



IL-17RB⁺ granulocytes are associated with airflow obstruction in asthma

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ABSTRACT

Background: Interleukin (IL)-25 (IL-17E) is a proinflammatory cytokine that plays an important role in the T-helper type 2 cell pathway. The effects of IL-25 are mediated by its specific receptor, IL-17RB. Previous studies have defined an IL-17RB⁺ granulocyte population known as type 2 myeloid (T2M) cells that express T-helper type 2 cell cytokines. The correlation of IL-17RB⁺ granulocytes, T2M cells, and asthma parameters is unknown.

Objective: To investigate the relation of IL-17RB⁺ granulocytes (and its subset, T2M cells) in patients with asthma with clinical parameters including spirometric values and the Asthma Control Test (ACT).

Methods: Peripheral blood from subjects with asthma and healthy controls was collected and analyzed by flow cytometry. Granulocytes were gated for IL-17RB⁺, T2M (CD11b⁺CD16⁺CD177⁺IL-17RB⁺), and eosinophil (CD16[−]) populations. Spirometry testing was performed on subjects with asthma. ACT scores and medical histories were collected by questionnaire and chart review. Correlations of IL-17RB⁺ cells and T2M cells with spirometry and ACT score were analyzed.

Results: Percentages of IL-17RB⁺ granulocytes and T2M cells were larger in subjects with asthma than in controls. Furthermore, percentages of the 2 cell populations were negatively correlated with degree of airway obstruction as measured by the ratio of percentage-predicted forced expiratory volume in 1 second to force vital capacity ($r = -0.17$, $P = .043$ for IL-17RB⁺ granulocytes; $r = -0.32$, $P = .03$ for T2M cells). There was no correlation with ACT score. The percentage of eosinophils was increased in subjects with asthma. However, IL-17RB⁺ eosinophil percentages were similar between subjects with asthma and controls and did not correlate with any clinical parameter.

Conclusion: IL-17RB⁺ granulocytes and T2M cells from peripheral blood were increased in subjects with asthma, and the 2 cell types correlated with degree of airflow obstruction.

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Introduction

Interleukin (IL)-25 (IL-17E) belongs to a family of IL-17 cytokines (IL17A–F) that are structurally related but with distinct functions.^{1–3} There has been increasing evidence for the important role of IL-25 in driving T-helper type 2 cell (T_H2) pathway responses implicated in multiple immune-mediated conditions, including asthma and allergy development and severity.^{4–6} In animal models, IL-25 and its

receptor, IL-17RB, are increased after parasitic infection,^{7,8} long-term allergen exposure,^{6,9–13} and respiratory syncytial virus infection.^{14–16} In humans, allergen provocation increases the expression of IL-25 and its receptor in the asthmatic bronchial submucosa.¹¹

Potential sources of IL-25 in human include lung epithelial cells, bronchial endothelial cells, eosinophils, basophils, and mast cells.^{11,17,18} Activated eosinophils and basophils from subjects with and without atopy were found to secrete bioactive IL-25, which augments the functions of T_H2 memory cells.^{8,12,18,19} Therefore, IL-25 is likely involved in the innate pathway that promotes T_H2 cytokine responses in allergic and asthmatic airway inflammation.

Several cell populations express the IL-25 receptor, IL-17RB, such as eosinophils, T lymphocytes, mast cells, bronchial endothelial, and lung epithelial cells.^{11,17} Previous research by our group defined a unique population of IL-25-responsive granulocytes that were termed type 2 myeloid (T2M) cells.¹⁰ T2M cells express a set of

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defining cell surface markers, including IL-17RB, CD11b, CD16, and CD177. This distinctive population of granulocytes responds to IL-25 stimulation with the production of type 2 cytokines (IL-4 and IL-13). In mice, the adoptive transfer of T2M cells reconstituted an IL-25–mediated allergic response in IL-17RB^{-/-} animals. In addition, these cells appeared to be resistant to corticosteroids, because high-dose steroid treatment did not decrease the IL-25–induced T2M cell-associated pulmonary response. In humans, peripheral T2M cell numbers were found to be increased in a small set of 9 patients with moderate to severe asthma compared with control subjects without asthma.¹⁰ Intracellular staining of this human granulocyte population showed the expression of IL-4 and IL-13. Therefore, T2M cells could play a proinflammatory role in asthma and contribute to the severity of the response.

The role and relative importance of the T2M cell to asthma severity is currently unknown. In this study, we sought to investigate the presence of IL-17RB⁺ granulocytes and T2M cell population in the peripheral blood of subjects with asthma and determine associations with clinical parameters. Our hypothesis was that T2M cells and IL-17RB⁺ granulocytes would be correlated with airflow obstruction and asthma control.

Methods

Subjects

Potential subjects at least 18 years old with a physician diagnosis of current asthma were recruited. Information on asthma clinical history and treatment and other medical comorbidities including allergic rhinitis was obtained through patient interview and chart review. The diagnosis of allergic rhinitis was confirmed through the review of previous skin test results and clinical history. Spirometry testing was performed on each subject in accordance with American Thoracic Society guidelines.²⁰ Subjects also provided a measurement of current asthma control through the Asthma Control Test (ACT) score.²¹ Exclusion criteria included smoking within the past 6 months, any significant cardiopulmonary disease other than asthma, and current use of any long-term immune suppressant administered systemically (including systemic corticosteroids). Adult volunteers without asthma and with similar exclusion criteria served as control subjects. All subjects provided written informed consent, and the study was approved by the institutional review board of the University of Michigan (Ann Arbor).

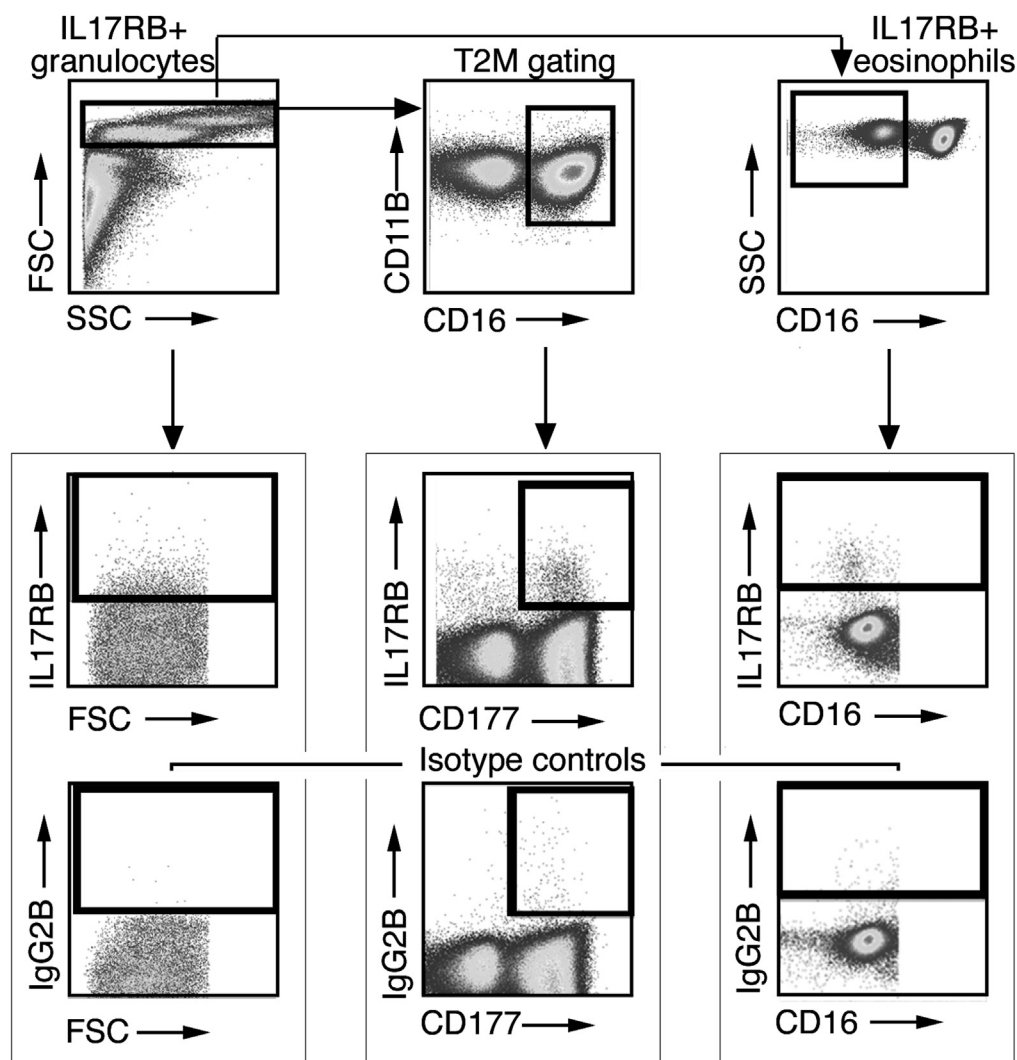


Figure 1. Granulocytes were isolated from peripheral blood samples donated by volunteers with ($n = 26$) and without ($n = 15$) asthma and analyzed with flow cytometry. Flow diagram shows the gating strategies for IL-17RB⁺ granulocytes, T2M cells (CD11B⁺CD16⁺CD177⁺IL-17RB⁺ granulocytes), and IL-17RB⁺ eosinophils (IL-17RB⁺CD16⁺ granulocytes). Granulocytes were selected and then gated for the subgroups. The bottom panel shows the correspondent isotype control for each cell type. FSC, forward-angle scatter; IL, interleukin; SSC, side-angle scatter; T2M, type 2 myeloid.

Granulocyte Isolation From Human Peripheral Blood

Peripheral blood was obtained from all subjects (20 mL). The erythrocyte layer was removed after Ficoll-Paque (GE Healthcare, Pittsburgh, Pennsylvania) separation. Granulocytes were isolated from the erythrocyte layer after erythrocyte sedimentation with 5% dextran (Sigma, St. Louis, Missouri) at room temperature for 30 minutes, followed by red blood cell lysis with 0.2% NaCl. The separated granulocytes were washed twice with FACS buffer (phosphate buffered saline with 0.5% fetal bovine serum).

Immunofluorescent Staining and Flow Cytometric Analysis

For cell surface marker staining, 1 million granulocytes were resuspended in 200 μ L of FACS buffer. FcR was blocked using purified human IgG at a final dilution of 1:500 (Sigma) for 15 minutes at 4°C. Then, cells were incubated for 30 minutes in the dark with the following anti-human monoclonal antibodies: CD177 fluorescein isothiocyanate, CD11b peridinin chlorophyll plus cyanine 5.5, CD16 biotin conjugated with Pacific Orange, IL-17RB phycoerythrin, and isotype mouse IgG2b phycoerythrin (all with 1:200 dilution, except for 1:100 dilution with IL-17RB antibody). All antibodies were from BioLegend (San Diego, California), except for anti-human IL-17RB and IgG2b isotype (R&D System, Minneapolis, Minnesota). After washing with FACS buffer, cells were resuspended in 1% paraformaldehyde and stored at 4°C. Data were collected on a BD Biosciences (San Jose, California) LSR II flow cytometer, with 6 parameters acquired: linear forward-angle light scatter, linear side-angle light scatter, log phycoerythrin, log fluorescein isothiocyanate, log Pacific Orange, and log peridinin chlorophyll plus cyanine 5.5. Data were processed with FlowJo software (Tree Star, Ashland, Oregon). Expressions of IL-17RB, T2M cells, and eosinophils were analyzed using stepwise gating (Fig 1).

Statistics

The significance of the differences between the asthmatic and control groups was analyzed using a nonparametric *t* test. Correlations between the percentage of specific cell populations and the ratio of forced expiratory volume in 1 second (FEV₁) to forced vital capacity (FVC), FEV₁, and ACT score were analyzed using a Pearson correlation analysis. Sample size calculations were based on our previous study of 9 subjects with asthma. Based on an absolute difference of 2% for T2M percentage between subjects with asthma and healthy controls, we calculated that we would need 38 subjects in a 2:1 asthma:healthy ratio to evaluate for differences in T2M and IL-17RB⁺ cells in a population of subjects with more severe asthma. This sample size also was judged to be adequate to determine correlations in clinical parameters, T2M cells, and IL-17RB⁺ cells.

Results

Forty-one subjects were enrolled in the study (26 with asthma and 15 control subjects without asthma). A summary of subject characteristics is presented in Table 1. Almost all subjects with asthma (92%) were using an inhaled corticosteroid on a daily basis, and approximately half (56%) had used a systemic steroid in the past year. Subjects with asthma were significantly more likely to have allergic rhinitis compared with controls (92% vs 20%, *P* < .001).

IL-17RB⁺ Granulocytes and T2M Cells

For peripheral granulocytes, the overall percentage of cells expressing the IL-25 receptor (IL-17RB⁺ cells) was significantly larger in subjects with asthma compared with control subjects (*P* = .03; Fig 2A). Furthermore, when examining the percentage of IL-17RB⁺ granulocytes that were of the T2M subtype (IL-17RB⁺CD177⁺CD11b⁺CD16⁺), subjects with asthma had

Table 1

Subject Characteristics

	Asthma (n = 26)	Control (n = 15)	P value
Women, n (%)	20 (77)	9 (60)	.17
Age (y), mean (SD)	48.00 (13.89)	40.71 (11.40)	.10
Race, n (%)			.01
White	22 (84.6)	11 (73.3)	
Black	4 (16)	0	
Asian	0	4 (26.7)	
Allergic rhinitis, n (%)	24 (92)	3 (20.0)	<.001
Age at asthma diagnosis, mean (SD)	25.88 (18.43)		
Current ICS use, n (%)	24 (92)		
Used systemic steroid in past year, n (%)	14 (56)		
Courses of systemic steroid in past year, average (SD)	2.8 (3.1)		
ACT score, mean (SD)	18.9 (4.2)		
FEV ₁ percentage predicted, mean (SD)	92.60 (13.64)		
FEV ₁ /FVC percentage predicted, mean (SD)	96.56 (10.43)		

Abbreviations: ACT, Asthma Control Test; FEV₁, forced expiratory volume in 1 second; FVC, forced vital capacity; ICS, inhaled corticosteroid.

significantly larger numbers than control subjects without asthma (*P* = .03; Fig 2B).

Clinical Correlation of IL-17RB⁺ Granulocytes and T2M Cells in Subjects With Asthma

We sought to identify any relation between traditional asthma parameters and IL-17RB⁺ granulocytes or T2M cells. As shown in Figure 3A, in subjects with asthma, a larger percentage of IL-17RB⁺ granulocytes was associated with a lower percentage-predicted FEV₁/FVC (*r*² = 0.17, *P* = .043). For T2M cells, the percentage of T2M cells was negatively correlated with percentage-predicted FEV₁/FVC (*r*² = 0.32, *P* = .0038; Fig 3B). There was no correlation between FEV₁ and percentage of IL-17RB⁺ granulocytes or T2M cells (data not shown). Likewise, there was no correlation between subjective symptoms reflected through ACT scores and T2M cells or IL-17RB⁺ granulocytes (*P* = .9 for IL-17RB⁺ granulocytes, *P* = .26 for T2M cells; Fig 3C,D).

Correlation of Eosinophils With Clinical Disease

Eosinophils comprise an important subset of granulocytes involved in asthma and were investigated in this study. Compared with controls without asthma, the percentage of eosinophils among granulocytes was larger in subjects with asthma (*P* = .002; Fig 4A). However, among eosinophils, the percentage of cells that expressed IL-17RB was similar between subjects with asthma and controls (Fig 4B). In subjects with asthma, the percentage of eosinophils was not correlated with FEV₁/FVC (Fig 4C). Furthermore, the percentage of IL-17RB⁺ eosinophils did not correlate with FEV₁/FVC (Fig 4D) or with FEV₁ or ACT scores (data not shown).

Discussion

As a chronic inflammatory disease of the airways, allergic asthma involves the interplay of many cells and molecular pathways. IL-25 and its receptor, IL17-RB, have been described as important regulators of type 2 immunity. Previous research has suggested that lung epithelial cell-derived IL-25, in addition to IL-33 and thymic stromal lymphopoietin, initiate and augment T_H2-type airway inflammation and remodeling, leading to pathogenesis and complications of asthma.^{22,23} Other investigators have found that IL-17RB is expressed in the asthmatic airway on various cells such as eosinophils, mast cells, and endothelial cells.¹¹

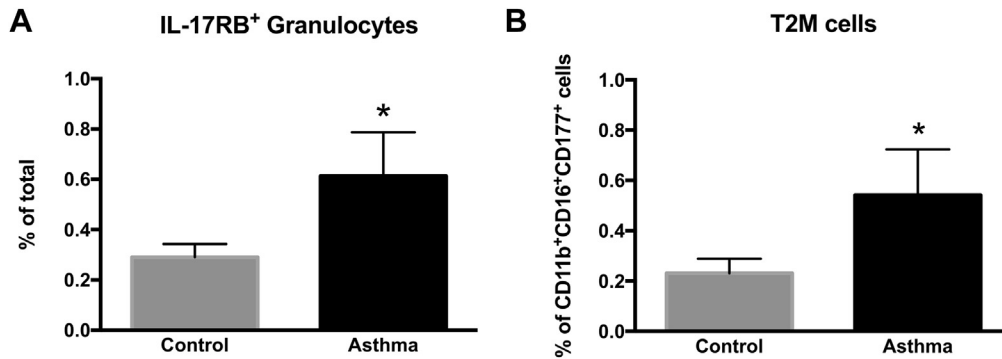


Figure 2. Results of flow cytometric analysis of peripheral blood samples donated by volunteers with ($n = 26$) and without ($n = 15$) asthma. (A) Percentage of IL-17RB⁺ granulocytes from subjects with asthma compared with control subjects ($*P = .03$). (B) Percentage of T2M cells from subjects with asthma compared with control subjects ($*P = .03$). Percentage of T2M is expressed as the percentage of IL-17RB⁺ cells among CD11b⁺CD16⁺CD177⁺ granulocytes. All data are presented as mean $\pm$ SEM. IL, interleukin; T2M, type 2 myeloid.

In our previous study, a small group of 9 patients with asthma showed increased expression of IL-17RB on peripheral granulocytes and T2M cells (IL-17RB⁺CD11b⁺CD16⁺CD177⁺ granulocytes) compared with controls without asthma. The present study supports and extends the previous observations by verifying that percentages of IL-17RB⁺ granulocytes and T2M cells are increased in subjects with asthma compared with healthy controls. We included an expanded number of 26 subjects with asthma, and asthma severity also was better characterized. Of subjects with asthma, nearly all were on an inhaled steroid (92%), and more than half needed a systemic steroid in the past year (56%), indicating more severe asthma.

More importantly, the present study provides novel data that IL-17RB⁺ granulocytes and T2M cells correlate with the severity of airway obstruction (percentage-predicted FEV₁/FVC). Our previous observations that T2M cells produce T_H2-type cytokines, IL-4 and IL-13,¹⁰ might help explain these findings. Specifically, IL-13 induces many features of allergic lung disease, including airway hyperresponsiveness, goblet cell metaplasia, and mucus hypersecretion, which contribute to airway obstruction.^{24–27} Previous studies on IL-25 and its cellular targets have focused mostly on local airway pathology through methods such as bronchial biopsy or cell culture of asthmatic lung tissue.^{11,18,28} The present study investigated the effects of IL-25 on systemic inflammation in asthma

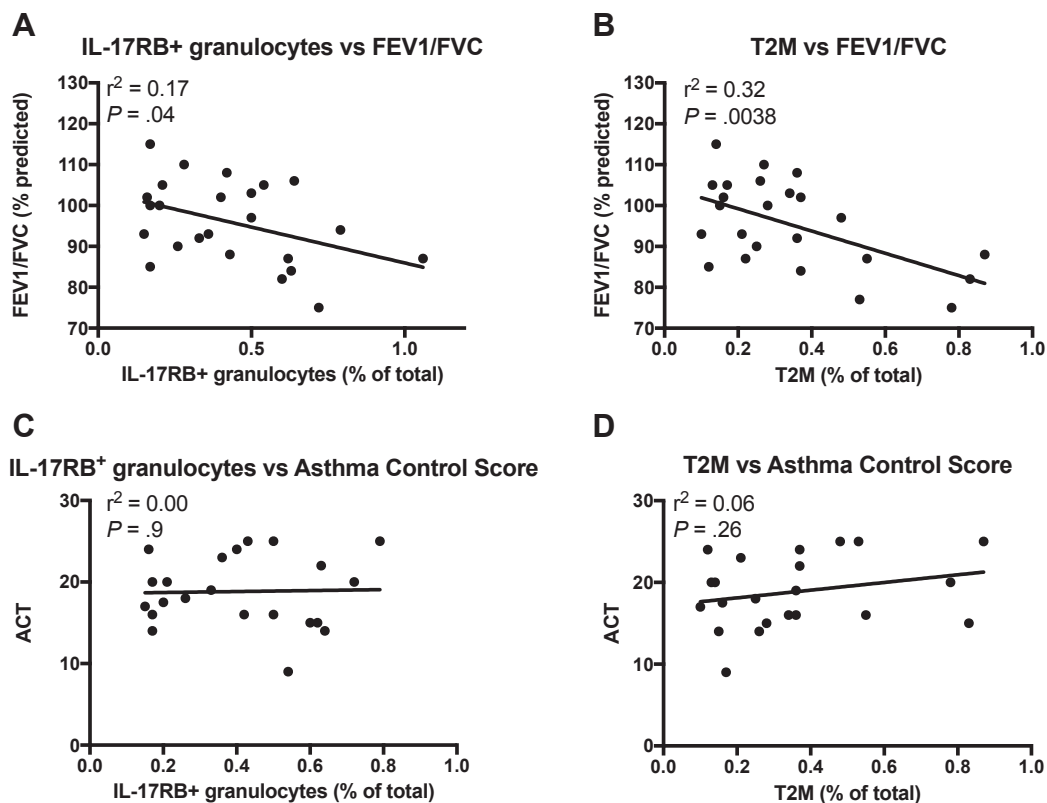


Figure 3. For subjects with asthma ($n = 26$), correlations were analyzed between cell percentage and spirometry parameter or ACT score. (A) Correlation analysis of IL-17RB⁺ granulocytes with FEV₁/FVC ($r^2 = 0.17$, $P = .043$). (B) Correlation analysis of T2M cells with FEV₁/FVC ($r^2 = 0.32$, $P = .0038$). (C) Correlation analysis of IL-17RB⁺ granulocytes with ACT score ($r^2 = 0$, $P = .9$). (D) Correlation analysis of T2M cells with ACT score ($r^2 = 0.06$, $P = .26$). ACT, Asthma Control Test; FEV₁, forced expiratory volume in 1 second; FVC, forced vital capacity; IL, interleukin; T2M, type 2 myeloid.

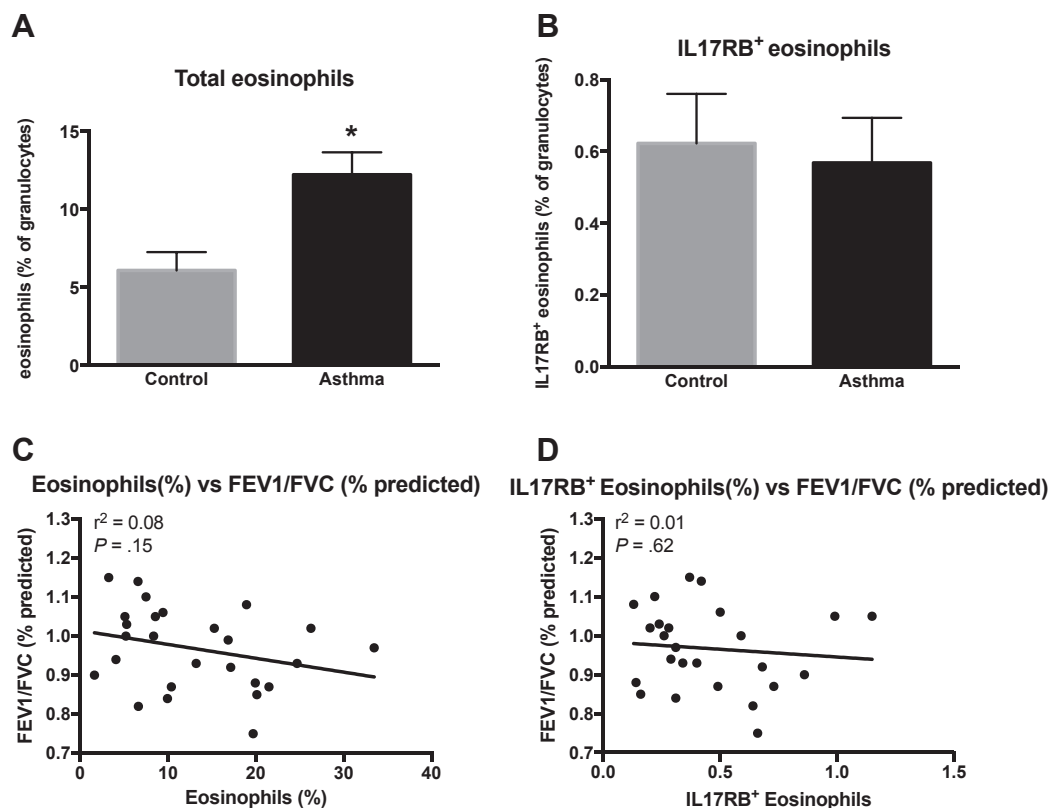


Figure 4. Percentages of eosinophil and eosinophil IL-17RB expression were analyzed in peripheral blood granulocytes from volunteers with ($n = 26$) and without ($n = 15$) asthma. (A) Percentage of eosinophils in granulocytes from subjects with asthma compared with control subjects ($P = .002$). (B) Percentage of IL-17RB⁺ eosinophils in granulocytes from subjects with asthma compared with control subjects ($P = .73$). (C) Correlation analysis of eosinophil percentage with FEV₁/FVC ($r^2 = 0.08$, $P = .15$). (D) Correlation analysis of IL-17RB⁺ eosinophil percentage with FEV₁/FVC ($r^2 = 0.01$, $P = .62$). FEV₁, forced expiratory volume in 1 second; FVC, forced vital capacity; IL, interleukin.

through an analysis of peripheral granulocytes. Overall, we found correlations of specific markers and cell populations in peripheral blood with degree of airway obstruction. This study did not show a correlation with the subjective symptomatology scoring as in the ACT. IL-17RB might correlate more with baseline airway remodeling, airflow obstruction, and hyperresponsiveness than with currently perceived symptom control. Future studies examining the correlation of T2M or IL-17RB⁺ cells with airway hyperresponsiveness through methacholine challenge could prove useful to explore this relation further. Eosinophils are believed to be an important factor related to airway damage through the release of various toxic granule proteins.^{19,29} In the analysis of granulocytes, we also assessed eosinophil percentage and used flow cytometric gating to identify the subset of eosinophils that were IL-17RB⁺. Similar to previous studies,^{30–32} the percentage of eosinophils was larger in subjects with asthma. However, for eosinophils, the percentages of cells that expressed IL-17RB were similar between subjects with asthma and controls. Furthermore, there was no correlation between percentage of IL-17RB⁺ eosinophils with pulmonary function parameters (FEV₁/FVC or FEV₁). Thus, the IL-17RB⁺ granulocytes responsible for the inverse correlation between FEV₁/FVC and IL-17RB⁺ cells appear to be a distinct granulocytic population rather than an eosinophilic population, and T2M cells could play a role in this relation. These findings provided insight into a distinct asthmatic endotype. In patients who express high IL-17RB, there might not be an eosinophil-predominant pathophysiology. Previous studies have suggested that eosinophilic airway inflammation predicts a better response to inhaled corticosteroids, whereas non-eosinophilic asthma is less responsive.^{33,34} Interestingly, high-dose dexamethasone treatment

did not decrease the IL-25–induced T2M pulmonary response in a previous murine study.¹⁰

Neutrophilic asthma has been described as a unique endotype.³⁵ T2M cells appear to be a steroid-resistant cell population, similar to neutrophils. However, the exact cellular designation of the T2M cell is not fully resolved. Although the overall surface receptor expressions are distinct based on microarray profile (suggesting the T2M cell represents a separate subpopulation of granulocytes), the relation between the T2M cell and the neutrophil is still being elucidated.

There are limitations to this study. Bronchoscopy and lung biopsy examination were not performed, and the findings of the peripheral blood might not accurately reflect the cellular physiology of the lung. We were unable to define steroid resistance in patients with limited data from chart review, although the average number of systemic steroid courses in the past year in subjects with asthma was 2.8, indicating a more severe asthma population. Because of the sample size, subjects with asthma were not stratified by asthma severity or use of oral corticosteroids. Future studies with a larger cohort might be able to detect an interaction effect for the T2M cell based on such strata. Furthermore, this is a cross-section study, and cause and effect cannot be conclusively stated as to whether a higher expression of IL-17RB or T2M might cause steroid resistance. A future prospective study could be helpful to find the relation. An important consideration for our study was that the patients who came to our asthma clinic were in a relatively stable condition. Patients with acute asthma exacerbation, such as those who present to emergency departments or urgent care centers, would have a greater upregulation of inflammatory cells and cytokines. The most common cause of severe

exacerbations has been identified as respiratory viral infections.³⁶ Our laboratory recently identified that respiratory syncytial virus infection elicits enhanced IL-25 production and increases T2M-type cells in the lungs of allergic animals in an IL-17RB-dependent manner. The same study also showed that inhibition of IL-25 or deletion of IL-17RB decreased T2M cells and attenuated the exacerbation responses.¹⁶ Thus, future studies examining these parameters in patients with asthma might provide further insight into the role of IL-17RB⁺ granulocytes in the progression of disease.

In summary, granulocytes expressing IL-17RB and T2M cells (a subset of IL-17RB⁺ granulocytes) are upregulated in subjects with asthma compared with controls and are correlated with clinical measurements of airway obstruction. IL-17RB⁺ granulocytes and T2M cells could serve as biomarkers and tools for clinical assessment and diagnosis and as potential therapeutic targets. Further investigations on a larger scale, in various clinical settings, would be important and helpful.

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