

Cytokine Production Profile of CD4+ T Cells from Patients with Active Churg-Strauss Syndrome Tends Toward Th17

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Key Words

Churg-Strauss syndrome · Pathogenesis · Tr1 cell · Th17 cell

Abstract

Background: Churg-Strauss syndrome (CSS) is a rare systemic necrotizing vasculitis that develops in some asthma patients. What types of asthma make patients prone to develop this condition remains unknown. We found that inhibition of regulatory T cells (Treg) differentiation, especially by Tr1 cells, due to a decreased ability of responder T cells to generate IL-2, is associated with the onset and pathogenesis of CSS. In contrast, recent evidence suggests that IL-17-producing Th17 cells play a crucial role in autoimmune inflammation. However, few studies have addressed the role of Th17 in the pathogenesis of CSS. **Methods:** Mononuclear leukocytes were obtained from healthy subjects, patients with bronchial asthma (BA), asthma-accompanying chronic eosinophilic pneumonia (BA+CEP) and CSS. The cells were stimulated for 4–5 h with PMA and ionomycin in the presence of brefeldin A to generate and accumulate cytokines. Intracellular cytokines were detected after fixation and permeabilization of these cells. Tr1 cells were evaluated as CD4+CD25+ T cells dominantly producing IL-10 and TGF- β , nTreg as CD4+CD25+ T cells expressing Foxp3, a master transcriptional factor, and Th17 were also evaluated as CD4+ T cells mainly producing IL-17. Patients with CSS were classified into an active group and an inactive group in accordance

with the disease state after treatment. **Results:** The frequency of Th17 in peripheral blood was significantly higher in active CSS patients than in healthy subjects, BA, BA+CEP, and inactive CSS patients. In contrast, the Tr1 cell detection frequency was remarkably decreased in active CSS in comparison with BA, BA+CEP, and inactive CSS patients. Also, there was a significant relation between the condition of a given CSS patient and the detection frequencies of both CD4+ helper T cells when CSS cases were divided into inactive and active stages. The Th17 detection frequency was increased in the active stage of CSS, while the Tr1 frequency decreased sharply but then reversed in the inactive stage of CSS. **Conclusion:** Active CSS patients have elevated intracellular IL-17 and decreased IL-10 levels, which correlate with measures of disease activity, suggesting that Th17 and Tr1 may play important roles in the pathogenesis of CSS.

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Introduction

Churg-Strauss syndrome (CSS), also known as allergic granulomatosis and angiitis, is a disease involving multiple organs, characterized by bronchial asthma (BA) and peripheral blood eosinophilia. The pathogenesis of CSS is unknown. CSS develops following repeated chronic eosinophilic pneumonia (CEP) prior to the onset, but not all asthma patients will develop CSS [1–3]. We reported that

in the patients who do not develop CSS even after repeat CEP, the frequency of CD4+CD25+ T cells mainly producing IL-10 and TGF- β_1 (Tr1) and the ability of CD4+CD25- T cells (responder T cells) to generate IL-2 do not decrease, while showing a remarkable decrease in CEP patients who develop CSS [4, 5]. In addition, the number of Tr1 cells in patients with CEP was significantly higher at disease onset than in CSS at disease onset and in relapse stages. Also, the number of Tr1 cells in patients with CSS in remission was significantly higher than that in CSS patients at disease onset, and then decreased again with disease recurrence. These results suggest that Tr1 cells in patients with CEP inhibit the development of CSS and that the suppressive activity of Tr1 cells in CEP functions naturally in the absence of systemic corticosteroids. Thus, a decrease in the number of Tr1 cells modifies the disease state and leads to CSS via suppression of Tr1 activity.

In contrast, it is well known that dysregulated production of IL-17 is associated with human autoimmune diseases, including multiple sclerosis, inflammatory bowel disease, and psoriasis [6–9]. Importantly, studies in a murine experimental arthritis model showed IL-17 to be involved in both the initiation and the progression of disease. Furthermore, IL-17 elevations were detected in synovial fluid from patients with rheumatoid arthritis, and osteoclast formation was inhibited by anti-IL-17 antibody, suggesting an effect on bone resorption [10–14]. CD4+ T cells producing IL-17 represent a distinct lineage (Th17), a lineage that produces neither IL-4 nor IFN- γ [15, 16]. The current models of Th17 differentiation are all derived from studies using murine cells. However, despite advances in our understanding of murine Th17 differentiation, a systematic assessment of factors that promote the differentiation of naive human T cells into Th17 cells has not been reported. Recently, involvement of regulatory T cells (Treg) and Th17 in the pathogenesis of some human autoimmune diseases has been suggested. Thus, it is reasonable to propose that skewing of responses towards Th17 or Th1 and away from Treg may be responsible for the development and/or progression of this disease [17]. However, few studies have addressed the involvement of Th17/Treg imbalance in the pathogenesis of CSS. The present study examined the possibility of Th17 and Treg involvement in the pathogenesis of CSS, in contrast to asthma and CEP. Although a decrease in the frequency and absolute number of Tr1 cells in peripheral blood was observed in patients with active stage CSS, the number of Th17 cells was conversely increased at the same disease stage. The Tr1 and Th17 ratio accounting

for all CD4+ T cells associated with this condition was examined in the same CSS patient, that is Th17 increased and Tr1 decreased in the active stage, while Tr1 increased and Th17 decreased in the inactive stage.

Subjects and Methods

Study Subjects

From our clinic at the Clinical Research Center of Sagamihara National Hospital, Sagamihara, Japan, we recruited 35 consecutive CSS (14 inactive stage, 5 male/9 female, 67.0 ± 7.9 years old; 21 active stage, 6 male/15 female, 51.0 ± 16.0) patients, 54 asthmatics (BA, 20 male/34 female, 52.0 ± 17.0), 14 asthmatics with accompanying CEP (BA+CEP, 5 male/9 female, 57.0 ± 12.0) and 9 healthy controls (5 male/4 female, 38.2 ± 11.9). CSS was diagnosed according to the classification criteria of the American College of Rheumatology. All 54 BA patients were given diagnoses according to the criteria of the Global Initiative for Asthma (GINA) guidelines [18], and the severity of their asthma was classified as follows, as defined by GINA guidelines: step 1, intermittent ($n = 7$); step 2, mild persistent ($n = 9$); step 3, moderate persistent ($n = 1$), and step 4, severe persistent ($n = 37$). Four of 37 severe persistent BA patients had received oral corticosteroids (OCS, mean 3.6 mg) and inhaled corticosteroids (ICS, mean 1,050 μ g). Only 5 of 14 BA+CEP patients had received OCS (mean 5 mg), and the remaining 9 patients had received ICS (fluticasone propionate 800 μ g). Fourteen inactive and 21 active CSS patients had received OCS (mean 4.6 and 11.1 mg), respectively. CSS patients were classified into an active group and an inactive group in accordance with the condition of the disease after treatment. The inactive state was defined as the absence of any clinical signs or symptoms of active vasculitis. The active state was defined as the recurrence of vasculitis symptoms with or without an increase in the proportion of eosinophils among whole blood cells after initial inactive state requiring the resumption of immunosuppressive therapy or an increased dose of immunosuppressant.

Reagents

FITC-conjugated mouse IgG1, anti-CD4, anti-CD25, PE-conjugated mouse IgG1, anti-CD4, anti-CD25, anti-CD3, anti-CD127, PE-Cy5-conjugated mouse IgG1 and anti-CD4 antibody were purchased from BioLegend (San Diego, Calif., USA). PE-conjugated anti-human FOXP3 antibody was obtained from eBioscience (Rikaken Co. Ltd., Tokyo, Japan). PE-conjugated anti-human IL-2, IL-5, IL-10, rat IgG2a, PerCP-conjugated mouse IgG1 and anti-CD4 antibody were purchased from BD Biosciences (Nihon BD, Tokyo, Japan) and PE-anti-human TGF- β_1 and PE-anti-human IL-17 were purchased from IQ-Products (Groningen, Netherlands) and R&D Systems (Cosmo Bio Co. Ltd., Tokyo, Japan), respectively.

Intracellular Cytokine Staining and FACS Analysis

Mononuclear leukocytes (1×10^5 cells) obtained from the patients were stimulated for 4–5 h with medium or 50 ng/ml PMA and 1 μ g/ml ionomycin at 37°C in the presence of 10 μ g/ml brefeldin A (MP Biomedicals, Inc., Wako, Osaka, Japan) to generate and accumulate cytokines. The cells were harvested and

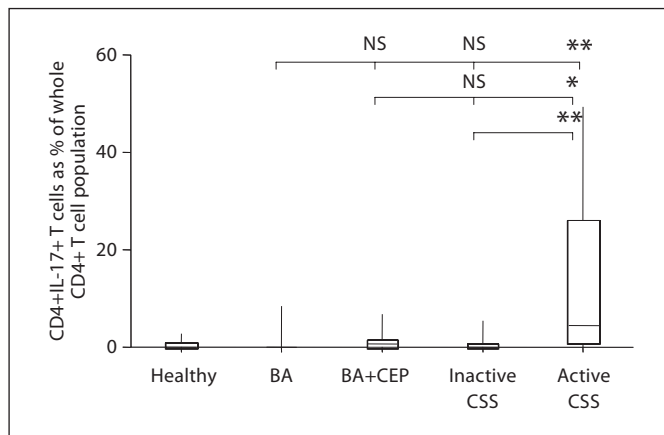


Fig. 1. Increased ability of CD4+ T cells from active CSS patients to produce IL-17. Mononuclear leukocytes derived from patients of each group were stimulated with PMA and ionomycin in the presence of 10 μ g/ml of brefeldin A to generate and accumulate IL-17. Statistical significance was tested using the Mann-Whitney U test. * $p < 0.05$; ** $p < 0.01$.

stained with PE-Cy5-conjugated anti-CD4 monoclonal antibody (mAb) and FITC-conjugated anti-CD25 mAb. Permeabilized cells were stained with PE-conjugated anti-IL-2 mAb, anti-IL-5 mAb, anti-IL-10 mAb, anti-TGF- β_1 mAb or anti-IL-17 mAb. Briefly, the cells were suspended in 0.25 ml of cold 4% paraformaldehyde fixative and incubated at 4°C for 20 min. Following fixation, cells were washed twice with an aqueous solution containing saponin (0.1%, w/v) and sodium azide (0.09%, w/v) (PermWash™, BD) by spinning at 300 g for 5 min, and the pellet was resuspended in 0.1 ml of the same buffer. After the addition of mAb (2 μ l/10⁵ cells) against each cytokine, the cells were incubated for 35 min at 4°C in the dark. Each tube of the cells was washed twice using 2 ml of PermWash buffer each time and resuspended in PBS containing 0.05% bovine serum albumin (BSA). Cells were analyzed using FACSCalibur™ with CellQuest Analysis software. Tr1 cells were evaluated as CD4+CD25+ T cells producing mainly IL-10 and TGF- β . Intracellular staining for FOXP3 in cells isolated from the patients was performed according to the manufacturer's instructions. Two- or three-color flow cytometry was performed using directly conjugated mAb against CD4, CD25 and CD127. Appropriate isotype-matched mAbs were used as T cells, phenotyped with mAb to controls for all immunoassays. The cells were gated electronically according to light scatter properties to exclude dead and contaminant cells.

Statistics

Nonparametric descriptive and analytical statistics were employed. The Mann-Whitney U test was used for comparisons of the data between the two patient groups as well as for comparisons with the healthy control group. The paired Student's t test was used for comparisons of the data between patients with active and inactive CSS. A value of $p < 0.05$ was considered to be statistically significant in all analyses.

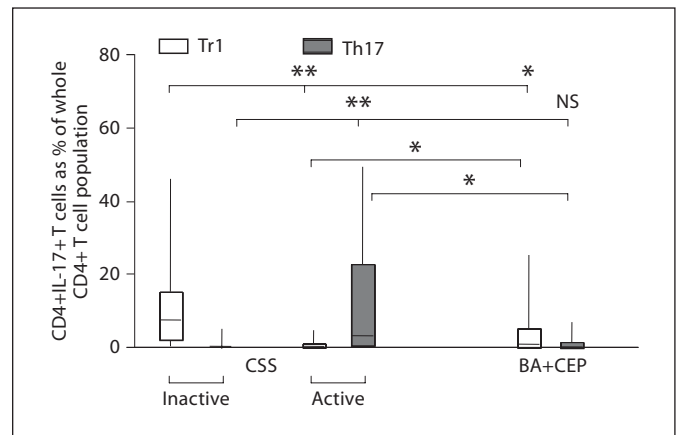
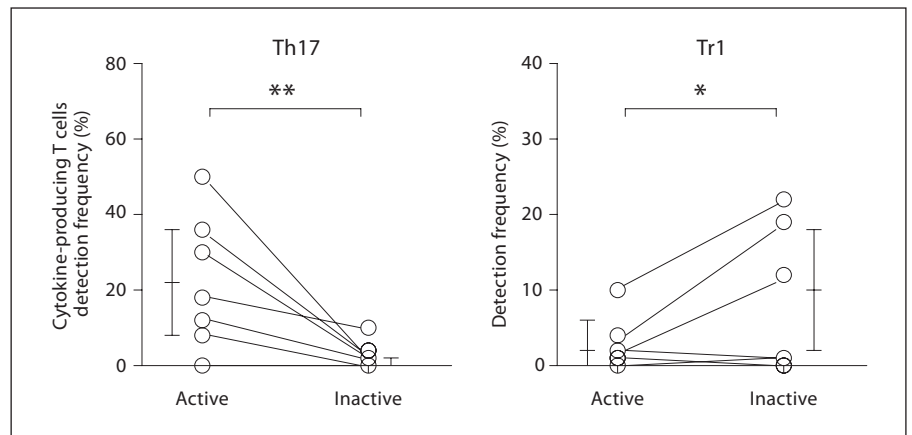


Fig. 2. Ability of CD4+ T cells to produce IL-17 and IL-10 skews toward the Th17 profile in the active CSS patient. Mononuclear leukocytes derived from patients of each group were stimulated with PMA and ionomycin to generate IL-17 and IL-10. The open and shaded columns represent CD4+CD25+ T cells producing IL-10 and CD4+ T cells producing IL-17, respectively. * $p < 0.05$; ** $p < 0.01$; NS = not significant.

Results

The roles of Tr1 and Th17 in the pathogenesis of CSS, and in severe asthmatics who did not develop CSS in spite of repeat CEP, were examined. No significant difference was observed in the number of naturally-occurring regulatory T cells (nTreg) among BA, BA+CEP, and all CSS patients, while a significantly lower number of nTreg cells was observed in healthy subjects (data not shown). The lowest number of CD4+ T cells was observed in inactive CSS patients (mean $\pm$ SD, 260.4 $\pm$ 140.1 cells/ μ l) in comparison to active CSS (531.5 $\pm$ 416.7 cells/ μ l, $p < 0.05$) and both BA (732.6 $\pm$ 694.7 cells/ μ l, $p < 0.001$) and BA+CEP (564.4 $\pm$ 253.6 cells/ μ l, $p < 0.001$) patients. A higher frequency of Th17 was detected only in the peripheral blood of CSS patients in the active stage (mean detection frequency: 12.5 $\pm$ 15.3%) in comparison to healthy subjects (0.64 $\pm$ 0.92%) and both BA (0.71 $\pm$ 1.9%) and BA+CEP (1.2 $\pm$ 1.7%) patients. In contrast, there were no differences among healthy subjects, BA, BA+CEP patients, and inactive CSS patients (0.74 $\pm$ 1.4%; fig. 1). The difference of the condition in CSS patients may be attributed to a difference of the absolute number of CD4+ T cells. As we have already reported, a significantly lower frequency of Tr1 was detected in active stage CSS patients (1.4 $\pm$ 3.7%) in comparison to BA+CEP (7.2 $\pm$ 8.1%), whereas the level of Tr1 was conversely higher in the inactive CSS (11.0 $\pm$ 13.7%) patients.

Fig. 3. Active CSS patients show Th17 elevations which correlated with disease activity. Patients were classified into active and inactive stages of CSS in accordance with the disease condition after treatment. Mononuclear leukocytes derived from the same patients in each state were stimulated with PMA and ionomycin to generate cytokines. The frequencies of both CD4+ and CD4+CD25+ T cells producing IL-17 and IL-10 were measured. * $p < 0.05$; ** $p < 0.01$.



The highest frequency of Th17 was detected in the active CSS patients, while none was observed in inactive CSS, BA, or BA+CEP patients (fig. 2). The detection frequencies of both Tr1 and Th17 were inversely related to disease activity in the same CSS patient. The lower frequency of Tr1 in the relapse stage of CSS was shown and then increased in the remission stage ($n = 7$, 2.3 ± 3.1 vs. $9.2 \pm 6.3\%$, $p < 0.05$). In contrast, the frequency of Th17 in the relapse stage of CSS was higher and then sharply decreased in the remission stage ($n = 7$, 22.3 ± 17.3 vs. $1.7 \pm 0.9\%$, $p < 0.01$; fig. 3).

Discussion

We have observed increased IL-17 and decreased IL-10 levels in active stage CSS patients as compared to patients who do not develop CSS, even after repeat CEP, and inactive CSS patients. Maintenance of immune homeostasis depends on the tightly controlled balance between activating and suppressing molecular events. Thus, defective counter-regulation and/or excessive activation of effector cells may each promote tissue-damaging immune responses [19]. We reported that in the patient who does not develop CSS even after repeat CEP, the frequency of Tr1 cells and the ability of responder/effector T cells to generate IL-2 do not decrease, but show remarkable decreases in the CEP patient who develops CSS [4, 5]. These results suggest that Tr1 cells in patients with CEP inhibit the development of CSS and that the suppressive function of Tr1 cells in CEP is a natural activity of these cells. Additionally, we also found active CSS patients to show Th17 elevations which correlated with disease activity, suggesting that Th17 may play an important role in the pathogenesis

of CSS. The detection frequency of Th17 did not have a significant difference among BA, BA+CEP, and inactive CSS patients. These results suggest that the appearance of Th17 does not depend on the difference of the immunological background of each disease, but on the doses of corticosteroids. In general, Th17 dominantly induces neutrophilic inflammation. How should we think about involvement of Th17 in eosinophilic inflammation in CSS patients? Wakashin et al. [20] report that IL-23 and Th17 not only induce Th17-cell-mediated neutrophilic airway inflammation, but also upregulate Th2-cell-mediated eosinophilic airway inflammation in mice. In addition, many cytokines and transcription factors that have shown a differentiation of naive T cells into Tregs or effector T cells, including Th1, Th2, Th3, and Th17 cells, are known. What is important, these Tregs and effector T cells are regulated exclusively mutually, and immunological homeostasis is maintained. An increase of Th17 in CSS patients in an active state may control differentiation to Tr1 cells from naive CD4+ T cells. A BA+CEP patient who neither develops CSS nor has an increase of Th17 maintains a normal differentiation induction of Tr1. CSS pathogenesis involves not only the remarkable decrease of Tr1 cells, which partially function as 'brakes', there is also a significant increase in Th17, which acts as an 'accelerator', contributing to tissue-damaging immune responses. This is the first report to demonstrate Th17 involvement in the pathogenesis of CSS.

Disclosure Statement

The authors declare that no financial or other conflict of interest exists in relation to the content of the article.

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