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Original Article

Effect of ginkgo biloba extract on splenic immune function of chronic ulcerative colitis in mice

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Abstract: Objective: Ulcerative colitis (UC) is a chronic nonspecific inflammatory bowel disease. *Ginkgo biloba* extract, EGB761, has therapeutic action on colitis in animals. In this study, we discussed effect of EGB761 on splenic immune function of chronic UC in mice. Methods: Dextran sulfate sodium-induced chronic UC in mice was used to test the effect of EGB761 on regulation of immunologic splenic function. Disease activity index (DAI), body weight (BW) changes, colon length, and pathological changes in colon were monitored. Changes in spleen shape, length, weight and pathological condition were observed. Single karyocyte from spleen was separated and enumerated then tested for positive rate using flow cytometer. Real-time quantitative PCR and immunohistochemistry were applied to test mRNA and protein expression of inflammatory factor IL-7, IL-6, TNF-α, and IFN-γ. Results: EGB761 could improve the general condition of mice with chronic UC; increase their body weight, colon length and colon weight; and slow down the pathology changes in chronic colitis. The amount of karyocytes and positive rate of CD4, CD8, and CD45R on karyocytes were reduced after applying EGB761. Real-time quantitative PCR further demonstrated that EGB761 could decrease the mRNA and protein expression of inflammatory factor IL-7, IL-6, TNF-α, and IFN-γ. Conclusion: EGB761 can ease inflammatory reaction on mice with chronic UC through depressing the activity of splenic immune cells and through down regulating the expression of splenic inflammatory factors.

Keywords: *Ginkgo biloba* extract, ulcerative colitis, spleen, inflammatory factor, dextran sulfate sodium

Introduction

Ulcerative colitis (UC) is a chronic nonspecific inflammatory bowel disease (IBD) with increasing morbidity in recent years [1-3]. Studies of UC have been focused on its mechanism. The onset of IBD is considered to be the result of interaction among genetic effects, environment factors, infection and self-immunity. The disorder of immunoregulation is the major cause of IBD. As the largest peripheral immune organ, spleen is an important locality for immunoresponses. There are various of immunocytes in spleen, including T lymphocytes, B lymphocytes, dendritic cells, macrophages, natural killer cells, and monocytes, that involve in variety of immunoresponses [4]. Spleen is the locality for T lymphocytes residing and responding immunoreactions, and has an important role in innate and adaptive immunity. Study showed that the amount of T lymphocytes in spleen had the positive correlation with immune responses of human body [5]. T lymphocytes can be grouped into subpopulations according to surface antigen expression, CD4+ subgroup and CD8+ subgroup, which is the key regulation in immune reaction [6]. The imbalance ratio of the two subgroups will result in immunofunction disorder or immune disease [1]. Th17 cells which is characterized of inducing the secretion of IL-7 by Th17 cells, is one subgroup of CD4+ helper T cells [7]. IL-17 is a proinflammatory cytokine with powerful ability to raise and activate neutrophils. IL-17 can also induce and activate T cells, macrophages and epithelial cells to secrete various proinflammatory

Table 1. DAI score criteria

Score	Weight loss (%)	Stool consistency	Stool occult blood
0	<1	Low amount, hard, dry, non-sticky, non-dispersive	Colorless
1	1--5	Low amount, hard, wet, sticky	Spotted color
2	6--10	High amount, soft, very sticky	Continuous blue
3	11--15	High amount, soft, dispersive	Dark stool, blue occult blood
4	>15	Loose stool	Bloody stool, dark blue

*DAI = (score of Weight Loss + score of Stool Consistency + Stool Occult Blood)/3.

Table 2. Macroscopical colon scoring criteria

Macroscopical appearance	Score
Normal colon appearance	0
Colonic wall mildly thicken, no congestion	1
Colonic wall moderate thicken, congestion	2
Colonic wall obviously thicken and harden, congestion	3
Colonic wall obviously thicken and harden, congestion, adhesion	4

mediators, such as IL-1, IL-6, TNF-α, et al, thus causing inflammation [7]. The function of spleen is closely related to UC. Both medication and surgery can improve the function of spleen in UC [8]. But this improvement is still depended on extent of UC.

Ginkgo biloba extract, EGB761, is extracted and purified from *Ginkgo biloba* leaves, containing 24% Ginko flavone glycosides and 6% terpene lactones. EGB761 has various biological and pharmacological functions, such as cleaning free radicals, anti-inflammation, regulating immune system, and so on [9]. Study showed that EGB761 can regulate the development of immune organs, cellular immunity and humoral immunity. EGB761 can effectively activate monocyte-macrophage to start non-specific immune response and promote antigen passed to T lymphocytes and B lymphocytes [10]. T lymphocytes play an important role in cellular immunity. EGB761 can increase T lymphocytes counts and its concentration in blood serum and strengthen T lymphocytes functions. The ratio of mature T cells (CD3+) and helper T cells (CD4+) can also be regulated by EGB761 [10]. Cytokines (CK) are proteins or peptides secreted by immune cells that pass information between cells, and regulate immune responses and effector function [11]. CK is one of important factors to assess one's immuno-competence. EGB761 can also improve one's immunocompetence by regulating the level of CK (for example, interleukins, tumor necrosis factors, etc) [11]. In this study, EGB761 was found to alleviate inflammation of chronic UC in experimental mice. The mechanism is to suppress the activity of splenic immune cells thus down-regulate the secretion of splenic inflammatory cytokines.

Materials and methods

Ethics statement

The mice and the protocol involved in the study had been approved by Institutional Animal Care and Use Committee (IACUC) of Hebei General Hospital. Approval ID: I07-038-3. All the mice were housed under standard conditions per protocols of IACUC and Hebei medical university vivarium in a barrier facility (GB14925-2001).

Animals and reagents

Male C57BL/6 mice (weight: 18-22 g; 7-12 weeks) were purchased from Vital River Laboratory Animal Technology Co. Ltd.. Dextran sulfate sodium (DSS) was bought from Sigma. *Ginkgo biloba* extract standard, Tebonin, was acquired from Dr. Willmar Schwabe Gmblt & Co. KG. CD3, CD4, CD8, CD45R and anti-mouse CD45R antibodies were purchased from Beckmen Coulter. Anti-mouse CD3e antibody, anti-mouse TNF-α antibody, anti-rabbit IL-17 antibody, anti-mouse IFN-γ antibody, anti-goat IL-6 antibody, and anti-goat GAPDH polyclonal antibody were bought from Santa Cruz Biotechnology, inc..

Modeling method

Three model groups were included in this study: control group; chronic UC model group (Model group), and EGB761 intervention group

Table 3. Histopathological changes scoring criteria

Histological appearance	Score
Inflammation (I)	
Non	0
Mild	1
Moderate	2
Severe	3
Depth (E)	
Non	0
Mucosa	1
Mucosa and submucosa	2
Through mucosa	3
Hyperplasia (R)	
Complete	0
Almost complete	1
With crypt	2
Incomplete epidermis	3
No tissue repair	4
Crypt damage (C)	
Non	0
1/3 base crypt damaged	1
2/3 base crypt damaged	2
Only complete surface epithelium	3
Crypt and epithelium completely damaged	4
Scope of lesions (P, %) S	
1--25	1
26--50	2
50--75	3
75--100	4

*Histological score = I+E+R+C+P.

Table 4. Immunohistochemistry analysis scoring criteria

	A-score for percentage of positive cells	B-score for color intensity of positive cells
0	Negative	Negative
1	1%--25% positive	Slightly positive
2	26%--50% positive	Positive
3	51%--75% positive	Strongly positive
4	76%-100% positive	

*Luminous integral = A×B.

(EGB761 group). Each group randomly got 10 male C57BL/6 mice and was observed for 28 days. Control group was fed with deionized water all the time. For Model group and EGB761 group, 2% DSS (10 g DSS dissolved in 500 mL DI water) was fed to replace DI water at Day 1 to Day 5, Day 8 to Day 12, Day 15 to Day 19, and Day 22 to Day 26. From Day 14, EGB761 group was intervened with EGB761 gavage at dosage of 100 mg/kg, one time per day, which lasted for 14 days. As control, model group and control group were intervened with phosphate buffer solution (PBS) gavage at the same volume. On Day 29, mice were sacrificed by dislocate cervical vertebrae for tests.

Disease activity index (DAI) measurement

Mental state, weight, activity, hair glossiness, appetite, and stool consistency of mice were monitored each day. According to DAI scoring criteria (**Table 1**), scores were recorded based on the weight, stool consistency and occult blood of mice [12].

Colon length and weight change measurement

Colon was dissected and its length and weight were measured and recorded. Scores were given based on the criteria given in **Table 2**.

Hematoxylin-eosin staining (H-E staining)

Paraffin-embedded colon tissue was sectioned to 5 μm-thick slices. Paraffin was washed off using dimethylbenzene. Gradient ethanol was used to dehydrate tissue. Rinse dehydrated tissue with DI water for 1 min. Stain the tissue with hematoxylin for 7 min then rinse it with water for 2 min, with 1% HCl-ethanol for 2 s, with water for another 2 min, with 1% ammonia for 30 s, with water for 4 min. Stain the tissue with 1% Eosin in ethanol for 30 s. Dehydrate the tissue with gradient ethanol, make the tissue transparent using dimethylbenzene. Seal the tissue with neutral balsam and observe it under microscope. Histopathological changes of colon tissue were scored based on H-E staining observation under microscope [13] (**Table 3**).

Spleen length and weight measurement

Anesthetize mouse by intraperitoneal injecting 10% chloral hydrate. Collect peripheral blood at 1500 rpm for 15 min and collect serum. Test calcium concentration in blood serum. Open abdomen at center and harvest spleen. Observe the macroscopical appearance of spleen and measure its length and weight. Calculate spleen index (SI) using weight of spleen ×100/

Table 5. Genes for PCR and primers sequences

Detected gene	PCR primer sequence	Propagated DNA fragment
TNF-α	Sense: 5' GGT TCT GTC CCT TTC ACT CAC T 3'	169 bp
	Antisense: 5' GAG AAG AGG CTG AGA CAT AGG C 3'	
IFN-γ	Sense: 5' ATG AAC GCT ACA CACC TGC ATC TT 3'	139 bp
	Antisense: 5' TTT CTT CCA CAT CTA TGC CAC TT 3'	
IL-6	Sense: 5' CAT GTT CTC TGG GAA ATC GTG 3'	141 bp
	Antisense: 5' TCC AGT TTG GTA GCA TCC ATC 3'	
IL-17	Sense: 5' TAT CCC TCT GTG ATC TGG GAA G 3'	161 bp
	Antisense: 5' ATC TTC TCG ACC CTG AAA GTG A 3'	
GAPDH	Sense: 5'-GAGACCTTCAACACCCCAGC-3'	263 bp
	Antisense: 5'-ATGTCACGCACGATTTCCC-3'	

The results of Real-time Q-PCR were concluded from threshold comparison. The amplification of target gene = $2^{-\Delta\Delta C(t)}$, C(t) is the cycle number of thermal cycler to reach fluorescence threshold. The amplification of target gene compared to control group was calculated as: $\Delta\Delta C(t) = \Delta C(t)_{experiment} - \Delta C(t)_{control}$, Where $\Delta C(t)_{experiment} = C(t)_{target\ gene} - C(t)_{GAPDH}$, and $\Delta C(t)_{control} = C(t)_{target\ gene} - C(t)_{GAPDH}$.

weight of mouse [12]. H-E stain spleen tissue and observe the slide under microscope.

Splenic monocytes separation and enumeration

Anesthetize mouse by intraperitoneal injection of 10% chloral hydrate and fix it on plate, exposing abdominal area for surgery. Sterilize surgical area with 70% ethanol then cut skin from bottom transversely. Blunt dissect peritoneum and open abdominal cavity along *linea alba* transversely. Cut ligaments around spleen and take out spleen, put it on ice. Place spleen in a petri dish and immerse it in 10 mL cold complete RPMI medium (1 bag of RPMI medium powder dissolved in 1 L sterile water, adding 2.0 g sodium bicarbonate, glutamine, sodium pyruvate, HEPES, 100 U/mL penicillin, and 100 U/mL streptomycin), then crush it with two glass slides rinsed ahead with the same medium. Filter tissue with 70 μm mesh and transfer filtrate into a 50 mL centrifuge tube. Repeat filtration several times until the 50 mL centrifuge tube is full. Centrifuge cell suspension 3000 rpm for 10 min at 20°C. Discard supernatant and add 2 mL red blood cell lysis buffer to sediment. Stand for 2 min then add 10 mL complete RPMI medium. Centrifuge above mentioned cell suspension 3000 rpm for 10 min and discard supernatant. Resuspend sediment with complete RPMI medium until the cell concentration is 10^6 cells/mL.

Flow cytometry analysis of splenic immunocytes positive rate

Separate splenic monocytes using aforementioned method and rinse twice with physiological saline. Resuspend monocytes with saline until the concentration reach 2×10^5/50 μL. The first tube of cell suspension was labelled with 5 μL of FITC-anti IgG and 12.5 μL of PE-Cy5-anti IgG2b. The second tube of cell suspension was labelled with anti-mouse-CD4, PE-Cy5-anti-mouse-CD3 and PE-anti-mouse-CD8, 2.5 μL for each. The third tube of cell suspension was labelled with FITC-anti-CD45R and IgG-Cy5-anti-CD3, 2.5 μL for each. The fourth tube of cell suspension was labelled as control without any incubation with fluorescence-conjugated antibodies. Each tube was incubated for 30 min in dark and rinse with saline once. Resuspend cells with 100 μL saline for flow cytometry analysis.

Immunohistochemistry (ICH) analysis

Spleen tissue was embedded in paraffin and sectioned into slices. Slices were dehydrated, dewaxed, followed by repairing antigens, then sealed. Primary antibodies, IL-7, IL-6, TNF-α, and IFN-γ were incubated at 37°C for 1.5 h, respectively, then rinsed with PBS five times. Secondary antibodies were incubated at 37°C for 30 min then rinsed with PBS for three times. The expression of IL-7, IL-6, TNF-α, and IFN-γ were observed under microscope. The percentage of positive staining area was analyzed using multifunctional true color cell image analysis system. IHC score was obtained according to following criteria (**Table 4**).

Real time quantitative PCR (Real-time Q-PCR) analysis of mRNA expression of IL-7, IL-6, TNF-α, and IFN-γ

All the procedures were conducted aseptically at low temperature. Grind spleen tissue in a mortar with liquid nitrogen and collect powder

into a 1.5 ml centrifuge tube. Add 1 mL of Trizol isolation reagent (Roche, USA), vortex rapidly then place it on ice. Add 0.2 mL of chloroform and homogenized mix for 15 s. Stand on ice for 5 min then centrifuge the tube at 1200 g for 15 min. Transfer supernatant to another 1.5 mL centrifuge tube and add 0.5 mL of pre-cooled isopropanol, then store the tube at -20°C for 1 h. Centrifuge the tube at 1200 g for 15 min and discard supernatant. Wash RNA sediment immediately with 75% ethanol and centrifuge it at 7500 g for 5 min. Discard supernatant and air dry RNA sediment for 15-20 min. Dissolve dried RNA in 20 μL diethypyrocarbonate (DEPC) processed MiliQ water and take 1 μL of RNA solution for integrity, purity, and concentration test. Keep the rest at -70°C. PCR primers of IL-7, IL-6, TNF-α, and IFN-γ were designed using Primer 5 (**Table 5**) (ABI, USA) and synthesized by Sango Biotech.

Statistical method

Quantitative data was expressed as "Average ± Standard deviation" ($\bar{x}$ ± s) and analyzed by SPSS 13.0 software. Comparison between multi groups was achieved using one-way ANOVA and inter-group comparison was analyzed using SNK, $P<0.05$ was considered to be statistically significant.

Results

Effects of EGB761 on chronic UC mice

General condition: After fed with 2% DSS for 1 day, mice in model group all gained weight and increased water and food intake. After 3 days of feeding with 2% DSS, mice in both model group and EGB761 group began to lose weight and water and food intake decreased, along with loosened and increased stool. Between Day 13 to Day 15, mice in both model group and EGB761 group showed significant weight loss and lack of spirit. Their hair was messy and stool was loose. With time come by, these symptoms become more obvious. For EGB761 group, intervention with EGB761 gavage began on Day 14. Weight loss, loose stool and low spirit last at the beginning of intervention. But on Day 17, mice in EGB761 group had stable weights and better spirit. From Day 18, mice in EGB761 gained weights, although not much. On Day 29, the weight of mice in EGB761 returned to original status and mental state was good (**Figure 1A**).

DAI score

DAI score of control group was 0.00 ± 0.00. Compared to control group, DAI score (1.31 ± 0.65, $P<0.01$) of Model group increased after feeding them with 2% DSS on Day 5. On Day 13, DAI score of model group reached peak value (4.00 ± 0.43, $P<0.01$). On Day 16, DAI score of EGB761 group was 3.30 ± 0.65. From Day 19, DAI score of EGB761 group (3.00 ± 0.85, $P<0.01$) began to be lower than it of model group. On Day 29, DAI score of EGB761 decreased to 0.30 ± 0.46, $P<0.01$, which was a little higher than it of control group but much lower than it of model group (**Figure 1B**). Thus it can be concluded that intake of EGB761 can lower DAI score.

Colon length and weight

Compared to control group, which had an average colon length of 5.90 cm ± 0.27 cm ($P<0.01$), average colon length of model group was shorter, 4.65 cm ± 0.63 cm ($P<0.01$). Compared to model group, average colon length of EGB761 group was lower, 5.56 cm ± 0.42 cm ($P<0.01$).

Compared to control group, which had an average colon weight of 0.73 g ± 0.12 g ($P<0.01$), average colon weight of model group was heavier, 0.92 g ± 0.15 g ($P<0.01$). Compared to model group, average colon weight of EGB761 was lighter, 0.81 g ± 0.10 g ($P<0.01$) (**Figure 1C-E**).

Pathological changes of colon

For control group, after H-E staining, complete intestinal mucosa epithelium was observed. Single epithelial and enteraden in lamina intestine arranged orderly. For model group, intestinal mucosa was impaired and enteraden was damaged or disappeared. Amount of goblet cells was low. Lymphocytic infiltration to mucosa, submucosa, even muscular layer was observed. After treatment with EGB761, pathological changes were alleviated compared to model group (**Figure 1F**).

Effect of EGB761 on mice colon general condition

Spleens in control group had uniform size, ruddy color and smooth surface. Spleens in

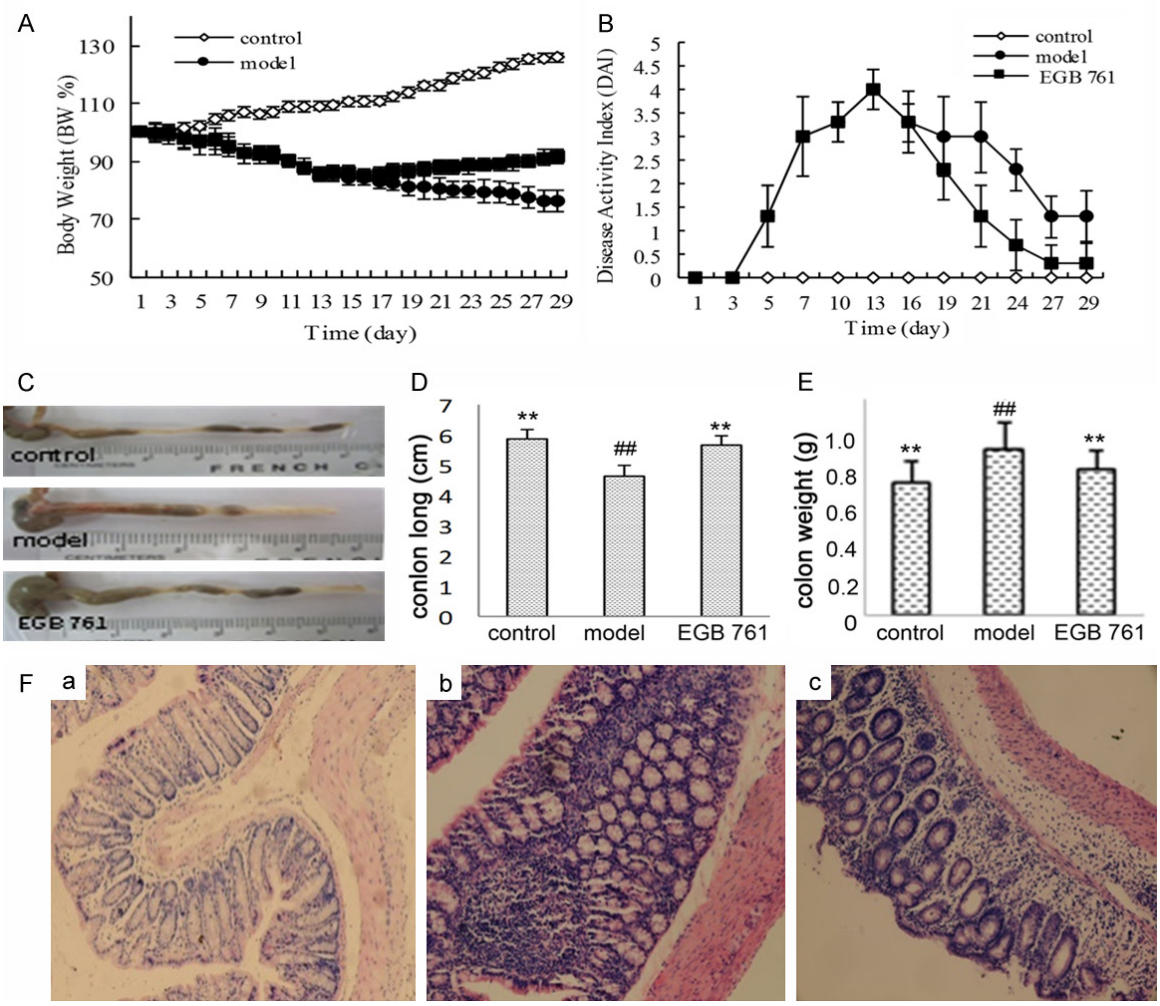

Figure 1. The inflammation in the DSS-induced model of chronic colitis. A. BW, shown as percentage of weight change, was assessed daily and represented from day 0 to day 29. Compared to the control group, the model group displayed a more weight loss of BW. B. DAI, comprised of weight loss, stool consistency and OB, was shown. C. Representative photographs of colon at the end of experiment were shown for control group, model group and EGB761 group. D. The length of the colon was quantitated and shown. E. The weight of the colon was quantitated and shown. F. Representative H&E stained histology from control group (a), model group (b) and EGB761 group (c) (H&E staining; original magnifications, ×200). Data were expressed as mean ± SD. $^{**}P<0.01$ *vs* model group; $^{\#\#}P<0.01$ *vs* control group.

model group swelled up with dark color. Spleens in EGB761 group had smaller size compared to model group (**Figure 2A**).

Average length of spleens in control group was 1.217 cm ± 0.12 cm, ($P<0.01$). Average length of spleens in model group was obviously larger (1.767 cm ± 0.17 cm, $P<0.01$). After EGB761 intervention, average length of spleen shortens to 1.417 cm ± 0.12 cm, ($P<0.01$), compared to model group (**Figure 2B**).

Average weight of spleens in control group was 0.059 g ± 0.017 g, (P<0.01). Average weight of spleens in model group was gained to 0.092 g ± 0.019 g, ($P<0.01$). After EGB761 intervention, average weight was decreased to 0.074 g ± 0.016 g, ($P<0.01$) in EGB761 group (**Figure 2C**).

For H-E staining, spleen white pulp had a clear vision of integrity. Splenic sinus and cords arranged orderly. Compared to control group, spleen in model group had disordered structure with blurry germinal center. Density of periarteriolar lymphoid sheath cells increased. Red pulp was congested with lymphocytes infiltration. Amount of multinucleated giant cells increased. For EGB761 group, splenic white pulp

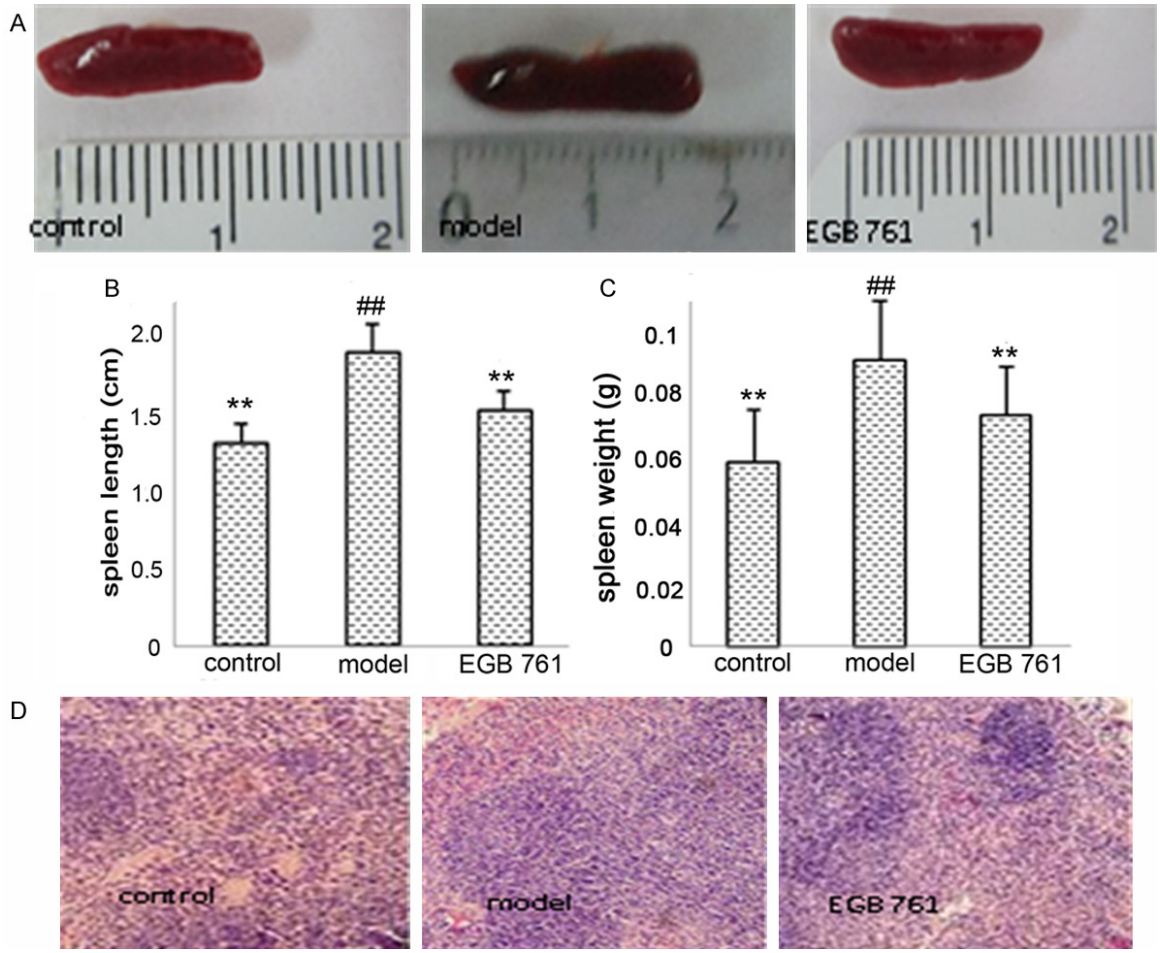

Figure 2. The changes of spleen in chronic colitis. A: Representative photograph of spleen at the end of experiment, the control group, the model group, EGB761 group. B: The changes of the spleen length. C: The changes of the spleen weight. D: Representative H&E stained histology of the spleen from control group, model group and EGB761 group (H&E staining; original magnifications, ×200). Data were expressed as mean ± SD. $^{**}P<0.01$ *vs* model group; $^{\#\#}P<0.01$ *vs* control group.

had orderly structure. Red pulps had malacic hemorrhage. Congestion and lymphocytes infiltration were less severe compared to model group (**Figure 2D**).

Effect of EGB761 on immune cells of UC mice

The concentration of monocytes in control group was 56×10^6/mL ± 15.2×10^6/mL. Model group had increased monocytes concentration as 108×10^6/mL ± 20.3×10^6/mL. After EGB761 intervention, monocytes concentration in EGB761 group was lower (76.8×10^6/mL ± 18.6×10^6/mL) compared to model group (**Figure 3A**).

In control group, the positive rate of CD3, CD4, CD8 and CD45R in splenic monocytes was 30.50% ± 0.90%, 18.22% ± 0.70%, 14.00% ± 0.73%, and 20.02% ± 0.81%, respectively. After intervention with EGB761, the positive rate of CD3, CD4, CD8 and CD45R was 50.01% ± 0.67%, 29.00% ± 0.68%, 15.82% ± 0.59%, and 48.40% ± 0.67%, respectively. All of them were lower than them in model group (**Figure 3B**, **3C**).

Effect of EGB761 on splenic cytokines of UC mice

Immunohistochemical staining images (**Figure 4A**) showed that the secretion of cytokines IL-17, IL-6, TNF-α, and IFN-γ were mainly located in splenic marginal zone and around splenic artery area. Multi neucleated giant cells in red pulp also had positive expression of these cy-

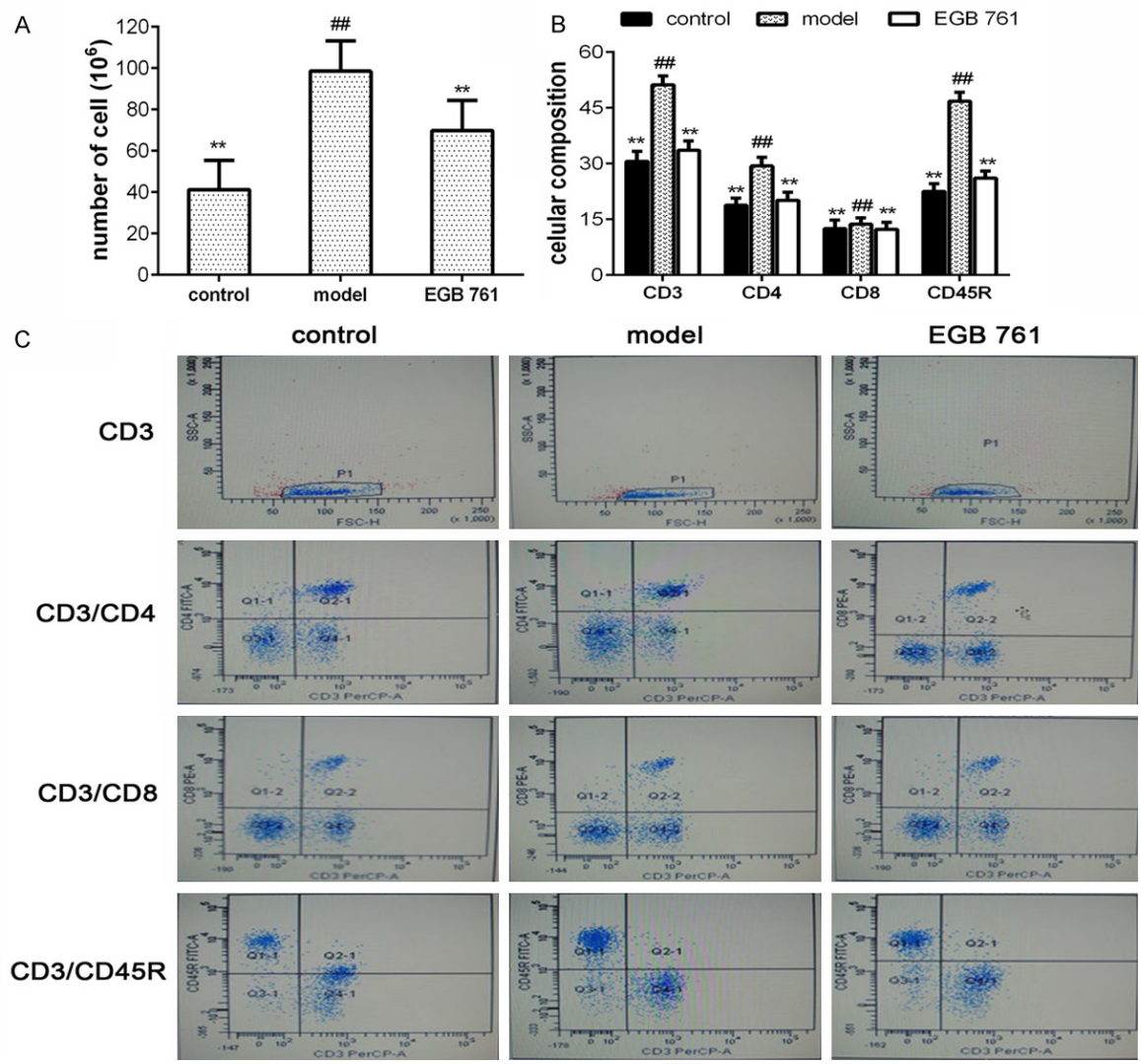

Figure 3. The changes of cellular composition in spleen. A: The total numbers of mononuclear cells from Spleen. B: The changes of CD3, CD4, CD8, CD45R in spleen mononuclear cell. C: Data changes of cellular composition in spleen. Data were expressed as mean ± SD. $^{**}P<0.01$ *vs* model group; $^{\#\#}P<0.01$ *vs* control group.

tokines. The expressions of IL-17, IL-6, TNF-α, and IFN-γ were not much in control group but increased in model group at level of 0.75 ± 0.02 vs 0.25 ± 0.02, 0.45 ± 0.03 vs 0.15 ± 0.02, 0.65 ± 0.03 vs 0.25 ± 0.03, and 0.37 ± 0.02 vs 0.10 ± 0.03, ($P<0.001$), respectively. The expression of IL-7, IL-6, TNF-α, and IFN-γ in EGB761 group were 0.45 ± 0.02; 0.25 ± 0.04, 0.35 ± 0.02, and 0.17 ± 0.02, ($P<0.001$), respectively, which were lower than them in model group (**Figure 4B**).

mRNA of above mentioned 4 types of cytokines were expressed more in Model group than them in control group, at a level of 6.11 ± 0.50 vs 1.25 ± 0.56 for IL-17 mRNA, 7.50 ± 0.65 vs 1.25 ± 0.44 for IL-6 mRNA, 6.65 ± 0.65 vs 1.25 ± 0.34 for TNF-α mRNA, and 3.37 ± 0.60 vs 1.15 ± 0.40 for IFN-γ mRNA ($P<0.001$). The expression of IL-17 mRNA, IL-6 mRNA, TNF-α mRNA, and IFN-γ mRNA were lower in EGB761 group compared to model group, at a level of 2.78 ± 0.49, 3.75 ± 0.56, 4.35 ± 0.55, and 2.17 ± 0.50, respectively (**Figure 4C**).

Effect of EGB761 on colon macroscopic appearance

Colon mucosa was smooth without congestion nor edema in control group, with a score of 0.00 ± 0.00. In model group, colon mucosa showed apparently congestion, edema, thicken

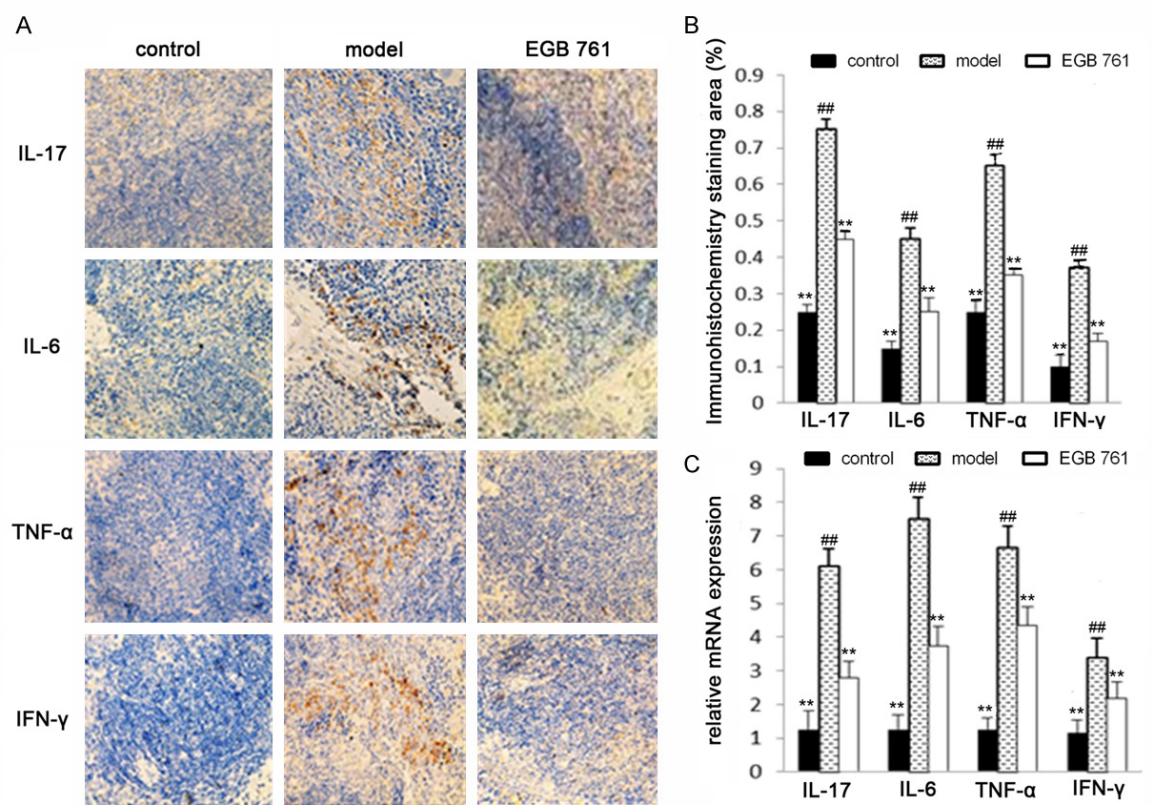

Figure 4. The expressions of IL-17, IL-6, TNF-α and IFN-γ examined by immunohistochemistry staining in the spleen (×400). A: Immunohistochemistry staining of IL-17 and IL-6, TNF-α, IFN-γ in spleen specimen. B: Immunohistochemistry score of IL-17 and IL-6, TNF-α, IFN-γ in the spleen. IL-17 and IL-6, TNF-α, IFN-γ expression increased significantly in the model group than that in the control group, and decreased in the EGB761 group than the model group. C: The relative expressions of IL-17, IL-6, TNF-α, IFN-γ mRNA in the spleen. The relative expressions of IL-17 and IL-6, TNF-α, IFN-γ mRNA were significantly increased in the model group compared with that in the Control group, and decreased in the EGB761 group. $^{**}P<0.05$ *vs* model group, $^{\#\#}P<0.01$ *vs* control group.

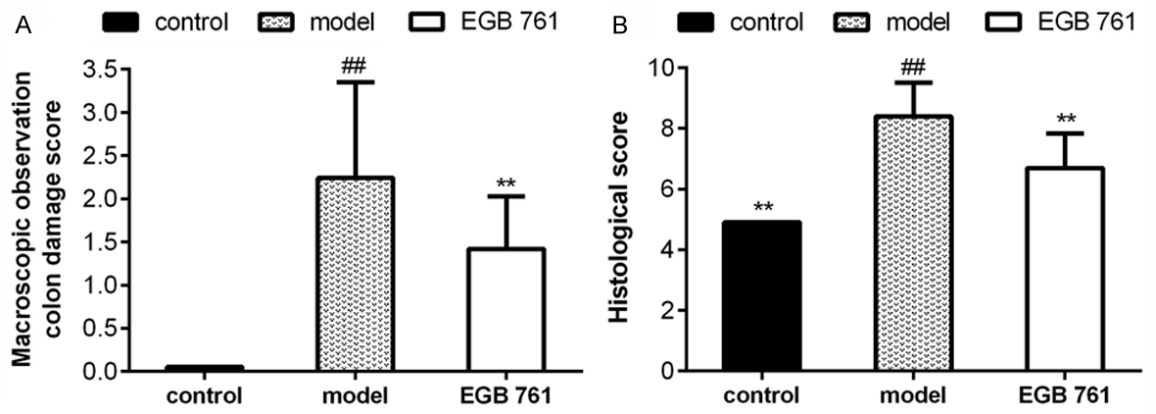

Figure 5. A: Scores of the macroscopic observation of the colonic damage. B: Macroscopic score of colonic damage. Data were expressed as mean ± SD. $^{**}P<0.01$ *vs* model group; $^{\#\#}P<0.01$, *vs* control group.

colonic wall and shorten colon length. Macroscopic score for model group was 2.17 ± 0.98. In EGB761 group, colon mucosa was more smooth, less congestion, without edema and less shorten length compared to model group, with a score of 1.33 ± 0.52 (**Figure 5A**).

Compared to control group, the histological score of Model group increased from 5.00 ± 0.00 to 8.33 ± 0.89. With EGB761 treatment, histological changes were alleviated and number of inflammatory cells decreased. The histological score of EGB761 group (6.89 ± 1.01) was lower than it of model group (**Figure 5B**).

Discussion

Ulcerative colitis (UC) is a chronic non-specific inflammation with an unclear mechanism. Study showed that UC may be caused by interactions among susceptibility genes, environmental factors, infections, and disorder of immune system [14]. In other words, environmental factors act on people with susceptibility gens; then intestinal immune and non-immune system begin to fight with the help from intestinal flora, which results in hyper-functional and non-controllable immunological inflammatory reactions [8]. In recent years, the occurrence of UC in China is increasing. Thus there is a need to study the mechanism of UC and final suitable medication to control UC development.

There is a network of inter-collaboration and inter-constrains among immune organs, cells and molecules. Once the balance broken, the immune response will be abnormal, which includes unbalanced ratio among T lymphocytes subgroup, disordered expression of cytokines, suppressed macrophage functions, damaged microstructure or immune organs, etc [15]. Especially, the uncontrollable release of cytokines can result in local even general lesion. Spleen is the largest immune organ and lymphatic organ in human body, with vast number of lymphocytes, macrophages, and various immunocytes and cytokines. It takes 25% of total lymphatic tissues [16]. The amount and function of T lymphocytes in spleen is positive related to immune responses. Spleen also is the location where T lymphocytes and B lymphocytes reside, proliferate and accept immuno-stimulation from antigens. Spleen consists of white pulp, red pulp and marginal zone. Marginal zone is the first place to contact antigens and respond to immune-stimulation. Red pulp consists of cord and sinus. It is the major location of phagocytosis. White pulp consists of periarterial lymphatic sheath and corpuscles. It is the major location of specific immune responses [17]. Spleen is directly involved in cellular immunity and regulates distribution of T lymphocyte subgroups in peripheral blood. T lymphocytes can be classified into two subgroups based on its surface antigen expression, CD4+ subgroup and CD8+ subgroup. The unbalanced ration between these two groups can lead to immunologic inadequacy and immunological diseases [18].

In 2005, Th17 cells were found to be one of subgroups of CD4+ T helper cells, characterized by secreting IL-17. It soon became a focus in immunology study to explain mechanism of UC. Naïve T cells differentiate to Th17 cells then secrete IL-17 under stimulation of IL-6. IL-17 mediates tissue inflammation and differentiation, chemotaxis and mature of neutrophils by promoting cytokines function. TNF-α plays dual roles in human body. In normal condition, TNF-α is mainly anti-tumor and anti-infection. If the concentration of TNF-α is high or secretion of TNF-α lasts long, TNF-α can also cooperate with IL-17 to amplify inflammatory caused by the latter. IL-6 is a core member in cytokines. It can promote proliferation and differentiation of T lymphocytes and B lymphocytes, and induce differentiation of killer T cells. Disordered level of IL-6 will result in immune and inflammatory diseases. Study showed that IL-6 and IL-17 had effects on UC development, which could serve as indexes of treatment and prognosis [19]. Treatment with IL-17 antibody alleviated intestinal inflammation of model animals and UC patients. Autoimmune diseases, such as experimental autoimmune encephalomyelitis, collagen-induced arthritis, etc, and allergic diseases, such as contact dermatitis, respiratory allergic disease, etc, can be significantly suppressed by knocking out IL-17 gene in mice [20-22]. Therefore, Th17 cells may play an important role in occurrence and development of autoimmune and allergic diseases.

Ginkgo biloba is gymnosperm. As one of China-specific ancient species, it is the only survival of *Ginkgosida*. It has existed widely on the northern hemisphere since Mesozoic time, one to two hundred million years ago, which is also the reason why it being called “living fossil”. Ginkgo biloba has served as medication in China for more than 500 years. It was used to treat asthma and bronchitis in Song Dynasty. EGB761 was first used in clinical treatment in 1975, France. Studies have been conducted to explore active ingredients in *Ginkgo biloba* worldwidely. *Ginkgo biloba* Extraction Injection

is the fourth generation preparation with active ingredients of ginkgolides and flavonoids. It can promote blood circulation and stimulate immune cells. Studies showed that EGB761 strengthen immunocompetence by regulating the ratio between CD4+ and CD8+ T lymphocytes thus promote recovery of damaged immunofunctions [23]. EGB761 cleared oxygen free reagents and reduced expression of TNF-α, and IFN-γ to alleviate colon inflammation of experimental mice [24]. Pretreatment with EGB761 can enforce proliferation activity of lymphocytes and improve immunosuppression in acute ischemic stroke. The degree of cerebral infraction can be lowered by fine controlling the ratio of CD4+ and CD8+ T lymphocytes in certain range. EGB761 down-regulates expression of IL-6 and TNF-α in a normal range, which is good for human body to maintain anti-infection ability in immunologic homeostasis. Therefore, on one hand EGB761 can treat and suppress excessive inflammation; on the other hand, EGB761 can maintain and promote immunological defense. EGB761 can also increase the weight of thymus and spleen, and improve the thymus- and spleen- index of immunological suppressed mice, thus to protect immune organs. In summary, EGB761 can improve nonspecific immunity, specific immunity (including cellular immunity and humoral immunity), and phagocytic index of immunological suppressed mice, indicating its improvement in phagocytosis of monocytes. EGB761 can also regulate the development of immune organs and differentiation of monocytes and natural killer cells.

In this study, 2% DSS was used to stimulate immune system response in mice to induce UC. Results showed that after 14 days of intake 2% DSS, experimental mice showed a series of inflammatory symptoms, such as low spirit, decreased water and food intake, lusterless and caducous hair, loose and bloody stool, etc. Meanwhile, the weight of mice decreased to the lowest value of 80% original weight. Further DAI evaluation showed that model group has apparently higher score than control group, indicating successful model building. Different degrees of diarrhea, bloody stool, weight loss and shortened colon appeared on model group mice. Histopathological changes included shortened and twisted intestinal glands and submucosa. With experiment time increasing, the weight loss in model group got more and more obvious. The lowest weight was only 20% of original value. Meanwhile mice in model group started to defecate bloody stool with a continuous blue of occult blood test paper. DAI score of model group was significantly higher than it of control group. After intervention with EGB761, DAI score began to rise. On Day 29, the weight of EGB761 group mice was 95% of original value. Dark and bloody stool changed to plenty of wet and soft stool. Continuous blue of occult blood test paper changed to spotted blue. DAI score significantly decreased to 0.30 ± 0.46 ($P<0.01$). EGB761 treatment also alleviated intestinal wall congestion, edema, shortened length and thickened wall. Mucosa improvements were consistent with literatures [25]. Therefore, EGB761 intervention can alleviate UC symptoms, including weight loss, diarrhea, bloody stool, shortened colon, congestion, edema, thus lower DAI value. EGB761 functions on intestinal inflammation of UC mice may be suppressing lymphocytes infiltration. Secretion of cytokine in model group increased thus proliferation of immune cells was enhanced, resulting in some immune organs other than intestine cooperating in system immune response. In this study, changes of cytokine secretion in spleen and peripheral immune system were investigated. Results showed that spleens in model group swelled up with dark color. Length, weight and index of spleen all increased. These data were statistically significant compared to control group. H-E results showed that in model group, area of white pulp increased with disordered arrangement. Structure of germinal center was not clear. Density of periarteriolar lymphoid sheath cells increased. Red pulp had congestion along with lymphocytes infiltration and increased amount of multinucleated giant cells, indicating that there were activation, proliferation and migration of immune cells in the process of building UC model. Spleen participated in immune response to fight against colon inflammation. Monocytes in spleen were separated. The amount of monocytes in Model group was larger than it in Control group. Expression of CD3, CD4, CD8, which indicated changes in T cells, and CD45R, which indicated changes in B cells, were analyzed using flow cytometer. Results showed that in Model group the total amount of monocytes and expression of CD3, CD4, CD8 and CD45R all increased

compared to Control group. After intervention with EGB761, all the indexes mentioned above decreased significantly, which indicated that EGB761 could regulate cellular and humoral immunity. This might be one mechanism for spleen participating in UC development.

Recent study showed that Th17 type cellular immunity was a major cause of UC [19]. The function of IL-17 is regulated by IL-6, IL-1β, TNF-α and chemokine. Th17 can stimulate epitheliums, endothelium, monocytes and fibrocytes to function in immunoregulation. IL-17 has powerful ability to recruit and activate neutrophils, induced activate T cells, and stimulate secretion of various proinflammatory factors by fibrocytes, macrophages and epitheliums. The expression of IL-17 mRNA, IL-6 mRNA, TNF-α mRNA, and IFN-γ mRNA were higher in model group than it in control group, which indicated changes in Th17 subgroup.

Conclusions

In conclusion, our study demonstrate that EGB761 regulates immune responses by reducing amount of immunocytes, restraining activity of immunocytes and suppressing secretion of cytokines, and further alleviate mucosa changes caused by inflammatory factors.

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Disclosure of conflict of interest

None.

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