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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 ± 0.090versus0.035 ± 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. Bartemes 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,

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