The study of the ratio and distribution of Th17 cells and Tc17 cells in asthmatic patients and the mouse model

Kaiyan Li, Zhengyun Wang, Yong Cao, Hansvin Bunjhoo, Jing Zhu, Ying Chen, Shengdao Xiong, Yongjian Xu and Weining Xiong

Summary

Background: Whether CD8$^+$ IL-17-producing T cells, namely Tc17 cells, play a role in asthma has not been determined. The aim of this study was to evaluate and compare the frequency of peripheral blood Th17 cells and Tc17 cells in asthmatic patients. In addition, the number, ratio and distribution of Th17 cells and Tc17 cells in the lung tissue and splenocytes of asthmatic mice were also investigated.

Methods: Th17 and Tc17 cells in the peripheral blood samples of asthmatic patients and in murine spleens were detected by flow cytometric analysis. Th17 and Tc17 cells in murine lung tissues were detected by double immunofluorescence stain. IL-17A levels in murine bronchoalveolar lavage were detected by ELISA.

Results: The result of the flow cytometric analysis showed the percentage of Th17 cells among CD3$^+$ T cell populations in patients with asthma was higher than that in healthy controls ($P < 0.01$), The percentage of Tc17 cells was also higher ($P < 0.05$). The percentages of Th17 and Tc17 cells in asthmatic mice were both much higher than that in control animals ($P < 0.01$). Frozen sections of lung tissue showed that the number of Th17 cells and Tc17 cells in the asthma group were all significantly higher than in the control group ($P < 0.01$).

Conclusions: Our findings suggest a functional disequilibrium of Th17 and Tc17 cell subsets in asthma that may contribute to the inflammatory process and provide novel insights into a hypothetical driving role of those cells in disease pathogenesis. (Asian Pac J Allergy Immunol 2013;31:125-31)

Key words: asthma, airway inflammation, Th17 cell, Tc17 cell, interleukin 17

Introduction

Asthma is characterized by allergic airway inflammation associated with intense eosinophil and CD4$^+$ T cells infiltrates. Over recent years, it has been demonstrated that CD4$^+$ Th2 cell–derived cytokines, such as IL-4, IL-5, IL-9, and IL-13, play critical roles in orchestrating and amplifying allergic inflammation in asthma. It is also now widely accepted that CD4$^+$ Th17 cells play a crucial role in autoimmune diseases by promoting chronic inflammatory responses, including allergic diseases such as asthma. IL-17 family cytokines (IL-17A and IL-17F) are thought to be the major cytokines derived from Th17 cells.

Although most studies have focused on characterization of CD4$^+$ Th17 cells, less is known about the development of CD8$^+$ IL-17-producing T cells, namely the Tc17 cells, and their role in immune responses. Nigam P et al. found the frequency of Tc17 cells in the colorectal mucosa of SIV-infected macaques with AIDS was lower than that in healthy macaques, and could be partially restored by antiretroviral therapy, suggesting a role for Tc17 cells in the mechanism of inflammatory immunity. Some other studies also suggested a role for Tc17 cells in the pathogenesis of autoimmune diseases, such as psoriasis, autoimmune colitis, allergic dermatitis, and multiple sclerosis. Zhao found that the IFN-γ (+)/IL-17(+) ratio among CD4(+)CD8(+) T cells was significantly increased in peripheral blood from patients with allergic asthma.
The aim of this study was to evaluate and compare the frequency of peripheral blood Th17 cells and Tc17 cells in asthmatic patients and in healthy control subjects. The number, ratio and distribution of Th17 cells and Tc17 cells in the lung tissue and splenocytes of asthmatic mice were also investigated.

**Methods**

**Patients and controls**

Twenty three patients with stable asthma fulfilling the guideline proposed by the American Thoracic Society criteria were recruited from the outpatients of the Department of Respiratory Diseases, Tongji Hospital (Wuhan, China), and twelve age-matched healthy controls were enrolled from volunteers in the hospital. Healthy volunteers were subjects without a history of allergy and asthma symptom scores. All subjects had no history of smoking or atopic dermatitis. Subjects who had a history of respiratory tract infection within 6 weeks before the study or who had received immunotherapy in the previous 5 years were excluded. Asthmatic patients were only allowed to use inhaled β2-agonists and did not use steroid therapy or leukotriene receptor antagonist therapy during the 4 weeks preceding the study. The study was approved by Ethics Committee of Tongji Hospital and all subjects were required to sign informed consent forms.

**Asthma Model**

All animal experiments were carried out according to the guidelines set by the Chinese Council on Animal Care and approved by the Tongji Medical College Committee on Animal Experimentation. The research protocol was reviewed and approved by the Tongji Medical College Committee. All animals were kept in SPF conditions at the animal facility of Tongji Medical College. Twenty four pathogen-free six to eight weeks’ old BALB/c female mice were obtained from the animal supply center at Tongji Medical College (Wuhan, Hubei Province, China). The animals were randomly divided into two groups: asthma group (n = 12) and control group (n = 12). The allergic mouse model was established as described previously. Briefly, the mice in the asthma group were sensitized via intraperitoneal injections with 40ug ovalbumin (OVA, Grade III, Sigma, St. Louis, USA) and 4.5mg adjuvant [Al(OH)₃] in 0.2 ml of saline three times at weekly intervals (Day 0, 7, 14). The mice in control group received adjuvant alone. On Days 21–23, mice received aerosolized challenges with 2% OVA for the asthma group or saline for the control group respectively, for 30 min/d via an ultrasonic nebulizer. All animals were killed on Day 24 and analyzed for the allergic parameters described below.

**Bronchoalveolar Lavage (BAL) Analysis**

Immediately after the mice were killed, cells in the airways were collected by flushing 1 ml of ice-cold sterile phosphate-buffered saline (PBS) into the lungs via the trachea three times. The total cell count was obtained by using a hemocytometer. BALF was centrifuged at 400g for 5 minutes at 4°C, and cell differentials were determined by counting cells in 10 fields stained with Wright-Giemsa solution. Cell-free BALF was preserved at –70°C for the detection of IL-17A level by ELISA.

**Flow Cytometric Analysis**

One 8 ml peripheral blood sample was collected from each participant on the day of inclusion and clinical evaluation. PBMC were isolated from the fresh anticoagulant treated blood by Ficoll-Hypaque centrifugation and were cultured in RPMI medium supplemented with 15% FCS. The single-cell suspensions were stimulated for 4–5h with PMA (50ng/ml; Sigma, USA) and ionomycin (1000ng/ml; Sigma, USA). Brefeldin A (50ng/ml; Sigma) was also added to block the release of cytokines from the cells. After incubation, the single-cell suspensions were harvested and washed immediately, then stained extracellularly with PEcy5–conjugated anti-CD3ε (mAb, BD Pharmingen, USA) and fluorescein isothiocyanate–conjugated anti-CD8 (mAb, BD Pharmingen) for 30 min at 4° in darkness. Following the surface staining, the cells were fixed, permeabilized with Cytofix/Cytoperm solution (BD Pharmingen), washed and then incubated at 4°C for overnight. The next day the cell suspensions were stained intracellularly with phycoerythrin–conjugated anti-IL-17 (mAb, BD Pharmingen) or phycoerythrin–conjugated monoclonal antibody isotype (mAb, BD Pharmingen) for 30 min at 4°C in darkness. Then the cell suspensions were washed. Samples were acquired and measured by using a FACS Calibur (BD Pharmingen, USA) and data were analyzed with Cell Quest-Pro software (BD Pharmingen, USA). Murine spleens were collected from all mice and single-cell suspensions were prepared by mechanical disruption in RPMI 1640 medium supplemented with 10% FCS, 100 IU/ml of...
penicillin, 100 mg/ml of streptomycin, 1 µM sodium pyruvate, 2.5 µM β-mercaptoethanol and 2 mM L-glutamine (R-10). Spleen cells were stimulated for 5 h with PMA (50 ng/ml; Sigma, USA) and ionomycin (750 ng/ml; Sigma, USA) in the presence of BFA (50 ng/ml; Sigma, USA). Before staining, Fc receptors were blocked with anti-CD16/32 antibody (2.4G2; BD PharMingen, USA). Standard intracellular cytokine staining was done as previously described. Cells were first stained extracellularly with PEcy5–conjugated anti-CD3ε (hamster anti-mouse 145-2C11; BD Pharmingen, USA) or fluorescein isothiocyanate–conjugated anti-CD8+ (rat anti-mouse 53-6.7; BD Pharmingen, USA), were fixed and permeabilized with Cytofix/Cytoperm solution (BD Pharmingen, USA) and then stained intracellularly with phycoerythrin–conjugated anti-IL-17A (rat anti-mouse TC11-18H10; BD Pharmingen, USA) or phycoerythrin-conjugated monoclonal antibody isotype (rat IgG1, κR3734; BD Pharmingen, USA). Samples were acquired on a FACS Calibur (BD, USA) and data were analyzed with CellQuest-Pro software (BD, USA). Lymphocytes were gated according to size in a forward and side scatter plot.

**Double Immunofluorescence stain**

The right lungs collected from the mice were immediately frozen in liquid nitrogen. Four-µm sections of the lung tissues, fixed with acetone at 4°C for 10 min followed by blocking with donkey serum for 30 min, were incubated with either a mixture of rat anti-mouse CD4 mAb (GK1.5; Abcam, UK) and goat anti-mouse IL-17 antibody (E-19; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or a mixture of rat anti-mouse CD8 mAb(YTS169.4; Abcam, UK) and goat anti-mouse IL-17 antibody (E-19; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. After washing, the sections were further incubated with a mixture of Rhodamine (TRITC) -conjugated donkey anti-goat IgG (Jackson ImmunoResearch, USA) and Fluorescein (FITC) -conjugated donkey anti-rat IgG (Jackson ImmunoResearch, USA) in the dark. For counter staining, the slides were incubated with DAPI (staining for nuclei of the cells) for 1 min. The slides were mounted in buffered glycerol containing 4, 6-diamidino-2-phenylindole. The slides were then examined by a fluorescence microscope and digital images were recorded. The cells were counted on the digital pictures after superimposition of the IL-17 staining (red) and T-cell (CD4+ or CD8+) staining (green) images. On average nine fields from each double-stained section were counted with a mean of 130 infiltrating cells per field.

**Cytokine levels**

BAL IL-17 levels were measured by ELISA according to the manufacturer’s instructions (R&D Systems, USA). Briefly, samples of BAL fluid and serum were aliquoted in duplicate into 96-well plates (50 µl/well) pre-coated with antibody to mouse IL-17 and assayed according to the manufacturer’s instructions. The optical density was measured at 450 nm. Cytokine concentrations were determined by comparison with known standards.

**Data analysis**

The data were expressed as mean ± standard deviation. The results were analyzed with Student’s t test. *P* < 0.05 was regarded as statistically significant.

**Results**

**Study population: asthma patients and healthy controls**

Thirty-five subjects were enrolled in the study, comprising 23 asthma patients (13 female, mean age 38.5 ± 3.8 years) and 12 controls. The patients all had controlled disease. Their mean predicted percentage of forced expiratory volume in the first second (FEV1%) was 84.32% ± 15.27%, and their mean asthma severity score was 7.09±2.03. Twelve sex- and age-matched healthy control subjects (NC group) (7 female, mean age 40.1 ± 5.0 years) were also included.

**The total and differential cell count of BALF from asthmatic mice and normal controls**

The total and differential cell counts of BALF in the two groups are shown in Figure 1. As seen from Figure 1, the total number of cells and the number of eosinophils in the asthma group were significantly higher than in the control group (*P* < 0.01).

**The levels of IL-17A protein in BALF from asthmatic mice and normal controls**

The levels of IL-17A protein in BALF are shown in Figure 2. The IL-17A content of BALF in the asthma group was 36.59±13.97 pg/ml, while that in the control group was 11.53±3.28 pg/ml. The difference was statistically significant (*P* < 0.0001).
Figure 1. The total cell number and differential counts in BAL from normal control and asthmatic mice, the total number of cells and eosinophils in BAL from asthmatic mice is significantly greater than that from normal controls (*P <0.01, n =12 per group). The data are expressed as mean ± standard deviation.

Figure 2. IL-17A cytokine protein levels in BAL from normal controls and asthmatic mice, as determined by ELISA (n=12 per group). The data are expressed as mean ± standard deviation. * P<0.0001

The frequency of peripheral Th17 and Tc17 cells from asthma patients and healthy controls

A representative result of the flow cytometric analysis is shown in Figure 3. As shown in Figure 3, the percentage of Th17 cells (IL-17+ CD3+ CD8- cells) among CD3+ T cell populations in patients with asthma was higher than that in healthy controls (4.56 ± 0.50% vs 1.17 ± 0.19%, P <0.01) and the percentage of Tc17 cells (IL-17+ CD3+ CD8+ cells) was also higher (1.21 ± 0.15% vs 0.72 ± 0.11%, P <0.05).

The frequency of Th17 and Tc17 cells from murine spleens CD3+ T cells

The percentage of Th17 cells (IL-17+ CD3+ CD8- cells) among CD3+ cell populations in asthmatic mice (4.67 ± 1.68%) was much higher than that in control animals (1.13 ± 0.69%, P <0.0001), as was the percentage of Tc17 cells (IL-17+ CD3+ CD8+ cells; 1.26 ± 0.65% vs 0.34 ± 0.35%, P <0.001).

The number and distribution of Th17 cells and Tc17 cells in lung tissue

Frozen sections of lung tissue were examined to show the distribution of Th17 cells (IL-17+ CD4+ cells) and Tc17 cells (IL-17+ CD8+ cells) in the asthma group. They were mainly localized in the peribronchial and perivascular area, compared to the control group, and the number of Th17 cells and Tc17 cells in asthma group were all significantly higher than those for the control group (P <0.01) (Figure 4, 5).

Discussion

An important role for the recently discovered Th17 cells as one of the primary drivers of asthma has been proposed. In the CD8+ T cell compartment, a Tc17 subset was newly discovered, but whether it might also play a role in asthma is unclear. Here, we evaluated the relative abundance of Th17 and Tc17 cells in asthma. We showed that an increased proportion of Th17 and Tc17 cells was present in the peripheral blood of asthma patients, and in asthmatic murine spleen CD3+ T cells. The exact role of Tc17 cells remains unclear; however, their presence has also been demonstrated in other immunological abnormalities, such as systemic lupus erythematosus. Tc17 cells display a greatly suppressed cytotoxic function and share some key features with the Th17 cells. We might speculate that Tc17 cells cooperate with Th17 cells in inflammatory conditions like asthma, just as they do in autoimmune diseases, infection, and antitumor immunity.

Recent studies using murine asthma models have shown that IL-17 may enhance the recruitment of inflammatory cells into the airways, including eosinophils. Th17 cell is a new cell subset of CD4+ T cells, which is different from Th1 cells and Th2 cells, and plays a biological role mainly by

DOI 10.12932/AP0268.31.2.2013
producing IL-17 and other inflammatory factors. In addition to Th17 cells, there are CD8 + T cells which also produce IL-17, named "Tc17 cells". Tc17 cells and Th17 cells have many similar characteristics, such as secretion of cytokine IL-17. Therefore we postulated that the increased level of IL-17 in the BALF of asthmatic mice was derived not only from Th17 cells, but also from Tc17 cells.

Active inflammation is often accompanied by local immune infiltration, including airway inflammation in asthma. Interestingly, in normal mice, the percentages of Th17 and Tc17 cells were relatively higher in the interstitial and lung tissues than in other compartments, suggesting that IL-17+ T cells may play a role in immune homeostasis in mucosal compartments. Another study showed that Th17 and Tc17 cells are all significantly increased in the lesions of allergic contact dermatitis, indicating that those cells are regular participants in the immunopathology of this allergic reaction. Zhao found that CD8(+) and γδ T cells might be involved in the pathogenesis of allergic asthma through the release of Th1-, Th2-, and Th17-type cytokines. Our results show that an increased in

**Figure 3.** The frequency of peripheral Th17 and Tc17 cells from asthma patients and healthy controls detected by flow cytometric analysis. The percentage of Th17 and Tc17 cells among CD3+ T cell populations in patients with asthma were higher than that in healthy control (*P <0.05). The data are expressed as mean ± standard deviation.

**Figure 4.** Th17 and Tc17 cells in lung sections from normal controls and asthmatic mice, as determined by double immunofluorescence. The lung sections are stained with IL-17 antibody (red) and anti-T-cell monoclonals (anti-CD4 IN (a) or anti-CD8 IN (b))(green). The nuclei are counterstained by DAPI(blue). Data are representative of four independent experiments in each group. The white arrows showed the IL-17+ CD4+ or IL-17+ CD8+ cells.

In summary, we found an increased proportion of Th17 and Tc17 cells in the peripheral blood of asthma patients compared to healthy controls, as well as in the spleen cells and lung tissue of asthmatic mice. In conclusion, our findings suggest a functional disequilibrium of Th17 and Tc17 cell subsets in asthma that may contribute to the inflammatory process and draw novel insights on a hypothetical driving role of those cells in disease pathogenesis. For further understanding the role of Th17 and Tc17 cells dynamics in driving asthma disease activity, more longitudinal studies are needed.
Figure 5. Percentage of Th17 and Tc17 cells in airway submucosal cells of asthma patients. The data are expressed as mean ± standard deviation. *P < 0.05.

Acknowledgment
None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

This work was supported by the National Natural Science Foundation of China (No. 81170021 and No. 30900647)

References


