm 0.008\%$) compared with the healthy controls ($p < 0.01$; Fig 2b). However, there was no significant difference in ILC2 levels between the 2 patient groups (Fig. 2b).

Correlation between ILC2 Levels and Eosinophil Percentage in Asthma with AR Patients

We conducted a correlation analysis to investigate whether there was a relationship between ILC2 levels and the eosinophil percentage. The percentage of eosinophils was correlated with ILC2 levels in asthma with AR patients ($p = 0.001$), but not in asthma patients (Fig. 3a, b). This was consistent with our above data showing that asthma with AR patients had higher eosinophil levels compared to asthma-only patients.

ILC2 Levels and Pulmonary Function

The pulmonary ventilation function in asthma patients is generally assessed by FEV₁. To investigate the relationship between the pulmonary ventilation function and ILC2s, the asthma patients and asthma with AR patients were classified based on FEV₁. Overall, 21 asthma patients, 22 asthma with AR patients, and all 15 of the

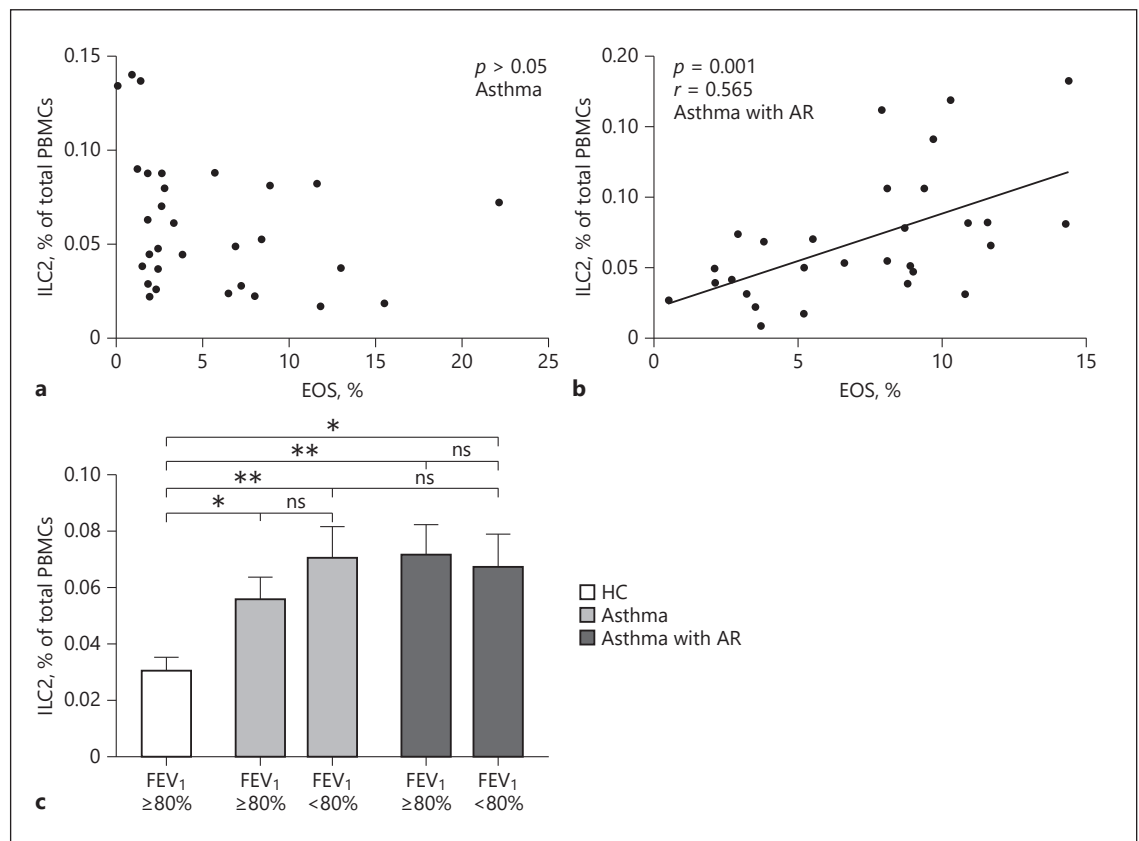


Fig. 3. The connection between ILC2 levels and pulmonary function. **a** Eosinophil percentages in peripheral blood were not correlated with ILC2 levels in asthma patients ($n = 30$). **b** Eosinophil percentages in peripheral blood were correlated with ILC2 levels in asthma with AR patients ($n = 30$). Each dot represents 1 patient.

c Statistical analysis of ILC2 levels in PBMCs for groups with $FEV_1 < 80\%$ (asthma, $n = 9$; asthma with AR, $n = 8$) and $FEV_1 \geq 80\%$ (healthy controls, $n = 15$; asthma, $n = 21$; asthma with AR, $n = 22$). Data are shown as the mean $\pm$ SEM. Nonparametric test: * $p < 0.05$, ** $p < 0.01$.

healthy controls had $FEV_1 \geq 80\%$, while 9 asthma patients and 8 asthma with AR patients had $FEV_1 < 80\%$. ILC2 percentages in PBMCs from the 3 groups were first compared in subjects with $FEV_1 \geq 80\%$. Asthma patients ($0.056 \pm 0.008\%$) and asthma with AR patients ($0.072 \pm 0.010\%$) showed significantly higher ILC2 levels than the healthy controls ($0.030 \pm 0.005\%$). In addition, ILC2 levels in asthma patients ($0.070 \pm 0.011\%$) and asthma with AR patients ($0.067 \pm 0.012\%$) with $FEV_1 < 80\%$ were also increased compared to the healthy controls ($p < 0.05$; Fig. 3c). However, there was no difference of ILC2 levels between the conditions of $FEV_1 \geq 80\%$ and $FEV_1 < 80\%$ in each patient group (Fig. 3c). Although weakened pulmonary ventilation function was a characteristic of asthma, no direct connection was observed between ILC2 levels and FEV_1 .

The Correlation of ILC2 Levels and the Severity of Asthma

The ACT is a validated assessment of asthma control or asthma severity which consists of 5 questions assessing the interference with activity, shortness of breath, nocturnal symptoms, rescue medication use, and self-rating of asthma control [19]. Because of the high sensitivity and specificity, and good correlation with expert assessment and pulmonary function tests, ACT was used to assess the newly diagnosed asthma patients. The ACQ-7 has 7 questions (the top-scoring 5 symptoms, FEV_1 % predicted, and daily rescue bronchodilator use). Patients are asked to recall how their asthma has been during the previous week and to respond to the symptom and bronchodilator use questions on a 7-point scale (0 = no impairment, 6 = maximum impairment). The ACQ-7 has strong measurement properties and has been fully validated for use in

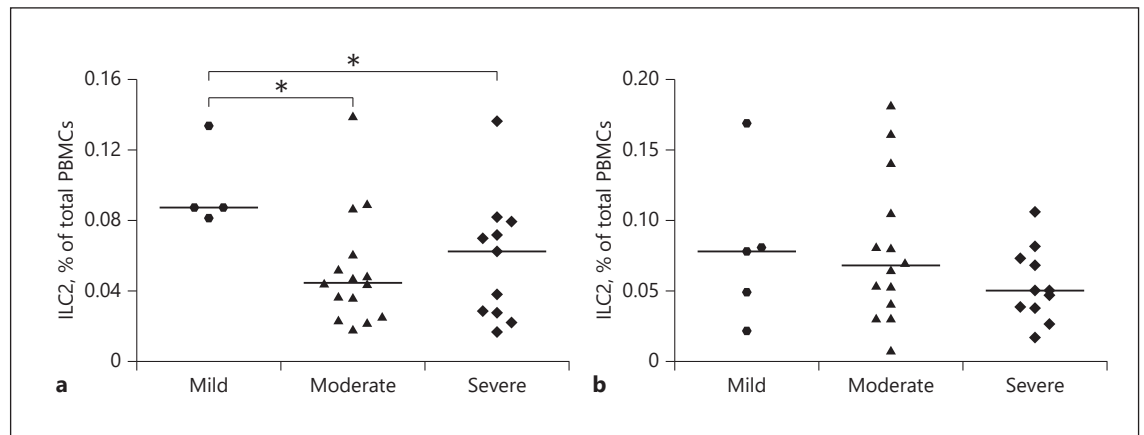


Fig. 4. The correlation of ILC2 levels and the severity of asthma. **a** ILC2 levels in PBMCs of the blood of mild ($n = 4$), moderate ($n = 15$), and severe ($n = 11$) asthma patients. **b** ILC2 levels in PBMCs of the blood of mild ($n = 5$), moderate ($n = 14$), and severe ($n = 11$) asthma with AR patients. Each symbol represents 1 patient; horizontal bars indicate the median for each group. Nonparametric test: * $p < 0.05$.

Table 2. Statistical FEV₁ (% predicted before bronchodilation) of the patients enrolled in this study

	Asthma patients	Asthma with AR patients
Mild	89.36±0.07	87.50±0.02
Moderate	87.17±0.09	81.38±0.04
Severe	84.08±0.04	74.05±0.08

both clinical practice and clinical trials. We found that there was a significant negative correlation between ACT and ACQ-7 scores in both groups of patients (online suppl. Fig S2). Therefore, to investigate whether ILC2s were associated with the severity of asthma, we divided the asthma control condition into 3 groups according to the ACT score: severe (<15), moderate (16–20), and mild (>21). FEV₁ was $84.08 \pm 0.04\%$ for severe asthma patients and $74.05 \pm 0.08\%$ for patients with asthma and AR patients (Table 2), suggesting not much severity for the patients in our study. For the 2 patient groups in this study, most subjects belonged to the moderate group, but the highest ILC2 levels were observed in the mild group of asthma patients (Fig. 4). In detail, asthma patients in the mild group ($n = 4$, $0.098 \pm 0.012\%$) had significantly increased ILC2s compared with those in the moderate ($n = 15$, $0.052 \pm 0.008\%$, $p < 0.05$) or severe ($n = 11$, 0.058 ± 0.011 , $p < 0.05$) groups (Fig. 4a). For asthma with AR patients, there was no significant difference of ILC2s between these 3 groups (Fig. 4b).

Discussion

In this prospective study, ILC2 levels in healthy individuals, asthmatic patients, and subjects with both asthma and AR were evaluated by flow cytometry. The ILC2 levels were significantly increased in the patient groups compared with the control group. The link between ILC2 levels and other clinical items, including FeNO level, eosinophil percentage, and pulmonary function, was also explored among the 3 groups. It was found that ILC2s were positively correlated with eosinophil percentage but not with pulmonary function. Also, ILC2 levels were inversely related to asthma severity.

Elevated levels of ILC2s in PBMCs of seasonal AR patients during the grass pollen season have been reported, but not outside of this season [20]. An influence on ILC2 levels has also been shown in patients sensitized to mugwort allergen [9]. However, it was also reported that ILC2s were increased in asthma but not in AR patients [6]. We found that AR patients sensitized to HDM had higher ILC2 levels than healthy controls [11]. In this study, ILC2 levels in the control and patient groups were consistent with previous findings [6–8, 14, 17]. One of the major findings in this study was the clear difference in innate type 2 responses between healthy participants and patients with asthma and AR. Patients with asthma and AR had higher eosinophil percentages and FeNO levels than the asthma-only patients and healthy controls. Although the differences between the 2 patient groups were not statistically significant, ILC2 levels in patients with

asthma and AR were slightly higher than in the asthma-only patients. It was found that human ILC2s were able to produce antigen-independent type 2 cytokines, which are induced by IL-33 or IL-25 [6]. However, the link between clinical indicators and ILC2s in asthma patients had not previously been established.

Asthma-related clinical indicators were collected from all participants in this study. The asthma patients and asthma with AR patients who were recruited were newly diagnosed and had not received any treatments. The representative data, including eosinophil percentages, FeNO levels, and severity of asthma, were analyzed comprehensively. Both patient groups had higher eosinophil percentages and FeNO levels than the healthy controls. Although FeNO is widely used as a biomarker of airway inflammation and its level is helpful to diagnose early-stage asthma [2, 3], the ILC2 levels in the patient groups were not related with the FeNO levels in this study.

Patients with both asthma and AR had higher eosinophil and ILC2 levels than patients with asthma alone. This suggests that combined upper and lower airway inflammation has more obvious levels of eosinophils in the peripheral blood. IL-5 and IL-13 secreted by ILC2s play an important role in stimulating the activation and aggregation of eosinophils. Therefore, there may be some correlation between the levels of eosinophils and ILC2 in patients with asthma and AR. The level of ILC2s in peripheral blood is extremely low. Even though the levels of ILC2 in patients with allergic diseases were significantly higher than in the normal control group, they still only represented trace amounts. Therefore, this may be one reason why no significant difference was found in the asthma group considering the numbers and other comparable indicators. On the other hand, the role of ILC2 in allergic inflammation is manifest in the increased levels and, more importantly, in the functional release of type II inflammatory cytokines.

It has been shown that the levels of IL-5-responsive eosinophils are higher in the blood of patients with allergic asthma compared with healthy control subjects [21]. Activated ILC2s secreted a large amount of IL-5, resulting in eosinophil accumulation in human peripheral tissues of nasal polyps [22]. ILC2s were linked with high tissue/blood eosinophilia and had a potential role in the activation/survival of eosinophils [15]. In this study, we found that patients with both asthma and AR had a higher percentage of eosinophils compared to asthma patients. More importantly, we identified that there was a positive correlation between ILC2 levels and eosinophil percentage in patients with asthma and AR, but not with the asthma-only patients.

This suggests that ILC2 levels in allergic diseases may have more correlation with eosinophil levels.

In our study, we found that there were higher levels of ILC2s in the mild asthmatic patients compared to severe patients, but not in patients with asthma and AR. The finding that a significantly greater number of ILC2s was detected in blood and sputum samples from patients with severe eosinophilic asthma compared with those from patients with mild atopic asthma was inconsistent with the study by Smith et al. [17]. However, our study was quite different to theirs. All of the patients enrolled in our study were newly diagnosed, with FEV₁ levels of $84.08 \pm 0.04\%$ for severe asthma patients and $74.05 \pm 0.08\%$ for the asthma and AR patients. More importantly, the patients in our study had no medical history. However, in the study by Smith et al. [17], FEV₁ measured in patients with severe asthma was $56.8 \pm 16.3\%$, which is much lower than in our group. This suggests that the asthmatic patients in their study had a more severe condition than the patients in our study. Furthermore, the patients with severe asthma and even mild asthma in that study had received medical treatment over a long period of time [17], which is quite different from the newly diagnosed patients in our study. Additionally, there were some differences in the evaluation of the severity of asthma between the 2 studies. Severe asthma was defined by sputum eosinophilia and level of pulmonary function in their study [17]. In our study, the severity of the patients was determined by ACT, and the ACT and ACQ-7 scores showed good consistency. This may be another reason for the difference between the 2 studies. Christianson et al. [8] demonstrated that ILC2 levels in bronchoalveolar lavage were significantly decreased in patients with controlled asthma compared with asthmatic patients; both of these groups had a history of medication. Previous studies showed that non-Th2 asthma, like adult-onset neutrophilic asthma, generally has a severely obstructed and reduced connection with eosinophils [23]. It is possible that a non-Th2 type of asthma, such as neutrophilic asthma, was present among our severe asthma patients. This might explain why the severe asthma patients had a low level of ILC2s. Of course, the inconsistent results and the possible reasons should be carefully considered in the future.

Generally speaking, ILC2s were increased in our asthma patients as well as in those with both asthma and AR. ILC2s were positively related to eosinophil percentages but not to pulmonary function. In this study, clinical data were systematically analyzed and compared with ILC2 levels. This offers a new interpretation of the pathogene-

sis of allergic airway inflammation and provides a new direction for the diagnosis and assessment of the disease. A potential new target for the treatment of allergic airway disease could possibly be found with further research.

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Statement of Ethics

This research was conducted in accordance with the World Medical Association Declaration of Helsinki. The study protocol was approved by the Ethics Committee of The First Affiliated Hospital, Sun Yat-Sen University (Approval No. 2015-48). Written informed consent was obtained from all participants.

Disclosure Statement

The authors declare that they have no conflicts of interest.

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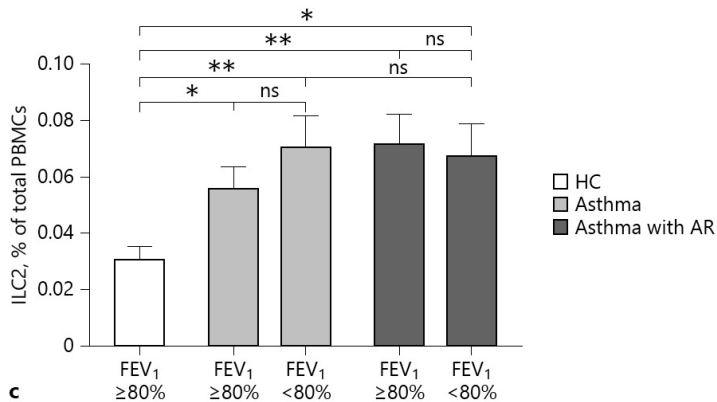
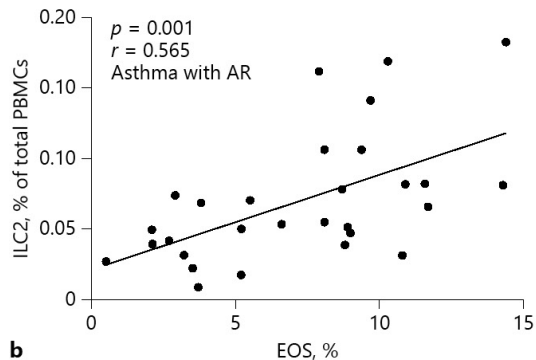
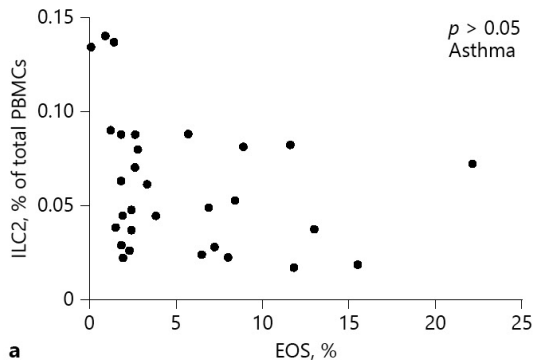
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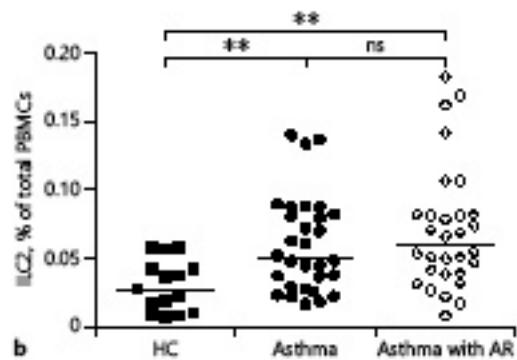
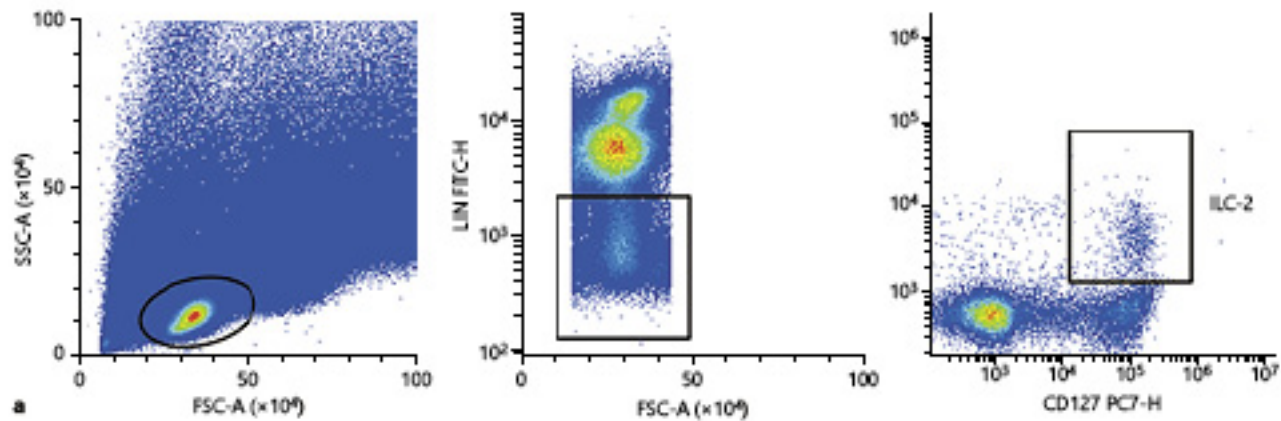
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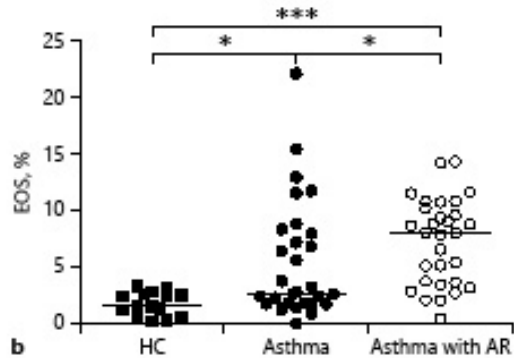
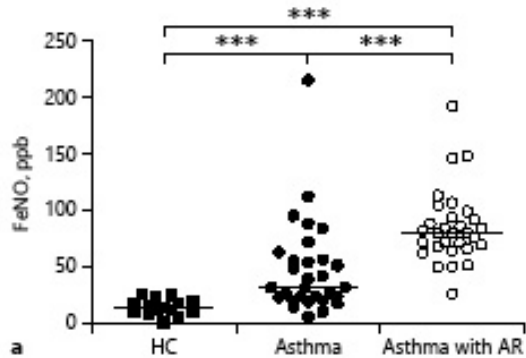
Author Contributions

Q.-N.Y.: collection and/or assembly of data, manuscript preparation and data analysis. W.-P.T.: collection and/or assembly of data. Y.-B.G.: collection and/or assembly of data. X.-L.F.: manuscript preparation and data analysis. Z.-L.Q. and C.-L.L.: data analysis. D.C., Z.B.L., and W.W.: collection of data. Q.-L.F.: concept and design, data analysis, manuscript preparation and final approval of the manuscript. All authors read and approved the final manuscript.

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	Asthma patients	Asthma with AR patients
Mild	89.36±0.07	87.50±0.02
Moderate	87.17±0.09	81.38±0.04
Severe	84.08±0.04	74.05±0.08

Characteristic	Controls (n = 15)	Asthma (n = 30)	Asthma with AR (n = 30)	p value
Age, years	34.3±2.2	38.8±2.6	36.6±2.6	0.55
Female/Male	10/5	21/9	21/9	0.97
Ever smoked, %	0	6.7	3.3	0.55
Specific IgE positive/subjects tested, %	0	23.3	100	<0.0001
Blood eosinophils, %	1.8±0.3	5.4±0.9	7.4±0.7	<0.0001
FEV ₁ , % predicted before bronchodilation	107.7±1.6	86.4±3.7	80.8±3.3	<0.0001
FEV ₁ /FVC ratio, %	91.9±1.8	77.7±2.1	71.4±1.9	<0.0001
FeNO, ppb	15.5±1.8	48.3±7.6	86.0±6.0	<0.0001
ACT	–	16.6±0.7	16.4±0.8	0.99
ACQ-7	–	1.5±0.2	1.4±0.1	0.78

ACT, asthma control test; ACQ-7, asthma control questionnaire 7; FeNO, fractional exhaled nitric oxide; FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity.