

Original Article

The scutellaria baicalensis stem-leaf total flavonoid regulates the balance of Th17/Treg in EAE rats

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Abstract: Multiple Sclerosis (MS), a common autoimmune-mediated disease, is associated with the imbalance of T helper (Th) 17 and regulatory T (Treg) cells. Previous reports have shown that Scutellaria baicalensis stem-leaf total flavonoid (SSTF) has obvious preventive effect on autoimmune disease. The aim of this study was to explore the therapeutic effect of the SSTF on experimental autoimmune encephalomyelitis, an well-established animal model of MS. We provided data showing that SSTF attenuated EAE disease severity, accompanied by enhanced Treg frequency and level of Treg-associated cytokines (IL-10 and TGF- β), as well as downregulated Th17 frequency and expression of Th17-related cytokines (IL-17 and IL-23). The results indicated that SSTF can regulate the balance of Th17/Treg in EAE rats.

Keywords: Multiple sclerosis, experimental autoimmune encephalomyelitis, scutellaria baicalensis stem-leaf total flavonoids, Th17 cells, Treg cells

Introduction

Multiple sclerosis (MS) is a chronic inflammatory, demyelinating and degenerative disease of the central nervous system (CNS) [1], affecting mostly young adults in the prime of their lives. Female preponderance in the incidence and prevalence of MS is firmly established [2, 3]. Clinically, MS is characterized by major motor and sensory deficits, and in addition patients develop cognitive dysfunction, e.g. memory impairment, which has a dramatic impact on their quality of life [4]. Experimental autoimmune encephalomyelitis (EAE) is a commonly used animal model for the study of MS [5]. And it has similar pathological and clinical manifestations to MS.

Numerous studies have shown the autoimmune responses of MS are caused mainly by CD4⁺ T cells and the generation of a T helper (Th) 1/Th2 imbalance [6]. Recently, a great deal of researches have indicated the Th17/Treg imbalance is a more critical factor in the mechanism underlying the neurological dysfunction of MS [7]. Th17 cells and Treg cells belong to CD4⁺ T cells subset. Th17 cells con-

tribute to the inflammatory diseases, whereas Treg cells play a pivotal role in maintaining the immune tolerance and immune homeostasis. It is well known that Th17 cells secrete pro-inflammatory cytokine interleukin (IL)-17 [8, 9], Treg cells secrete soluble molecules interleukin (IL)-10 and transforming growth factor (TGF)- β [10]. Retinoic acid-related orphan receptor (ROR) γ and forkhead box P3 (FoxP3) are the transcriptional factors of Th17 cells and Treg cells, respectively [11-13]. However, an effective therapy for regulating Th17/Treg balance has not been found yet.

Interferon- β , methylprednisolone, glatiramer acetate, mitoxantrone and natalizumab have been approved for treating MS [14]. Despite their promising efficacy, therapeutic response is rarely optimal. Furthermore, adherence to parenteral forms of these drugs is challenging, which has implications for the effectiveness of treatment [15]. Traditional Chinese medicine (TCM) can be used to treat the complex and varied presentations of MS, with fewer side-effects [16]. Scutellaria baicalensis stem-leaf total flavonoid (SSTF) is the active ingredient of the aerial part of Radix Scutellariae, which has obvi-

ous preventive effect on rheumatoid arthritis (RA). However, none of efforts has been made to understand the preventive effect of SSTF on the treatment of MS. The aim of this study was to explore the regulatory effect of SSTF on Th17 and Treg cells in EAE rats.

Materials and methods

Materials

Complete Freund's adjuvant (CFA) was purchased from Sigma-Aldrich, St. Louis, MO, USA. SSTF and Prednisone acetate were purchased from Jilin province northeast Asia pharmaceutical co., LTD and Zhejiang XianJu pharmaceutical co., LTD, respectively. IL-17, TGF- β 1 and Foxp3 polyclonal antibodies were from Beijing Bioss Biological Technology co., LTD. IL-17, IL-23, IL-10 and TGF- β enzyme linked immunosorbent assay (ELISA) kits were from Wuhan Colorful Gene Biological Technology Co., LTD. Anti7rat CD4 FITC, anti-rat CD25 APC, anti-rat IL-17 PE and anti-rat Foxp3 PE were from eBioscience, San Diego, CA, USA. Red Blood Cell Lysis Buffer was from Solarbio Science & Technology Co., Beijing, China. Trizol, Prime-Script reagent Kit and SYBR Green Premix Ex Taq were purchased from TaKaRa, Dalian, China.

Animals

Female Wistar rats (6-8 weeks old, weight 180 g-200 g) were obtained from the Experimental Animal and Animal Experiment Centre of Qingdao, Shandong, China. Female guinea pigs (6-8 weeks old, weight 350 g-400 g) were obtained from the Experimental Animal and Animal Experiment Centre of Qingdao, Shandong, China. Animals were housed with a standard 12 h light/12 h dark cycle in the animal experiment centre of the Qingdao University, with temperature and relative humidity 22°C and 65-70%, respectively. After 1 week's adaptation and being fed with a standard-mixed feed, the animals were used for experiments.

EAE induction

EAE was induced as described previously [17], universally accepted procedures. Rats were immunized under anesthesia in the four foot-pads and neck with a mixture of 0.5 ml guinea pig spinal cord homogenate and an equal volume of complete Freund's adjuvant (CFA)

containing 6 mg/ml Bacillus Calmette-Guérin vaccine. All procedures were approved by the Bioethics Committee of Qingdao University Medical College. Animals were weighed and monitored daily by two independent observers for clinical signs of acute EAE using the following criteria [18]: 0, no clinical signs; 1, limp tail; 2, hind limb weakness; 3, hind limb paralysis; 4, forelimb paralysis; 5, moribund or death.

Treatment of EAE

Immunized rats were randomly assigned to four groups for different treatments. The EAE group consisted of 10 immunized rats that received saline intragastrically in the amount of 6.7 ml/kg from day 1 until day 17 after immunization. Meanwhile 10 non-immunized normal rats that received the same amount of saline served as naive control. Briefly, SSTF was dissolved in Phosphate Buffer Solution (PBS) and administered intragastrically daily, either at a low dose (100 mg/kg; SSTF-L) or high dose (200 mg/kg; SSTF-H). Prednisone acetate, the positive control drug, was dissolved in saline and given daily at 6 mg/kg, from day 1 until day 17.

Histological analysis of EAE disease severity

On day 17, rats were sacrificed, the blood was harvested from the heart for serum preparation and, then, spinal cords were harvested after extensive perfusion with normal saline. The lumbar enlargement of spinal cords was fixed in 4% paraformaldehyde and embedded in paraffin. After embedded in paraffin, 5 μ m slices were sectioned and stained with hematoxylin-eosin (H&E) for inflammatory infiltration [17] or with Luxol fast blue (LFB) to evaluate loss of myelin. Histopathological examination was performed and scored in a blinded fashion as follows [17]. For inflammation: 0, no inflammatory cells; 1, a few scattered inflammatory cells; 2, organization of inflammatory infiltrates around blood vessels; 3, extensive perivascular cuffing with extension into adjacent parenchyma, or parenchyma. For each rat, 3 histological sections were analyzed and their average scores were calculated. Demyelination of the spinal cord was assessed by LFB staining and scored as described [19, 20], with 0 indicating normal, 1 indicating one small focal area of demyelination, 2 indicating two or three areas, 3 indicating one to two large areas of demyelination, and 4 indicating extensive demyelination involving $\geq 20\%$ of the white matter.

Immunohistochemistry analysis of IL-17, TGF- β 1 and Foxp3

Immunohistochemistry was performed with 5 μ m paraffin sections as described above, deparaffinized in xylene, and rehydrated in graded ethanol solutions. Immunohistochemical SP method was used to detect the expressions of IL-17, TGF- β 1 and Foxp3. The tissue sections were first incubated a specific antibody (anti-IL-17, anti-TGF- β 1 and anti-Foxp3) overnight at 4°C, washed in Phosphate Buffer Solution (PBS) for three times, and then was incubated with biotinylated secondary antibody (anti-immunoglobulin of the animal species from which the specific antibody was obtained) for 20 minutes at 37°C. After washed in PBS, the sections were incubated with avidin conjugated to HRP for 20 min at 37°C, then colored with 3,3-diaminobenzidine (DAB). After counterstained with haematoxylin for 5 mins, tissue sections were washed and then dehydrated with ethanol from 70% to 80%, 90%, 95%, 100%, then transformed into xylene for 3 times. Finally, the sections were covered with coverslips by neutral gums and observed by microscope.

Image Pro plus 6.0 (Media Cybernetics, Bethesda, MD, USA) was used to quantify immune-staining results. This software was designed to select stained nuclei or cells based on color intensity and nuclear shape. Brown staining was considered positive. The chromatic area and the strength (light density values) of positive cells were calculated and represented as a percentage of total positively stained cells with integral light density values (integral optical density).

ELISA analysis of IL-17, IL-23, IL-10 and TGF- β

Concentrations of IL-17, IL-23, IL-10 and TGF- β in sera were assayed by ELISA using a rat IL-17, IL-23, IL-10 and TGF- β ELISA Kit. Testing samples and standard were added into each well following the instructions of manufacturer and then anti-IL-17, IL-23, IL-10 and TGF- β HRP-conjugated antibody was added. After incubation (30 min, 37°C) and washing, chromogen solution A (50 μ l) and chromogen solution B (50 μ l) were added into each well, followed by Stop Solution (50 μ l) to end the reaction. The results were read using a microtiter plate reader within 15 min at 450 nm.

Flow cytometry analysis of Th17 and treg cells

Each rat was taken with the anticoagulant whole blood for 100 μ l per tube, after lysis by Red Blood Cell Lysis Buffer were resuspended in PBS. The cells were washed with washing buffer and then stained with FITC-conjugated anti-CD4 Ab in the dark on ice for 30 min. APC-conjugated anti-CD25 Ab was also required for the detection of Treg cells. After surface staining, the cells were resuspended in fixation/permeabilization solution and then stained intracellularly with PE conjugated anti-IL-17 in the dark at room temperature for 20 min. When Treg cells were detected, PE conjugated anti-Foxp3 was used in this step. After extensive washing, stained cells were analysed using a FACS Calibur flow cytometer (BD Biosciences, Mountain View, CA, USA). Isotype controls and unstimulated controls were utilized to confirm antibody specificity and to enable correct compensation. Stained cells were analyzed using Accuri C6 software.

Statistical analysis

All data were presented as mean \pm SD. Data were analyzed with SPSS 19.0 software. Multiple comparisons were evaluated by repeated measures analysis of variance (ANOVA) followed by the LSD-t test, or Kruskal-Wallis test. Significant difference was determined when *P* value was less than 0.05.

Results

Clinical symptoms and scores

The EAE group of rats showed the first signs of EAE on day 9, while the SSTF-L group on day 11, and the SSTF-Hgroup on day 12. Compared to the EAE group, the mean clinical scores in the two SSTF-treated groups were significantly decreased ($P < 0.05$), and the mean clinical scores in the two SSTF-treated groups were also significantly different in a dose-dependent manner ($P < 0.05$). The weight change of rats in each group was same as above (**Figure 1**).

Histopathological analysis

Inflammatory infiltration and demyelination were evaluated by H&E and LFB staining, respectively. Histopathologic examination showed that SSTF suppressed inflammatory cell infiltration and reduced demyelination in spinal cords of EAE rats. Compared with EAE rats,

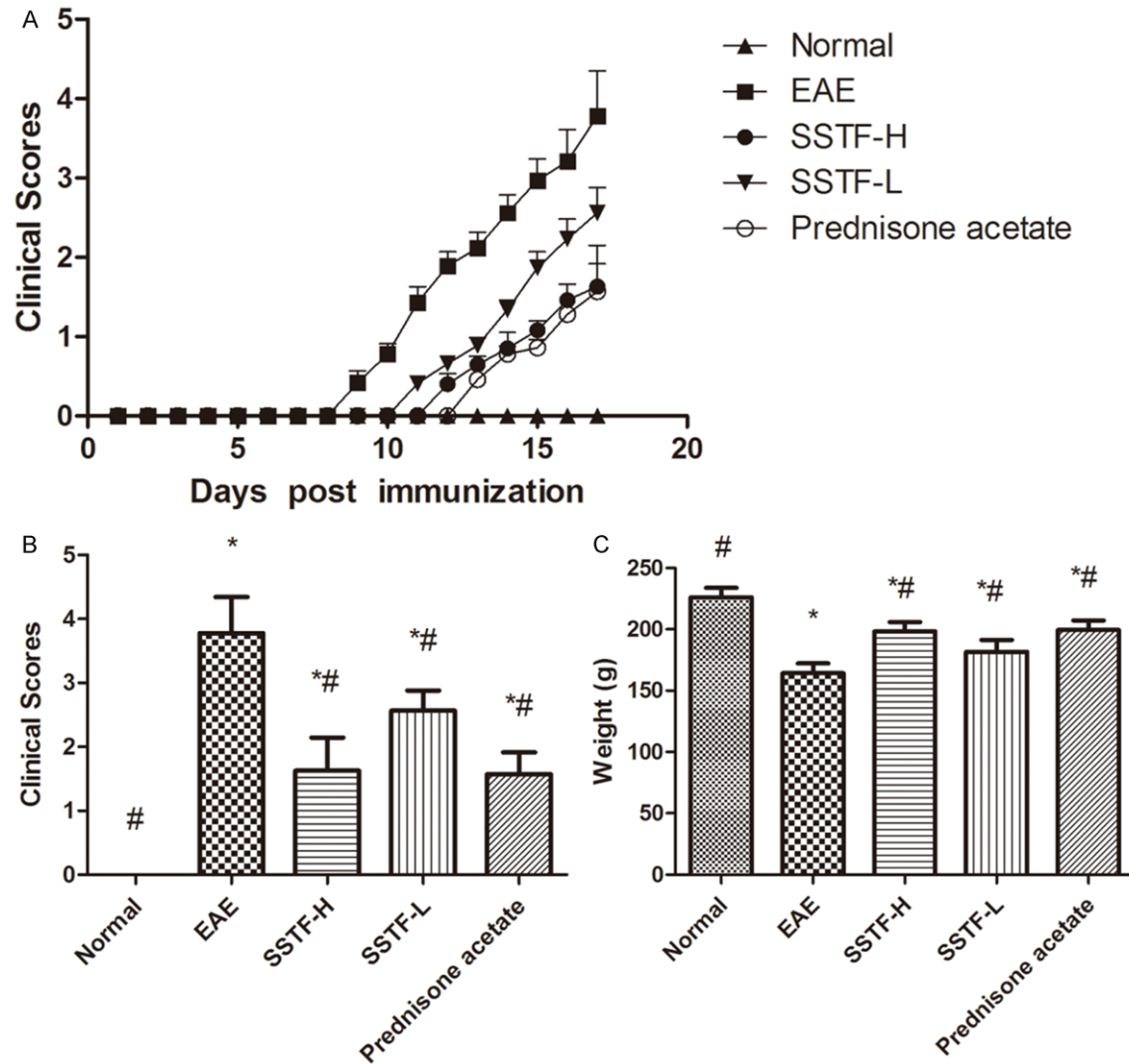


Figure 1. SSTF attenuates clinical course of EAE. A. Clinical scoring was implemented daily from days 1 to 16. Clinical EAE was scored according to a 0-5 scale daily post-immunization. B. Histogram of the mean maximum clinical scores. * $P < 0.05$, as compared to normal group. # $P < 0.05$, as compared to EAE group. C. The mean final weight are expressed as mean \pm SD. * $P < 0.05$, as compared to normal group. # $P < 0.05$, as compared to EAE group.

treatment group rats had significantly lower inflammation scores and demyelination scores ($P < 0.05$) (Figure 2).

Expression of IL-17, TGF- β 1 and Foxp3 proteins

The expression IL-17, TGF- β 1 and Foxp3 in the spinal cords were analyzed by immunohistochemistry. As shown in Figure 3A, the numbers of positive cells of IL-17 in the spinal cords of the EAE group were significantly higher than in the SSTF-treated groups, while the numbers of positive cells of TGF- β 1 and Foxp3 in EAE group were lower than in the SSTF-treated groups. As shown in Figure 3B, EAE rats exhibited the highest OD level of IL-17 positive cells

among all the groups, while this expression was significantly reduced after SSTF treatment ($P < 0.05$). Similarly, the expression of IL-17 was also reduced after Prednisone acetate treatment ($P < 0.05$, compared to EAE group). However, the expression of TGF- β 1 and Foxp3 were contrary to the above.

Serum concentration of Th17/treg cytokines IL-17, IL-23, IL-10 and TGF- β

To test the in vivo immunomodulatory effect of SSTF in the treatment of EAE, we harvested sera from all groups of rats, and determined the concentration of Th17/Treg cytokines IL-17, IL-23, IL-10 and TGF- β by ELISA. A high level

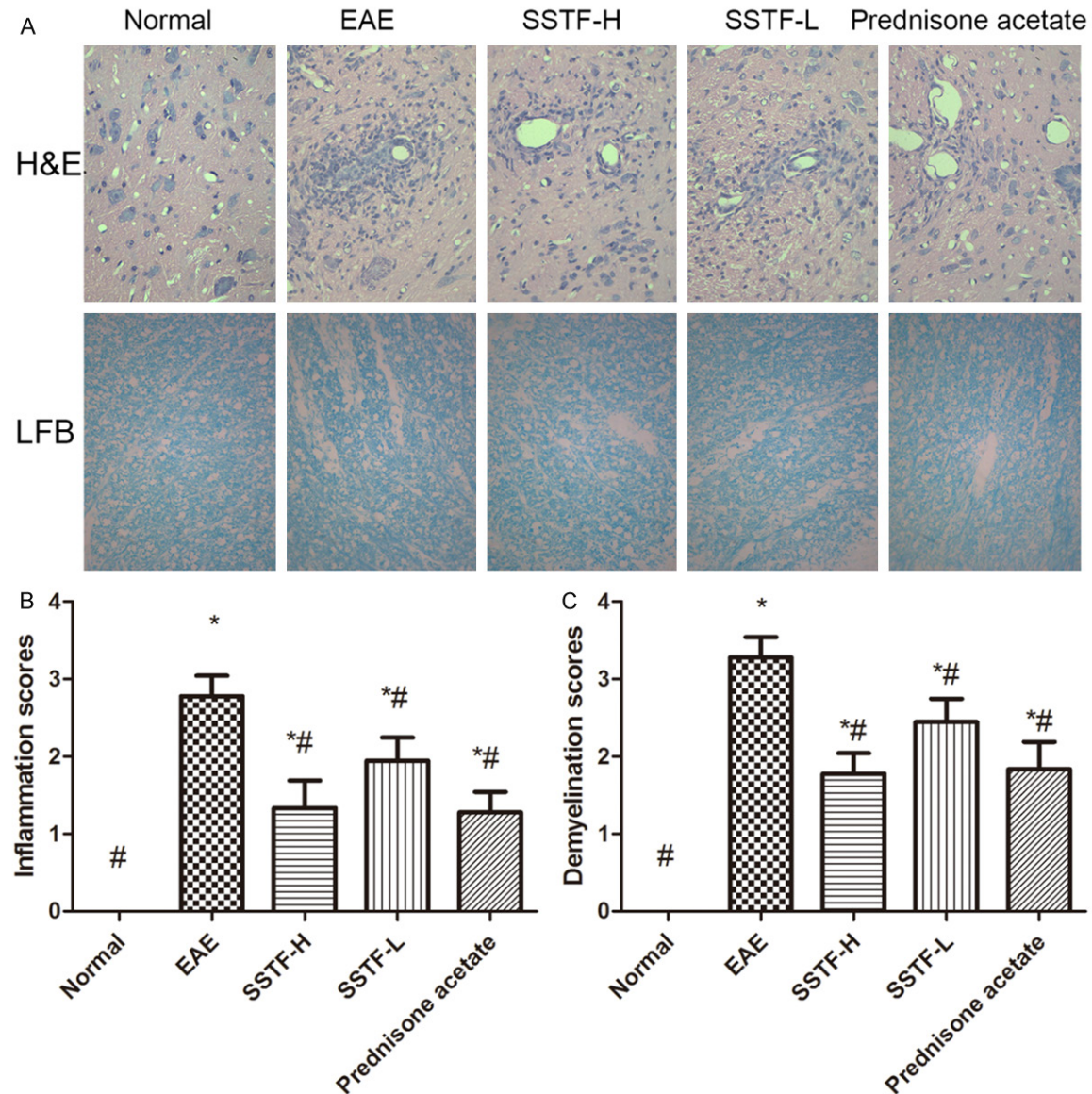


Figure 2. SSTF reduced inflammation and demyelination scores. A. Spinal cord tissues were harvested at day 17, HE and LFB staining were performed in the paraffin sections. Digital images and shown (40 × magnification). B. Quantitative analysis and statistics of HE staining. Inflammation was scored according to a 0-3 scale in a blinded manner. The values are the mean ± SD. * $P < 0.05$, as compared to normal group. # $P < 0.05$, as compared to EAE group. C. Demyelination scores. The values are the mean ± SD. * $P < 0.05$, as compared to normal group. # $P < 0.05$, as compared to EAE group.

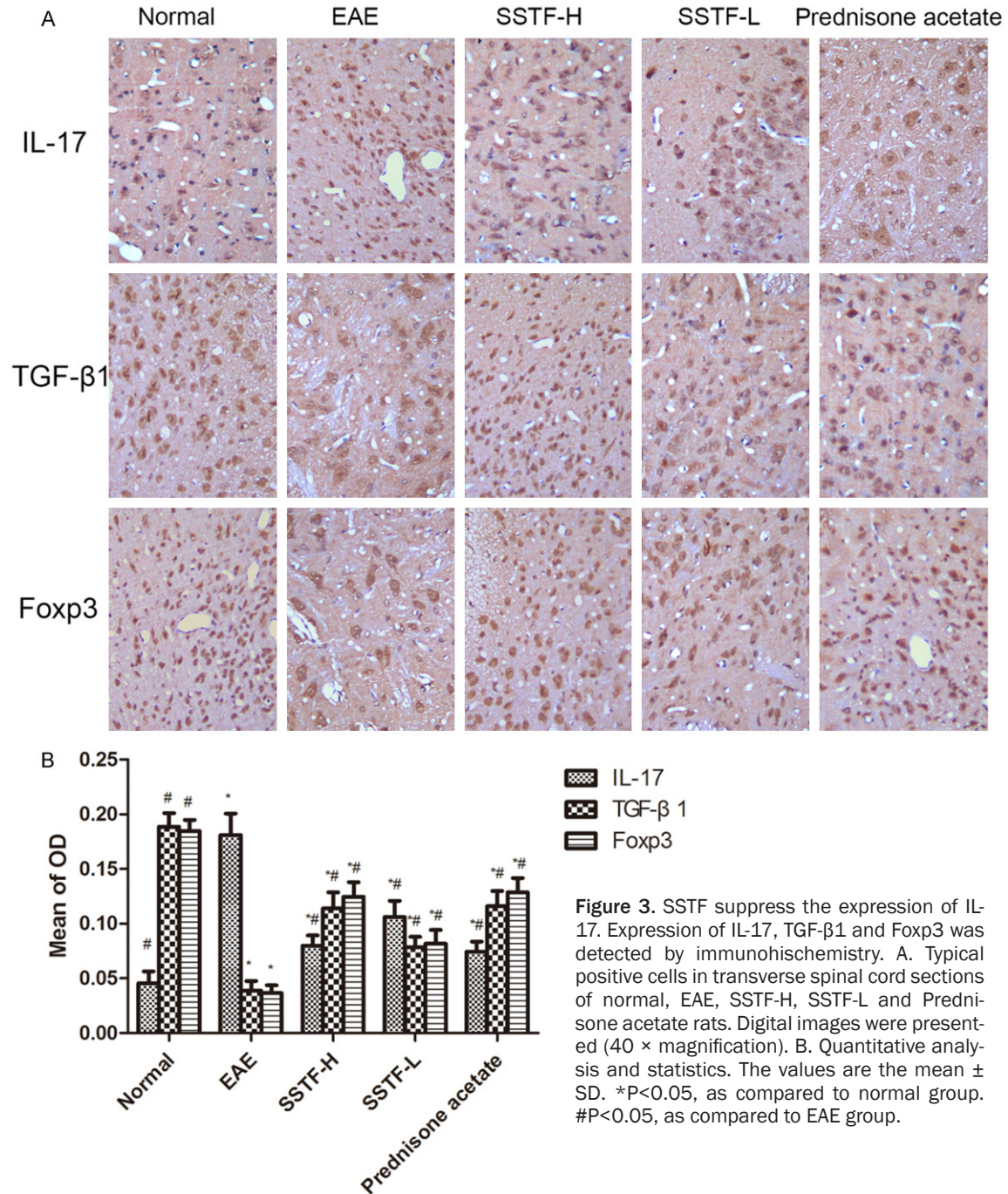
of serum IL-17 and IL-23 concentration was observed in EAE rats, while it was significantly decreased in both SSTF-treated groups ($P < 0.05$), and serum concentration of IL-17 and IL-23 in the SSTF-L group was higher than in the SSTF-H group ($P < 0.05$) (Figure 4).

We then determined the serum concentrations of Treg-related cytokines TGF- β and IL-10 by ELISA. As shown in Figure 4, a significant increase of these two cytokines was observed in

SSTF-treated groups compared to the EAE group ($P < 0.05$), with a more profound increase in the SSTF-H group compared to the SSTF-L group ($P < 0.05$). No difference was found in the TGF- β and IL-10 level between SSTF-H and Prednisone acetate groups (Figure 4).

The expression of Th17 and treg cells

By analyzing the number of IL-17-positive cells and CD25-Foxp3-positive cells in CD4⁺ T sub-



set, we could demonstrate whether Th17/Treg imbalance was responsible for exacerbating EAE. As shown in **Figure 5A-C**, the number of Th17 cells dramatically increased in EAE rats than in SSTF-treated rats ($P<0.05$), while the number of Treg cells were decreased in EAE rats than in SSTF-treated rats. Thus, our results provided evidence that SSTF treatment can regulate the balance of Th17/Treg cells.

Discussion

The results of this study showed the EAE models induced by guinea pig spinal cord homogenate and CFA had neurological deficits associated with demyelination in the spinal cord. Compared with EAE group, the SSTF-treated group was manifested having an improved neurological function, and reduced inflammation

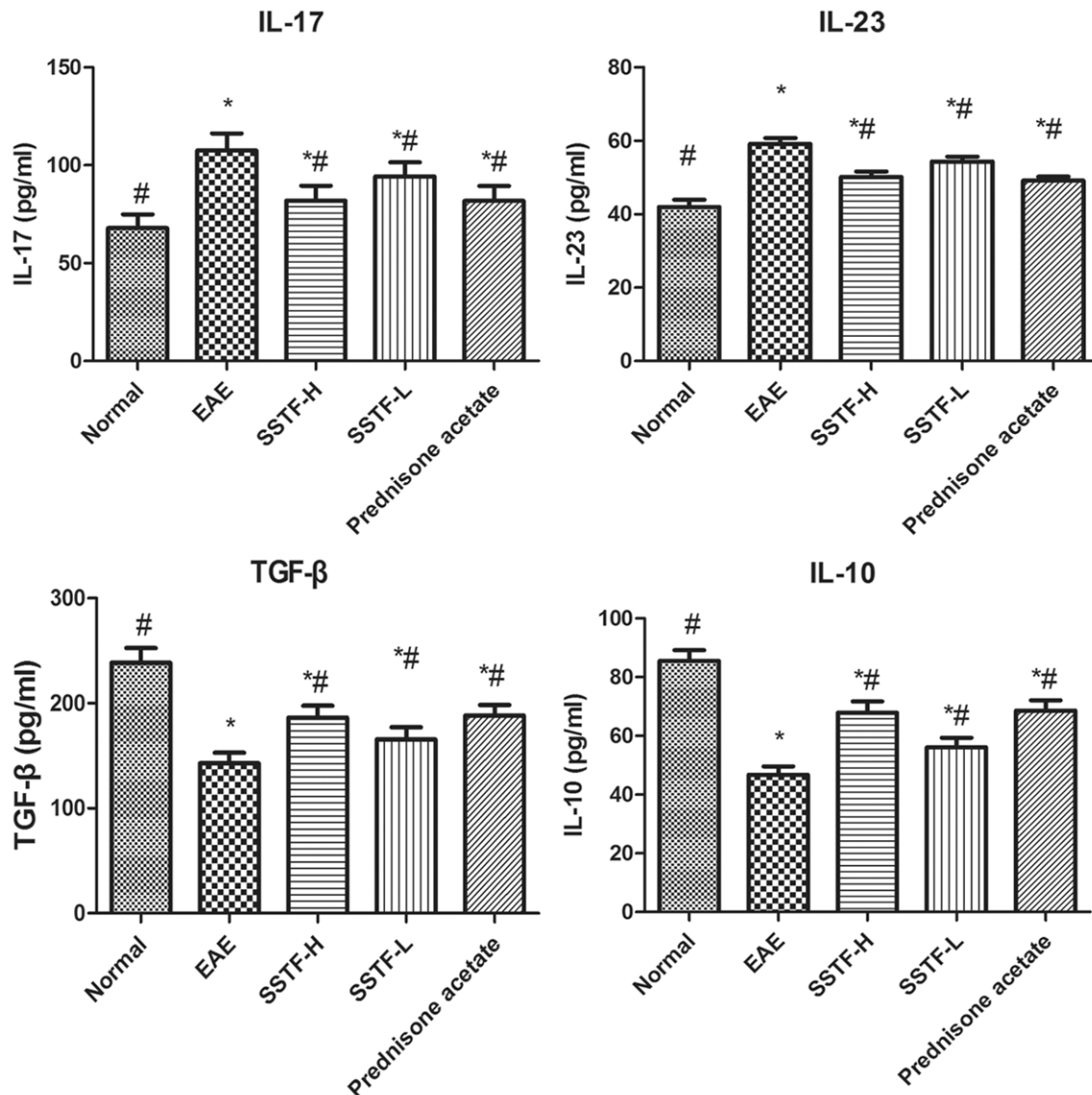


Figure 4. Serum production of IL-17 and TGF-β in each group rats. Sera from each individual rat were taken on day 17, when animals were sacrificed, and the concentrations of IL-17, IL-23, IL-10 and TGF-β in the sera were measured by ELISA. The values are the mean ± SD. *P<0.05, as compared to normal group. #P<0.05, as compared to EAE group.

and demyelination in the CNS. The data demonstrated that EAE rats exhibited significantly increased peripheral Th17 frequency and Th17-related cytokines (IL-17 and IL-23) as well as dramatically decreased Treg frequency, Treg-related cytokine (TGF-β1 and IL-10) and transcription factor (Foxp3) levels. In addition, the impaired balance of Th17/Treg cells in EAE group is evidently indicated.

CD4⁺ T lymphocytes play a critical role in the pathogenesis of multiple sclerosis and its animal model, EAE [21]. Th17 cells are commonly

considered as a pathogenic population of lymphocytes, as they have been detected at the onset of EAE in mice [22]. In 2003, Cua et al. showed that IL-23 plays an essential role in autoimmune inflammation of the CNS [23]. Lots of studies have shown that IL-17A plays a vital role in EAE, as in most studies lack of IL-17A bioactivity led to mitigated disease course and improved recovery [24-30]. In our study, the number of Th17 cells in the SSTF-treated group was significantly downregulated compared to the EAE group. Moreover, we found the levels of IL-17A mRNA and inflamma-

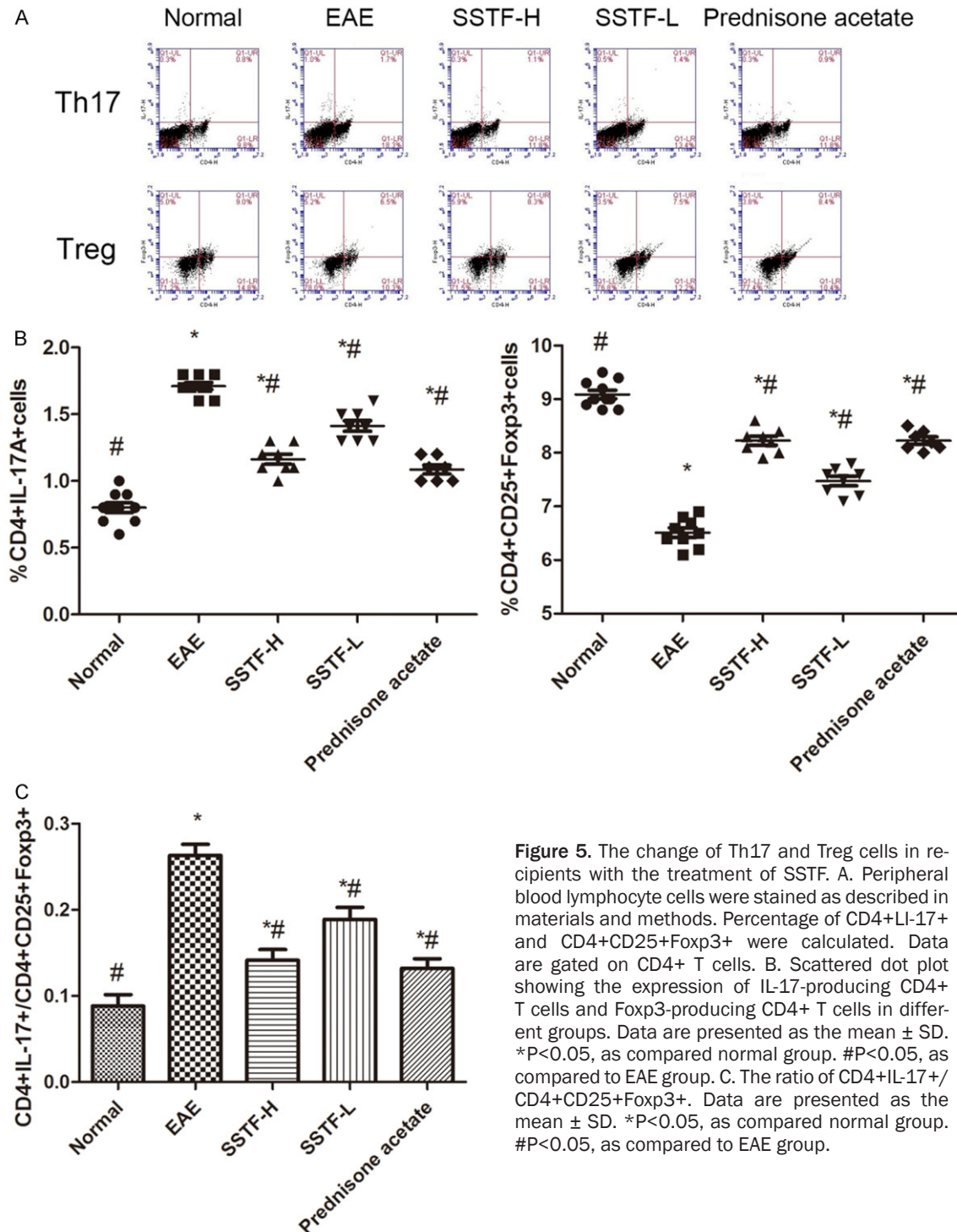


Figure 5. The change of Th17 and Treg cells in recipients with the treatment of SSTF. A. Peripheral blood lymphocyte cells were stained as described in materials and methods. Percentage of CD4+IL-17+ and CD4+CD25+Foxp3+ were calculated. Data are gated on CD4+ T cells. B. Scattered dot plot showing the expression of IL-17-producing CD4+ T cells and Foxp3-producing CD4+ T cells in different groups. Data are presented as the mean \pm SD. * $P < 0.05$, as compared normal group. # $P < 0.05$, as compared to EAE group. C. The ratio of CD4+IL-17+/CD4+CD25+Foxp3+. Data are presented as the mean \pm SD. * $P < 0.05$, as compared normal group. # $P < 0.05$, as compared to EAE group.

tion-related cytokines such as IL-17 and IL-23 decreased in SSTF group compared to EAE group, which suggested SSTF can inhibit the secretion of these inflammatory cytokines.

The regulatory T (Treg) cells are a subset of CD4⁺ cells suppressing immune responses to

maintain unresponsiveness to self-antigens and prevent excessive immune responses to foreign antigens [31]. Accumulation of these T-regs in the CNS has already been reported during recovery in mice EAE models [32-34]. The results of our study showed that the frequency of Treg cells in the SSTF-treated group

was improved than in the EAE group. We also observed that Treg-associated anti-inflammatory cytokines (IL-10 and TGF- β) were increased in SSTF group compared to EAE group. The content of SSTF treatment group was higher than that of EAE group. Moreover, we also analyzed Foxp3 and obtained similar results. Different populations of Treg cells have also been reported on the basis of high expression of CD25 and forkhead family transcription factor 3 (Foxp3) or on the basis of the production of immunosuppressive cytokines, such as interleukin (IL)-10 or transforming growth factor (TGF)- β [31]. CD4+CD25+Foxp3+ cells are the most characterized Treg cells [35]. IL-10 is a critical regulator of EAE severity [36, 37]. Forkhead box protein 3 (Foxp3) is a master transcription factor that confers regulatory function to some, but not all, types of Tregs [38].

Scutellaria baicalensis stem-leaf total flavonoid (SSTF) is the main ingredient of the aerial part of Radix Scutellariae, and the flavonoids are the main components of SSTF. In our study, we found that SSTF had a protective effect on EAE rats and it can regulate the balance of Th17 and Treg cells. This study provides a basis for further research the treatment of SSTF in MS, as the underlying mechanism as to how SSTF modulates the differentiation and quantity of Th17 and Treg cells needs to be further investigated.

Disclosure of conflict of interest

None.

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