

IMMUNOPATHOGENESIS

Altered Expressions of Helper T Cell (Th)1, Th2, and Th17 Cytokines in CD8⁺ and $\gamma\delta$ T Cells in Patients with Allergic Asthma

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Background. T cells play an important role in orchestrating allergic asthma by producing various cytokines; however, little information is available on the phenotype of cytokine-producing T cells, especially CD8⁺ T and $\gamma\delta$ T cells in humans. **Objective.** The aim of this study was to investigate the role of cytokine expressions from circulating CD4⁺, CD8⁺, and $\gamma\delta$ T cells in patients with allergic asthma. **Methods.** Peripheral blood mononuclear cells were isolated from patients with allergic asthma ($n = 29$) and healthy controls ($n = 12$). The percentage of helper T cell (Th)1-, Th2-, and Th17-type cytokines interferon- γ (IFN- γ), interleukin (IL)-4, and IL-17, respectively, of CD4⁺, CD8⁺, and $\gamma\delta$ T cells was analyzed by flow cytometry. **Results.** The percentages of IL-4⁺ and IL-17⁺ CD4⁺ T cells increased, whereas IFN- γ ⁺ CD4⁺ T cells decreased in patients with allergic asthma compared with healthy controls. The frequency of IL-17⁺ CD4⁺ T cells tended to increase with the severity of allergic asthma. Higher frequency of IL-4-producing and lower frequency of IFN- γ -producing CD8⁺ and $\gamma\delta$ T cells were found in patients with allergic asthma compared with healthy controls. The IFN- γ /IL-17⁺ ratio among CD4⁺, CD8⁺, and $\gamma\delta$ T cells was significantly decreased in patients with allergic asthma compared with healthy controls. **Conclusion.** CD8⁺ and $\gamma\delta$ T cells might be involved in the pathogenesis of allergic asthma through the release of Th1-, Th2-, and Th17-type cytokines.

Keywords allergic asthma, CD8⁺, Th17, $\gamma\delta$ T

INTRODUCTION

Allergic asthma is a complex and chronic inflammatory airway disease characterized by the infiltration of inflammatory cells such as T cells, eosinophils, neutrophils, and mast cells. Among them, T cells play an important role in orchestrating the disease by producing various cytokines. According to the type of antigen-recognition receptors (T-cell receptor, TCR), T cells can be categorized into $\alpha\beta$ T cells and $\gamma\delta$ T cells. The former can be subdivided into CD4⁺ T cells and CD8⁺ T cells based on their distinct phenotype. Most studies in the past have focused on the functions of CD4⁺ T cells, especially the imbalance of their subgroup T helper (Th)1/Th2 cells, in the pathogenesis of allergic asthma (1). Recently, Th17 cells, a novel CD4⁺ subset characterized by the secretion of interleukin (IL)-17, are also found to be relevant in the initiation, development, and persistence of allergic asthma (2, 3). However, little information is known about the role of Th1, Th2, and Th17 cytokine-producing T cells, especially CD8⁺ T cells and $\gamma\delta$ T cells in humans.

CD8⁺ T cells can be classified into two subsets: T cytotoxic (Tc)1 and Tc2 cells. Interestingly, Tc1 cells predominantly secrete Th1 cytokine interferon (IFN- γ), whereas Tc2 produce Th2 cytokine IL-4 (4). There was evidence showing that CD8⁺ T cells were also

involved in the immune responses of asthma. Tc1 cells may act to alleviate inflammation and suppress airway hyperresponsiveness within the asthmatic lung (5, 6), whereas Tc2 could exacerbate asthma in a similar manner to Th2 cells within established asthma (7, 8). Additionally, Stanciu et al. suggested that CD8⁺ T cells from asthmatic patients might be an important source of IL-4 (9), and CD8⁺ T cells might contribute to the pathology of asthma death (10). However, the potential role of Tc1 and Tc2, especially that of the Tc1/Tc2 imbalance, has not been extensively studied.

As for $\gamma\delta$ T cells, studies in both humans and mice show that $\gamma\delta$ T cells also play a role in the process of allergic asthma. Higher levels of $\gamma\delta$ T cells have been observed in the bronchi alveolar lavage fluid (BALF) of asthmatic patients compared with those in healthy controls (11). It was reported that $\gamma\delta$ T cells were the first IFN- γ -producing T cells in ontogeny in both mice and humans, and they had essential functions in host protection in early life (12, 13). It was remarkable that $\gamma\delta$ T cells were also essential for inducing IL-4-dependent IgE and IgG responses as well as for Th2-mediated airway inflammation (14), suggesting that they may participate in the development of allergic asthma.

Furthermore, CD8⁺ T and $\gamma\delta$ T cells can also produce IL-17 under certain conditions (15, 16). $\gamma\delta$ T cells may secrete more IL-17 than CD4⁺ T cells during *Mycobacterium tuberculosis* infection. In this case, $\gamma\delta$ T cells isolated from the lung are the main source of IL-17 (17). These IL-17-producing $\gamma\delta$ T cells share some common features with Th17 cells (18). However,

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there are few studies concerning IL-17 production in the circulating CD8⁺ and $\gamma\delta$ T cells. Therefore, it is essential to investigate the functions of these IL-17-producing cells in the pathogenesis of allergic asthma.

In this study, the individual percentages of CD4⁺, CD8⁺, and $\gamma\delta$ T cells which secreted Th1-type cytokine IFN- γ , Th2-type cytokine IL-4, and Th17-type cytokine IL-17 were found from the peripheral blood of both patients with allergic asthma and healthy controls. Then the number of these cells was analyzed to correlate with the severity of allergic asthma. Finally, the IFN- γ ⁺/IL-17⁺ ratio among the three subsets was evaluated to figure out the balance between IFN- γ -producing and IL-17-producing T cells in both subject groups.

MATERIALS AND METHODS

Subjects

Twenty-nine patients with allergic asthma (17 women, 12 men) were recruited from outpatients and inpatients of Renmin Hospital and Zhongnan Hospital of Wuhan University. Pulmonary functions were assessed by spirometry according to the American Thoracic Society standards (19). The diagnostic criteria and severity assessment of asthma were evaluated on the basis of the Global Initiative for Asthma criteria (20). The asthmatic patients were divided into three groups: mild (mild intermittent, $n = 6$; mild persistent, $n = 7$), moderate ($n = 7$), and severe ($n = 9$) asthmatics. All the patients were allowed to inhale glucocorticoid if the disease got worse during the 4 weeks prior to the study, but no systemic steroids were used. In total, 10 patients were treated with inhaled glucocorticoid: 500 μ g/day fluticasone or equivalent (mild = 3, moderate = 3, severe = 4). All patients had positive skin-prick tests, defined as a >5-mm diameter skin-wheal response to at least 1 of the 10 common allergens (*Dermatophagoides pteronyssinus*, *D. farinae*, mixed grass pollen, mixed tree pollen, dog hair, feather, cat fur, fluffed cotton, cockroach, and alternaria). The control group included 12 sex- and age-matched healthy volunteers (7 women, 5 men) who had normal spirometry tests. Both groups were chosen on the basis of (1) being nonsmokers; (2) having no other allergic symptoms and no history of

upper or lower airway diseases for 2 months prior to the study as well as no chronic heart and/or pulmonary diseases; (3) having not received immunotherapy in the past 5 years; having not received oral or intravenous steroids in the past 4 weeks, but allowed to receive inhaled steroids; having not received theophylline, long-acting β 2-agonists, leukotriene antagonists, or antihistamines in the past 2 months before the study (for all subject characteristics, see Table 1). All the subjects were fully informed about the purpose and nature of the study, which was approved by the medical ethics committee of Renmin Hospital and Zhongnan Hospital of Wuhan University.

Cell Separation

From both patients with allergic asthma and healthy controls, 12 ml peripheral venous blood was collected. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation within 1 h of collection. The blood samples were heparinized, 1:2 diluted with phosphate-buffered saline (PBS), then laid on Lymphoprep ($d = 1.077$ mg/ml; Nycomed, Oslo, Norway), and centrifuged at 2000 rpm for 20 min at room temperature. PBMCs were collected and washed twice with PBS. Cell viabilities were more than 95% as examined by trypan blue dye exclusion assays.

Cell Culture

PBMCs were resuspended in RPMI 1640 medium (Sigma-Aldrich, St Louis, MO, USA) containing L-glutamine, HEPES buffer, and 10% fetal calf serum (R10) to a concentration of 1×10^6 cells/ml. Cells were activated with 50 ng/ml phorbol-12-myristate-13-acetate (PMA, Sigma-Aldrich) and 250 ng/ml ionomycin (Sigma-Aldrich) for 4 h at 37°C in a 5% CO₂ atmosphere, then stimulated for an additional 2 h in the presence of 1 μ g/ml Brefeldin A (Fluca, Buchs, Switzerland). Cells were harvested for intracellular staining and flow cytometry analysis.

Flow Cytometry Assay

Cytokine-producing cells were sorted by surface antigen and intracellular cytokine staining using a flow cytometry (EPICS ALTRA α ; Beckman, Seattle, WA, USA). The CD3⁺CD8[−] cells were defined as CD3⁺CD4⁺ cells due to the downregulation of surface CD4 molecules

TABLE 1.—Characteristics of the subjects.

Characteristics	Healthy controls ($n = 12$)	Allergic asthmatics ($n = 29$)		
		Mild ($n = 13$)	Moderate ($n = 7$)	Severe ($n = 9$)
Age (year)	33.75 $\pm$ 7.84	36.43 $\pm$ 8.83	46.10 $\pm$ 10.5	42.25 $\pm$ 11.2
Sex (male/female)	5/7	6/7	3/4	3/6
Inhaled glucocorticoid	0	3	3	4
FEV ₁ pred.%	104.92 $\pm$ 5.19	88.14 $\pm$ 6.08 ^a	71.00 $\pm$ 6.19 ^b	47.33 $\pm$ 8.80 ^c

Data are expressed as means $\pm$ SD. FEV₁ pred.%, predicted percentage of forced expiratory volume in the first second. Significant differences were compared with ^ahealthy controls, ^bmild asthma, and ^call other groups ($p < .05$).

after stimulation with PMA and ionomycin. Half of the cells were incubated with phycoerythrin (PE)-cy5-labeled anti-human CD3 and fluorescein isothiocyanate (FITC)-labeled anti-human CD8 (eBioscience, USA), while the remaining cells were stained with FITC-labeled anti-human $\gamma\delta$ TCR (eBioscience). Both batches of cells were incubated for 30 min at 4°C in the dark. After washing, cells were fixed and permeabilized with fixation solution and permeabilization buffer (eBioscience, San Diego, CA, USA) for 20 min at room temperature in the dark. The cells were then washed twice with permeabilization buffer and incubated with PE-labeled anti-human cytokine antibodies (IFN- γ , IL-4, and IL-17, eBioscience), respectively, for 20 min at room temperature in the dark. Nonspecific FITC-, PE-, and PE-cy5-conjugated antibodies (eBioscience) were used as controls. Cells were resuspended in PBS and analyzed on flow cytometer. Flow cytometric analysis was performed with scatter gates on the lymphocyte fraction. Results are expressed as a percentage of positive cells.

Statistics

All data were presented as means $\pm$ standard deviation. Differences of clinical characteristics were analyzed by one-way analysis of variance. Differences between patients with allergic asthma and healthy control were assessed by Mann-Whitney *U*-test. For comparison of paired data within the groups, Kruskal-Wallis *H*-test was used. Difference is considered to be significant when $p < .05$. All analyses were performed using the Statistical Package for the Social Sciences statistical software for Windows, version 11.5 (SPSS Inc., Chicago, IL, USA).

RESULTS

Characteristics of Subjects

In this study, 12 healthy individuals and 29 patients with allergic asthma were enrolled. Characteristics of participants are given in Table 1. The mean predicted percentage of FEV₁ (FEV₁ pred.%) was significantly lower in patients with allergic asthma than in healthy controls ($p < .05$), and the FEV₁ pred.% of asthmatics declined significantly with the severity of the disease ($p < .05$).

Percentages of Th2 and Th17 Cytokine-Producing CD4⁺ T Cells in PBMCs Increased in Patients with Allergic Asthma

Isolated PBMCs from patients with allergic asthma and healthy controls were stimulated with PMA and ionomycin. The percentages of Th1, Th2, and Th17 cytokine-producing CD4⁺ T cells were measured by flow cytometry. The CD3⁺CD8⁻ cells were defined as CD3⁺CD4⁺ cells. Representative cytometric profiles of cytokines-positive CD3⁺CD8⁻ T cells from patients with allergic asthma and healthy controls are shown in Figure 1A. Percentages of IL-4⁺ and IL-17⁺ CD3⁺CD8⁻ T

cells in PBMCs were higher in patients with allergic asthma compared with healthy controls ($5.18 \pm 2.77\%$ vs. $1.59 \pm 0.56\%$, $p < .01$ and $2.32 \pm 1.15\%$ vs. $0.95 \pm 0.31\%$, $p < .05$). In contrast, the percentage of IFN- γ ⁺ CD3⁺CD8⁻ T cells was lower in asthmatics than in healthy controls ($2.48 \pm 2.37\%$ vs. $12.23 \pm 4.32\%$, $p < .01$) (Figure 1B). The percentage of IL-17⁺ CD3⁺CD8⁻ T cells tended to increase with the severity of allergic asthma ($p < .05$) (Figure 1C).

Percentages of Th2 Cytokine-Producing CD8⁺ T Cells in PBMCs Increased in Patients with Allergic Asthma

Representative cytometric profiles of cytokines-positive CD8⁺ T cells from an allergic asthma patient are shown in Figure 2A. The frequencies of IL-4-producing CD8⁺ T cells were higher in patients with allergic asthma than in healthy controls ($5.06 \pm 1.06\%$ vs. $1.54 \pm 0.38\%$, $p < .01$). In contrast, the percentages of IFN- γ ⁺ CD8⁺ T cells were lower in asthmatics than in healthy controls ($2.64 \pm 1.85\%$ vs. $15.40 \pm 3.05\%$, $p < .01$). There were no differences in the percentages of IL-17-producing CD8⁺ T cells between the two groups ($p > .05$) (Figure 2B).

Percentages of Th2 Cytokine-Producing $\gamma\delta$ T Cells in PBMCs Increased in Patients with Allergic Asthma

Representative cytometric profiles of IFN- γ ⁺, IL-4⁺, and IL-17⁺ $\gamma\delta$ T cells from an allergic asthma patient are shown in Figure 3A. More IL-4-producing $\gamma\delta$ T cells were observed in patients with allergic asthma than in healthy controls ($2.38 \pm 0.81\%$ vs. $1.49 \pm 0.50\%$, $p < .01$). On the contrary, less IFN- γ ⁺ $\gamma\delta$ T cells were detected in asthmatics than in healthy controls ($4.60 \pm 1.61\%$ vs. $6.14 \pm 1.23\%$, $p < .01$). No differences in frequencies of IL-17-producing $\gamma\delta$ T cells were found between the two groups ($p > .05$) (Figure 3B).*

The Ratio of Percentages of IFN- γ ⁺/IL-17⁺ Cells in CD4⁺, CD8⁺, and $\gamma\delta$ T Cells All Decreased in Patients with Allergic Asthma

It was shown that IL-17 production was negatively regulated by IFN- γ (21), therefore we calculated the ratio of percentages of IFN- γ ⁺ cells to IL-17⁺ cells among CD4⁺, CD8⁺, and $\gamma\delta$ T cells in PBMCs. As shown in Figure 4, the IFN- γ ⁺/IL-17⁺ ratio among CD4⁺, CD8⁺, and $\gamma\delta$ T cells were all markedly decreased in patients with allergic asthma compared with healthy controls (all $p < .01$). Furthermore, we explored whether there was a significant correlation between IL-17-producing cells and IL-4-producing T cells, but no relationship was found (data not shown).

DISCUSSION

In this study, we demonstrated altered productions of IFN- γ , IL-4, and IL-17 in CD4⁺, CD8⁺, and $\gamma\delta$ T cells,

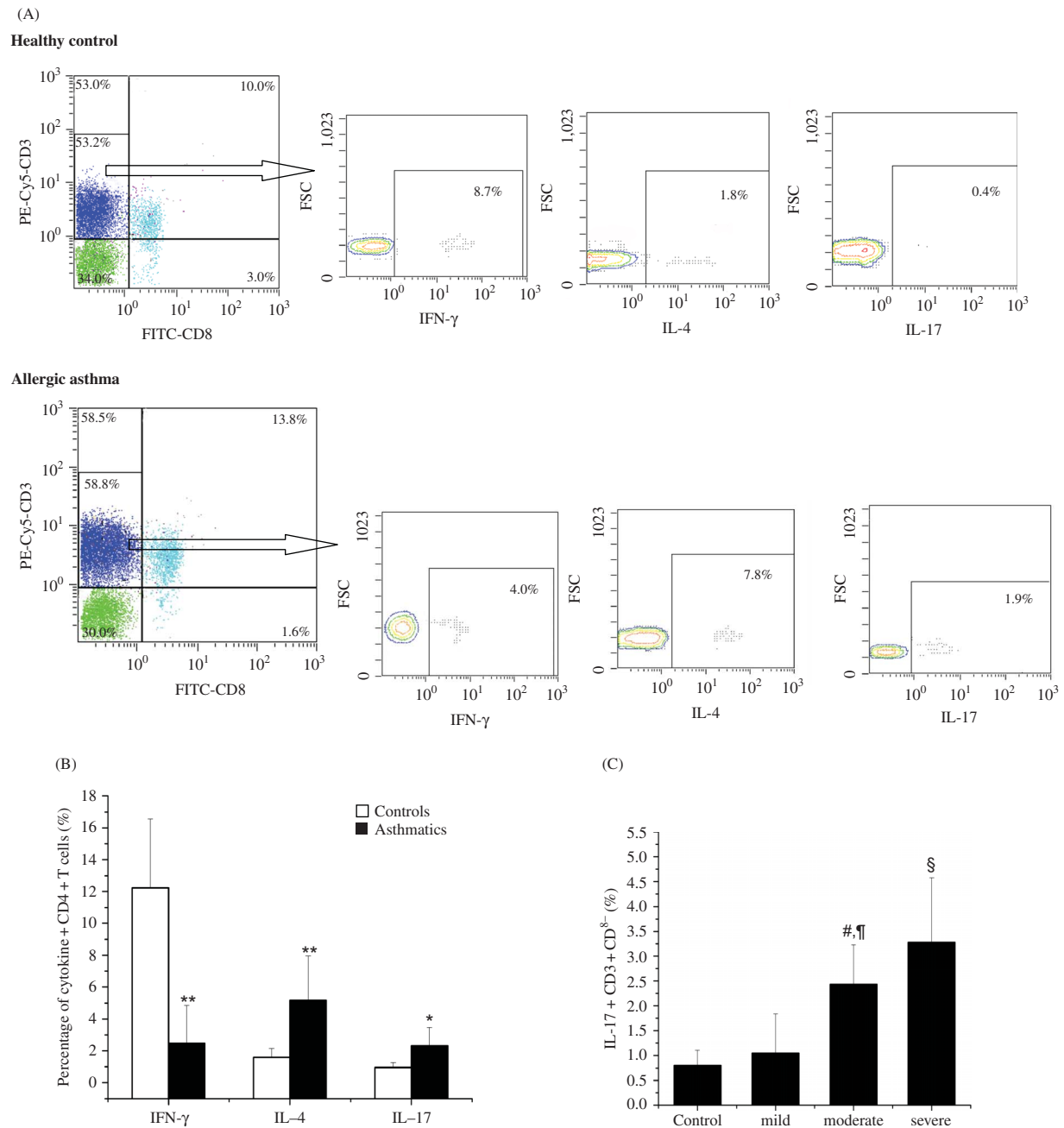


FIGURE 1.—The frequencies of IFN- γ (Th1)-, IL-4 (Th2)-, and IL-17 (Th17)-expressing CD4⁺ cells in PBMCs from both allergic asthmatics ($n = 29$) and healthy controls ($n = 12$). PBMCs were stimulated with PMA and ionomycin for 6 h, and then stained with fluorescence-labeled antibodies against CD3, CD8, and the indicated intracellular cytokines. (A) Representative flow cytometric profiles of the IFN- γ -, IL-4-, and IL-17-producing CD3⁺CD8⁻ T cells from an allergic asthma patient and a healthy control. (B) Percentages of CD3⁺CD8⁻ T cells with positive intracellular staining for IFN- γ , IL-4, and IL-17 in PBMCs from allergic asthmatics and healthy controls. Percentages of the indicated cells are shown in the quadrant areas. ** $p < .01$ compared with control subjects. (C) The percentage of Th17 cells was associated with severity of allergic asthma. Columns represent mean values * $p < 0.05$ as compared with control subjects, ($\pm$ SD). FSC, forward scatter; # $p < .05$ versus healthy controls, ¶ $p < .05$ versus mild asthma, and § $p < .05$ versus all other groups. Mild ($n = 13$), moderate ($n = 7$), and severe ($n = 9$).

respectively, in peripheral blood in allergic asthmatics, which suggested that these three subsets of T cells were involved in the systemic immune response of allergic asthma dependent on Th1, Th2, and Th17 cytokines.

It has been well established that CD4⁺ T cells and the imbalance of Th1/Th2 cells are critical for the initiation, progression, and persistence of allergic asthma (1). However, besides CD4⁺ T cells, CD8⁺ and $\gamma\delta$ T cells also play a role in the pathogenesis of allergic asthma.

In this study, IL-4-producing CD8⁺ T cells increased whereas IFN- γ -producing CD8⁺ T cells decreased in patients with allergic asthma compared with healthy controls. Therefore, an imbalance of Tc1/Tc2 cells existed in patients with allergic asthma, similar to the imbalance of Th1/Th2 in CD4⁺ T cells, indicating that CD8⁺ T cells contribute to the pathogenesis of allergic asthma partly through the Th1- and Th2-type cytokines. Our findings were consistent with previous studies which

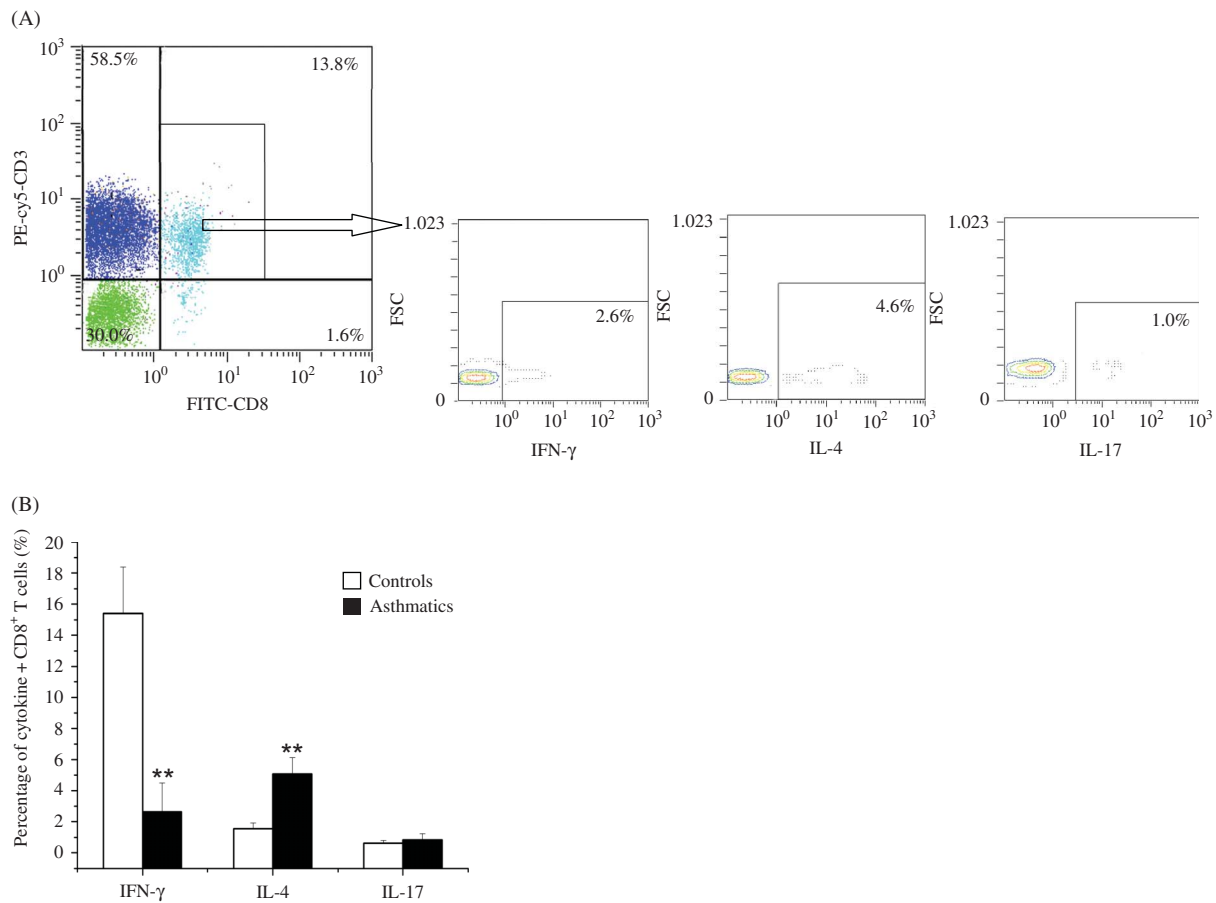


FIGURE 2.—The frequencies of IFN- γ (Th1)-, IL-4 (Th2)-, and IL-17 (Th17)-expressing CD8⁺ T cells in PBMCs from allergic asthma ($n = 29$) and healthy control subjects ($n = 12$). (A) Representative flow cytometric profiles of the IFN- γ -, IL-4-, and IL-17-producing CD8⁺ T cells from an allergic asthma patient. (B) Percentages of CD8⁺ T cells with positive intracellular staining for IFN- γ , IL-4, and IL-17 in PBMCs from allergic asthmatics and healthy controls. Percentages of the indicated cells are shown in the quadrant areas. Columns represent mean values ($\pm$ SD). FSC, forward scatter; ** $p < .01$, compared with control subjects.

observed decreased productions of IFN- γ in CD8⁺ T cells for patients with allergic asthma (22). However, other studies reported a higher (23, 24) or even normal (25) percentage of IFN- γ -producing CD8⁺ T cells in patients with allergic asthma. These deviations might be due to the difference of methodologies applied in studies, the characteristics of enrolled patients, as well as the severity of the disease. For example, intracellular cytokines could be detected by flow cytometry only after the cells were stimulated and activated *in vitro*. This may lead to a shift of the cytokine repertoire toward Th1 type, especially in patients with allergic asthma (26). On the other hand, patients enrolled in these studies were at different disease stages and with different severities, for example, at birth, in childhood, or early in the disease process (27, 28). In this study, we mainly focused on adult patients with different severities, with some of them severe. In addition, the observation in circulating CD8⁺ T cells may not necessarily reflect those in local airways. Therefore, further functional studies are needed to evaluate the role of CD8⁺ T cells in asthmatic patients.

$\gamma\delta$ T cells constitute a minor fraction of the total T cells. They are enriched in many tissues, including

sub-epithelium of alveolar and nonalveolar regions of the lung (29). They play an important role in the immune response against inhaled particles, such as microbial pathogens and toxic substances (20). Activated $\gamma\delta$ T cells exhibit extensive cytotoxic ability and can produce a range of cytokines and chemokines including Th1- and Th2-type cytokines (30). Higher levels of IL-4-producing and lower levels of IFN- γ -producing $\gamma\delta$ T cells have been observed in the BALF of asthmatic patients (11, 28). Similar changes of IL-4-producing and IFN- γ -producing $\gamma\delta$ T cells were also found in systemic circulation in our study. Therefore, it indicated that Th1 and Th2 cytokine-producing circulating $\gamma\delta$ T cells might participate in the onset of allergic reactions and in the development of asthma in patients.

Furthermore, IL-17 can stimulate the release of neutrophil-mobilizing cytokines, and then induce neutrophilic airway inflammation, predominantly in acute exacerbations of asthma or severe asthmatics (31). And IL-17-producing $\gamma\delta$ T cells played an important role in some autoimmune and atopic diseases (18, 32). In accordance with previous work (31), our study demonstrated that the levels of IL-17-producing CD4⁺ T cells increased in patients with allergic asthma and were

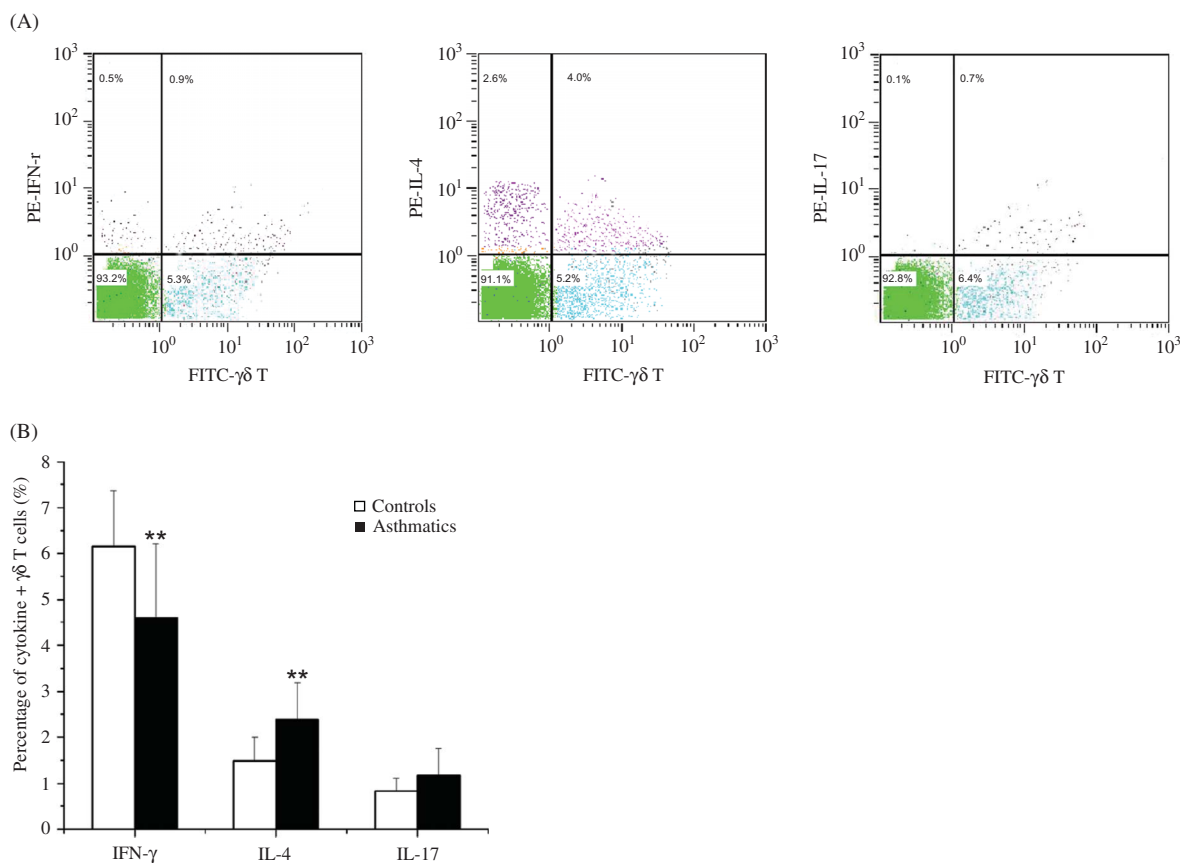


FIGURE 3.—The frequencies of IFN- γ (Th1)-, IL-4 (Th2)-, and IL-17 (Th17)-expressing $\gamma\delta$ T cells in PBMCs from allergic asthma ($n = 29$) and healthy control subjects ($n = 12$). (A) Representative flow cytometric profiles of the IFN- γ -, IL-4-, and IL-17-producing $\gamma\delta$ T cells from an allergic asthma patient. (B) Percentages of $\gamma\delta$ T cells with positive intracellular staining for IFN- γ , IL-4, and IL-17 in PBMCs from allergic asthmatics and healthy controls. Percentages of the indicated cells are shown in the quadrant areas. Columns represent mean values ($\pm$ SD). FSC, forward scatter; ** $p < .01$, compared with control subjects.

correlated with disease severity. However, no differences in IL-17 production in CD8 $^{+}$ and $\gamma\delta$ T cells were found between these two groups. Based on these evidences, we hypothesized that the increase in production of IL-17 in circulation of asthmatic patients was predominantly from CD4 $^{+}$ T cells. These results also suggested that CD8 $^{+}$ and $\gamma\delta$ T cells contribute to the process of asthma by mechanisms other than the secretion of IL-17.

In addition, the production of IL-17 was negatively regulated by IFN- γ in many diseases such as experimental autoimmune encephalomyelitis (3) and TB (33). Here, we calculated the ratio of IFN- γ^{+} cells to IL-17 $^{+}$ cells in CD4 $^{+}$, CD8 $^{+}$, and $\gamma\delta$ T cells, respectively. We found that the ratio of IFN- γ^{+} to IL-17 $^{+}$ cells in the three T subsets all markedly decreased in patients with allergic asthma compared with healthy controls. However, we did not find any correlation between IL-17-producing and IL-4-producing T cells. Therefore, we hypothesized that besides Th1/Th2 cytokine imbalance, the Th1/Th17 cytokine imbalance may represent another mechanism involved in the pathogenesis of asthma.

In conclusion, we demonstrated a Th1/Th2 cytokine imbalance in circulating CD8 $^{+}$ and $\gamma\delta$ T cells from patients with allergic asthma and the IFN- γ^{+} /IL-17 $^{+}$ ratio markedly decreased in CD4 $^{+}$, CD8 $^{+}$, and $\gamma\delta$ T cells in patients with allergic asthma compared with

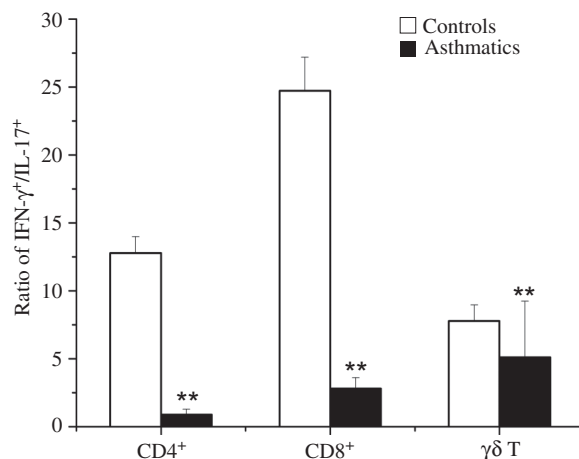


FIGURE 4.—The ratio of percentages of IFN- γ^{+} cells to IL-17 $^{+}$ cells among CD4 $^{+}$, CD8 $^{+}$, and $\gamma\delta$ T cells in PBMCs from allergic asthma ($n = 29$) and healthy control subjects ($n = 12$). ** $p < .01$, compared with control subjects.

healthy controls. These results indicated that besides Th1/Th2 cytokine imbalance, the Th1/Th17 cytokine imbalance may represent another mechanism involved in the pathogenesis of asthma. An increased production of IL-17 was detected in CD4 $^{+}$ T cells, but not in CD8 $^{+}$ or $\gamma\delta$ T cells, correlating this increase with the severity

of allergic asthma. Our results shed some light on the functional role of these T subsets in the process of allergic asthma. Further studies on the exact mechanism of these T subsets, employing other stimuli such as specific allergens and recruitment of more patients, are required.

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DECLARATION OF INTEREST

The authors report no conflicts of interests. The authors alone are responsible for the content and writing of this paper. And this paper received no funding from outside agencies, organizations, or companies. We confirm that the manuscript has been submitted solely to this journal and is not published, in press, or submitted elsewhere and that all the research meets the ethical guidelines, including adherence to the legal requirements of the study country.

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