Epigallocatechin-3-gallate-induced inhibition of interleukin-6 release and adjustment of the regulatory T/T helper 17 cell balance in the treatment of colitis in mice

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Abstract. Epigallocatechin-3-gallate (EGCG) has a promising therapeutic effect for ulcerative colitis (UC), but the treatment mechanism has yet to be fully elucidated. The aim of the present study was to investigate the mechanism of EGCG in the treatment of UC. Experimental colitis mouse models were prepared. The mice were randomly divided into four groups: Normal control, model (MD), 50 mg/kg/day EGCG treatment and 100 mg/kg/day EGCG treatment. The daily disease activity index (DAI) of the mice was recorded, changes in the organizational structure of the colon were observed and the spleen index (SI) was measured. In addition, levels of interleukin (IL)-6, IL-10, IL-17 and transforming growth factor (TGF)-β1 in the plasma and hypoxia-inducible factor (HIF)-1α and signal transducer and activator of transcription (STAT) 3 protein expression in colon tissues were evaluated. Compared with the MD group, the mice in the two EGCG treatment groups exhibited decreased DAIs and SIs and an attenuation in the colonic tissue erosion. EGCG could reduce the release of IL-6 and IL-17 and regulate the mouse splenic regulatory T-cell (Treg)/T helper 17 cell (Th17) ratio, while increasing the plasma levels of IL-10 and TGF-β1 and decreasing the HIF-1α and STAT3 protein expression in the colon. The experiments confirmed that EGCG treated mice with experimental colitis by inhibiting the release of IL-6 and regulating the body Treg/Th17 balance.

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Introduction

Ulcerative colitis (UC) is a chronic, non-specific inflammatory disease that involves the rectum and colon of the body; however, the etiology and pathogenesis of the disease have yet to be fully elucidated. Studies have suggested that UC is associated with a variety of factors, such as genetics, infection, the immune system, and environmental and intestinal dysbiosis, as well as damage to the intestinal tissues caused by immune cells and inflammatory cytokines in the inflammatory signaling cascade (1,2). In colonic mucosal lesions, inflammation, mucosal congestion, edema and ulcers may be visible, as well as the infiltration of immune cells (lymphocytes and neutrophils) in the acute exacerbation stage (3). Following the administration of 5% dextran sulfate sodium ($C_6H_7Na_3O_{14}S_3$, DSS), mice gradually exhibit the symptoms of colitis, such as loose stools, bloody diarrhea and weight loss, thus forming an experimental colitis model similar to human UC (4).

Epigallocatechin-3-gallate (EGCG) is a type of natural compound that can be extracted from green tea. EGCG has no toxic effects but has a high biological activity and is known to exert antibacterial, anti-inflammatory, anti-tumor, antioxidation and anti-aging effects (5-8). Furthermore, it has been demonstrated that EGCG can modulate the immune system to treat autoimmune disease (9-11). EGCG is known to effectively treat experimental colitis in mice, but its therapeutic mechanism underlying this treatment is unclear (9,12). In a previous study it was found that an imbalance in the number and function of regulatory T cells (Tregs)/T helper 17 cells (Th17s) and the secretion of certain cytokines, such as interleukin (IL)-6, IL-10, IL-17 and transforming growth factor (TGF)-β1, had a crucial role in the development of UC (13). The aim of the present study was therefore to investigate the mechanism of EGCG in the treatment of UC by measuring the levels of proinflammatory [interleukin (IL)-6 and IL-17] and anti-inflammatory [IL-10 and transforming growth factor (TGF)-β1] cytokines in the plasma and the colonic protein expression of hypoxia-inducible factor (HIF)-1α and signal transducer and activator of transcription (STAT) 3 in a mouse model of colitis. The Treg/Th17 balance was also examined.

Table I. Murthy scoring system (10).

Scores	Weight loss rate (%)	Stool stickiness	Presence of blood in stool
0	(-)	Normal (granular, with form)	Normal
1	1-5		
2	6-10	Soft (mushy, not adhering to the anus)	Occult blood (+)
3	11-15		
4	>15	Diarrhea (watery, adhering to the anus)	Bloody stools (+)

Materials and methods

Experimental animals. Forty male BALB/c mice (age, 6-7 weeks; weight, 22-26 g) of specific pathogen-free status were obtained from the China Experimental Animal Center of Southern Medical University (Guangzhou, China; certificate of conformity no. SCXK Guangdong 2011-0015) for use in the present study. The mice were housed in clean animal houses at a controlled temperature (22-25˚C) and a relative humidity of 55%, and were subjected to a 12-h light/dark cycle. Food and drinking water were available *ad libitum*. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the Second Clinical Medical College of Jinan University (Shenzhen, China).

Treatment program. The 40 mice were fed with adaptability for one week, and then randomly divided into four groups (n=10/group): Normal control (NC), model (MD), EGCG (Qiyun Biotechnology Co., Ltd., Guangzhou, China) 50 mg/kg/day treatment (ELD) and EGCG 100 mg/kg/day treatment (EHD). The NC group was given free access to sterile distilled water for 14 days, while the MD and EGCG treatment groups received 5% DSS (Qiyun Biotechnology Co., Ltd.) continuously for seven days; the DSS was replaced by distilled water for seven days following the successful establishment of the model. On day 8 of the experiment, the mice in the NC and MD groups were given a daily gavage of 0.2 ml 0.5% ethanol for seven days, while the ELD and EHD groups were administered EGCG at doses of 50 and 100 mg/kg/day for each mouse, respectively. A total of 0.2 ml 0.5% ethanol was then used to dissolve the EGCG for gavage treatment for seven days. On the day 14 the mice were administered 100 mg/kg pentobarbital anesthesia, and eyeball blood was obtained following the sacrifice of the mice. Blood plasma and spleen and colon tissues were collected under sterile conditions.

Macroscopic and microscopic assessment. The disease activity index (DAI) and spleen index (SI) of the mice were measured and recorded daily, with reference to the Murthy scoring system (14). Details of the scoring system are shown in Table I. The DAI was calculated with the following formula: DAI = (weight loss rate score + stool consistency score + presence of blood in stools score)/3. The spleens of the mice were washed with saline, and then dried and weighed for the calculation of the SI using the following formula: SI = spleen weight (mg)/body weight (g).

Specimen collection and pathology assessment. The mice were sacrificed following pentobarbital anesthetization and the abdominal cavity was opened. Measuring from the anus, a 10-cm section of colon was cut along the longitudinal axis of the mesentery and rinsed repeatedly with iced saline. The section was then cut vertically into two parts, taking the lesion as the center in the longitudinal axis. One colon sample was immediately placed in 10% formalin solution at room temperature and fixed for 48 h, prior to undergoing tissue dehydration, clearing, embedding and staining with hematoxylin and eosin. The colon tissue morphology was subsequently observed by microscopy. For the second section of the colon tissue, the lesion was excised, dried by filter paper, weighed and frozen at -70˚C for tissue specimen tests.

Lymphocyte extraction from spleen tissue. Under sterile conditions, the spleen of each mouse was obtained for lymphocyte separation. A 5-ml volume of lymphocyte separation medium (Shenzhen Lvshiyuan Biotechnology, Co., Ltd., Shenzhen, China) was added to 35-mm petri dishes with homogenized spleen tissue. Following the separation of the suspension, spleen cell fluid was immediately transferred to a 15-ml centrifuge tube with 200-500 μl RPMI-1640 medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Samples were centrifuged at 800 x g for 30 min at room temperature. The lymph cell layer was removed by suction and 10 ml RPMI-1640 medium was added for reverse washing. The samples were centrifuged at 250 x g for 10 min at room temperature, and the lymphocytes were subsequently collected. The number of lymphocytes was then adjusted to $2x10^6$/reaction tube.

Flow cytometry. The lymphocyte samples ($2x10^6$/reaction tube) were analyzed using flow cytometry with a Th17/Treg kit (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. A total of 50 ng/ml phorbol 12-myristate 13-acetate (for the lymphocytes), 1 μg/ml ionomycin and monensin were used to stimulate the cells for 5 h. After 5 h, the cells were collected, stained, fixed and lysed in strict accordance with the kit instructions, and 20 μl streaming antibodies were added to each tube in turn, mixed and incubated for 30 min. The main antibodies used in the flow cytometry included mouse cluster of differentiation 4 (CD4)-PerCP-Cyanine5.5, IL-17A-phycoerythrin (PE) and Alexa Fluor 647-forkhead box protein P3 (Foxp3). A

parallel negative control group was set, and 0.2 mg/l PE-rat immunoglobulin (Ig)G1 and 0.2 mg/l Alexa Fluor 647-Rat IgG2b were added. The samples were loaded and the data were analyzed.

ELISA. Blood was obtained from the mice and treated with heparin to prevent coagulation, prior to the supernatant being centrifuged for 5 min at 1,000 x g (4˚C). A total of 50 mg mouse colon tissue was weighed using an electronic analytical balance and cut into pieces as soon as possible with a small pair of ophthalmological scissors. Following cutting, 1,200 μl physiological saline at 0˚C was added and the tissue was fully homogenized on ice using a glass homogenizer and centrifuged for 10 min using a refrigerated high-speed centrifuge (Sigma, St. Louis, MO, USA) at 4˚C (1,000 x g). The supernatant was obtained, and protein quantification was then performed using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). According to the specifications of the ELISA kit (Dakota Biotech Co. Ltd., Beijing, China), the IL-6, IL-10, IL-17 and TGF-β1 concentrations in the plasma and colon tissues of the mice were detected.

Western blot analysis. A total of 100 mg mouse colon tissue was accurately weighed using an electronic analytical balance, and plasma and nuclear proteins were extracted from the cells using a nucleus and cytoplasm tissue extraction kit in accordance with the manufacturer's instructions (Thermo Fisher Scientific, Inc.). The protein samples were quantified using a NanoDrop 2000 spectrophotometer and the protease inhibitor phenylmethylsulfonyl fluoride was added. The proteins were then separated using SDS-PAGE (10%) and transferred to a nitrocellulose membrane (Beijing Bayer Di Biotechnology, Co., Ltd., Beijing, China). The membrane was blocked with Western blocking buffer (P0023B) and incubated with the primary and secondary antibodies, for 1 h each, and subsequently developed using enhanced chemiluminescence for analysis. Western washing liquid (P0023C) was used to wash the membrane between incubations. Anti-mouse polyclonal STAT3 (1:500; Bioworld Technology, Inc., St. Louis Park, MN, USA) and HIF-1α (1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA) primary antibodies were used to analyze the nuclear HIF-1α and cytoplasmic STAT3 protein levels. Goat anti-rabbit horseradish peroxidase secondary antibodies (1:2,000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) were incubated with the membranes with phosphate-buffered saline, containing 0.1% Tween-20, 5% w/v skimmed milk powder and 2% BSA diluent/blocking solution, for 1 h at room temperature. In addition, levels where tested against lamin or β-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).

Statistical analysis. Experimental data are expressed as the mean ± standard deviation. The statistical analysis of the experimental data was performed using SPSS statistical software, version 20.0 (IBM SPSS, Armonk, NY, USA). The differences between the groups were compared using single-factor analysis of variance, and P<0.05 was considered to indicate a statistically significant difference.

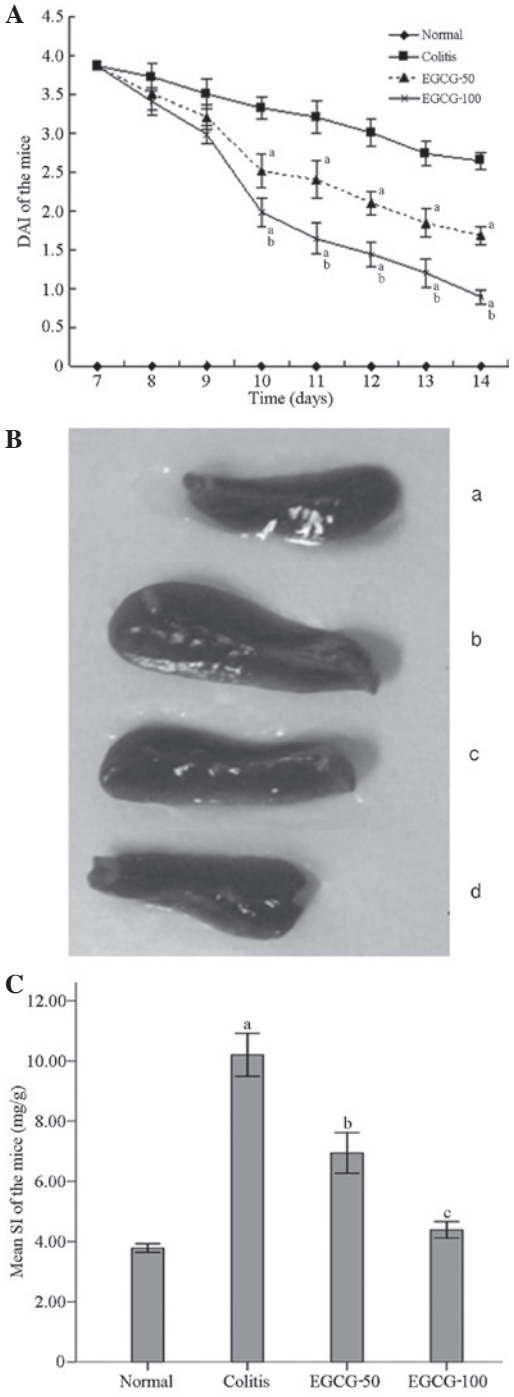

Figure 1. Effect of EGCG on the DAI and SI of mice with experimental colitis. (A) DAIs of the mice in the four groups. [a]P<0.05 vs. the colitis group; [b]P<0.05, vs. the EGCG-50 group. (B) Images of spleens from mice in the four groups: (a) Normal, (b) colitis (model), (c) EGCG-50 and (d) EGCG-100 groups. (C) SIs of the mice in the four groups. [a]P<0.05 vs. the normal group; [b]P<0.05 vs. the colitis group; [c]P<0.05 vs. the EGCG-50 group. Data are presented as the mean ± standard deviation. EGCG, epigallocatechin-3-gallate; EGCG-50, EGCG at 50 mg/kg/day; EGCG-100, EGCG at 100 mg/kg/day; DAI, disease activity index; SI, spleen index.

Results

Assessment of DAI and SI. No animals died during the experiment. During the course of the experiment the activity, body weight, stool consistency and presence of blood in the stools of the mice were observed and recorded daily. The score of the

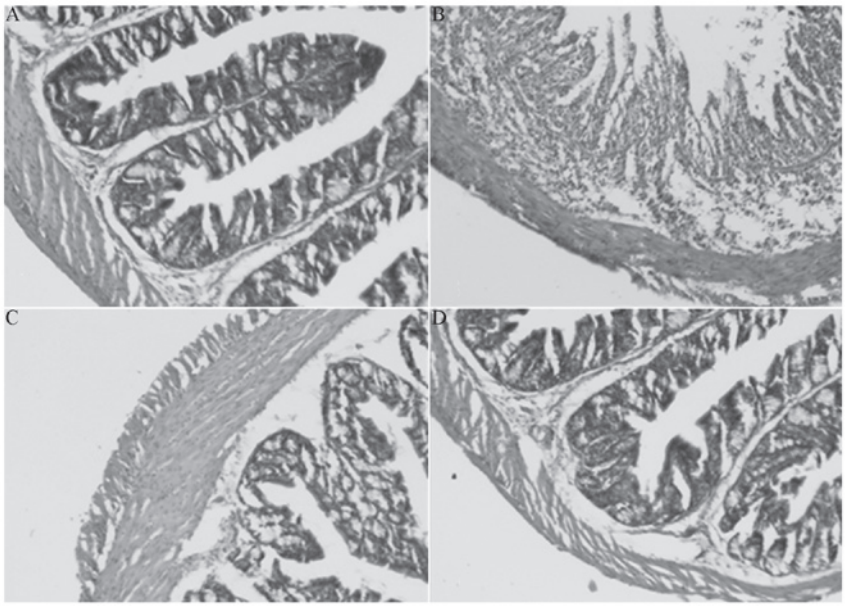

Figure 2. Effect of EGCG on the morphology of colon tissue from mice in the four groups (magnification, x200): (A) Normal group; (B) model group; (C) EGCG-50 group; (D) EGCG-100 group. EGCG, epigallocatechin-3-gallate; EGCG-50, EGCG at 50 mg/kg/day; EGCG-100, EGCG at 100 mg/kg/day.

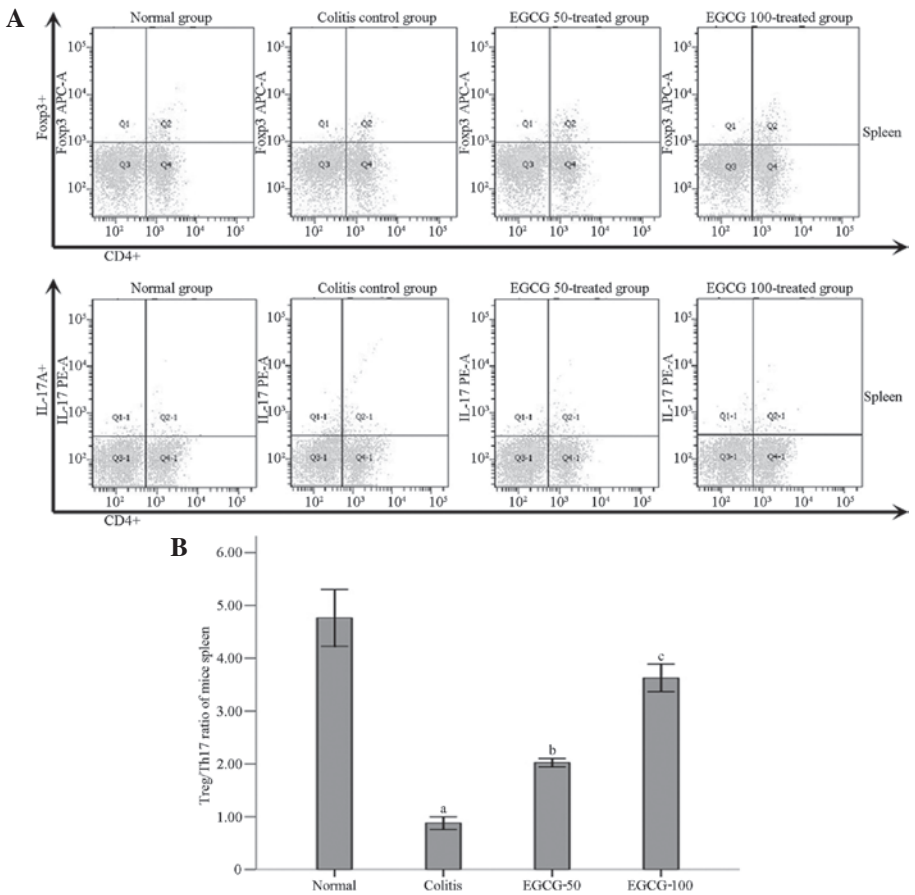

Figure 3. Effect of EGCG on Tregs, Th17s and the Treg/Th17 ratio. (A) Effect of EGCG on Tregs and Th17s. (B) Effect of EGCG on the Treg/Th17 ratio. [a]P<0.05 vs. the normal group; [b]P<0.05 vs. the colitis group; [c]P<0.05 vs. the EGCG-50 group. Data are presented as the mean ± standard deviation. EGCG, epigallocatechin-3-gallate; EGCG-50, EGCG at 50 mg/kg/day; EGCG-100, EGCG at 100 mg/kg/day; Treg, regulatory T cell; Th17, T helper 17 cell; Foxp3, forkhead box protein P3; CD4, cluster of differentiation 4; IL-17, interleukin 17.

NC group was 0. Blood, weight loss and significant decreases in activity were visible in the MD, ELD and EHD groups following the establishment of the model using 5% DSS for seven days. As shown in Fig. 1A, the presence of blood in the stools, the rate of weight loss and the DAI scores were significantly reduced in the ELD and EHD groups between

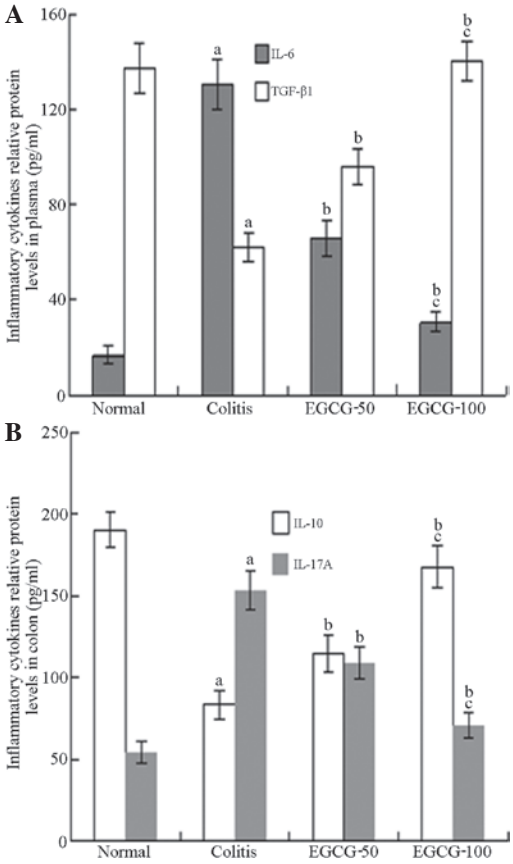

Figure 4. Effect of EGCG on (A) IL-6 and TGF-β1; (B) IL-10 and IL-17A. [a]P<0.05 vs. the normal group; [b]P<0.05 vs. the colitis group; [c]P<0.05 vs. the EGCG-50 group. Data are presented as the mean ± standard deviation. EGCG, epigallocatechin-3-gallate; EGCG-50, EGCG at 50 mg/kg/day; EGCG-100, EGCG at 100 mg/kg/day; IL, interleukin; TGF-β1, transforming growth factor-β1.

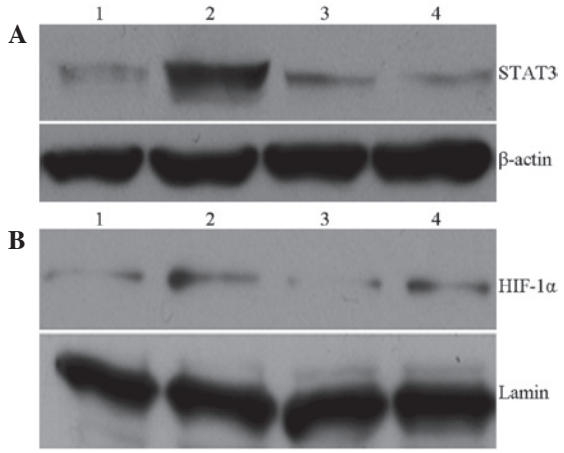

Figure 5. Effect of EGCG on cell signaling pathway proteins: (A) STAT3 and (B) HIF-1α. (A) Lane 1, normal group; lane 2, colitis (model) group; lane 3, EGCG-50 group; lane 4, EGCG-100 group. (B) Lane 1, normal group; lane 2, colitis (model) group; lane 3, EGCG-100 group; lane 4, EGCG-50 group. EGCG, epigallocatechin-3-gallate; EGCG-50, EGCG at 50 mg/kg/day; EGCG-100, EGCG at 100 mg/kg/day; STAT3, signal transducer and activator of transcription 3; HIF-1α, hypoxia-inducible factor-1α.

days 10 and 14 compared with those of the MD group. Compared with the score in the ELD group, the DAI was decreased more significantly in the EHD group. The spleen in the MD group was enlarged compared with that in the NC group, while the spleen size and SI were significantly reduced in the ELD and EHD groups as compared with the MD group (Fig. 1B and C). The SI in the EHD group was decreased to a greater extent than that in the EDL group (Fig. 1C).

Pathological assessment. Compared with the NC group (Fig. 2A), the MD group (Fig. 2B) exhibited visible pathological manifestations, including acute inflammation accompanied by mucosal erosion, as well as edema, crypt reduction and the infiltration of inflammatory cells, such as neutrophils, in the muscularis propria and mucosa. Compared with the MD group, the colonic mucosal inflammatory cell infiltration, erosion and edema in the ELD (Fig. 2C) and EHD (Fig. 2D) groups were significantly improved.

Treg/Th17 ratio. Compared with the NC group, the $CD4^+IL\text{-}17^+$ (Th17)/$CD4^+$ lymphocyte ratio was significantly elevated in the MD group, while the $CD4^+Foxp3^+$ (Treg)/$CD4^+$ lymphocyte ratio and the Treg/Th17 ratio were decreased significantly (P<0.001) (Fig. 3). Compared with the MD group, the levels of Th17s in the ELD and EHD groups were decreased and the Treg/Th17 ratio was significantly increased (P<0.001). The difference in the Treg/Th17 ratio between the EHD and NC groups was not statistically significant (P=0.674), and a statistically significant difference was also found in the ratio between the ELD and NC groups (P=0.006). Compared with the ELD group, the Treg/Th17 ratio in the EHD group showed a significant increase (P<0.001).

Cytokines. Compared with the NC group, the IL-6 and IL-17 levels of the MD group were significantly increased, while the IL-10 and TGF-β1 levels were significantly decreased. Compared with the MD group, the IL-6 and IL-17 levels in the ELD and EHD groups were decreased while the IL-10 and TGF-β1 levels were significantly increased. Compared with the ELD group, the IL-6 and IL-17 levels in the EHD group were decreased to a greater extent, while the IL-10 and TGF-β1 levels were increased to a greater extent (Fig. 4).

Western blot analysis. As presented in Fig. 5, the nuclear HIF-1α and cytoplasmic STAT3 protein expression in the MD group was significantly increased compared with that in the NC group. Compared with the MD group, the expression of the proteins in the ELD and EHD treatment groups was decreased significantly, with a greater decrease observed in the EHD group.

Discussion

UC is a chronic inflammatory disease of the intestine. The exact pathogenesis of the disease is not fully understood, but it is believed to involve the abnormal activation of the innate and adaptive immune systems. The immune inflammatory response exhibits excessive hyperactivity, which negatively affects the self-limiting nature of the reaction (15,16). Immune cells and cell factors involved in intestinal tissue are imbalanced and lead to immune injury (15,16). It has previously been suggested that UC is mediated by Th2 cells (17-21),

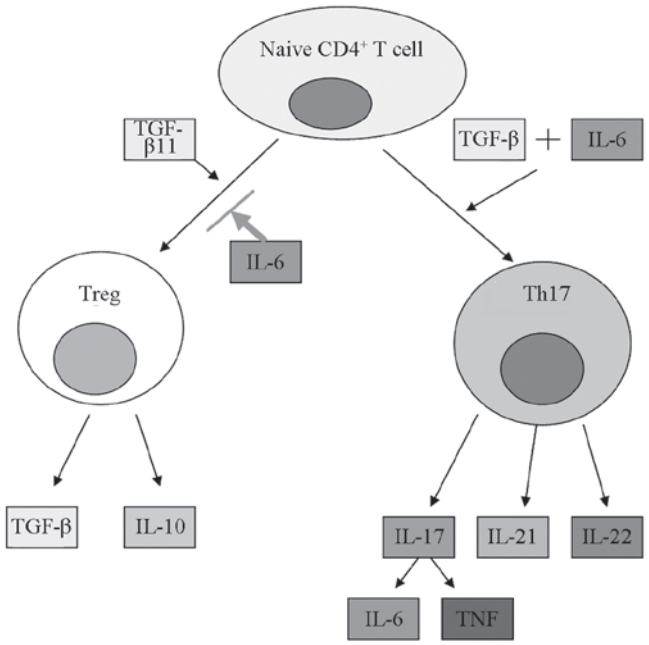

Figure 6. Differentiation of naive CD4+ T cells into Tregs following induction by TGF-β1 alone. TGF-β1 and IL-6 promote jointly the differentiation of the naive CD4+ T cells into Th17s, while IL-6 inhibits the TGF-β-induced Treg generation. Th17s secreted IL-17 to further promote the release of cytokines such as IL-6 and TNF and other inflammatory cell activators, leading to a waterfall-like overlay effect. Treg, regulatory T cell; Th17, T helper 17 cell; CD4, cluster of differentiation 4; IL, interleukin; TGF-β1, transforming growth factor-β1; TNF, tumor necrosis factor.

while other studies have also indicated that the Thl/Th2 axis is fundamental to the pathogenesis of UC (22-25); however, the identification of Th17s confirmed the immune bypass in UC and explained the anomalies of the traditional Th1/Th2 imbalance (26-28). The rebalancing of the Treg/Th17 ratio is considered to be an important treatment strategy for UC (29,30). It is known that Treg/Th17 imbalance is common in a variety of autoimmune disorders, and that human inflammatory bowel disease (IBD) is associated with Tregs, Th17s and the secreted cytokines. The Treg/Th17 imbalance may therefore be a possible target for the treatment of IBD (2,31,32). IBD includes UC and Crohn's disease. The findings of the present study further confirmed the existence of a Treg/Th17 ratio imbalance in the mouse model of experimental colitis, a model reflecting human UC, and were consistent with the results of previous reports (33-35). Previous studies have found that EGCG can treat UC, but the exact mechanism has yet to be fully elucidated. We hypothesized that EGCG could treat UC by mediating the rebalancing of the Treg/Th17 ratio, and attempted to explore the specific mechanisms underlying the EGCG-induced adjustments in the Treg/Th17 ratio.

Th17s are involved in the immune pathological process of UC, possibly by releasing IL-17, IL-21, IL-22 and other inflammatory cytokines (36). Tregs are a CD4+ T-cell subset with immunosuppressive activity. These cells inhibit the intestinal mucosal inflammation cascade and inflammatory reaction amplification effect by regulating the secretion of IL-10, TGF-β1 and other anti-inflammatory cytokines and maintain intestinal immune balance (37,38). Tregs act antagonistically with Thl7s to maintain the relative stability of the body's immune status, so that the body is held in a delicate and complex balance. Tregs undergo a similar differentiation process to Thl7s. It has been demonstrated that naive CD4+ T cells can differentiate into Tregs following stimulation from TGF-β1 alone, but, in the presence of IL-6, TGF-β1 and IL-6 act together to induce the expression of the transcription factor retinoic acid-related orphan receptor γt (RORγt), thus suppressing the production of Tregs and promoting the naive CD4+ T cells to differentiate into Th17s (39-41) (Fig. 6). At low concentrations, TGF-β1 acts synergistically with IL-6 to induce RORγt generation; at high concentrations, TGF-β1 can regulate the expression of Foxp3. The balance between Foxp3 and RORγt could determine whether the naive T cells differentiate into Th17s or Tregs following stimulation by antigens (42). In the absence of IL-6, TGF-β1 does not induce the differentiation of the naive T cells into Th17s, but instead promotes the differentiation to Tregs (43). In the absence of TGF-β, Th17s can also be generated following stimulation by IL-6 or the combination of IL-23 and IL-1β (44). IL-6 can additionally inhibit the TGF-β-induced generation of Tregs (45).

It has previously been shown that two pathways, hypoxia-mammalian target of rapamycin-HIF-1α-Th17 and IL-6-STAT3-HIF-1α-Th17, have a crucial role in Treg/Th17 imbalance (26). In the first pathway, UC leads to a sustained hypoxic state in the intestine, and the local tissue hypoxia induces HIF-1α synthesis and promotes its entry into the nucleus to exert its biological functions, resulting in the upregulation of Th17 activity, the promotion of Foxp3 binding with ubiquitin for degradation, and then the downregulation and inhibition of Tregs (31,32,46). In the second pathway, UC causes increases in the levels of inflammatory cytokines, such as IL-6, leading to the upregulation and activation of HIF-1α by the IL-6/STAT3 signaling pathway. STAT3 can also inhibit the ubiquitination and degradation of HIF-1α and prolong its half-life, in addition to increasing the protein expression of HIF-1α; as a consequence, increased HIF-1α is available to activate Th17s (47-49).

IL-6 promotes the differentiation of Th17s (39,50-52), and the activated Thl7s secrete proinflammatory cytokines such as IL-17 (53-55). IL-17 can further promote the release of cytokines, such as IL-6 and TNF, and the activation of other inflammatory cells (56). The waterfall-like inflammatory cell and cytokine cascade may be central to the disease progression of patients with UC (13). Reducing the concentration of IL-6 could reduce the induced expression of RORγt and inhibit the IL-6-STAT3-HIF-1α-Th17 pathway, thereby reducing the generation of Th17 cells (39,57). The waterfall-like effects could thus be interrupted and the inhibition of Treg generation could be reduced, thereby regulating the Treg/Th17 balance. EGCG has been found to reduce the generation of IL-6 in patients with IBD (58,59). Since IL-6 plays such an important role in the mechanism of Treg/Th17 imbalance, we speculated that EGCG could regulate the Treg/Th17 balance by reducing IL-6 levels in a mouse model of experimental colitis.

In the present study it was found that, compared with the MD group, the IL-6 levels in the ELD and EHD groups were significantly reduced, with a more significant decrease in the EHD group. This demonstrated that EGCG could significantly reduce the release of IL-6 in mice with experimental

colitis. It could be concluded that EGCG reduced the induced expression of RORγt by downregulating the cytokine IL-6 and inhibiting STAT3 and HIF-1α protein expression, thus reducing the generation of Th17s and simultaneously reducing the inhibition of IL-6 on Tregs, and ultimately leading to the rebalancing of the Treg/Th17 ratio and treating mice with experimental colitis. This mechanism would explain the findings in the present study. The experiments also confirmed that the effect of EGCG treatment was dose-related, as the high-dose therapy produced superior results to the low-dose therapy. In conclusion, EGCG represents a natural medicine that exhibits potential for clinical application in the treatment of UC.

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