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Original Article

Enhanced circulating ILC2s accompany by upregulated MDSCs in patients with asthma

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Abstract: Group 2 innate lymphoid cells (ILC2s) are considered to be the most significant mediators during the orchestration of immune responses in asthma. Myeloid-derived suppressor cells (MDSCs) has received a great deal of attention for their immunosuppressive activity, and our early studies indicate that the increased Th2 cytokines are associated with MDSCs. In this study, we sought to determine whether MDSCs are also participation in immune imbalance and its relationship with ILC2s in asthma. The data showed that the circulatory ILC2s or MDSCs and their characteristic cytokines or transcription factors were significantly enhanced in asthmatic patients, as well as in chronic obstructive pulmonary disease (COPD) or respiratory viral infections (RVI). Meanwhile, a Th2-dominated phenotype was found in patients with asthma which closely related to the expression levels of ILC2s and MDSCs associated moleculars. These findings indicated that Th2 polarization was close related to synergistically increased ILC2s and MDSCs, it may allow to further the comprehension of the contribution of these cells to the inflammatory response involved in asthma or other respiratory tract inflammatory diseases, such as COPD and RVI, as well as to develop novel therapeutic strategies.

Keywords: Asthma, ILC2s, MDSCs, Th2 cells

Introduction

Asthma is one of the most common airway chronic inflammatory diseases, which is characterized by constriction of airway smooth muscle, hyper secretion of mucus, edema and airway hyper responsiveness (AHR), mucus secretion and thickening of the basement membrane underlying the airway epithelium [1]. Similarities in asthma and COPD have been confirmed, and previous studies suggested that asthma may be a risk factor for the development of COPD [2], meanwhile respiratory viral infections (RVI) are common and usually self-limiting illnesses in healthy adults and a major cause of exacerbation in patients with asthma and/or COPD, and have long been regarded as one of the causal factors for asthma and COPD. However the precise mechanisms of immune modulation in the progression of these diseases remain elusive.

During the past decade, a novel innate lymphoid cells (ILCs) have been identified. These cells are a heterogeneous group of cell types and can be categorized into three groups mainly by their expression of cell surface markers, transcription factors and effector cytokines [3, 4]. Group 2 ILCs are capable of producing large amounts IL-13 and IL-5 when stimulated by IL-25 and IL-33 in vitro because of the membrane expression IL-17RB or ST2 [5, 6]. These cells require the transcription factor called retinoic acid receptor related orphan receptorα or ROR_{α}, and highly express the master Th2 transcription factor *GATA3* [3, 7, 8] which is required for the Th2 cytokine produced by ILC2s. ILC2s are recently shown to mediate the immune pathology of asthma even without adaptive immunity, and can be found in human respiratory and gastrointestinal tissue as well as in skin [5, 6, 9-13].

MDSCs are an innate heterogenous population of cells with strong immunosuppressive activity which consists of myeloid progenitor cells and immature myeloid cells (IMCs). Murine MDSCs can be broadly classified into two groups: $CD11b^{+}Gr\text{-}1^{high}$ granulocytic MDSC (which can also be identified as $CD11b^{+}Ly\text{-}6G^{+}Ly6C^{low}$ MDSC) and $CD11b^{+}Gr\text{-}1^{low}$ monocytic MDSC (which can also be identified as $CD11b^{+}Ly\text{-}6G^{-}Ly6C^{high}$ MDSC) [14]. Due to the heterogeneity of MDSCs, there are on specific markers to define these cells in human. However large amounts of reports have defined that the phenotypic markers for human MDSCs are $Lin^{-}HLA\text{-}DR^{-}CD33^{+}$ or $CD11b^{+}CD14^{+}CD33^{+}$, the latter sometimes is $CD15^{+}$ [15]. Notably, the hallmark of all MDSCs is their ability to suppress immune responses, which have recently been discussed in a comprehensive review [15], and provides an alternative means of further understanding their roles in normal physiology and disease. To date, most of the attention has been focused on cancer-associated MDSCs in human [16-18], whereas few studies have reported the activation, phenotype and especially the role of MDSC in a number of different non-malignant settings such as infections, allograft rejection, autoimmunity and hypersensitivity [15, 19-23]. Normally, the regulatory function of MDSCs is mainly in adaptive immunity via suppressed the activation of T cells, but few know how performance of MDSCs in asthma which accompanied by Th2 polarization. In this study, the frequencies of ILC2s and MDSCs in PBMCs, the expression levels of ILC2s, MDSCs and Th2 cells-related transcription factors and cytokines were detected. In addition, the correlations of expression levels between ILC2s or MDSCs and Th2 cells characteristic moleculars were analyzed, with the goal to understand the significance of ILC2s and MDSCs in asthma.

Methods

Patients and healthy controls

23 asthma patients from the Affiliated People's Hospital of Jiangsu University were included in the study, 16 male and 7 female, age ranged from 41 to 63 years (average age, 53.7 years), they were diagnosed based on commonly accepted clinical and laboratory criteria. All the patients were untreated for their condition at the time of blood collection. 30 healthy volunteers were studied simultaneously as control, including 21 males and 9 females ranging in age from 45 to 62 years (average age, 56.3 years). In addition, 27 COPD patients (21 males and 6 females, age ranged from 58 to 72 years, mean age 65.9 years) and 27 RVI patients (21 males and 6 females, age ranged from 58 to 72 years, mean age 65.9 years) were also included. This study was approved by the Ethical Committee of the Affiliated-People's Hospital of Jiangsu University, and written informed consent was obtained from all individuals.

Cell preparation and Flow cytometric quantification

Peripheral blood samples were collected form patients and healthy controls in lithium heparin tubes and peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll-Hypaque density-gradient centrifugation. Plasma was stored at -80°C for subsequent analysis. The PBMCs were divided into two equal aliquots, one was used for flow cytometric quantification immediately, and then added 1 ml Trizol (Invitrogen, USA) into the other aliquots and was cryopreserved at -80°C for extracting total RNA. For flow cytometric quantification, ILC2s population was defined as Lineage (CD2, CD3, CD14, CD16, CD19, CD56, and CD235a) $Lineage^{-}/ICOS^{+}/IL\text{-}17RB^{+}$, MDSCs population was defined as $Lineage^{-}/HLA\text{-}DR^{-}/CD33^{+}$. PBMCs were stained with Lin-FIEC, CD33-PE, HLA-DR-PE-Cy5, IL-17RB-PerCP or ICOS-APC (eBioscience, San Diego, CA, USA and R&D Systems, USA) at room temperature for 30 min. After incubation, the samples were washed with phosphate buffered saline (PBS), the pellets were resuspended in 250 μL of PBS. The isotype control antibody was used in all cases. The labeled cells were quantified with an Accuri C6 flow cytometer (Becton-Dickinson), and analyzed by Flow Jo software (Tree Star, Inc.).

RNA extraction, cDNA synthesis and quantitative real-time PCR

Total RNA was extracted from individual PBMCs following the manufacturer's instructions, and it was reverse-transcribed to cDNA using the RT reagent kit (TaKaRa, Ohtsu, Japan) according to the manufacturer's instructions. For quantitative real-time PCR, reverse transcribed cDNA (1 μL) was amplified by real-time PCR with the

Table 1. Primers used in real-time PCR

Gene	Sequence (5'-3')	Length
IL-33	U: ATCCCAACAGAAGGCCAAAG	198
	L: CCAAAGGCAAAGCACTCCAC	
ROR_{α}	U: CTGACGAGGACAGGAGTAGG	204
	L: GTGCGCAGACAGAGCTATTC	
GATA3	U: TTGTGGTGGTCTGACAGTTC	294
	L: AGTACAGCTCCGGACTCTTC	
T-bet	U: CGGGAGAACTTTGAGTCCAT	115
	L: ACTGGTTGGGTAGGAGAGGAG	
IL-13	U: GGCTGAGGTCTAAGCTAAGG	370
	L: GACAGCTGGCATGTACTGTG	
IL-5	U: ACTCTCCAGTGTGCCTATTC	102
	L: CTGCTGATAGCCAATGAGAC	
IL-4	U: GACATCTTTGCTGCCTCCA	99
	L: TACTCTGGTTGGCTTCCTTCA	
Arg1	U: CAAGAAGAACGGAAGAATCAGC	149
	L: TTGTGGTTGTCAGTGGAGTGTT	
iNOS	U: CTTTCCAAGACACACTTCACCA	236
	L: TATCTCCTTTGTTACCGCTTCC	

U: upper; L: lower.

SYBR Green Premix EX Taq kit (TaKaRa, Ohtsu, Japan). Each sample was analyzed in duplicate with the CFXA96 Cycler (Thermal) and the normalized signal level for each target gene was calculated based on the ratio to the corresponding β-actin signal. All primer sequences were shown in **Table 1**.

Enzyme-linked immunosorbent assay

The concentrations of IL-33, IL-13 and IL-5 in plasma were measured by enzyme-linked immunosorbent assay (ELISA) following the manufacturer's protocols (eBioscience, San Diego, CA, USA). Hemolyzed and lipoidaemia samples were excluded. All samples were run in batches to minimize inter assay variability and in triplicate, and the mean absorbance was calculated from the standard curve.

Statistical analysis

Statistical comparisons between groups were performed using the Student's unpaired or paired t-test. The correlation between two continuous variables was analyzed by the Spearman test. The Graph Pad Prism, Version 5.0 software (San Diego, CA, United States) was used to perform these calculations. *P*-value < 0.05 was considered to be statistically significant.

Results

Elevated percentage of ILC2s and MDSCs accompanied by increased IL-33 in PBMC from asthmatic patients

Human ILC2s have been identified by Mjösberg in healthy fetal gut, lung and peripheral blood [5], in search of whether there exist a changed number of these cells in patients with asthma compared with healthy controls, we stained single-cell suspensions derived from PBMCs with a 'cocktail' of lineage-specific antibodies, anti-IL-17RB and anti-ICOS. As shown in **Figure 1A-E**, a significantly increased frequency of individual ILC2s was found in patients with asthma.

Asthma and COPD are both characterized by chronic airway inflammation and airflow limitation [1]. Similarities in asthma and COPD have been confirmed, and previous studies suggested that asthma may be a risk factor for the development of COPD [2]. Meanwhile, RVI has been regarded as one of the causal factor for asthma and COPD. In view of this, the circulating ILC2s in PBMCs from COPD and RVI patients were also analyzed, the results showed in **Figure 1**.

Considering the beneficial surroundings of ILC2s in the peripheral blood, we then measured the plasma concentration of IL-33. Our data showed an obviously significantly increased both in mRNA (**Figure 1G**) and protein level (**Figure 1H**) in patients compared to healthy controls besides the mRNA expression of IL-33 in patients with RVI.

The function of MDSCs is mainly in adaptive immunity via suppressed the activation of T cells, but few know how performance of MDSCs in asthma which was accompanied by Th2 polarization. In the present study, we detected the frequency of circulating MDSCs in asthma, COPD and RVI patients, the data showed there were also significantly elevated (**Figure 2**).

Different expression levels of ROR_{α}, GATA3 and T-bet in PBMC from patients with asthma

ROR_{α} and *GATA3* are the main transcription factors which essential for the development and function of human ILC2s. *GATA3* was also Th2 specific transcription factor. For further studied the Th1/Th2 balance state in asthma patients,

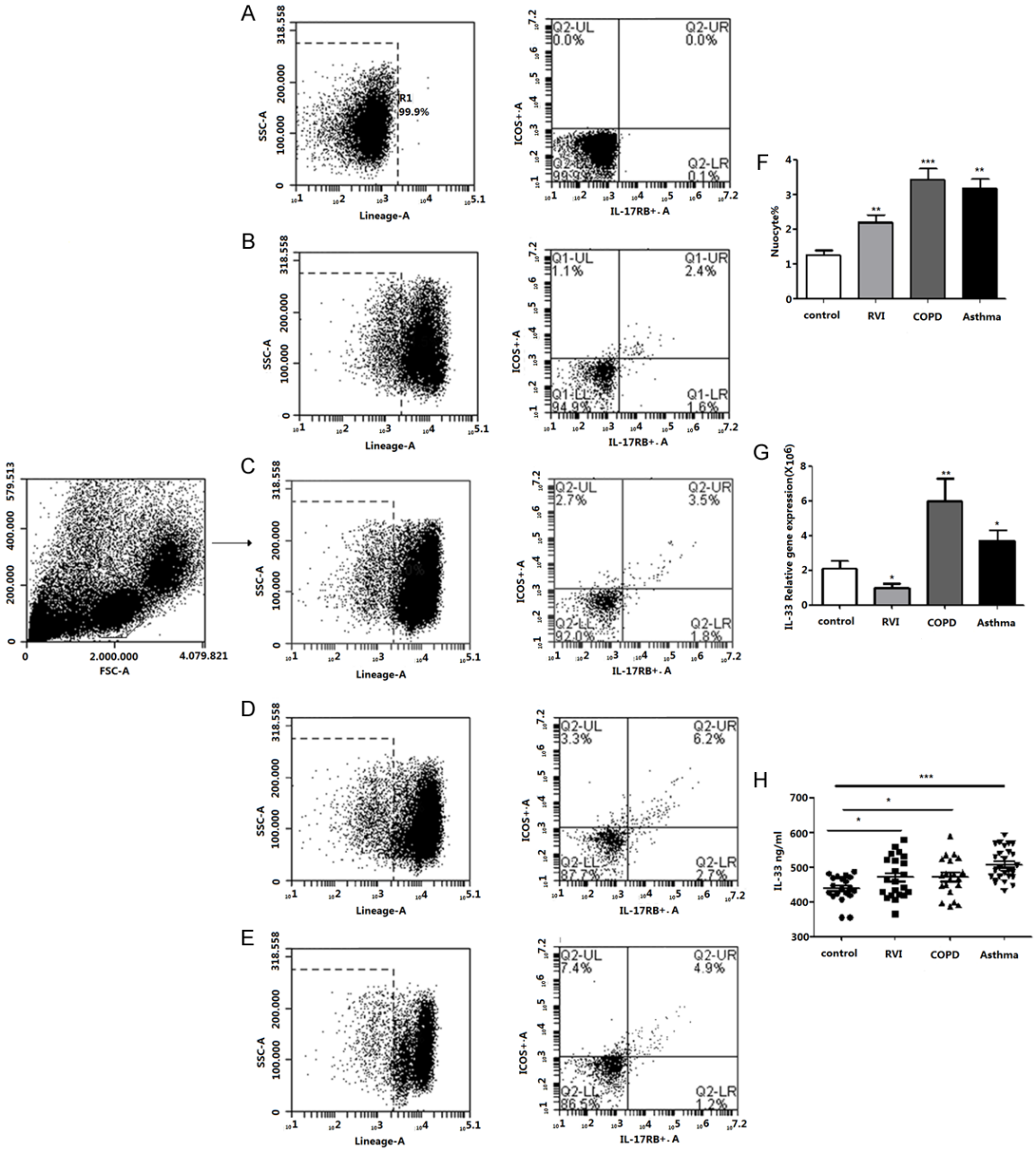

Figure 1. The frequency of ILC2s (Lineage-/ICOS+/IL-17RB+) and IL-33 expression in peripheral blood from patients and healthy controls. (A-D) Representative diagrams of flow cytometry analysis for circulating ILC2s in isotype control (A), healthy control (B), RVI (C), COPD (D) and asthma (E) respectively. (F) The frequency of ILC2s in PBMC from asthma patients was significantly increased compared with healthy control, the similar results were obtained in RVI and COPD. The IL-33 expression was obviously increased both in mRNA (G) and protein level (H) in patients compared to healthy controls. Data were analyzed by the Student's t-test. $***P < 0.001$, $**P < 0.01$, $*P < 0.05$.

and analyzed the relationship between Th1/Th2 balance and ILC2s polarization, we detected the levels of *T-bet*, *GATA3* and ROR_{α}. As shown in **Figure 3**, there was increased mRNA expression of *GATA3* in patients with asthma, but decreased in patients with RVI or COPD; the expression level of T-bet was obviously decreased in all patients. At the same time, the ROR_{α} mRNA was increased in asthma, RVI or COPD patients.

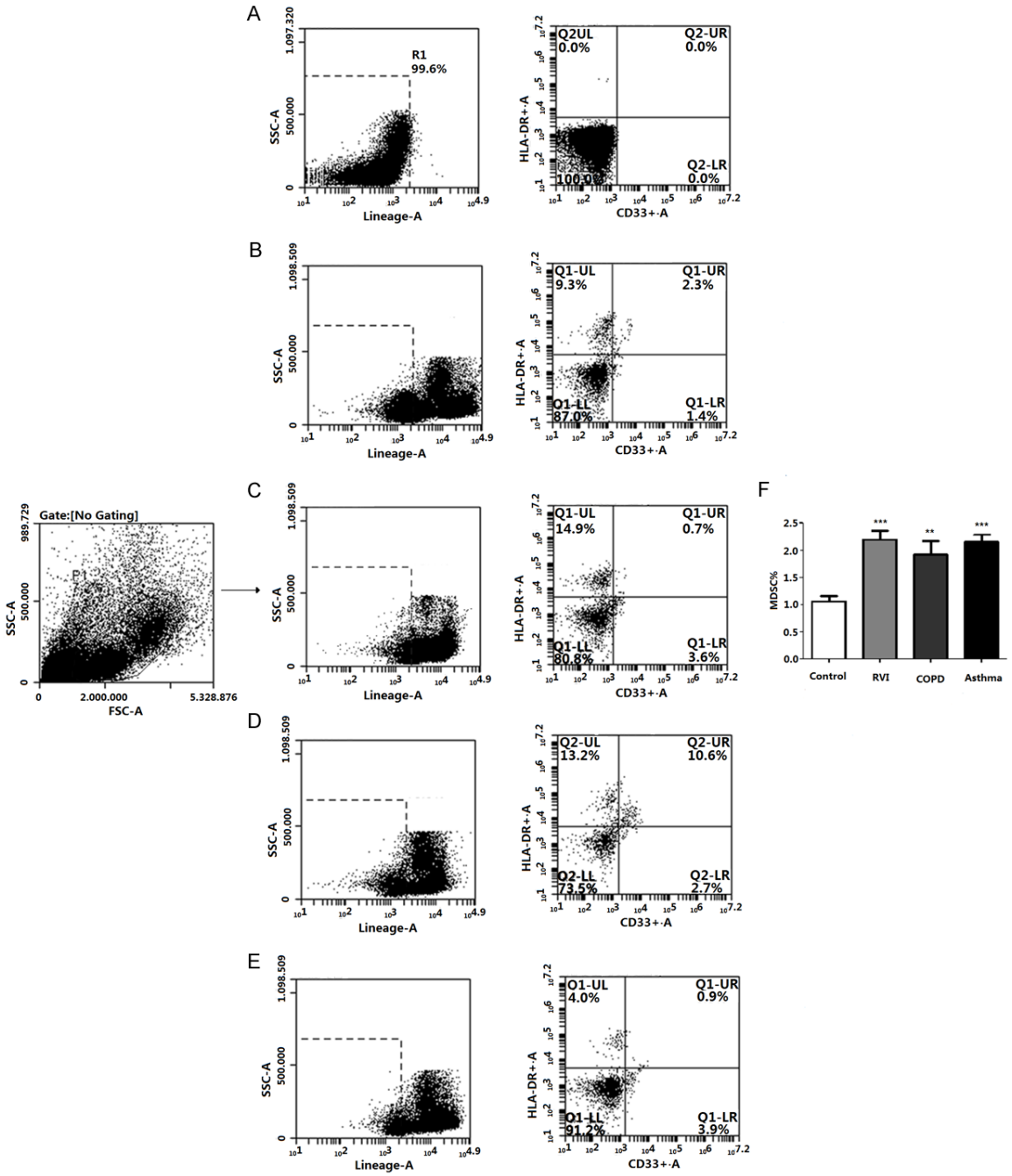

Figure 2. Elevated frequency of MDSCs (Lineage-/HLA-DR-/CD33+) in PBMCs of patients. (A-D) Representative diagrams of flow cytometry analysis for circulating MDSCs in isotype control (A), healthy control (B), RVI (C), COPD (D) and asthma (E) respectively. (F) The frequency of MDSCs in PBMC from asthma patients was significantly increased compared with healthy controls, the similar results were obtained in RVI and COPD. $^{**}P < 0.01$, $^{*}P < 0.05$.

Collaborative elevated expression levels of ILC2s and MDSCs characteristic cytokines in peripheral blood from asthmatic patients

ILC2s can provide IL-5 and IL-13, which are also the early source of Th2 cells related cytokines. Arg1 and iNOS are two signature cytokines related with MDSCs. We used the qRT-PCR to analyze the mRNA expression levels of IL-5, IL-13, Arg1 and iNOS in PBMC, and performed the ELISA to evaluate the protein levels of these signature cytokines in plasma. Our data indi-

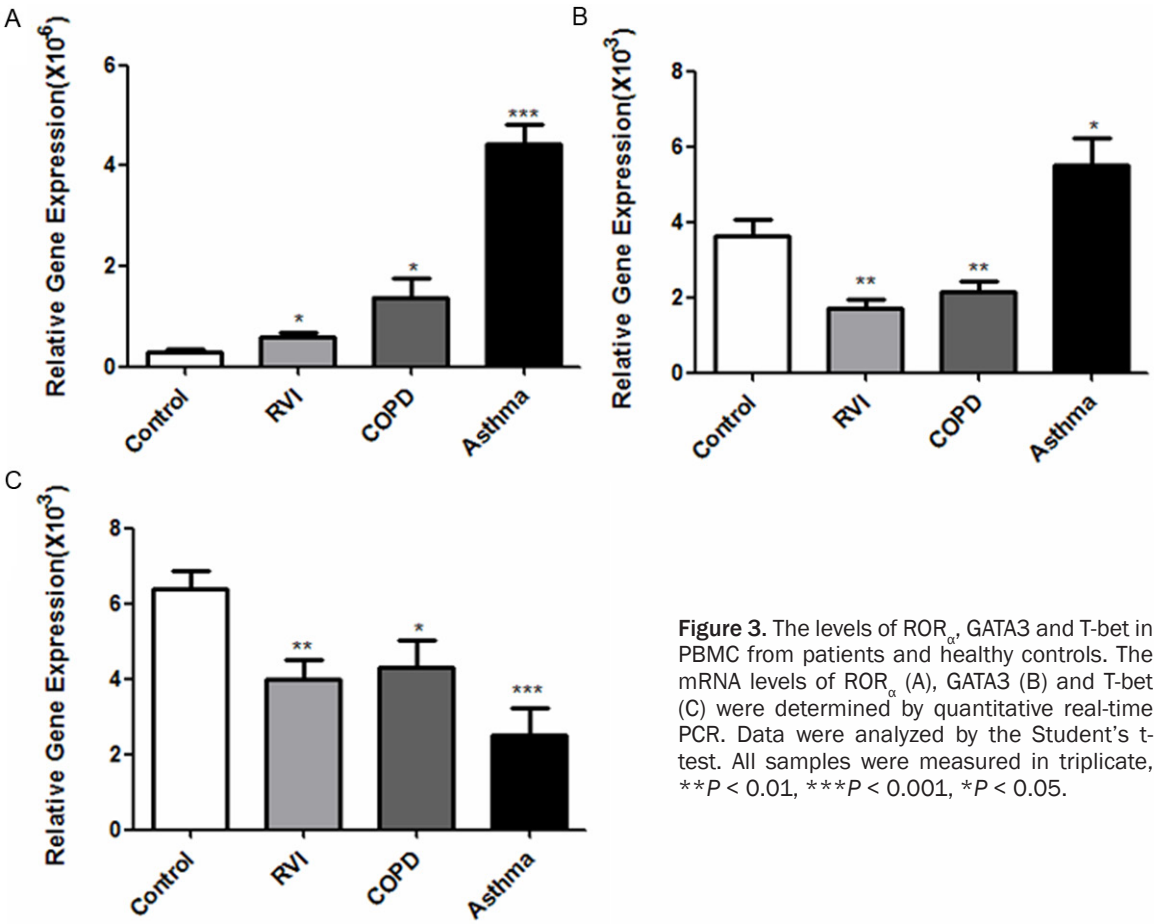

Figure 3. The levels of ROR_{α}, GATA3 and T-bet in PBMC from patients and healthy controls. The mRNA levels of ROR_{α} (A), GATA3 (B) and T-bet (C) were determined by quantitative real-time PCR. Data were analyzed by the Student's t-test. All samples were measured in triplicate, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{*}P < 0.05$.

cated that the mRNA expression levels of the ILC2s and MDSCs related cytokines, IL-5, IL-13 and Arg1 were obviously increased in patients with asthma, as well as in COPD and RVI. In addition, increased expression levels of IL-13 and IL-5 in serum were also found in patients with asthma, COPD and RVI (**Figure 4**).

IL-4, a Th2 characteristic cytokine, it was increased in all patients with asthma, COPD and RVI (**Figure 4**). Combined with the expression of *GATA3*, it could be indicated that there was a Th2 polarization in these diseases.

Correlations between the expression levels of ILC2s or MDSCs and Th2 related cytokines in asthma

IL-5, IL-13 and Arg1 or iNOS are the signature cytokines produced by ILC2s and MDSCs respectively. Correlation analysis showed that the expression levels of ILC2s signature cytokines (IL-5 and IL-13) in asthma were associated with MDSCs related cytokines (Arg1 and iNOS), there were significantly positive correlations between them. Although some of these cytokines were also increased in COPD and RVI, the positive correlations were only found between IL-5 and Arg1 or iNOS in COPD (**Figure 5**).

In this study, three kinds of diseases were all related to the upregulation of Th2 molecules. We further analyzed the correlations between the expression levels of ILC2s or MDSCs and Th2 cells associated molecules, the results showed that the expression levels of ILC2s signature cytokines (IL-5 and IL-13) or MDSCs related cytokines (Arg1 and iNOS) were associated with Th2 specific molecules (IL-4 and *GATA3*) in asthma and COPD, there were significantly positive correlations between them. However, the positive correlation was only found between ILC2s signature cytokines (IL-5 or IL-13) and Th2 transcription factor (*GATA3*) in RVI (**Figure 6**).

Discussion

Recently identified natural helper cells, nuocytes, and innate helper 2 cells, currently termed group 2 innate lymphoid cells (ILC2s)

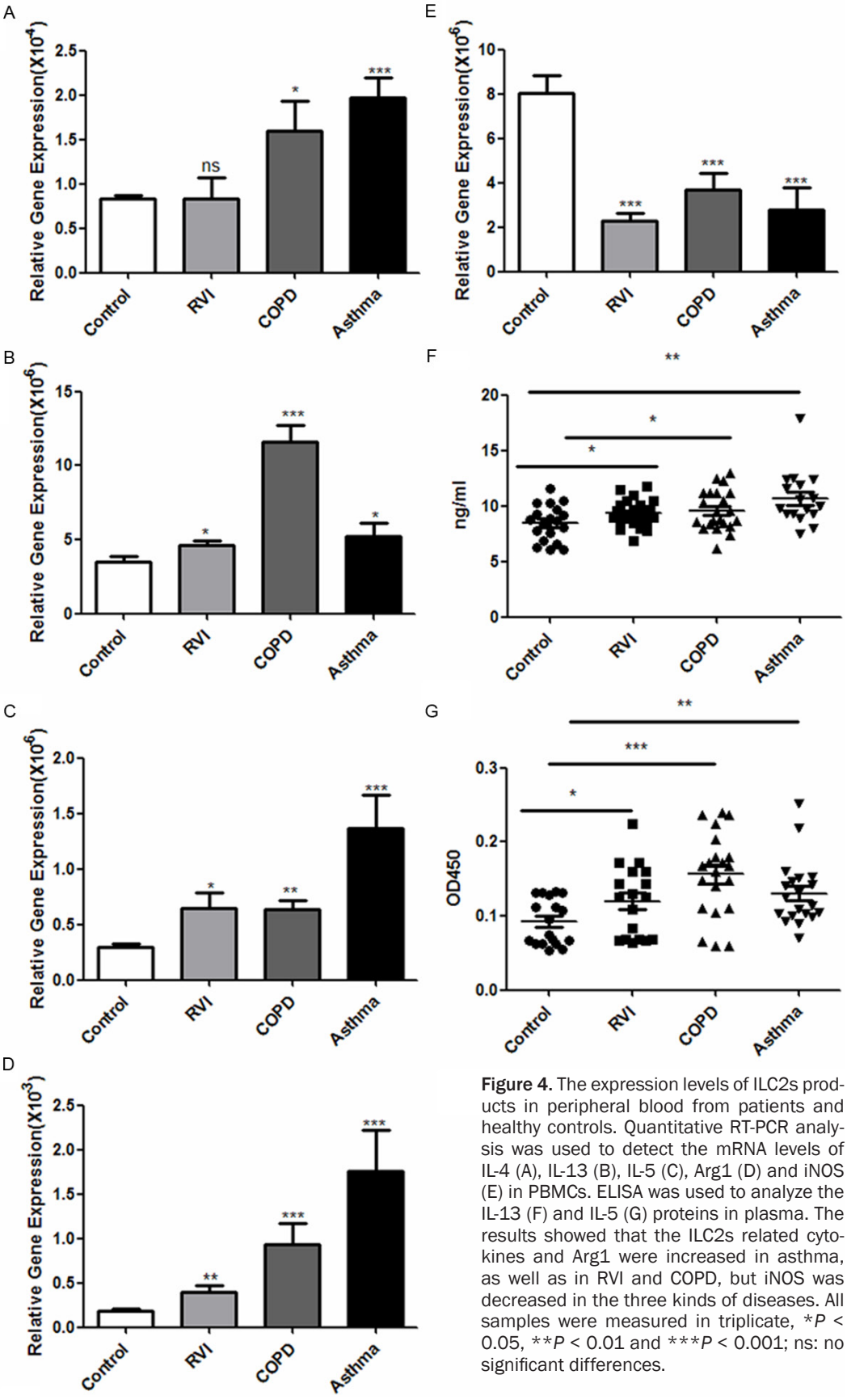

Figure 4. The expression levels of ILC2s products in peripheral blood from patients and healthy controls. Quantitative RT-PCR analysis was used to detect the mRNA levels of IL-4 (A), IL-13 (B), IL-5 (C), Arg1 (D) and iNOS (E) in PBMCs. ELISA was used to analyze the IL-13 (F) and IL-5 (G) proteins in plasma. The results showed that the ILC2s related cytokines and Arg1 were increased in asthma, as well as in RVI and COPD, but iNOS was decreased in the three kinds of diseases. All samples were measured in triplicate, $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$; ns: no significant differences.

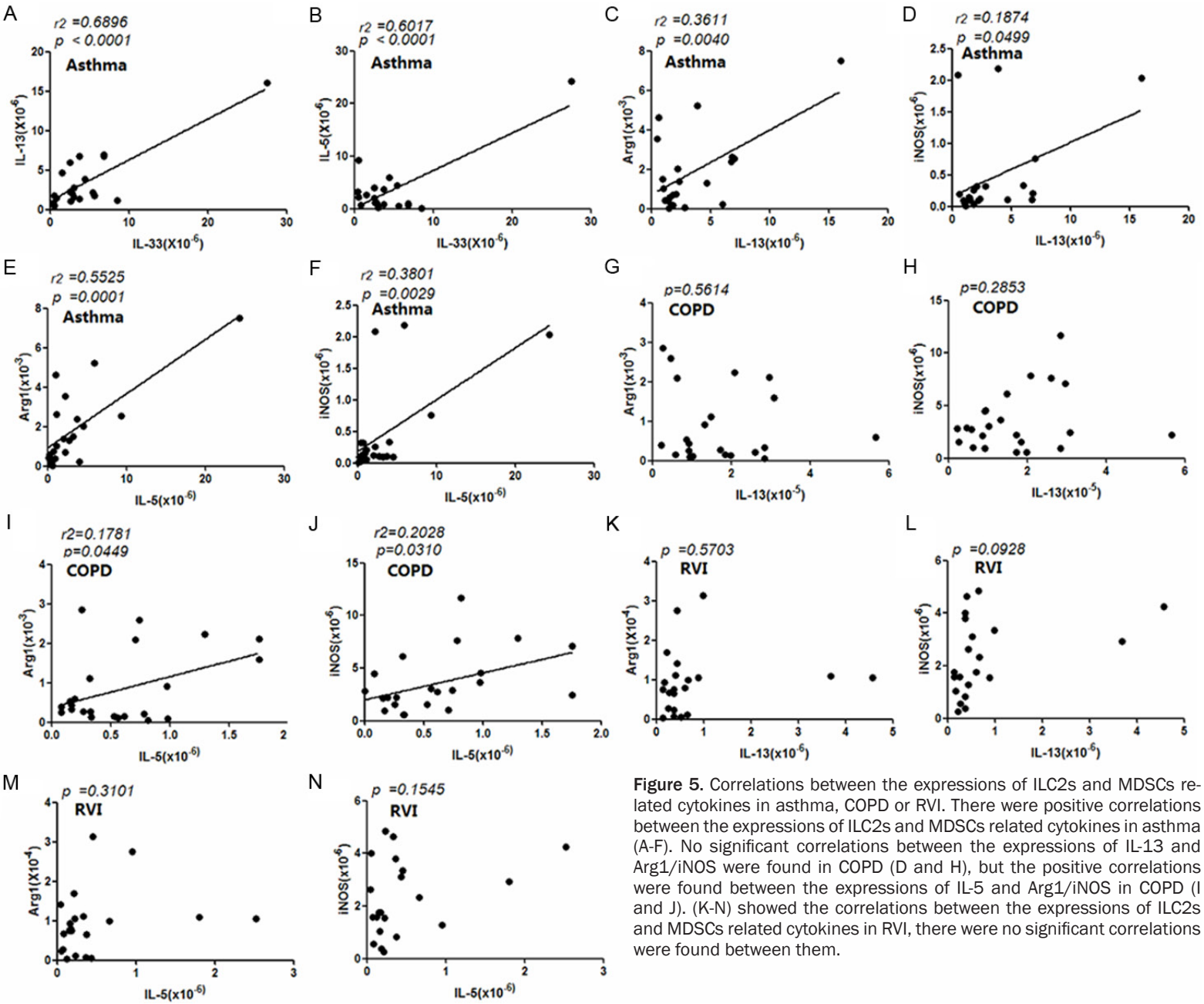

Figure 5. Correlations between the expressions of ILC2s and MDSCs related cytokines in asthma, COPD or RVI. There were positive correlations between the expressions of ILC2s and MDSCs related cytokines in asthma (A-F). No significant correlations between the expressions of IL-13 and Arg1/iNOS were found in COPD (D and H), but the positive correlations were found between the expressions of IL-5 and Arg1/iNOS in COPD (I and J). (K-N) showed the correlations between the expressions of ILC2s and MDSCs related cytokines in RVI, there were no significant correlations were found between them.

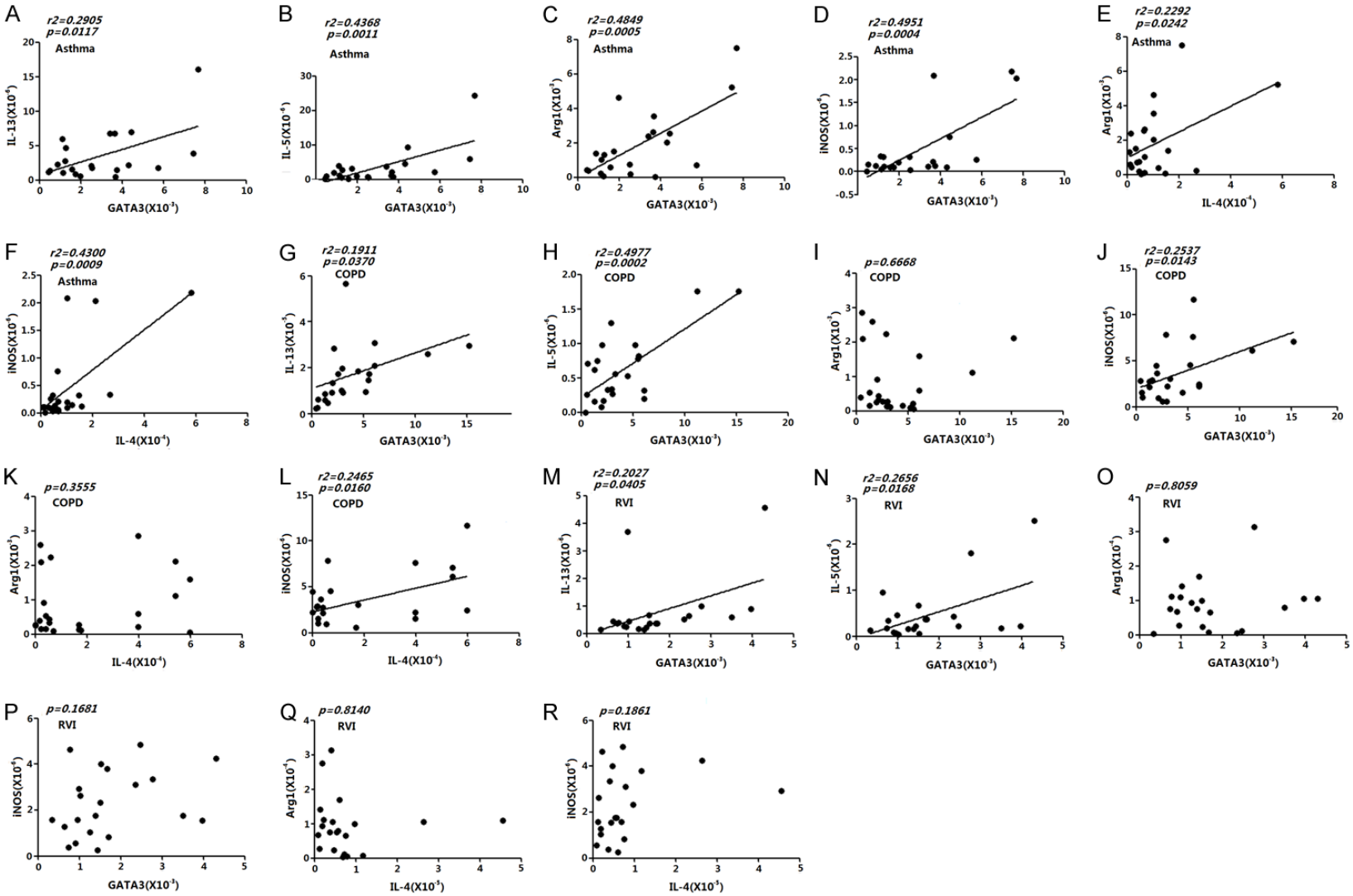

Figure 6. Correlations between the expression levels of ILC2s or MDSCs and Th2 related cytokines in asthma, COPD or RVI. There were significantly positive correlations between the mRNA expressions of IL-13/IL-5 and GATA3 (A and B), Arg1/iNOS and GATA3/IL-4 (C-F) in asthma. (G-L) the correlations between IL-13/IL-5 and GATA3, Arg1/iNOS and GATA3, and Arg1/iNOS and IL-4 in COPD. (M-R) the correlations between IL-13/IL-5 and GATA3, Arg1/iNOS and GATA3, and Arg1/iNOS and IL-4 in RVI.

[19-22], which are negative for lineage markers, express ST2 (IL-33R) and IL-17RB (IL-25R) and produce large amounts of IL-5 and IL-13 after induced by IL-33 as well as IL-25. Previous studies have shown that ILC2s serve as a crucial link between innate immunity and the type 2 adaptive immune response and play important roles in the pathogenesis of allergic asthma, airway infection with H3N1 induced airway hyperreactivity [6], tissue repair and perhaps including COPD [9, 10].

Immune system will be activated in pathological condition including the activation of T cells, Solito et al have shown that strongly activated T cells are more easily suppressed by MDSC, and the presence of activated T cells is necessary for in vitro generated MDSC to proliferate and maintain their immature and suppressive phenotype, suggesting that factors derived from strongly stimulated T cells contribute to MDSC activation [23]. Clearly, inflammatory pathways are at work during cancer development and progression. The overlap of these pathways with mechanisms regulating normal immune response suggests that MDSCs may also contribute to the development of asthma, RVI and COPD, they are all inflammatory diseases. Therefore, it was thought that these two heterogeneous groups may co-exist in the inflammatory immune microenvironment in these diseases.

In the present study, our data confirmed that the circulating ILC2s (Lineage$^-$/ICOS$^+$/IL-17RB$^+$) were significantly increased in peripheral blood in patients with asthma, as well as in RVI or COPD. This result might due to the respiratory tract inflammation and epithelial cells pathological changes. Some studies have shown that airway epithelial cells phagocytose inflammatory cells and secrete the effective anti-inflammatory cytokine depend on the signal transduction of Rac1, loss of Rac1 expression in airway epithelial cells can lead to a defect on phagocytosis and produce inflammatory cytokines (possibly including IL-33) in epithelial cells [24, 25]. Rac1 expression in airway epithelial cells is closely related to a variety of airway inflammation and airway high reaction (AHR), the down-regulation of its expression will contribute to the increased expression of IL-33 which promotes ILC2 polarization [26]. In addition, our data also showed that ILC2s signature cytokines IL-5 and IL-13 were elevated both in mRNA and protein expression levels in asthma, RVI or COPD, and the mRNA of transcription factor ROR_α was obviously increased, which further indicated might exist the ILC2 polarization in these diseases.

Hypersensitivity may be due to the body's immunity too strong to injure normal tissues and organs, but immune deviation in some aspects or immune imbalance, perhaps this is the role of MDSCs which inhibit Th1 differentiation and promote Th2 polarization [27-31]. In this study, the MDSCs related cytokines and Th2 specific molecules in patients with asthma were detected. The results showed that the MDSCs related cytokines were increased not only in asthma, but also in RVI and COPD. A positive correlation between the expression levels of MDSCs and Th2 specific molecules was found in asthmatic patients. In the patients with RVI and COPD, MDSCs related molecules were increased, but Th2 specific transcription factor was no significant change. Asthma is an allergic disease, related to Th2 cell polarization or immune imbalance. Our previous study showed that MDSCs could promote the differentiation of Th2 cells. Thus it was not difficult to understand that increased MDSCs in the process of the occurrence and development of asthma, and there was a positive correlation between the expression levels of MDSCs and Th2 specific moleculars. Two other diseases were not or partially correlated with Th2 polarization state, it may due to RVI and COPD secreted distinctive patterns of cytokines which lead to remarked different pattern of inflammation in the respiratory tract.

IL-33 and IL-25 were previously implicated in the initiation and regulation of innate immune responses associated with ILC2s-mediated inflammatory diseases, and the function of IL-33 is more forcible which has been confirmed by Mjösberg [5]. Furthermore, an influenza-induced airway hyper-reactivity model also confirmed the idea of IL-33 is more potent in the induction of ILC2s [32, 33]. Li et al also illuminated the important role of IL-33-IL-13 axis in a murine biliary injury model [34]. Recent study has showed that IL-33 was a primary influence on the innate immune axis that drives IL-13 dependent lung disease both in mouse model and in human [35]. Consistent with these previous reports, our data demonstrated that the patients with asthma, RVI and COPD all exhib-

ited a remarkable increased level of IL-33 both in mRNA and in protein, as well as transcription factor ROR_{α}, while the expression of transcription factor T-bet was reduced. This might due to the increased circulating MDSCs executed their inhibitory function on Th1 cells by some unknown mechanisms. These results indicated that increased IL-33 was a common danger signal for respiratory inflammation including asthma, RVI and COPD, and also related to upregulation of ILC2s and MDSCs. ILC2s and MDSCs contributed to the Th2 polarization, while MDSC played an inhibitory effect on Th1 cells.

In conclusion, we demonstrated significant increases in circulating ILC2s and MDSCs, accompanied by elevated expression level of IL-33 in patients with asthma, as well as RVI or COPD. The positive correlations between the expression level of Th2 and ILC2s or MDSCs characteristic moleculars indicated that upregulation of ILC2s and MDSCs contributed to Th2 phenotype predominant in asthma, which may lead to new immunotherapy approaches for asthma based on the associated metabolites and cytokines.

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Disclosure of conflict of interest

None.

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