



Type 2 innate lymphoid cells: A novel biomarker of eosinophilic airway inflammation in patients with mild to moderate asthma



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ABSTRACT

Background: Eosinophilic airway inflammation can predict the exacerbation of asthma, and we can improve the management of asthma by monitoring the eosinophilic airway inflammation. Although induced sputum and sputum eosinophil count is the gold standard test for diagnosing eosinophilic asthma, a more accessible and receptive method is needed for clinical practice. Type 2 innate lymphoid cells (ILC2) have recently been proposed to play a crucial role in eosinophilic inflammation and have been identified in peripheral blood from patients with asthma.

Objectives: We sought to identify simple and feasible biomarkers which can predict eosinophilic airway inflammation in asthmatic patients.

Methods: Sputum was induced for the assessment of eosinophils in 150 asthmatic patients. In parallel, the proportion of ILC2s of peripheral blood lymphocytes (%ILC2), blood eosinophil counts, total immunoglobulin E (IgE), fractional exhaled nitric oxide (FeNO) and lung function tests were measured. 42 healthy donors served as controls.

Results: 126 patients finished sputum induction and produced adequate sputum. The ILC2 level was significantly increased in eosinophilic asthmatic patients compared with non-eosinophilic asthmatic patients (0.117 ± 0.090 versus 0.035 ± 0.021 , $p < 0.001$). A multiple regression model, including age, sex, BMI, blood eosinophil counts, FeNO, IgE and %ILC2, showed that %ILC2, blood eosinophil counts and FeNO were correlative factors of sputum eosinophil counts ($p < 0.001$, $p = 0.037$, $p < 0.001$, respectively) and % ILC2 was the most significant subset of airway eosinophilic inflammation (Estimate = 11.385). A receiver operating characteristic (ROC) analysis showed a sensitivity of 67.7% and a specificity of 95.3% for %ILC2 of 0.076 to distinguish eosinophilic asthmatic patients from non-eosinophilic asthmatic patients.

Conclusion: ILC2 is a surrogate marker of airway eosinophilic inflammation in patients with mild to moderate asthma and has great potential advantages for selecting the asthmatic patients most likely to benefit from therapeutics targeting Th2 inflammation.

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1. Introduction

Asthma is a genetically complex and heterogeneous disease with various phenotypes [1]. Although certain phenotypes of

asthma have already been recognized for some time, the biomarkers of these different phenotypes have not been identified yet [2]. Petsky et al. found that asthma treatment based on inflammation phenotypes can help to improve the disease outcomes [3]. So, it is useful for us to find out accurate biomarkers of different inflammation phenotypes of asthma.

Eosinophilic asthma often has poor disease control and high risk of exacerbation; however, it responds well to corticosteroid therapy [2]. Induced sputum can be used to identify eosinophilic airway inflammation. Monitoring sputum eosinophils can lead to reduction of asthma exacerbation and improve responsiveness to anti-inflammation therapy. However, sputum induction is time-extensive, laborious and many patients are unable to endure the

Abbreviations: ILC2, type 2 innate lymphoid cell; %ILC2, the proportion of ILC2s of peripheral blood lymphocytes; IgE, total immunoglobulin E; FeNO, fractional exhaled nitric oxide; BMI, body mass index; ROC, receiver operating characteristic; AUC, area under the curve; PPV, positive predictive value; NPV, negative predictive value.

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sputum induction and produce adequate sputum. So, there is a need to discover simpler methods to evaluate airway eosinophilic inflammation. Many other noninvasive markers such as peripheral blood eosinophil counts, FeNO, IgE and serum periostin have been reported as surrogates for sputum eosinophils in asthma, but their diagnostic accuracy to detect eosinophilic inflammation in asthma remains controversial [4,5].

ILC2s have recently been identified as a population of lineage-negative cells (lacking known lineage markers of T, B, NK cells) that produce large amounts of Th2 cytokines [6]. It is reported that ILC2s are responsible for eosinophilic inflammation in humans, such as nasal polyps and atopic dermatitis [7,8]. Kathleen R. Barmes has also found that ILC2s are present in human peripheral blood and are increased in asthma patients [9]. To extend this research and to detect whether peripheral blood ILC2s can be used as a surrogate of eosinophilic asthma, we aimed to compare the mutual relationships of ILC2s, blood eosinophils, FeNO and IgE with sputum eosinophils.

2. Materials and methods

2.1. Subjects

We recruited 150 patients with mild to moderate asthma in the Respiratory Clinic of Qilu Hospital, Shandong University (Jinan, Shandong, China) (2014.05–2015.05). All of the asthmatic patients were required to meet the following eligibility criteria: a history of wheezing, cough, chest, tightness and/or dyspnea, airflow variability with a 12% and 200 ml increase in FEV1 and/or airway hyper-responsiveness (PC20 methacholine <8 mg/ml). All of the subjects were first seen at our hospital and they had not received regulatory treatment previously. None of them used antiasthma medication (oral or inhaled corticosteroids, long-acting or short-acting bronchodilators, leukotriene modifiers, or antihistamines) at least 24 h before enrollment. All the patients were nonsmokers without complications of chronic obstructive pulmonary disease. Patients who had any symptoms of respiratory infection for at least 4 weeks when they are recruited were excluded. 42 healthy donors served as controls.

The research was approved by the Ethics Review Committee for Human Studies at Qilu Hospital, Shandong University, and all the participants gave their written informed consent.

2.2. Study design

Sputum was induced by hypertonic saline. Lung function test, FeNO and blood sampling were performed in the asthmatic patients.

3. Measurements

3.1. Lung function test and FeNO

To assess lung function in subjects, spirometry was performed by using a spirometer (Jaeger Co, Hoechberg, Germany) according to the American Thorax Society and European Respiratory Society (ATS/ERS) recommendations [10]. FeNO concentration was measured with a Niox Mino unit (Aerocrine, Solna, Sweden) based on the Standardization procedure [11,12]. Subjects were instructed to avoid food intake, exercise and smoking at least 1 h before testing. The participants were asked to empty the lungs as much as possible and then inhaled to total lung capacity through a NIOX filter. Expiration was carried out with a constant flow rate of 50 mL/s for at least 6 s. The average levels of at least 3 acceptable measurements were used.

3.2. Sputum induction and processing

Prebronchodilator FEV1 was measured firstly. If FEV1% predicted $\leq 70\%$, we did not process the sputum induction. Before commencing, the patients should inhale 200ug salbutamol. After 10 min, we measured postbronchodilator FEV1. Induced sputum examination was performed by inhalation of 3% hypertonic saline through a small ultrasonic nebulizer (yuwell, Jiangsu, China) at 5-min intervals for 20 min [5,13]. The FEV1 were measured at the end of each induction interval. The induction will be stopped if there is a fall in FEV1 of $\geq 20\%$ compared to the postbronchodilator FEV1 or if symptoms occur. The patients were asked to cough and spit after 5, 10, 15 and 20 min of induction or whenever they get the urge to do so.

We processed the sputum sample within 30 min. The sputum plugs were diluted with an equal volume of 0.1% dithiothreitol (Sigma, MO, USA) for 30 min at 37 °C. Then the dispersed sputum sample was centrifuged at 700g for 10 min, the sputum cell pellet was resuspended in PBS to 1 mL. The cell cytospin was prepared for eosinophils counts after Wright's staining. Eosinophil counts were expressed by the percentage of non-squamous cells, based on 500 non-squamous cells. We choose a sputum eosinophil count of 3% as the cut off for determining eosinophilic or non-eosinophilic asthma [14].

3.3. Markers in peripheral blood

IgE and peripheral blood eosinophil counts were measured by the clinical laboratory of Qilu Hospital, Shandong University (Ji'nan, Shandong, China). Peripheral blood ILC2s (Lin[−]CD127⁺CRTH2⁺ cells) were detected by flow cytometry. Heparinized peripheral blood (150ul) was firstly depleted of red blood cells by adding 1 ml RBC lysis solution (Solarbio, Beijing, China) for 10 min. Then centrifuge cells 10 min at 400g, 4 °C, and discard supernatant. The cell pellet was then diluted in PBS to 400ul and the cell suspension was incubated with an mAb to CD16/CD32/CD64 (eBioscience, CA, USA) for 10 min to block Fc receptors. To detect ILC2s, cells were stained with a FITC-conjugated monoclonal antibodies (mAbs) against human lineage cocktail (CD2, CD3, CD14, CD16, CD19, CD56, CD235a, eBioscience, CA, USA), PE-conjugated antibodies against human CD127 (eBioscience, CA, USA), and APC-conjugated antibodies against human CRTH2 (CD294) (Biolegend, CA, USA) at room temperature in the dark for 30 min. Flow cytometry was performed using a BD FACS machine after the sample was filtered into a single cell suspension. Stained cells were analyzed by flow cytometric analysis using a FAC Scan cytometer equipped with CellQuest software (BD Bioscience, CA, USA). We used the % ILC2 of lymphocytes to express the percentage of ILC2.

3.4. Statistical analysis

SPSS 17.0 software (SPSS, Chicago, IL, USA) was used for data analysis. Sputum eosinophils, blood eosinophils, FeNO, %ILC2 were expressed as the mean $\pm$ SD. Comparison of %ILC2 between eosinophilic asthma and non-eosinophilic asthma was performed using unpaired t-test. The association between %ILC2 and other factors was analyzed using Pearson's correlation test. A multiple regression model was used to evaluate the biomarker variables as correlative factors of airway eosinophilia.

The receiver operating characteristic (ROC) analysis was generated to determine the biomarker that best identified a sputum eosinophil $\geq 3\%$. To detect whether the area under the curve (AUC) of different biomarkers differ significantly, comparisons of ROC curves were performed using Medcalc statistical software (version 12.7.0, MedCalc, Mariakerke, Belgium). The cut-off point, specificity,

sensitivity, positive predictive value (PPV) and negative predictive value (NPV) were calculated.

4. Results

4.1. Baseline characteristics

There were 42 healthy controls and 126 asthmatic patients finally finished our research. The 126 asthmatic patients were divided into two groups by sputum eosinophil counts of $\geq 3\%$ or $< 3\%$ (eosinophilic asthma, $n = 62$; non-eosinophilic asthma, $n = 64$). The patient characteristics are described in Table 1. There were no significant differences between the asthmatic groups in mean age, sex, BMI and FEV1%predicted. However, the levels of sputum eosinophils, FeNO, blood eosinophil counts, IgE and blood ILC2 were higher in eosinophilic asthma group relative to non-eosinophilic asthma group, and the differences were statistically significant.

4.2. The level of peripheral blood %ILC2 was higher in eosinophilic asthmatic patients compared with non-eosinophilic asthmatic patients

We identified ILC2s as lineage-negative lymphocytes that express CRTH2+ and CD127 + cells as previously mentioned in the methods and previously reported. ILC2s are rare population of lymphocytes that comprise 0.01–0.03% of total PBMCs in peripheral blood [9]. In our studies, ILC2s were gated as the Lin-CD127 + CRTH2+ population (Fig. 1, A–C). The asthmatic patients have higher level of ILC2 in peripheral blood relative to healthy controls (0.076 ± 0.077 versus 0.020 ± 0.013 , $p < 0.01$). Moreover, the level of ILC2s (%ILC2 of lymphocytes) was increased in the eosinophilic asthmatic group compared with the non-eosinophilic asthmatic group (0.117 ± 0.090 versus 0.035 ± 0.021 , respectively, $p < 0.01$) (Fig. 1, D). And we also found the %ILC2 positively correlated with sputum eosinophils ($r = 0.624$, $p < 0.01$), peripheral blood eosinophils ($r = 0.617$, $p < 0.01$) and FeNO ($r = 0.336$, $p < 0.01$). In contrast, the %ILC2 negatively correlated with FEV1% predicted ($r = -0.295$, $p = 0.02$) and did not correlate with IgE ($p = 0.88$) (Fig. 2).

4.3. Comparison of ILC2 with blood eosinophil counts, FeNO and IgE as eosinophilic asthma biomarkers

In Table 1, we can find that the eosinophilic asthmatic subjects had higher levels of blood eosinophils, FeNO, IgE and ILC2. To

compare the relationships of blood eosinophils, FeNO, IgE and ILC2 with airway eosinophilia and to select all possible subsets, we applied a multiple regression model incorporating age, sex, BMI, blood eosinophil counts, FeNO, IgE and %ILC2. The analysis revealed that %ILC2, blood eosinophil counts and FeNO were correlative factors of sputum eosinophil counts ($p < 0.001$, $p = 0.037$, $p < 0.001$, respectively) and %ILC2 was the most significant correlative factor of airway eosinophilic inflammation (Estimate = 11.385). (Table 2).

To determine the biomarker that best identified an airway eosinophilic inflammation (a sputum eosinophil counts $\geq 3\%$) and to find the diagnostic criterion, we performed ROC analysis. We found that ILC2 performed favorably to blood eosinophil counts, whereas FeNO and IgE performed less well. ILC2 has an improved combination of sensitivity and specificity to detect eosinophilic asthma. The diagnostic accuracy of %ILC2, described as ROC AUC, was 0.88 ($p < 0.001$, 95%CI 0.810 to 0.931) (Fig. 3). Using $\geq 0.076\%$ ILC2 as a cutoff value, eosinophilic and non-eosinophilic airway inflammation was well differentiated with a sensitivity of 67.7% and a specificity of 95.3% (Table 3). The diagnostic accuracy of blood eosinophils to distinguish eosinophilic inflammation from non-eosinophilic airway inflammation, described as ROC AUC, was 0.84 ($p < 0.001$, 95%CI 0.761 to 0.897) (Fig. 3). There is no significant difference between %ILC2 and blood eosinophils in ROC AUC ($p = 0.254$) (Table 4). Blood eosinophils of $\geq 0.33 \times 10^9/L$ provided a sensitivity of 71% and a specificity of 85.9%. However, the accuracy of FeNO measurement to detect eosinophilic inflammation from non-eosinophilic inflammation, described as ROC AUC, was 0.79 ($p < 0.001$, 95%CI 0.707 to 0.856). This ROC AUC performed less well compared with the ROC AUC of ILC2 ($P = 0.042$) (Table 4). IgE was not able to differentiate eosinophilic inflammation and non-eosinophilic inflammation (ROC AUC = 0.60, $p = 0.054$, 95%CI 0.508 to 0.685).

5. Discussion

The present study showed several novel findings. First, we demonstrated that ILC2s were increased in eosinophilic asthmatic patients compared with non-eosinophilic asthmatic patients. Secondly, we assessed the usefulness on ILC2 measurement for an initial evaluation of asthmatic patients with eosinophilic airway inflammation.

Although there are many inflammatory pathways involved in the development of asthma, eosinophilic inflammation plays a pivotal role in the pathogenesis of this disease [15]. Patients with eosinophilic inflammation have worse disease control, more serious symptoms and greater risk of exacerbations than patients

Table 1
Patient characteristics.

	Healthy controls	Eosinophilic asthma	Non-eosinophilic asthma	P value
Number of patients	42	62	64	
Age (y)	37.6 $\pm$ 9.8*	36.7 $\pm$ 9.26	37.1 $\pm$ 9.15	0.862 ^{a,c}
Sex (M/F)	19/23*	24/38	31/33	0.271 ^{b,c}
BMI	21.3 $\pm$ 1.02*	21.8 $\pm$ 1.2	21.6 $\pm$ 1.3	0.724 ^a
FEV1 (%predicted)	–	93.4 $\pm$ 15.0	101.5 $\pm$ 18.2	0.063 ^{a,c}
Sputum eosinophils (%non-squamous)	–	5.35 $\pm$ 1.31	1.68 $\pm$ 0.93	0.027 ^{a,c}
FeNO (ppb)	–	51.3 $\pm$ 24.2	28.2 $\pm$ 14.9	<0.01 ^{a,c}
Blood eosinophils ($10^9/L$)	–	0.52 $\pm$ 0.34	0.21 $\pm$ 0.15	<0.01 ^{a,c}
Serum IgE (IU/ml)	–	71.7 $\pm$ 48.1	52.4 $\pm$ 21.7	<0.01 ^{a,c}
Blood IL C2 (%lymphocytes)	0.020 $\pm$ 0.013	0.117 $\pm$ 0.090	0.035 $\pm$ 0.021	<0.01 ^{a,c}

Data expresses as mean $\pm$ SD.

* $p > 0.05$ [Comparison of healthy control group vs. Asthma group (Eosinophilic Asthma group and Non-Eosinophilic Asthma group)]. BMI, body mass index; FeNO, fractional exhaled nitric oxide; IgE, immunoglobulin E; ILC2, type 2 innate lymphoid cell.

^a Unpaired t-test.

^b χ^2 –test.

^c Comparison of Eosinophilic Asthma group vs. Non-Eosinophilic Asthma group.

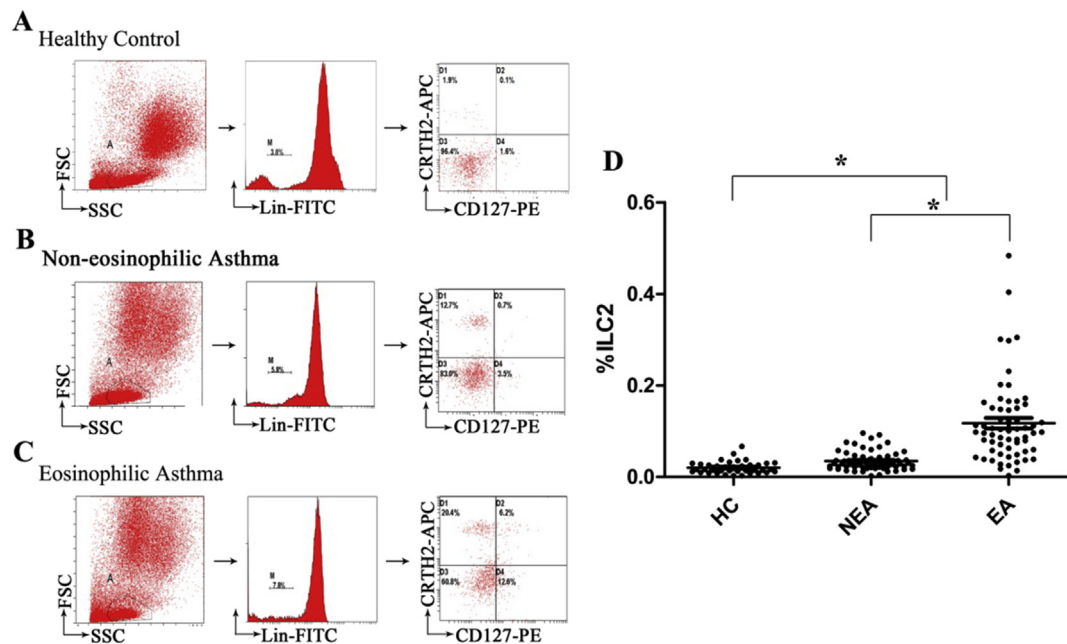


Fig. 1. Peripheral blood ILC2s are selectively enriched in the eosinophilic asthmatic patients. Single cell suspensions were stained for Lin, CD127 and CRTH2. ILC2 were identified as Lin⁻CD127⁺CRTH2⁺ cells. Representative examples shown from (A) healthy controls, (B) non-eosinophilic asthmatic patients and (C) eosinophilic asthmatic patients. (D) %ILC2 in healthy controls (n = 42), non-eosinophilic asthmatic patients (n = 64) and eosinophilic asthmatic patients (n = 62). %ILC2 defined as %Lin⁻CD127⁺CRTH2⁺ cells of peripheral blood lymphocytes. * p < 0.01.

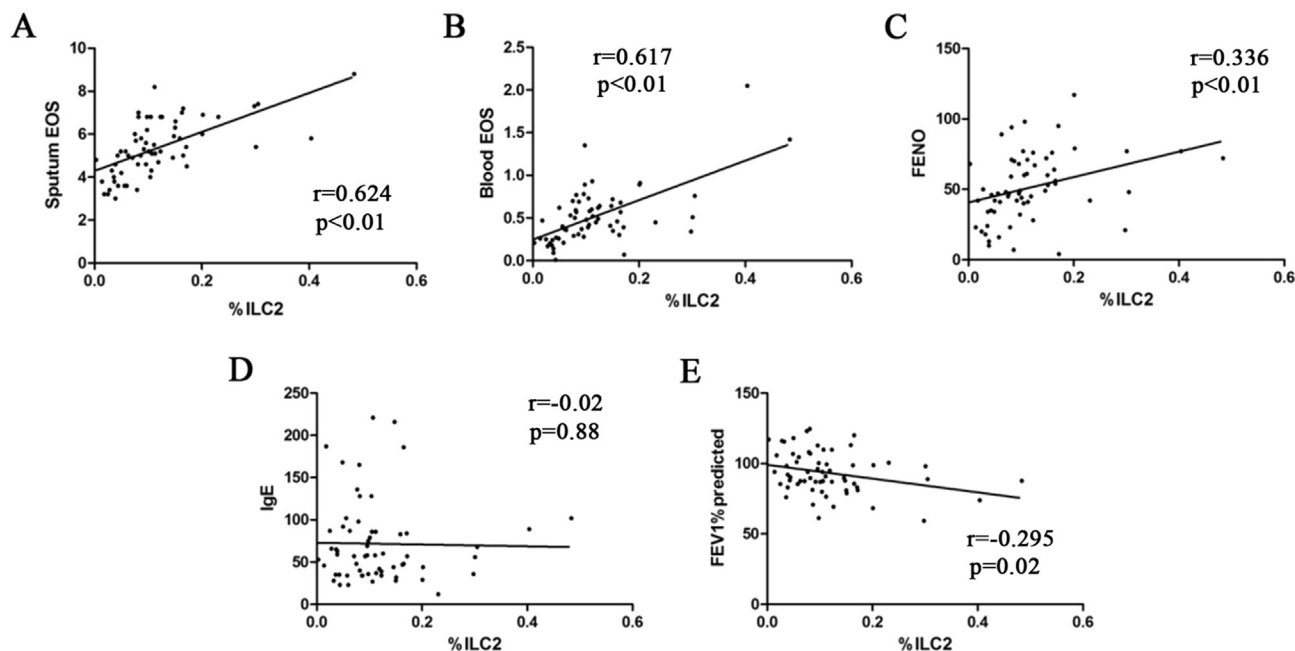


Fig. 2. Correlation analysis between %ILC2 and other factors in eosinophilic asthmatic patients (n = 62). %ILC2 positively correlated with sputum eosinophils (r = 0.624, p < 0.01), blood eosinophils (r = 0.617, p < 0.01) and FENO (r = 0.336, p < 0.01) (A,B,C). It did not correlate with IgE (p = 0.88) and negatively correlated with FEV1%predicted (r = -0.295, p = 0.02) (E).

with other phenotypes and they are more sensitive to inhaled corticosteroid (ICSs) [2,16]. Th2-driven cytokines IL-4, IL-5, IL-13 are associated with eosinophilic inflammation. As noted, IL-5 is responsible for eosinophilic inflammation and it is a specific and strong stimulus for eosinophil differentiation. Otherwise, with the actions of IL-4 and IL-13, mature eosinophils leave the bone marrow and are attracted to the sites of inflammation [16–19].

Many antagonists of Th2 cytokines are under development as eosinophilic asthma therapeutics, such as anti-IL-5 monoclonal antibody mepolizumab [17], so it has become more necessary to identify the asthma phenotype most likely to benefit from the targeted therapies [20].

ILC2 was first described by Fort et al., in 2001 and they are a new population of lineage-negative cells that can produce large

Table 2
Multiple regression statistics for sputum eosinophil counts (n = 126).

	Estimate	SE	t-value	P-value
Age	<0.001	0.014	0.025	0.980
Sex	0.131	0.261	0.502	0.617
BMI	0.015	0.105	0.144	0.886
IgE	0.003	0.004	0.868	0.387
Blood eosinophils	1.320	0.627	2.105	0.037
%ILC2	11.385	2.381	4.781	<0.001
FeNO	0.028	0.007	4.026	<0.001

We fit a multiple regression model to evaluate the biomarker variables as correlative factors of sputum eosinophil counts.

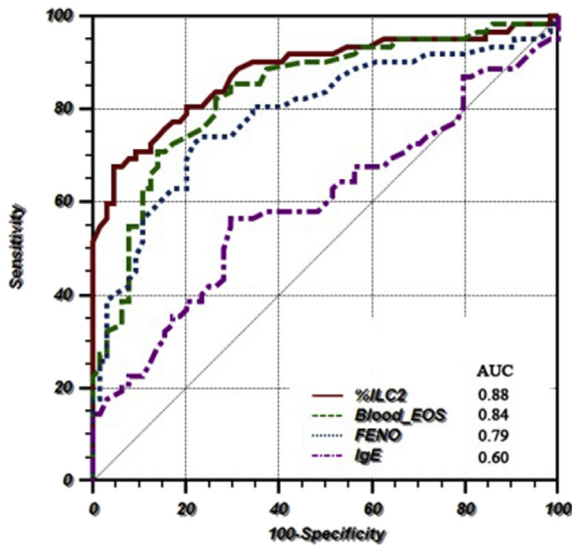


Fig. 3. Receiver operating characteristic curve analysis of %ILC2, blood eosinophils, FeNO and IgE levels for the diagnosis of airway eosinophilic inflammation. AUC, area under the curve.

amounts of Th2 cytokines [6,21]. In humans, mature ILC2s have been found in nasal polyps, sinus epithelium, peripheral blood, lung and gastrointestinal tract [22]. Hannah H. Walford et al. have found that the percentage of ILC2 was higher in eosinophilic nasal polyps from allergic individuals compared to non-eosinophilic nasal polyps from individuals with unknown allergic status [7]. ILC2 can be activated by airway epithelial cytokines IL-25, IL-33, TSLP as well as the lipid mediator leukotriene D4 and then become the major source of type 2 cytokines IL-4, IL-5 and IL-13 [23,24]. In

addition, ILC2 may influence the polarization and activation of Th2 cells by its ability to possibly uptake antigens and present them to naive T cells via MHC II [6]. Then the activated Th2 cells can also produce type 2 cytokines. So we indicated that ILC2s are responsible for the producing of Th2 cytokines and they play an important role in eosinophilic airway inflammation. A recent study reported that the prevalence of ILC2s in peripheral blood of allergic asthma patients was increased compared with healthy individuals and allergic rhinitis patients. Peripheral blood is useful for evaluating innate type 2 immunity in humans [9]. In the present study, we used flow cytometry and found that the proportion of ILC2s in peripheral blood lymphocytes of eosinophilic asthmatic patients was higher than that in non-eosinophilic asthmatic patients. A positive correlation was found between the proportion of ILC2 and airway eosinophilic inflammation in asthma patients, providing evidence that the ILC2s in peripheral blood is a reflection of eosinophilic inflammation in the airway. Thus, our work further supports the role of peripheral blood ILC2 in the eosinophilic asthma.

Our studies also demonstrated the novel finding that ILC2 as well as blood eosinophil numbers had the high accuracy in the identification of eosinophilic airway inflammation. To the best of our knowledge, this is the first time to show that ILC2 in peripheral blood is an accurate biomarker of eosinophilic airway inflammation in asthmatic patients, with an area of under curve of 0.88. And the cut-point was 0.076%, this yielded a sensitivity of 67.7% and a specificity of 95.3%. In our study, blood eosinophil count is also a good predictor for eosinophilic inflammation with the ROC AUC 0.84, which is nearly similar to several previous studies [5,25]. Whereas, our data is not in line with the study of Jia G et al. [20], which demonstrated the lower ROC AUC for blood eosinophil numbers. However, the latter study included patients with corticosteroid-refractory asthma only, and a daily ICS dose was at least 1000ug of fluticasone dipropionate or equivalent. Corticosteroids may have an effect on the blood eosinophil counts. The third-best predictor of eosinophilic inflammation is FeNO with an ROC AUC 0.79, which also supports the results of previous studies [5,20,26,27]. Other studies showed FeNO levels were associated with airway inflammation and can predict ICS responsiveness in asthma patients [28,29]. But Nair P et al. found that FeNO levels have a limitation to reflect eosinophilic inflammation in asthma with severe steroid-dependent patients [30]. The above studies demonstrate that blood eosinophils and FeNO levels may be not reliably reflect airway eosinophilic inflammation of asthmatic patients when they use high dose corticosteroid.

One of the potential limitations of our research is that we could not get adequate sputum in all patients. For example, we recruited

Table 3
Criterion, sensitivity, specificity, PPV and NPV of different biomarkers to diagnose eosinophilic airway inflammation.

	Criterion	Sensitivity (%)	Specificity (%)	PPV	NPV
%ILC2	0.076	67.7	95.3	93.3	75.3
Blood eosinophils ($10^9/L$)	0.33	71	85.9	83	75.3
FeNO (ppb)	37	74.2	76.6	75.4	75.4
IgE (IU/ml)	55	56.5	70.3	64.8	62.5

PPV, positive predictive value; NPV, negative predictive value.

Table 4
Pairwise comparison of ROC curves.

	Difference between areas	SE	Z statistic	P value
ILC2-blood eosinophils	0.0426	0.0374	1.140	0.254
ILC2-FeNO	0.0909	0.0448	2.030	0.042
ILC2-IgE	0.281	0.0624	4.496	<0.001

150 patients at first, but only 126 of them finished sputum induction and produced adequate sputum. The individuals in our study were only mild to moderate asthmatic patients and they had not received regulatory treatment before entering this study, so we do not know the relationship between ILC2 levels and the use of ICS.

Nonetheless, our study demonstrates that blood ILC2 can reflect airway eosinophilic inflammation in mild to moderate asthmatic patients, and this provides a new insight for the biomarker of eosinophilic asthma. Several studies indicated that blocking ILC2-derived cytokines can reduce eosinophilia and improve asthma control, as highlighted by studies of anti-IL-5 treatment [31–33]. The level of ILC2 might help identify and select those patients most likely to respond to these biological therapeutics, especially those steroid-refractory asthmatic patients may benefit a lot from these treatments. However, future studies are needed to identify whether ILC2s can guide these biological therapies in eosinophilic asthmatic patients.

Conflict of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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