Increased circulating IL-9-producing CD8+ T cells are associated with eosinophilia and high FeNO in allergic asthmatics

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Abstract. Allergic asthma is a chronic airway disorder mediated by Th2 cells. It has been shown that IL-9-producing CD8+ cytotoxic T (Tc9) cells promote the subsequent onset of allergic airway inflammation in mice mediated by abnormal Th2 immunity. Whether Tc9 cells are associated with the immunopathogenesis of asthmatic patients remains unknown. In the present study, peripheral blood mononuclear cells (PBMCs) were separated by Ficoll-Hypaque gradient centrifugation from all subjects. The frequency of Tc9 cells was measured by flow cytometry. Serum IL-9 levels were assessed by enzyme-linked immunosorbent assay (ELISA). mRNA expression levels of IL-9, STAT6, and IRF4 in PBMCs from healthy controls and asthmatic patients were detected by reverse transcription-quantitative polymerase chain reaction. The results showed that the numbers of Tc9 cells in allergic asthmatics were significantly increased, compared with healthy controls (P<0.0001). Notably, IL-9 protein and mRNA levels were increased in allergic asthmatics and STAT6 and IRF4 mRNA levels were elevated, as compared with healthy controls. In addition, circulating numbers of Tc9 cells were positively correlated with blood eosinophil counts and fractioned exhaled nitric oxide (FeNO) levels in asthmatic patients. Moreover, the number of Tc9 cells and serum IL-9 levels in asthmatic patients were significantly decreased after treatment with glucocorticoids (P<0.05). These findings suggest that increased circulating Tc9 cells are associated with eosinophilia and high FeNO of allergic asthma, and that abnormal Tc9 immunity may contribute to the pathogenesis of allergic asthmatics.

Introduction

Asthma is a chronic airway disease characterized by the infiltration of various inflammatory cells. The most common form, allergic asthma, is thought to be associated with abnormal CD4+ T helper (Th) 2 immunity (1). A classical Th2 cell response may lead to IgE production, eosinophils recruitment, goblet cell hyperplasia, and mucus overproduction by producing Th2 cytokines, such as interleukin (IL)-4, IL-5, and IL-13 (2). IL-9, which is a pleiotropic cytokine, was initially considered to be a Th2-specific cytokine. More recently, a distinct subset of CD4+ Th9 cells was found to secrete IL-9 (3,4). It has been reported that Th9 cells are involved in human and murine atopic disease and immunity to intestinal parasites via IL-9 (5,6). Moreover, IL-9-producing CD4+ T (Th9) cells are able to enhance IgE production and regulate mast cell accumulation in the lungs during allergic inflammation (7).

Similar to CD4+ Th cells, CD8+ cytotoxic T lymphocytes (Tc) may also have an important role in allergic asthma pathology (8,9). Previous studies have found that Tc2 cells aggravate asthma by secreting type 2 cytokines (10,11). On the other hand, Tc1 cells have been demonstrated to be beneficial for airway inflammation by secreting interferon (IFN)-γ (11). Under Th9-polarizing conditions, naïve CD8+ T cells regulated by the transcription factors signal transducer and activator of transcription 6 (STAT6) and interferon regulatory factor 4 (IRF4) are able to differentiate into IL-9-producing CD8+ T (Tc9) cells, a unique CD8+ T cell subset (12). It has been reported that tumor-specific Tc9 cells elicit great antitumor responses depending on IL-9 production after adoptive transfer (13). Tc9 cells are also increased in mice and humans with atopic dermatitis and are able to promote Th2-mediated airway inflammation in mice (12). However, whether Tc9 cells are abnormal in asthmatic patients remains unknown, and it is also unclear whether Tc9 cells have a role in allergic asthma.

The present study investigated the frequency of Tc9 cells and IL-9 expression levels in asthmatic patients and analyzed their association with disease clinical features.

Materials and methods

Subjects. The study protocol was approved by the Ethics Committee of Zhongnan Hospital of Wuhan University (Wuhan, China), and all subjects signed informed consent. A total of 28 allergic asthmatic patients (13 male, 15 female; mean age, 32.71±5.23), recruited between January 2015 and March 2015 from the Outpatient Department of Zhongnan

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Hospital of Wuhan University, and 20 healthy controls (9 male, 11 female; mean age, 30.35±4.55) were recruited in this study. Diagnosis of asthma was established according to the Global Initiative for Asthma (14): i) clinical history of current symptoms; ii) reversible airway obstruction; iii) positive skin prick tests (≥3 mm) to at least one of 10 common allergens (including *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, mixed grass pollens, mixed tree pollens, dog hair, cat fur, fluffed cotton, feather, cockroach, and *Alternaria*) (15); iv) no a history of smoking; v) no other allergic diseases, autoimmune or neoplastic diseases; and vi) no history of using systematic steroids in the past month. Asthma severity was determined according to international European Respiratory Society/American Thoracic Society guidelines (16). Clinical characteristics of subjects are presented in Table I. Peripheral blood samples from all subjects were collected into sterile vacutainers with ethylene-diaminetetraacetic acid. Blood eosinophil count was measured using automatic hematology analyzer. Prior to the lung function test, fractioned exhaled nitric oxide was measured by a portable nitric oxide analyzer at an exhalation flow rate of 50 ml/sec. FeNO measurements were performed according to the American Thoracic Society and European Respiratory Society 2005 guidelines methods (17). Normal FeNO values were set as 5-35 ppb for healthy adults.

Cell isolation and culture. Heparinized peripheral blood samples from all subjects were collected. Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll-Hypaque gradient centrifugation (d=1.077 g/ml; Haoyang Biological Products Technology Co., Ltd., Tianjin, China). PBMCs were harvested and washed twice in phosphate-buffered saline (PBS), and cell viability was assessed by Trypan Blue dye assay (>95%). Serum samples were stored at -70°C for subsequent use. Isolated PBMCs were seeded (final concentration, $1x10^6$/ml) in RPMI 1640 media (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany). Cells were activated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) and 500 ng/ml ionomycin (both Sigma-Aldrich; Merck Millipore,) for 2 h at 37°C in a humidified atmosphere containing 5% CO_2, and then further cultured for 4 h following the addition of 3 μg/ml brefeldin A (eBioscience, San Diego, CA, USA). Finally, cells were harvested and washed prior to flow cytometry assay.

Flow cytometry. The percentage of Tc9 cells was determined by surface molecule and intracellular cytokine staining using a flow cytometer. Briefly, cells were incubated with a cocktail of phycoerythrin (PE)-Cy5 anti-human CD3 (cat. no. 15-0038-42) and fluorescein isothiocyanate anti-human CD8 (cat. no. 11-0086-42) (eBioscience) for 30 min in the dark at 4°C. To analyze intracellular IL-9 production, cells were further fixed, permeabilized with fixation and permeabilization buffer, and then stained with PE-conjugated anti-human IL-9 (cat. no. 12-7098-42; eBioscience) for 30 min in the dark at room temperature. Following treatment, all stained cells were analyzed by flow cytometry using Expo32 software (version 1696954304; Beckman Coulter, Fullerton, CA, USA).

Table I. Clinical characteristics of subjects.

Characteristic	Healthy controls (n=20)	Allergic asthmatics (n=28)
Age (years)	30.35±4.55	32.71±5.23
Sex (male/female)	9/11	13/15
FEV_1 (% of predicted)	112.65±7.74	75.68±16.99[a]
Serum IgE (ng/ml)	239.68±126.76	1553.60±653.11[a]
Blood eosinophils (10^9/l)	0.17±0.09	0.42±0.19[a]
FeNO (ppb)	19.8±6.30	43.75±13.85[a]

Data are presented as mean ± standard deviation. [a]P<0.001 vs. healthy controls. FEV_1, forced expiratory volume in one second; IgE, immunoglobulin E; FeNO, fractioned exhaled nitric oxide.

Table II. Primer sequences for quantitative polymerase chain reaction analysis.

Gene	Primer sequences (5'→3')
IL-9	F: GTGCCACTGCAGTGCTAATGT
	R: CTCTCACTAAGCATGGTCTGG
STAT6	F: CCTCGTCACCAGTTGCTT
	R: TCCAGTGCTTTCTGCTCC
IRF4	F: TGGACATCTCAGACCCGTACAAAG
	R: ATGGACATCTGCGGGTCCTC
GAPDH	F: GGTGTGAACCATGAGAAG TATGACA
	R: GTCCTTCCACGATACCAAAGTTGT

IL-9, interleukin-9; F, forward; R, reverse; STAT6, signal transducer and activator of transcription 6; IRF4, interferon regulatory factor 4; GAPDH, glyceraldehyde phosphate dehydrogenase.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from PBMCs using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and cDNA was subsequently synthesized using the ReverTra Ace® qPCR RT kit (Toyobo Co., Ltd., Osaka, Japan), according to the manufacturer's instructions. mRNA expression levels of target genes were examined by qPCR using SYBR *Premix Ex Taq*™ (Takara Bio, Inc., Otsu, Japan). Relative expression levels of each gene were normalized to the expression of glyceraldehyde phosphate dehydrogenase (GAPDH) using the $2^{-\Delta\Delta Cq}$ method (18). qPCR was performed on a Bio-Rad iQ5 real-time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) under the following conditions: Initial denaturation at 95°C for 30 sec, followed by 35 cycles at 95°C for 5 sec, 58°C for 15 sec and 72°C for 15 sec. Primer sequences were used as described previously (19-21) and are shown in Table II.

Detection of serum IgE and IL-9 levels. Serum IgE and IL-9 levels were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocols (IgE and IL-9 ELISA kits; cat. no. BMS2097 and BMS2081,
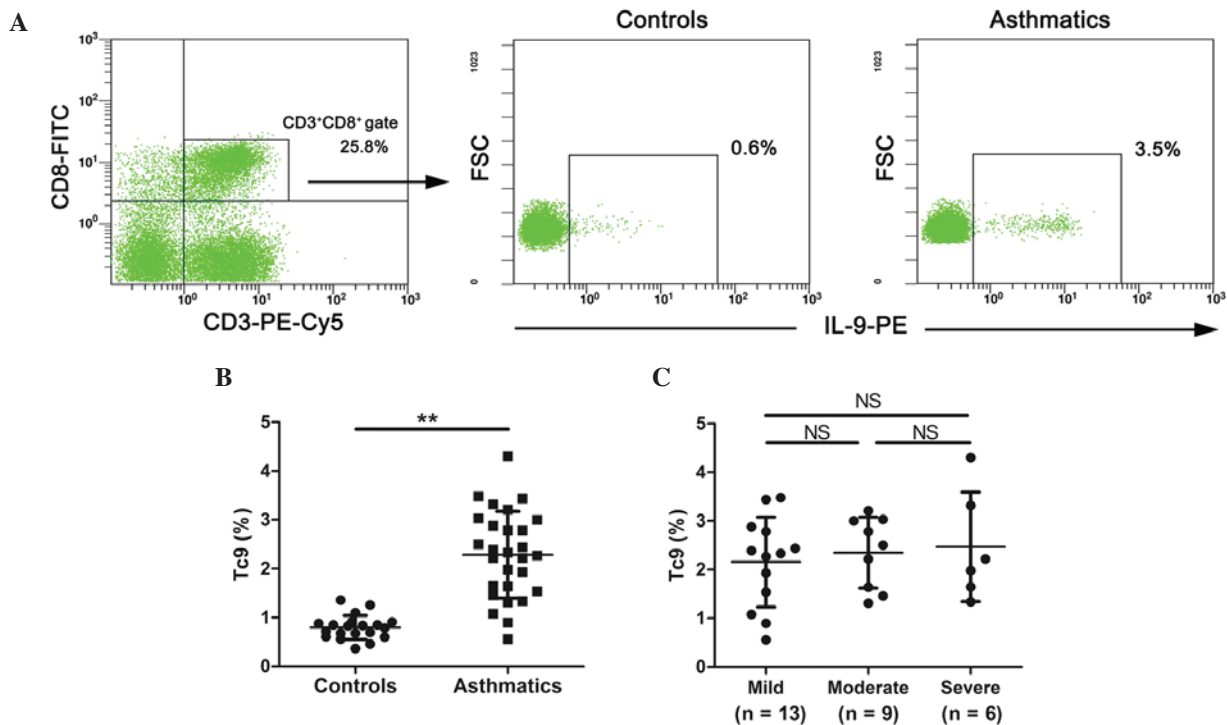
Figure 1. Increased numbers of IL-9-producing CD8^{+} T cells in PBMCs from patients with allergic asthma. (A) Representative dot plots of IL-9-producing CD8^{+} T (Tc9) cells from a healthy control and an asthmatic patient. (B) Percentage of Tc9 cells in PBMCs from healthy controls and asthmatic patients. (C) Frequency of Tc9 cells in asthmatic patients with differing severities. [**]P<0.01. PBMC, peripheral blood mononuclear cells; IL, interleukin; CD8, cluster of differentiation 8; FITC, fluorescein isothiocyanate; PE, phycoerythrin; NS, not significant.

respectively; eBioscience). All samples were tested in duplicate.

Statistical analysis. Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). All data were expressed as mean ± standard deviation. Differences between groups were assessed using unpaired Student's t-test or factorial analysis of variance. Correlation analysis was performed by Pearson's test. P<0.05 was considered to indicate a statistically significant difference.

Results

Clinical characteristics of studied subjects. Table I shows the detailed clinical characteristics of subjects. A total of 28 asthmatic patients and 20 age- and gender-matched healthy controls were recruited. The FEV$_1$% of the predicted value was significantly decreased in asthmatics compared with controls (P<0.001). Total serum IgE levels were significantly higher in asthmatic patients than in healthy controls (P<0.001). Moreover, blood eosinophil counts and fractioned exhaled nitric oxide (FeNO) levels were significantly increased in asthmatic patients (P<0.001).

Tc9 cells are increased in patients with allergic asthma. The frequency of Tc9 cells, which are IL-9-producing CD8^{+} T cells, in PBMCs from healthy controls and asthmatic patients was measured by flow cytometry. Representative cytometric profiles of Tc9 from healthy controls and allergic asthmatics are shown in Fig. 1A. As depicted in Fig. 1B, significantly

increased numbers of Tc9 cells were detected in peripheral blood from patients with allergic asthma (P<0.01). The frequency of Tc9 cells were subsequently analyzed in asthmatic patients with differing severities. The results revealed that there was no correlation between the numbers of Tc9 cells and asthma severity (Fig. 1C).

IL-9 expression levels are increased in asthmatic patients. ELISA and RTqPCR were used to examine IL-9 expression levels in healthy controls and asthmatic patients. The results showed that serum IL-9 protein levels in asthmatics were increased compared with healthy controls (P<0.01; Fig. 2A). Similarly, the mRNA expression levels of IL-9 in PBMCs were significantly higher in asthmatic patients than in healthy controls (P<0.01; Fig. 2B).

Correlations between the frequency of circulating Tc9 cells and clinical features of asthmatics. To determine the potential role of Tc9 cells in allergic asthmatic patients, whether the frequency of circulating Tc9 cells was correlated with clinical features (serum IgE levels, blood eosinophil counts and FeNO levels) was investigated in allergic asthmatics. There was no significant correlation between the frequency of Tc9 cells and serum IgE levels (Fig. 3A). However, circulating numbers of Tc9 cells were positively correlated with blood eosinophil counts or FeNO levels (P=0.0088, r=0.4859 and P=0.0120, r=0.4680, respectively; Fig. 3B and C).

Glucocorticoids significantly decrease the number of Tc9 cells and IL-9 protein levels in asthmatic patients. Glucocorticoids can greatly reduce cytokine synthesis and
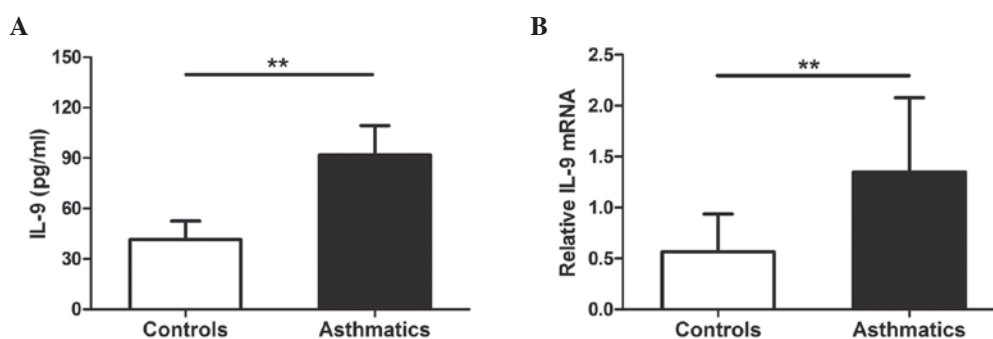
Figure 2. Increased IL-9 expression levels in asthmatic patients. (A) Serum IL-9 protein levels in allergic asthmatics and healthy controls. (B) Relative mRNA expression levels of IL-9 in PBMCs from allergic asthmatics and healthy controls. **P<0.01. PBMC, peripheral blood mononuclear cells; IL, interleukin.
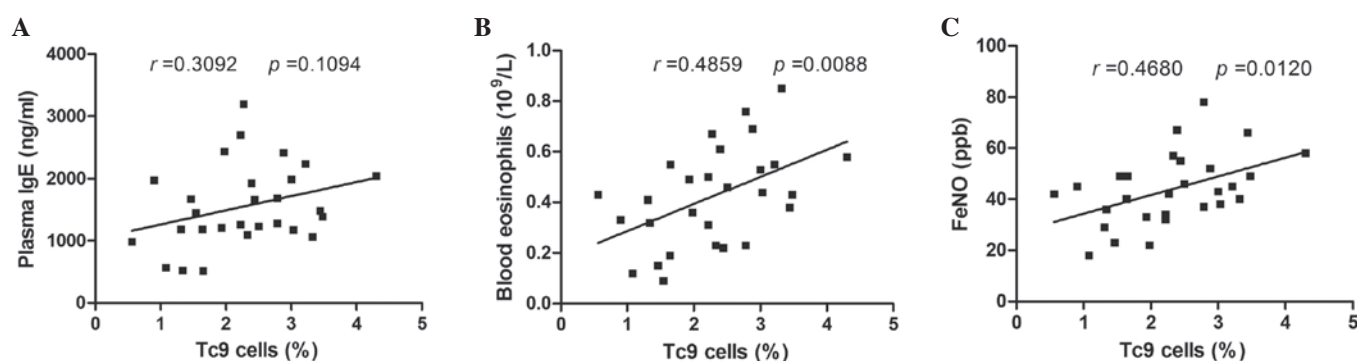

Figure 3. Correlations between the frequency of circulating Tc9 cells and clinical features of asthma, assessed by Pearson's correlation analysis. (A) Correlation between the frequency of Tc9 cells and serum IgE levels. (B) Correlation between the frequency of Tc9 cells and blood eosinophil counts. (C) Correlation between the frequency of Tc9 cells and FeNO levels. IgE, immunoglobulin E; FeNO, fractioned exhaled nitric oxide.
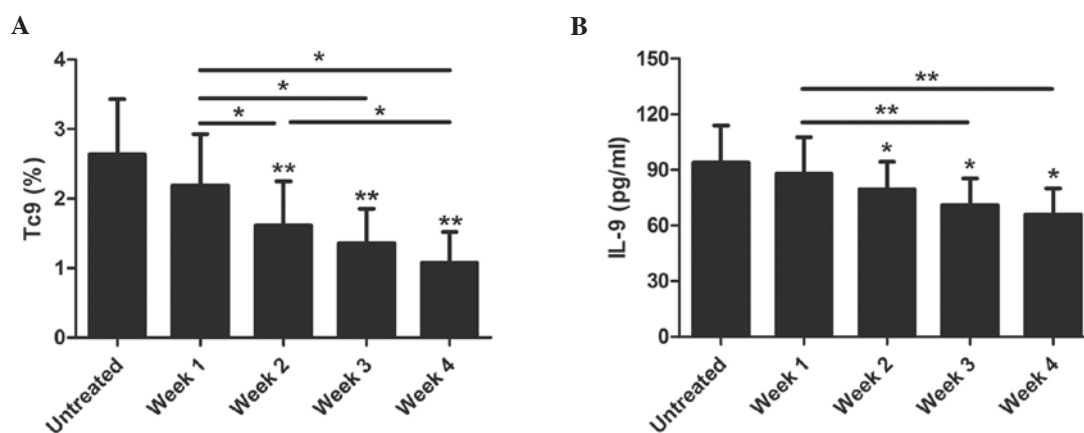

Figure 4. Effects of glucocorticoids on circulating numbers of Tc9 cells and IL-9 protein levels in asthmatics. A total of 15 patients received ICS treatment (160 μg budesonide, twice daily) according to asthma severity. (A) Frequency of Tc9 cells in asthmatics at 1, 2, 3 and 4 weeks following treatment with ICS. **P<0.01 vs. untreated asthmatic patients; *P<0.05. (B) Serum IL-9 protein levels in asthmatics at 1, 2, 3 and 4 weeks following ICS treatment. *P<0.05 vs. untreated asthmatic patients; **P<0.01. IL, interleukin; ICS, inhaled corticosteroid.

alleviate allergic airway inflammation. Therefore, the effects of glucocorticoids on Tc9 cells and IL-9 protein levels were analyzed in asthmatics. Accordingly, 15 patients in the present study received treatment with inhaled corticosteroid (ICS; 160 μg budesonide, twice daily) according to asthma severity. The results showed that the numbers of Tc9 cells and IL-9 protein levels in these patients were significantly decreased after two weeks of inhaled corticosteroid treatment (P<0.05; Fig. 4). Moreover, the percentage of Tc9 cells

and IL-9 protein levels in these asthmatic patients gradually declined at 2, 3, and 4 weeks following glucocorticoids treatment (P<0.05; Fig. 4).

mRNA expression of STAT6 and IRF4 is upregulated in asthmatics. STAT6 and IRF4 are key transcription factors for Tc9 cell differentiation. To assess whether STAT6 and IRF4 may contribute to regulate Tc9 development, the mRNA expression levels of STAT6 and IRF4 in PBMCs from peripheral blood
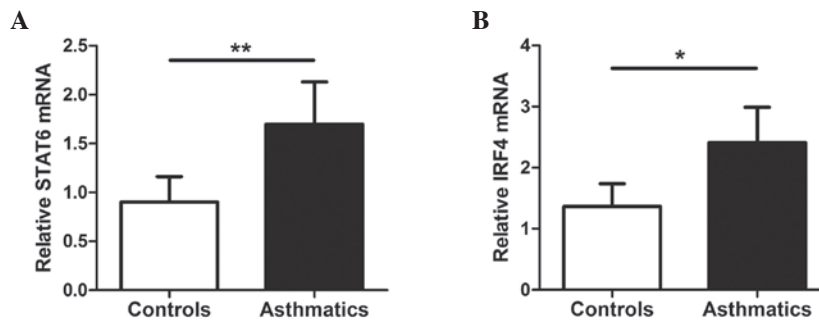
Figure 5. mRNA expression levels of STAT6 and IRF4 in healthy controls and asthmatics. (A) Relative mRNA expression levels of STAT6 in PBMCs from asthmatics and healthy controls. (B) Relative mRNA expression levels of IRF4 in PBMCs from asthmatics and healthy controls. *P<0.05; **P<0.01. STAT6, signal transducer and activator of transcription 6; IRF4, interferon regulatory factor 4; PBMC, peripheral blood mononuclear cells.

were detected by RT-qPCR. Notably, the results revealed elevated STAT6 and IRF4 mRNA levels in PBMCs from allergic asthmatics (P<0.05; Fig. 5).

Discussion

CD8^{+} T cells are thought to contribute to asthma pathology (8). Tc9 cells, which are newly discovered IL-9-producing CD8^{+} T cells, have been reported to promote the subsequent onset of allergic airway inflammation mediated by pathogenic Th2 immunity in mice (12). Nevertheless, it is not clear whether Tc9 cells are involved in the immunopathogenesis of asthmatic patients. In the present study, the frequency of Tc9 cells was markedly increased in asthmatic patients, and IL-9 protein and mRNA expression levels were significantly elevated in asthmatics. It was also observed that the percentage of Tc9 cells and IL-9 protein levels in asthmatics gradually declined following glucocorticoids therapy. Notably, circulating numbers of Tc9 cells were positively correlated with blood eosinophil counts and FeNO levels. These findings suggest that Tc9 cells may contribute to the pathogenesis of allergic asthmatics.

IL-9, a pleiotropic cytokine, has been reported to have an important role in atopic asthma (22,23). Studies have found that naïve CD8^{+} T cells are also able to differentiate into Tc9 cells under similar Th9-polarizing conditions (12,24). It has been demonstrated that tumor-specific Tc9 cells may elicit antitumor responses dependent on IL-9 production (13). Moreover, Tc9 cells promote Th2-mediated airway inflammation in mice (12). In the present study, significantly increased numbers of Tc9 cells were detected in the peripheral blood of allergic asthmatics, which was consistent with the results of Visekruna et al (12). Furthermore, elevated serum IL-9 and IL-9 mRNA expression levels were detected in asthmatic patients, suggesting that Tc9 cells may have a crucial role in the pathogenesis of allergic asthma. However, the exact mechanisms of increased Tc9 cells in allergic asthma remain unclear. It has been demonstrated that STAT6 and IRF4 are key transcription factors for Tc9 cells differentiation (12,24). They are essential for IL-9 production at the protein and mRNA levels (12). Herein, it was observed that allergic asthmatics had elevated STAT6 and IRF4 mRNA levels, indicating that increased Tc9 cells exist in asthmatics at least at the transcriptional level. Previous studies have shown that Th9 cells increase mucus production, airway hyperreactivity and peribronchial fibrosis mediated by IL-9 (25-27), which

is considered deleterious to lung function in asthmatics and increases the severity of asthma. Accordingly, the percentage of Tc9 cells in asthmatics with differing severities were analyzed. No significant difference was found in the numbers of Tc9 cells between them. This indicates that Th9 cells, but not Tc9 cells, may be associated with airway remodelling.

IgE overproduction and increased eosinophils have been recognized as cardinal features of Th2-mediated allergic asthma. FeNO is an indirect biomarker of airway inflammation in asthmatic patients (28). FeNO is often increased in steroid-sensitive asthmatics, and is correlated with eosinophilia (29,30). In particular, consistent with these previous studies, the present study exhibited increased serum IgE levels in asthmatics, as compared with healthy controls. Blood eosinophil counts and FeNO levels were also significantly increased in asthmatic patients. Notably, the present study found that circulating numbers of Tc9 cells were positively correlated with blood eosinophil counts and FeNO levels. Therefore, we hypothesize that increased circulating Tc9 cells may be an important feature of allergic asthma, and that Tc9 cells may activate Th2-mediated allergic inflammation, including eosinophilia and high FeNO, directly or/and through innate immune cells in asthmatics, which is in combination with the previous findings that Tc9 cells may elicit key features of allergic airway inflammation, such as eosinophilia (12).

Glucocorticoids are powerful anti-inflammatory factors used to treat asthma that can induce immune effects and affect cytokine production by activating receptors. It has been reported that glucocorticoids greatly reduced cytokine synthesis and alleviated allergic airway inflammation in asthmatics (31). Therefore, the effects of glucocorticoids on Tc9 cells and IL-9 protein levels were investigated in asthmatics. Notably, the number of Tc9 cells and serum IL-9 protein levels in asthmatics were significantly decreased after two weeks of inhaled corticosteroid treatment. Moreover, the percentage of Tc9 cells and IL-9 protein levels gradually declined with therapy. Thus, similar to the effects of glucocorticoids on eosinophils, glucocorticoids may downregulate Tc9 cell populations as well as inhibit IL-9 expression in asthmatics. Accordingly, we hypothesize that glucocorticoids may dampen the subsequent onset of allergic asthma by inhibiting the immunopathogenic role of Tc9 cells, which should be further investigated in future studies.

In conclusion, the present results demonstrated increased circulating Tc9 cells, IL-9-producing CD8^{+} T cells, in allergic

asthmatics, which are paralleled with eosinophilia and high FeNO. Elevated mRNA expression levels of transcription factors STAT6 and IRF4 may contribute to the abnormal Tc9 immunity in asthmatics. These findings suggest that targeting Tc9 cells in human asthma may be a promising therapy in the future.

Acknowledgements

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