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Effects of Bletilla Striata Polysaccharide on Rat Ulcerative Colitis Model by Rebalancing Th1/Th2 and T17/Treg Subsets

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Objective: Ulcerative colitis is an inflammatory disease that affects the lining of the colon and rectum. This study was to investigate the potential utility of *Bletilla striata* polysaccharide (BSP) for ulcerative colitis (UC) treatment and explore its immunoregulatory mechanisms.

Methods: Male Wistar rats were assigned to four groups at a ratio of 1:1:1:1. The four groups of rats were treated with no intervention, 2,4,6-trinitrobenzenesulfonic acid (TNBS), high-performance liquid chromatography (HPLC)-purified BSP (60 mg/kg), sulfasalazine (100 mg/kg). Symptom severity was assessed. Flow cytometry and quantitative real-time polymerase Chain Reaction (qRT-PCR) were performed to assess T cell responses, including the proportions of cluster of differentiation 4^+ ($CD4^+$) T cells and particular cytokines. Autophagic markers microtubule-associated protein 1A/1B-light chain 3 (LC3) II and sequestosome 1 (p62), and granzyme (Grz)-related proteins, Granzyme M (GrzM), macrophage inflammatory protein 1 (MIP-1α) and macrophage migration inhibitory factor (MIF) were estimated by Western blot.

Results: Significant elevations of T helper 1 (Th1) and Th17 and declines of Th2 and regulatory T cells (Treg) were observed in the UC group versus the controls ($p < 0.001$). These alterations could be reversed significantly in UC rats treated with BSP, in contrast to UC group ($p < 0.001$). T cell percentage alterations were validated by mRNA expression of cytokines associated to distinct T cell subgroups. BSP therapy resulted in increased LC3 II and decreased p62, as well as an increase in GrzM and MIP-1 and a decrease in MIF. Except for LC3 II, significant differences were observed in these protein levels from those in the UC group ($p < 0.001$).

Conclusion: BSP demonstrates great potential for UC management via rebalancing of Th1/Th2 and T17/Treg subsets. The study provides a certain theoretical basis for the treatment of UC with traditional Chinese medicine, but the results might be biased due to the small sample size, and future studies are required to further explore.

Keywords: *Bletilla striata* polysaccharide; T helper 1/T helper 2 responses; inflammatory bowel disease; ulcerative colitis; T helper 17/regulatory T

Introduction

Inflammatory bowel disease (IBD) is a several chronic and idiopathic inflammatory disorder of the gastrointestinal tract, including Crohn's disease and ulcerative colitis (UC). UC is a group of intestinal inflammatory diseases with lesions mainly involving the colon, with intermittent bloody stools and abdominal pain as the main clinical manifestations, some of which may have extraintestinal manifestations such as anemia, but rarely accompanied by intestinal perforation [1]. The primary complaints of IBD patients are colonic symptoms such as pain, cramping, diarrhea, bleeding, and weight loss because of colonic tissue damage caused by gastrointestinal inflammation, and results of histology examination of IBD patients show increased infiltration of immune cells, crypt abscesses and ulcers [2]. Despite an elusive pathogenesis of IBD, environmental and genetic factors are major contributors to the onset of IBD. At present, specific UC intervention is unavailable, and the suggested drugs are used to reduce inflammation and maintain remission. Glucocorticoids, aminosalicylic acid and immunosuppressive drugs are commonly used in the clinical management of UC. However, their efficacy is inconsistent among patients and may result in certain side effects.

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Recurrent attacks of UC aggravate the physical and mental burden of patients, so there is an urgent need to discover new treatment methods. Moreover, 10%–30% UC cases fail to obtain effective UC control, for whom surgical resection of affected colon is required [3]. Given the potential risks of complications, the need for optimal available medical therapies and especially new disease-modifying therapies is emphasized by the Crohn's and Colitis Foundation of America [4].

Ulcerative colitis belongs to the category of "dysentery" in traditional Chinese medicine. It is recorded in "Nanjing" that small bowel discharge is accompanied by pus and blood when urinating, and large bowel discharge is tenesmus after tenesmus, which is in line with the clinical manifestations of ulcerative colitis in modern medicine. In "Three Causes, Extremes and One Disease Syndrome", Chen Wuze attributed the internal cause of dysentery to "the stagnation of visceral qi and its occurrence", suggesting that the main pathogenesis of this disease is the invasion of external pathogens, internal injury to diet, affecting the spleen and stomach function; and the evil qi gathers in the large intestine, leading to stagnation of qi and blood, and imbalance of qi and blood. Physicians of later generations supplemented and perfected the etiology, pathogenesis and treatment principles and methods on this basis. Although dysentery was complicated due to complex syndromes and varied syndromes, its pathogenesis was always inseparable from the factors of qi and blood disharmony. Bletilla striata is excavated in summer and autumn, the roots are removed, washed, dried, and used raw. Bletilla striata is bitter, sweet, astringent, slightly cold, and belongs to the lung, stomach, and liver meridians, and has the effects of astringent, hemostasis, swelling and muscle regeneration. "Compendium of Materia Medica" records: "Bletilla striata, astringent qi, seeps phlegm, the essential medicine for eliminating carbuncle, and has the wonderful effect of detoxification and regeneration." It is said to be able to "enter the lungs to stop bleeding, build muscle and stop sores", and its pharmacological activities mainly include hemostasis, wound healing, anti-oxidation, anti-cancer, anti-virus, antibacterial and so on. Clinically, Bletilla striata can be used to treat traumatic hemorrhage, skin chapped, sore swelling, hemoptysis, hematemesis, tumor embolism, anorectal diseases, gynecological fibroids, and postoperative recovery of prostate.

Bletilla striata polysaccharide (BSP), which was composed of α-mannose, β-mannose as well as β-glucose, is extracted from *Bletilla striata*, one traditional Chinese medicine that has long been used for ulcers, bleeding, burns and bruises [5]. *Qingre Zaoshi Lianxue Fang*, a Chinese medicinal herb formula, is clinically effective and is commonly prescribed for UC therapy in China [6]. *Bletilla striata* is one of the five herbs contained in that herbal medication. The therapeutic mechanism of *Bletilla striata* has been investigated in a rat UC model, suggested the involvement of the delta-opioid receptor/β-arrestin 1/B-cell lymphoma 2 (Bcl-2) signaling pathway, which is a major participant of the cell death pathway [7]. Furthermore, lamina propria T cells isolated from UC patients showed that Bcl-2 family protein expression with a high Bcl-x (L)/Bax proportion appeared to encourage resistance to apoptosis rather than the typical vulnerability of activated T cells to it, thereby contributing to mucosal inflammation in UC [8]. The evidence indicates the aberrant regulation of cell death mechanisms and highlights the role of immunoregulation dysfunction in UC pathogenesis, in addition to the potential therapeutic effects of *Bletilla striata*.

T helper 1 (Th1), Th17, and Th2 immune responses have been intensively researched in UC. Studies have concluded that the imbalance of these different types of activated cluster of differentiation 4^+ ($CD4^+$) T cells subsets may contribute to UC development [9,10]. Furthermore, with reference to a strong relationship between the Th17/regulatory T cells (Treg) balance and autophagy in other immune dysfunction diseases found in prior studies [11], it is predicted herein that such alterations in $CD4^+$ T cell subsets and autophagy may involve in UC development and that assessment of the major components of Th17/Treg and autophagy facilitates the evaluation of the BSP mechanism. Besides, granzyme (Grz), particularly Granzyme M (GrzM), are linked to autophagy and UC [12]. Macrophage migration inhibitory factor (MIF) as well as macrophage inflammatory protein 1 (MIP-1α) have been reported to be the downstream molecule of GrzM in bacterial infection [13] and the possible upstream molecule of GrzM in keratinocytes [14], respectively. Accordingly, in this study, the changes of $CD4^+$ T cell proportions, the concentrations of specific cytokines, the expression of key molecules of autophagy, and GrzM related proteins before and after the treatment of BSP were evaluated.

In the present study, a well-established 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced UC rat model was used for the investigation of the potential efficacy of BSP on UC and to explore the possible underlying immunoregulatory mechanisms.

Methods

Animals

Male Wistar rats (160 g–180 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing Vital River Laboratory Animal Technology Co., Ltd., Beijing, China), housed in one animal facility with free access to water as well as standard rat chow based on a 12-hour light/dark cycle. The animals were given 7 days for adaption of laboratory conditions prior to experiments. All experiments were ratified by the Institutional Animal Care and Use Committee of Dongzhimen Hospital, Beijing University of Chinese Medicine. Rats were assigned to four groups at a ratio of 1:1:1:1 (8 rats in

each group) as follows: Rats in the control group received no interventions and served as healthy controls. The UC group was the 2,4,6-trinitrobenzenesulfonic acid (TNBS) (Thermoscientific, Waltham, MA, USA)-induced UC rat model. The remaining rats were in assigned to two treatment groups: Rats with successful induction of UC for 7 days received high-performance liquid chromatography (HPLC)-purified BSP (Nantong Feiyu Biological Technology Co., Ltd., Jiangsu, China) with 60 mg/kg in 2 mL once daily (BSP group) or 100 mg/kg of sulfasalazine (Shanghai Xinyi Tianping Pharmaceutical Co., Ltd., Shanghai, China) (Sulfasalazine group) through intragastrical administration for 14 days. The rats in control and UC groups received intragastric administration of normal saline for 14 days. The general condition of rats was monitored, including food and water intake and body weight. In the event of abnormalities, the disease activity index (see below) of the rat(s) was re-checked. Euthanasia was performed as appropriate when the index exceeds 3.

The trial was conducted according to Animal experiment management practices. The protocol was approved by the institutional review boards or independent ethics committees at each site. An independent data monitoring committee monitored safety and efficacy data. The experimental scheme has been approved by the Ethics committee of Beijing University of Chinese Medicine, and the ethics number was MI-PO20200201.

Construction of UC Rat Model

UC was induced through intra-colonic administration of TNBS according to the method published by Morris *et al*. [15].

Disease Activity Index (DAI) Assessment

DAI was evaluated after 0, 1, 5, 9, and 14 of treatment and calculated as the sum of the scores of weight loss in contrast to stool consistency, initial weight as well as bleeding, then divided through 3. Scores are defined: weight loss: 0 (no loss), 1 (1–5%), 2 (5–10%), 3 (10–15%), and 4 (>15%); bleeding: 0 (no blood), 2 (hemoccult), and 4 (gross bleeding); stool consistency: 0 (normal), 2 (loose stool), and 4 (diarrhea) [16].

Macroscopic Observation

Rats were sacrificed on day 14 of the treatment via cervical dislocation after 24-h fasting and anesthesia with 100 mg/kg pentobarbital sodium (Sigma Aldrich, St. Louis, MO, USA) via intraperitoneal injection. The colon was dissected from 4 cm proximal to the anus to the caecum, longitudinally opened and rinsed with iced phosphate buffered saline (PBS) buffer. The macroscopic morphology was observed, and the length of the excised colon was measured and recorded.

Histological Examination

Colon tissues were immobilized via 4% formaldehyde (Beyotime, Shanghai, China), followed by dehydration in alcohol (MacKlin, Shanghai, China), and embedding in paraffin (Solarbio, Beijing, China), and sliced into 3 μm-thick sections. After deparaffinization, immersion and rinsing in phosphate buffered saline (PBS), the sections were dyed through hematoxylin and eosin (HE) (Beyotime, Shanghai, China). Results were observed under an Olympus-CX light microscope (Olympus, Tokyo, Japan) at ×40.

Flow Cytometry

Colonic intraepithelial lymphocytes were isolated for flow cytometric analysis. Colon tissues were excised into small pieces with 1.0 cm and vortexed in Hank's Balanced Salt Solution via 1 mM ethylenediaminetetraacetic acid (EDTA) (Sigma Aldrich, St. Louis, USA) and 1 mM dithiothreitol (DTT) (Sigma Aldrich, St. Louis, USA). The supernatant was filtered using one 150-μm nylon filter (Beyotime, Nanjing, China) and treated with collagenase 0.4 mg/mL (Beyotime, Nanjing, China) and DNase 100 mg/mL (Beyotime, Nanjing, China) at room temperature for 60 min to isolate colonic intraepithelial lymphocytes. Cells were cultured in a roswell park memorial institute (RPMI) 1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37 °C, 5% CO_2 for 4 h. After centrifugation, 1×10^6 cells were resuspended in 1 mL 5% fetal calf serum-contained PBS with and labeled through anti-differentiation 4+ ($CD4^+$)-APC and CD25-Human Phytohemagglutinin-activated T Cells (FITC) (both, BioLegend, Inc., San Diego, CA, USA) for 30 min (4 °C). For intracellular staining, cells were incubated with Cyto-Fast™ Fix/Perm Buffer Set (BioLegend, Inc., CA, USA) and staining through anti- forkhead box protein 3 (FOXP3)-PE (eBioscience, San Diego, CA, USA), antiantihuman interferon-γ-FITC (IFNγ-FITC) (BioLegend, Inc., CA, USA), anti-IL-4-FITC (Bioss Antibodies, Boston, MA, USA) or anti-IL-17-FITC (eBioscience, San Diego, CA, USA) for 40 min (4 °C). The labeled cells were quantified through a Guava easyCyte 6HT-2L flow cytometer (Merck Millipore Co., Billerica, MA, USA).

Quantitative Real-Time Polymerase Chain Reaction

Total RNA from colon tissues was acquired via the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and DNA was collected using the Rnase-Free Dnase Set (Qiagen, Valencia, CA, USA). cDNA was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) under the manufacturer's guidelines. RNA samples were denatured in a metal bath at 65 °C for 5 min, then cooled on ice. We then used Nanodrop 2000 to measure RNA concentration (Applied Biosystems, Foster City, CA, USA). 2 μg RNA, 4 μL 5× RT Master Mix (Applied Biosystems, Foster City, CA,

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Table 1. Primer sequence of quantitative real-time polymerase Chain Reaction (qRT-PCR).

Gene	Forward	Reverse
Tumor necrosis factor alpha (*TNF-α*)	CCAGGTTCTCTTCAAGGGACAA	GGTATGAAATGGCAAATCGGCT
Interferon-γ (*IFN-γ*)	CCAGGCCATCAGCAACAACATAA	CACCGACTCCTTTTCCGCTTC
IL4 interleukin 4 (*IL-4*)	CCACCTTGCTGTCACCCTGTT	TCCGTGGTGTTCCTTGTTGC
IL-17	TACCTCAACCGTTCCACTTCACC	GCACTTCTCAGGCTCCCTCTTC
IL-13	GTGGCCCTCAGGGAGCTTAT	TGTCAGGTCCACGCTCCATA
IL-23	GGACCAGCTTCATACCTCCCTA	TCAGAGTTGCTGCTCCGTGG
IL-10	CACTGCTATGTTGCCTGCTCTT	GTCTGGCTGACTGGGAAGTGG
TGF-β	GGCGGTGCTCGCTTTGTA	TCCCGAATGTCTGACGTATTGA
β-actin	TGCTATGTTGCCCTAGACTTCG	GTTGGCATAGAGGTCTTTACGG

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USA) and nuclease-free water were added to every 20 µL reaction system. The volume of RNA required to be added is 2 µg RNA to RNA concentration ratio. The samples were then placed in a 37 °C water bath for 30 min and then in a 95 °C metal bath for 5 min to inactivate reverse transcriptase. The cDNA was used as the template for quantitative real-time polymerase Chain Reaction (qRT-PCR), done by StepOnePlus Real-Time PCR Systems using SYBR Green master mix (both, Applied Biosystems, Foster City, CA, USA). A 20 µL reaction system was then prepared, consisting of 1 µL forward primer, 1 µL reverse primer, 10 µL SYBR Green Realtime PCR, 1 µL cDNA, and 7 µL deionized water. The gene expression was quantitatively determined by quantitative real-time polymerase Chain Reaction (qRT-PCR). The primer sequences are summarized in Table 1.

The results of qRT-PCR were calculated by $\Delta\Delta Ct$ method. $\Delta Ct = Ct_{Target} - Ct_{reference}$, $\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{calibrator}$. The relative expression level of the gene was $Relative\ Expression = 2^{-(\Delta\Delta Ct)}$.

Western Blot (WB)

The total protein of the colon tissue was acquired using Radioimmunoprecipitation assay (RIPA) buffer (Beyotime Biotechnology, Jiangsu, China) with phenylmethanesulfonyl fluoride (Beyotime Biotechnology) on ice for 30 minutes. Followed by centrifugation, protein concentration was tested via Bradford assay with the Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad, Hercules, CA, USA), under the manufacturers' guidelines. WB was done through 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels, 10 µg protein/lane. The proteins were then moved to polyvinylidene difluoride (PVDF) membrane (Bio-Rad) which was immersed in 4% milk with 0.1% Tween-20 overnight at 4 ºC, and then incubated (4 ºC) with the primary antibodies overnight. The primary antibodies included rabbit polyclonal anti-GrzM (1:1000, Abcam, Cambridge, UK), rabbit polyclonal anti-MIP-1α (1:3000, Abcam), rabbit polyclonal anti-MIF (1:1000, Affnity, Cincinnati, OH, USA), rabbit polyclonal anti-light chain 3 (LC3) (1:1000, Affnity), rabbit polyclonal anti-sequestosome 1 (p62) (1:1000, Affnity) and rabbit polyclonal anti-β-actin (1:1000, Affnity). The membranes were then incubated with Horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibodies (1:10,000, Dianova, Hamburg, Germany) and visualized through the Clarity Western enhanced chemiluminescent (ECL) Substrate Kit and analyzed by Gel Doc systems (both, Bio-Rad).

We used Image J software (National Institutes of Health (NIH), Bethesda, MD, USA) to measure the gray scale of Western Blot strips and the number of markers in IHC, and analyzed and processed the above data using GraphPad Prism 8.0 (GraphPad Software, Inc., San Diego, CA, USA).

Statistical Analyses

Statistical analyses were conducted via SPSS 22.0 (SPSS Inc, Chicago, IL, USA). Measurement data are tested for normality, and data that do not conform to the normal distribution are normality transformed. The measurement data were expressed as mean ± standard deviation, and two independent samples *t*-test were used for comparison between the two groups. For multi-group comparisons (three or more), Analysis of Variance (ANOVA) and post-hoc testing were used. All statistical evaluations were two-sided and $p < 0.05$ was established as significance in a statistical sense.

Results

Fig. 1A shows HE staining of normal colon tissue, Fig. 1B shows the macroscopic morphology of the resected colon with significant edoema and congestion in the UC group. In contrast to rats in the Control group, the UC group showed a lower increase in body weight by time (Fig. 1C). The BSP group showed a significantly higher increase of body weight than the UC group but a lower increase than the Sulfasalazine group. Fig. 1D shows the DAI on days 1, 5, 9 and 14 after starting treatment. The two treatment groups showed notably higher DAIs than the UC group on day 14.

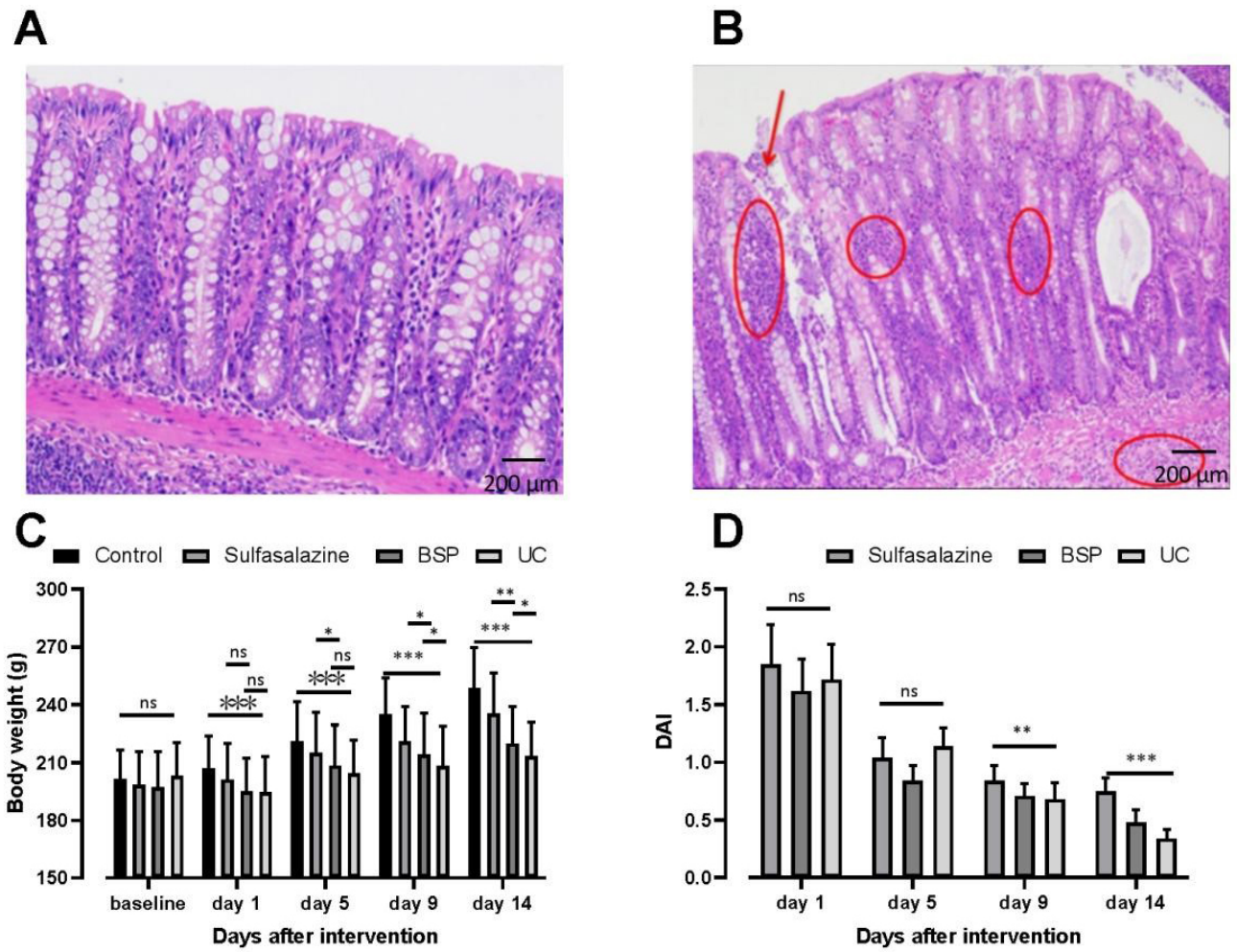

Fig. 1. *Bletilla striata* polysaccharide attenuated the body weight loss, disease activity index and colonic damage in the ulcerative colitis (UC) rat model. (A,B) Hematoxylin and eosin (HE) staining results for confirmation of successfully established UC rat model; the red circles indicate immune cell infiltration, and the red arrow indicates disorganized cell layers. Significant differences between groups were shown in (C) body weight (D) disease activity index. Data are expressed as mean ± SD, N = 3. * indicated $p < 0.05$; ** indicated $p < 0.01$; *** indicated $p < 0.001$; ns, not significant.

The UC group had a shorter colon length than the control group (Fig. 2). After treatment, both BSP and sulfasalazine increased the length of colon. The results of HE staining in UC rats demonstrated that both treatments reduced inflammatory cell infiltration, the irregular organisation of mucosa glands, the loss of mucus propria, and the decrease of goblet cells. The colon tissues of rats from the BSP and Sulfasalazine groups displayed structured layers of intestinal cells that were more closely approaching colon tissue shape than those from the Control group (Fig. 3).

Significant increases in the proportions of Th1 ($CD4^+IFN\text{-}\gamma^+$) and Th17 ($CD4^+IL\text{-}17^+$) (Fig. 4A,B, Fig. 5A) and decreases in the proportions of Th2 ($CD4^+IL\text{-}4^+$) and Treg ($CD4^+CD25^+FOXP^{3+}$) were shown in the UC group in contrast to those in the control group ($p < 0.01$, Fig. 4C,D and Fig. 5B). These changes were reversed significantly in rats treated with BSP or sulfasalazine. Compared to the UC group, Th1 and Th17 cells decreased ($p < 0.01$) and Th2 and Treg cells increased ($p < 0.05$ for BSP group and $p < 0.01$ for Sulfasalazine group). The ratio of Th1 and Treg did not differ between the BSP and Sulfasalazine groups; however, more Th17 and fewer Th2 cells were shown in the BSP group than those in the Sulfasalazine group ($p < 0.05$) (Fig. 4B,D and Fig. 5A,B). Th1/Th2 and Th17/Treg ratios also indicated that both treatments reversed the changes caused by UC. Overall, the $CD4^+$ T cell percentages of Sulfasalazine group were closer to those of the control group than to those of the BSP group.

The mRNA expression of specific cytokines further demonstrated the Flow cytometry (FC) findings. The expressions of Tumor necrosis factor alpha (TNF-α), IFN-γ (for Th1), IL-17 and IL-23 (for Th17) elevated notably in rats of the UC group than those of the control group (Fig. 6A–D). BSP or sulfasalazine treatments lowered the expression of Th1- and Th17-related cytokines significantly when compared to UC groups ($p < 0.001$). In contrast to

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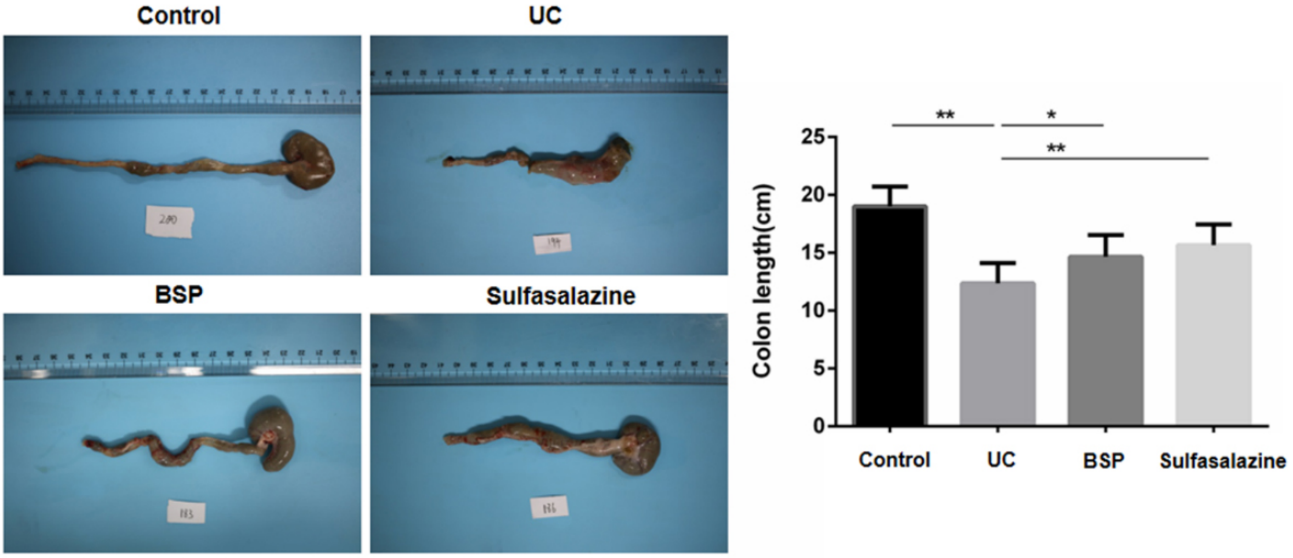

Fig. 2. Macroscopic morphology of the excised colon of Control, UC, BSP and Sulfasalazine groups. BSP, *Bletilla striata* polysaccharide; UC, ulcerative colitis. Data are expressed as mean ± SD, N = 3. *: $p < 0.05$; **: $p < 0.01$.

the control group, IL-13, IL-4 (for Th2), IL-10 and TGF-β (for Treg) decreased when UC was induced, and treatment with BSP or sulfasalazine significantly increased the IL-13, IL-4, IL-10 and TGF-β levels ($p < 0.01$) (Fig. 6E–H).

In colon tissue from rats of the UC group, the protein expression of GrzM, MIP-1α and LC3 II decreased and that of MIF and p62 increased compared to control group levels (Fig. 7a,b,d). Comparisons between the UC and Sulfasalazine groups revealed that all alerations were significantly attenuated with the treatment of sulfasalazine ($p < 0.01$) (Fig. 7). Comparisons between the UC and BSP groups showed that BSP treatment also increased GrzM, MIP-1α and LC3 II expression and decreased MIF and p62 expression (Fig. 7a,b,d); however, the change in expression of LC3 II did not reach statistical significance (Fig. 7d).

Discussion

In the pathogenesis of UC, current research suggests that UC is caused by a disruption of the balance between the composition of the microflora in the intestine and the immune barrier of the intestinal mucosa, resulting in an abnormal immune activation response of the body. The barrier role of the colonic epithelium, the anti-inflammatory regulatory role of commensal bacteria, and several other mechanisms have been found to function in maintaining normal immunity in the intestine. Dysfunction or disruption of the intestinal epithelial barrier function due to various causes allows a large number of pathogenic microorganisms and food antigens to cross the epithelial barrier in the intestine, resulting in a variety of inflammatory cell infiltrations and abnormal immune response of the body, and thus the onset and development of UC.

Traditional Chinese medicine classifies peptic ulcer as “abdominal pain”, “stomach pain”, “acid swallowing”, “spit acid” and other categories. In the treatment, the *Tong* method is often used to promote the spleen and stomach to absorb and transport, therefore lifting and lowering return to normal. At present, the treatment of ulcerative colon is based on drug therapy, the application of TCM in the treatment of UC is effective. In TCM, UC is a manifestation of heat and toxicity in the large intestine due to dampness and heat on the basis of congenital deficiency of endowment and poor spleen and stomach function, or external cold and dampness, and damage to lipid membrane and blood circulation. Therefore, UC treatment requires clearing heat and detoxifying and cooling blood to disperse blood stasis. The pharmacological activities of Bletilla are mainly hemostatic, wound healing, antioxidant, anticancer, antiviral and antibacterial. Clinically, Bletilla can be used to treat traumatic bleeding, skin chapping, sores and sores, hemoptysis, vomiting, tumor embolism, anal and intestinal diseases, gynecological fibroids, and post-prostate surgery recovery.

In the present study, the TNBS-induced UC rat model demonstrated the therapeutic efficacy of BSP through the attenuation of body weight loss, intestinal damage and DAI. The immunoregulatory effects of BSP may involve a rebalancing of the Th1/Th2 and Th17/Treg ratios from a UC status to a healthy state, as evidenced by decreased Th1 and Th17 and elevated Th2 and Treg. Changes in the levels of specific cytokines were synchronized with the percentage changes of $CD4^+$ T cells subsets of the colonic intraepithelial lymphocytes. The Th1 cytokines TNF-α and IFN-γ, and the Th17 cytokines IL-17 as well as IL-23 all increased significantly in UC rats and decreased after BSP treatment. The Th2 cytokines IL-13 and IL-4, the Treg cytokines IL-10 and TGF-β all decreased after induction of UC and in-

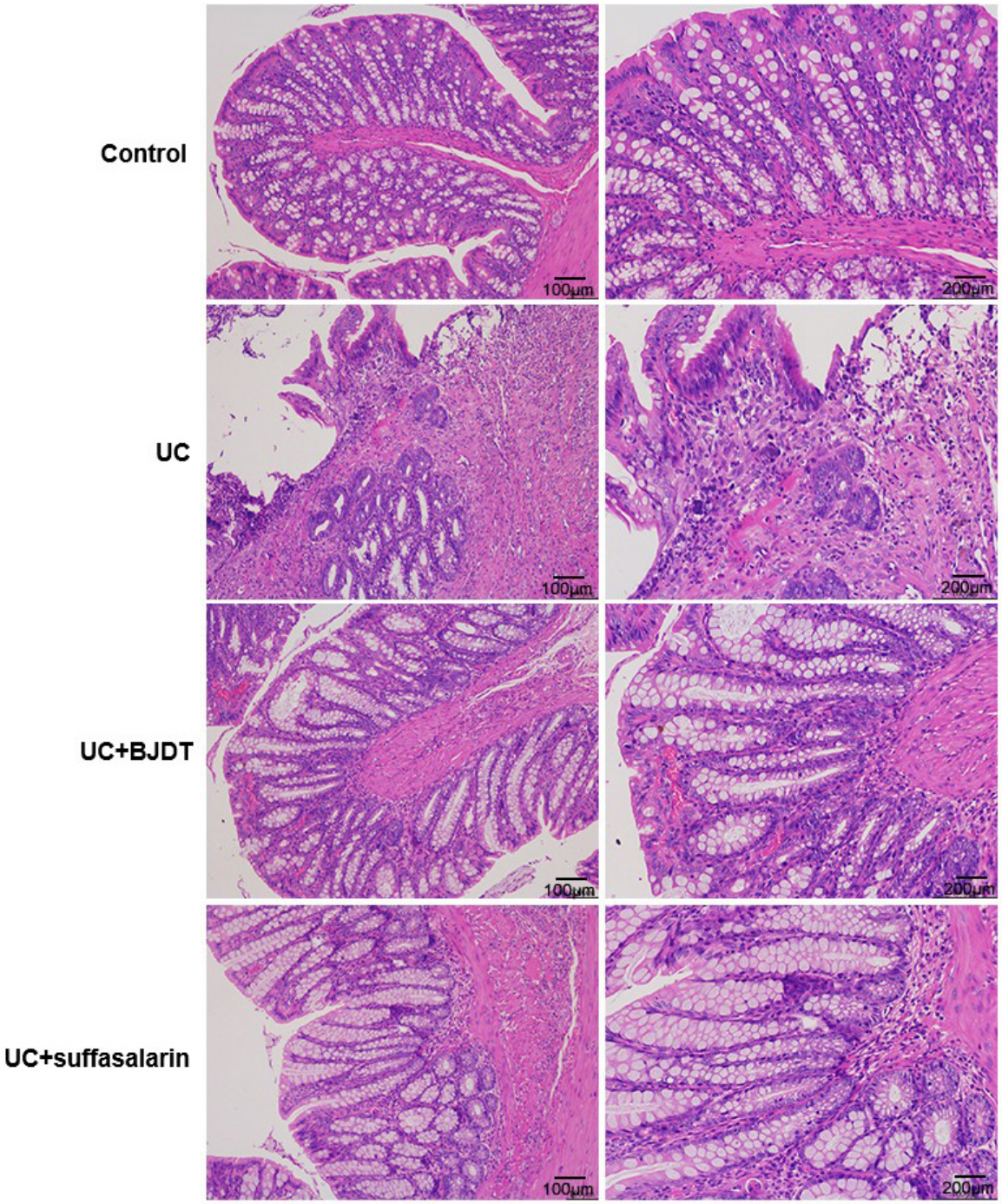

Fig. 3. Colon tissues from rats of Control, UC, BSP and Sulfasalazine groups. BSP, *Bletilla striata* polysaccharide; UC, ulcerative colitis. Data are expressed as mean ± SD, N = 3.

creased when receiving BSP treatment. In the UC group, a decrease in LC3 II expression and an increase in p62 expression suggested the involvement of autophagy, while BSP treatment results in an increase in LC3 II expression and a decrease in p62 expression. UC also changes the expression levels of GrzM and associated proteins MIP-1α and MIF. GrzM and MIP-1 rose while MIF decreased in BSP rats compared to UC rats.

The core process in UC pathogenesis is the accumulation of T cells in the inflammatory intestinal tissue attracted by the secreted inflammatory cytokines [17]. Intensive evaluation of T cell responses in several autoimmune diseases, including UC, revealed the associated of Th1, Th2, Th17 and Treg immune responses with the pathogenesis of UC [18]. Zhu *et al*. [19] found that the ratio of Th1 and Th17 increased in peripheral blood mononuclear cells (PBMCs) separated from UC patients. It was shown that

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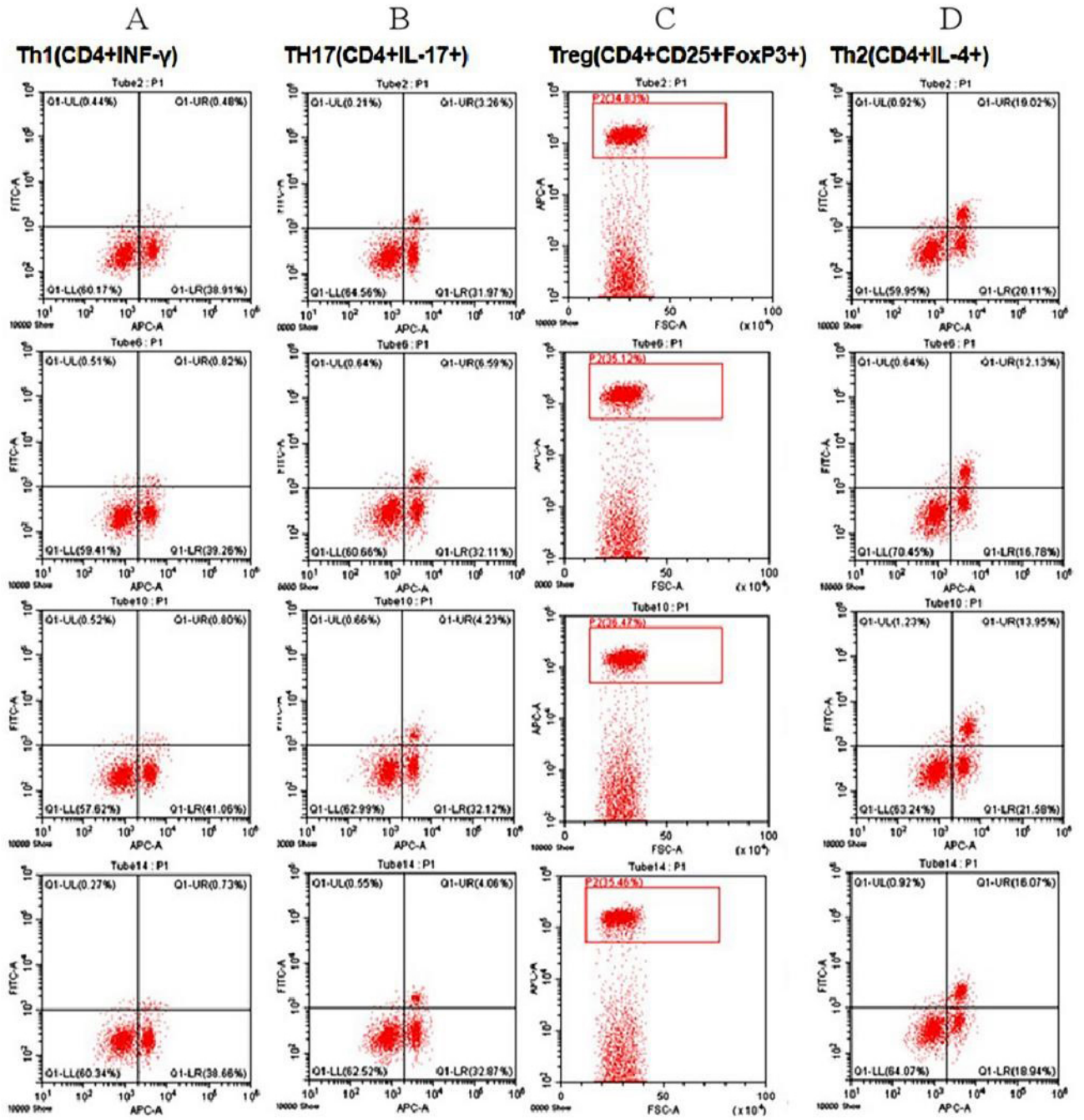

Fig. 4. Represent images of the flow cytometry of imbalances in Th1/Th2 and Th17/Treg ratios ulcerative colitis (UC) rats were reversed after treated by *Bletilla striata* polysaccharide. BSP, *Bletilla striata* polysaccharide; UC, ulcerative colitis. Data are expressed as mean ± SD, N = 3.

the addition of IL-23 to PBMCs from heathy donors significantly increases Th1/Th17 responses but decrease Th2 responses [19]. Results of the present research are similar to those of these previous studies, and, through the evaluation of specific cytokines and percentages of $CD4^+$ T cell subsets, have demonstrated that BSP is a candidate for the effective treatment of UC via rebalancing of Th1/Th2 and Th17/Treg immunity. Th cells can differentiate into four cell subtypes, Th1, Th2, Th17 and Treg, in response to various signaling molecules, thus performing different biological functions in the immune process. Th1 cells mainly clear intracellular pathogens, Th2 cells protect the body against allergic reactions caused by parasitic infections, Th17 cells clear extracellular infections and fungi, and Treg cells regulate other immune cells and promote damaged tissue repair. The effective management of UC by rebalancing Th1/Th2 and Th17/Treg immunity is a promising direction for research (Fig. 5).

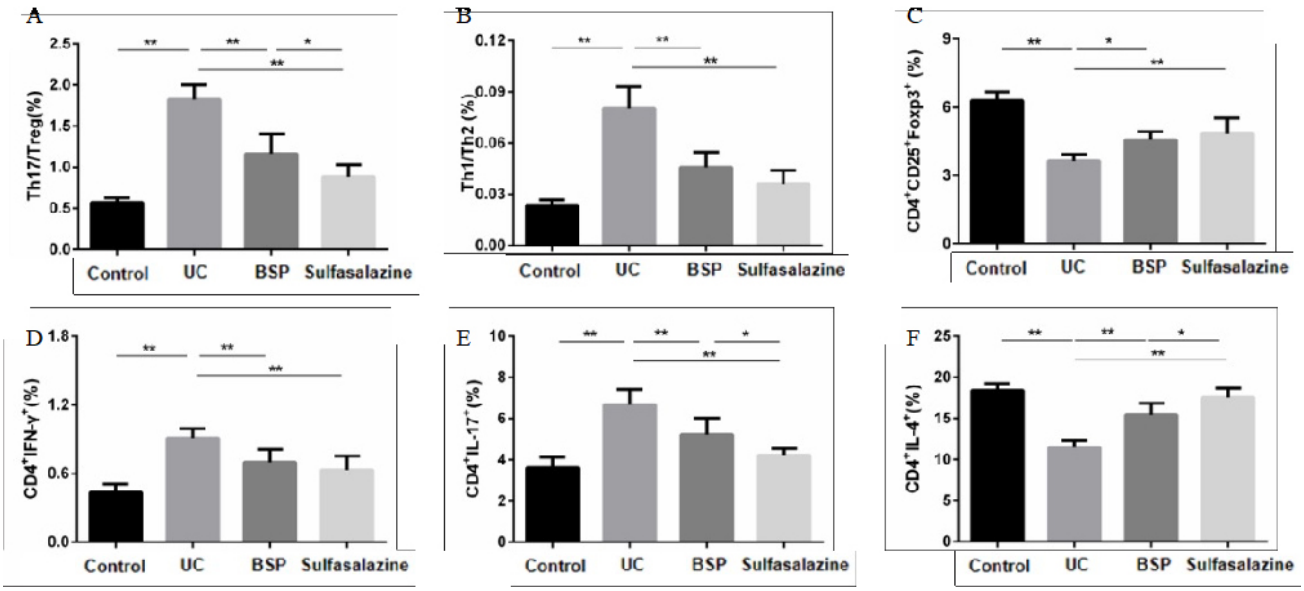

Fig. 5. Quantitative analysis of the results of flow cytometry of imbalances in Th1/Th2 and Th17/Treg ratios ulcerative colitis (UC) rats were reversed after treated by *Bletilla striata* polysaccharide (A–F). Data are expressed as mean ± SD, N = 3. *: $p < 0.05$; **: $p < 0.01$.

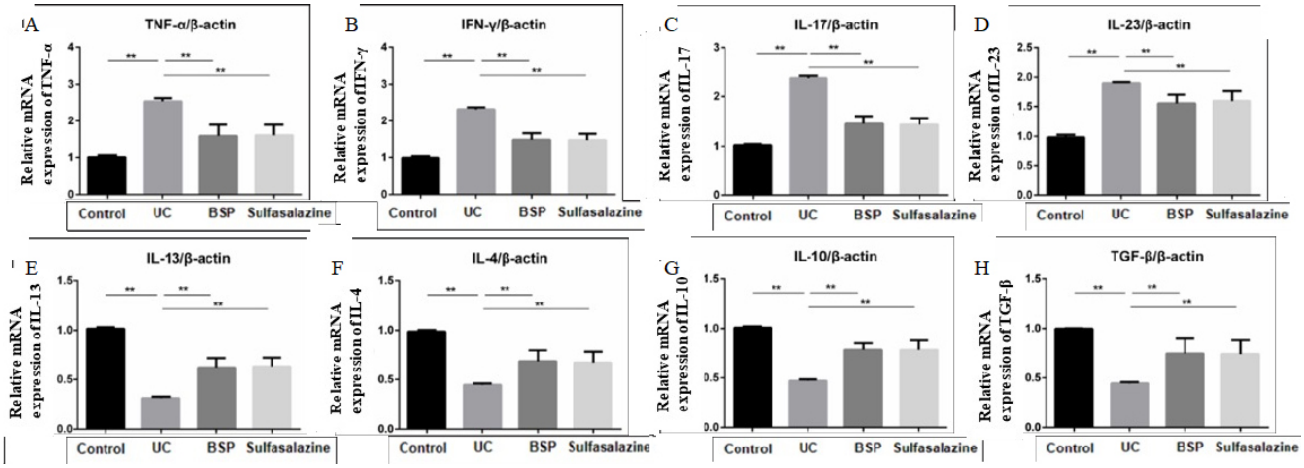

Fig. 6. The cytokines expression of specific cluster of differentiation 4$^+$ (CD4$^+$) T cells determined by qRT-PCR shows the reversal of ulcerative colitis after *Bletilla striata* polysaccharide treatment (A–H). BSP, *Bletilla striata* polysaccharide; UC, ulcerative colitis. Data are expressed as mean ± SD, N = 3. *: $p < 0.05$; **: $p < 0.01$.

Reduction in the susceptibility of cell apoptosis and intestinal mucosa homeostasis have also been observed in UC [20,21]. Autophagy may involve UC pathogenesis by affecting the homeostasis mechanism in T cells, which impact multiple essential cellular processes, including innate and adaptive immunity [22]. Also, activated autophagy was shown to contribute to the imbalance of Th17/Treg immune response in systemic lupus erythematosus (SLE) [23]. Interestingly, the role of autophagy in UC may be different than that in SLE. In patients with SLE, LC3 II increased and p62 decreased [23], which is in contrast to our results where rats in the UC group had down-regulated LC3 II expression and up-regulated p62 expression. Defective autophagy is reported to be strongly bound up with UC pathogenesis, and evidence shows that enhanced autophagy through modulation of inflammation and clearance of intestinal pathogens may be therapeutically beneficial [24].

In the present study, sulfasalazine was used as the active control. Sulfasalazine is a first-line drug to induce and maintain remission in mild to moderate cases of UC. The mechanism of sulfasalazine for UC treatment is still poorly understood. In the present study, BSP and sulfasalazine showed similar effects on the alteration of T cell responses; nonetheless, BSP fails to attenuate body weight loss and DAI to the same levels as sulfasalazine can. This might be explained by the different effects of these two medi-

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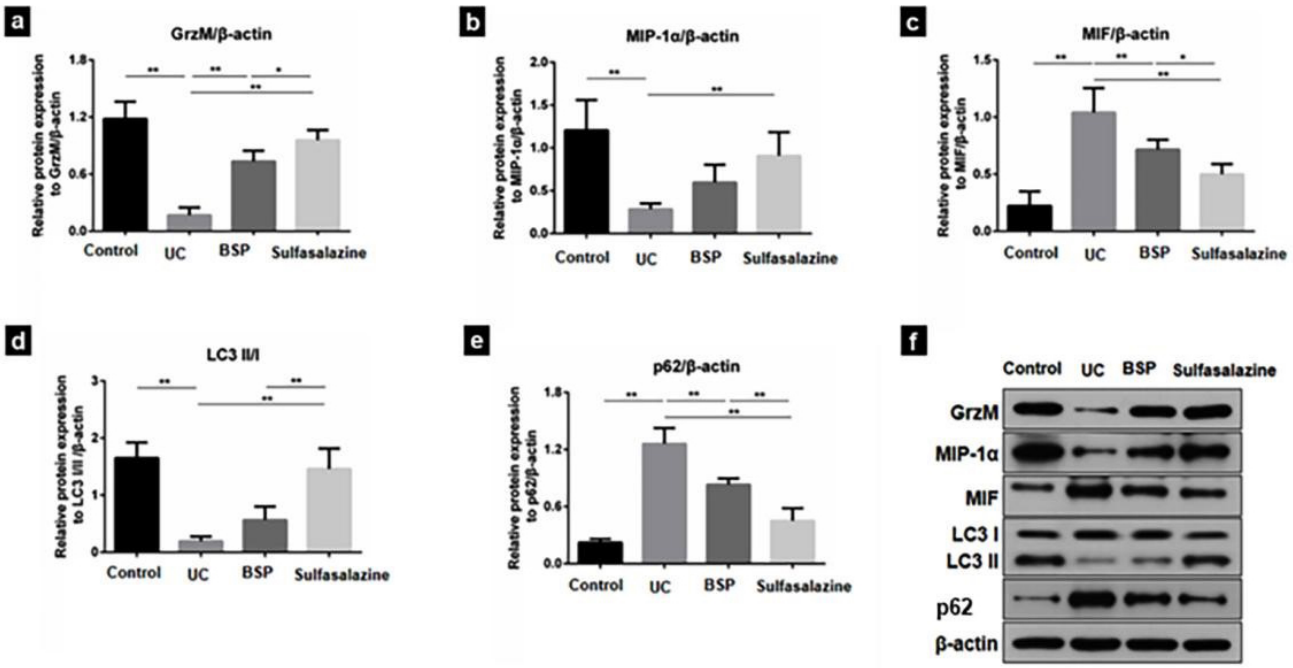

Fig. 7. Expression of granzyme M and downstream macrophage inflammatory protein 1 (MIP-1α) proteins decreased and expression of macrophage migration inhibitory factor (MIF) protein increased after inducing ulcerative colitis in rats, and was then reversed while rats were treated with *Bletilla striata* polysaccharide. Expression of autophagic markers indicates that *Bletilla striata* polysaccharide activates autophagy. (a–e) Bar-chart of the results of quantitative analysis; (f) representing Western Blot (WB) images. BSP, *Bletilla striata* polysaccharide; UC, ulcerative colitis. Data are expressed as mean ± SD, N = 3. *: $p < 0.05$; **: $p < 0.01$.

cations on the autophagic markers LC3 II and p62. Both sulfasalazine and BSP increased LC3 II while decreasing p62, activating autophagy; however, the difference in LC3 II did not achieve statistical significance in the BSP group. This disparity may be attributed to the variations in the approaches of the two medications on the same or distinct signal transduction pathways. Sulfasalazine has been reported in several cancer models to decrease autophagy via NFκB inhibition and induce autophagic cell death via inhibition of the Akt pathway [25]. Further experiments are necessitated to investigate the differences between the mechanisms of BSP and sulfasalazine for UC management.

Granzymes induce cell death in cytotoxic pathways of lymphocytes, and five different granzymes have been identified in humans, including GrzA, B, H, K and M [26]. Recently, GrzM has been evaluated for its critical role in mediating the early stages of the intestinal mucosal immune response in UC [27]. GrzM expression was increased in colon tissue samples from UC patients, and GrzM-deficient mice showed more severe neutrophil infiltration and impaired intestinal permeability after UC induction [28]. Therefore, the effects of sulfasalazine and BSP on GrzM and the related proteins MIP-α and MIF were evaluated herein, since the actual function of GrzM in UC is unknown. Similar to the results of autophagic markers, although BSP treatment significantly increased GrzM and MIP-α expression and decreased MIF expression compared to expression in the UC group, the differences between sulfasalazine and BSP were not statistically significant, suggesting that the effects of GrzM may not be part of the major role of the therapeutic mechanism of BSP.

This study has some limitations. Only single dose of BSP was tested in the present study, for establishing appropriate dosage and extension the application of BSP for treating UC clinically, the effects of more BSP doses should be evaluated, and clinical evaluation in UC patients must also be done in the future. To completely understand the mechanism of BSP therapy, more research into particular biochemical markers, such as inflammatory components in circulation and specific damaged organs by UC, is required. In addition, a functional assessment of the large intestine and a pain score might be undertaken to link the current animal model results to clinical features. Since the therapeutic effect of BSP may not be as strong as that of the active control drug sulfadiazine for UC management, the combination of BSP with other available drugs may be considered and studied by appropriate design.

Chinese medicine monomer contains multiple compounds, but this study is different from the conventional Chinese medicine monomer experiment. The traditional Chinese medicine compound *Bletilla striata* polysaccharide is used to explore the rebalance of Th1, Th2 and T17 and Treg subsets in ulcerative colitis model through animal experiments. It provides a certain research direction

for the treatment of ulcerative colitis with traditional Chinese medicine. It is expected that further experiments in combination with *in vitro* and *in vivo* studies will be carried out in the future, providing more basis for the promotion of traditional Chinese medicine.

Conclusions

In conclusion, BSP demonstrates great potential for first-line treatment of UC or as an adjuvant therapy in combination with currently available drugs. BSP may provide additional benefits to patients with complications in response to other drugs. Additional investigation is needed to further explore the underlying mechanisms of BSP in treating UC. In the UC group, Th1 and Th17 were significantly increased, and Th2 and Treg were significantly decreased. Th1, Th1, Th2 and Treg in UC can reflect the degree of UC to a certain extent, which serves as a route to observe whether the drug is effective.

Availability of Data and Materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Author Contributions

WZ, GL, DML designed the research study. LZ, HCY performed the research. HYS, ZCQ conducted experiments, analyzed the data. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

This study was approved by the ethics committee of Dongzhimen Hospital Beijing University of Chinese Medicine, No. MI-PO20200201. Informed consent was obtained from all study participants. All the methods were carried out in accordance with the Declaration of Helsinki.

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Conflict of Interest

The authors declare no conflict of interest.

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