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ORIGINAL ARTICLE

Basic Study

Faecalibacterium prausnitzii supernatant ameliorates dextran sulfate sodium induced colitis by regulating Th17 cell differentiation

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Abstract

AIM: To explore the preventive and therapeutic effects of *Faecalibacterium prausnitzii* (*F. prausnitzii*) supernatant on dextran sulfate sodium (DSS) induced colitis in mice.

METHODS: Forty C57BL/6J male mice were randomly

divided into four groups: control group, model group, treatment group, and prevention group. Mice were weighed daily. On day 10, the colon length was measured, the colorectal histopathologic damage score (HDS) was assessed, and plasma interleukin (IL)-17A, IL-6, and IL-4 levels were detected by enzyme-linked immunosorbent assay. The expression of transcription factor retinoic acid-related orphan receptor-γt (RORγt) and IL-17A in colon inflammatory mucosa tissue were determined by immunohistochemical assay, and the expression levels of RORγt mRNA, IL-17A mRNA, and IL-6 mRNA were detected by real-time quantitative polymerase chain reaction (PCR). The proportion of Th17 in mononuclear cells in spleen was assayed by fluorescence activated cell sorter.

RESULTS: When compared with the model group, the colon length ($P < 0.05$) and body weight ($P < 0.01$) in the treatment and prevention groups were significantly increased, and the colon HDS was decreased ($P < 0.05$ and $P < 0.01$). There was no statistical difference between the treatment group and prevention group. After treatment with *F. prausnitzii* supernatant, the plasma levels of IL-17A and IL-6 ($P < 0.05$), the protein and mRNA expression of IL-17A and RORγt, and the Th17 cell ratio of spleen cells ($P < 0.01$) were significantly decreased compared to the model group. Plasma IL-4 level in the prevention group was significantly higher than that in the model group ($P < 0.05$), but there was no significant difference between these two groups in the expression of IL-6 in both the plasma and colon mucosa tissues.

CONCLUSION: *F. prausnitzii* supernatant exerts protective and therapeutic effects on DSS-induced colitis in mice, probably *via* inhibition of Th17 differentiation and IL-17A secretion in the plasma and colon mucosa tissues. It can also improve colitis in mice by downregulating IL-6 and prevent colitis by upregulating IL-4.

Key words: *Faecalibacterium prausnitzii*; Ulcerative colitis; Animal model; Th17 cell; Treatment; Prevention

Core tip: *Faecalibacterium prausnitzii* (*F. prausnitzii*) supernatant has anti-inflammatory and immune regulatory activity. This study showed that the preventive and therapeutic use of *F. prausnitzii* supernatant could ameliorate dextran sulfate sodium (DSS)-induced colitis in mice by inhibiting Th17 cell differentiation and inflammatory cytokines release.

Huang XL, Zhang X, Fei XY, Chen ZG, Hao YP, Zhang S, Zhang MM, Yu YQ, Yu CG. *Faecalibacterium prausnitzii* supernatant ameliorates dextran sulfate sodium induced colitis by regulating Th17 cell differentiation. *World J Gastroenterol* 2016; 22(22): 5201-5210 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i22/5201.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i22.5201

INTRODUCTION

Inflammatory bowel diseases (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), are multifactorial ailments characterized by intestinal inflammation. Although the precise etiology and pathogenesis of IBD are not fully elucidated, multiple factors contribute to IBD, including genetic background, environment, intestinal flora imbalance, and immune disorder[1-4]. It has been hypothesized that an undesired intestinal mucosal immune response to intestinal flora imbalance contributes to the onset of IBD in genetically susceptible individuals.

A T helper (Th)17 cell is defined as a cell producing the cytokine interleukin (IL)-17A, but it also can secrete many other cytokines, such as IL-17F, IL-6, and IL-23, during an inflammatory response[5]. Th17 cells are characterized by the expression of the transcription factor retinoic acid-related orphan receptor (RORγt), and there is growing evidence that Th17 cells are paramount in the development of human autoimmune diseases, including IBD[6-8]. In the intestine of IBD patients, elevated numbers of Th17 cells and increased RORγt and IL-17 levels are found[9]. The differentiation of Th17 cells from naïve CD4+ T cells is known to be affected by multiple cytokines, such as transforming growth factor (TGF)-β, IL-6, IL-4, and IL-23[10,11]. IL-6 plays a key role in cooperating with TGF-β to initiate Th17 differentiation, while IL-4 inhibits Th17 differentiation.

Faecalibacterium prausnitzii (*F. prausnitzii*) is the major bacterium of the Clostridium leptum group, and is one of the most abundant anaerobic bacteria in the human gut[12]. *F. prausnitzii* plays an important role in maintaining the intestinal health and providing energy to the colonocytes[13]. A recent study indicated that *F. prausnitzii* levels were decreased in IBD patients compared with healthy controls[14]. Previously, we confirmed in animals that both the bacteria and its supernatant relieved trinitro-benzene-sulfonic acid induced colitis in rats[15]. Nevertheless, the specific mechanism is largely unclear.

Dextran sulfate sodium (DSS) induced colitis is a well-established animal model for IBD pathogenesis, and it has been used in preclinical studies for over two decades[16,17]. Furthermore, it has been shown that the clinical features and pathological changes of DSS-induced colitis in mice were similar to human UC[18]. Here, we determined whether the *F. prausnitzii* supernatant could relieve DSS-induced colitis in mice by reducing Th17 cells and inflammatory cytokines.

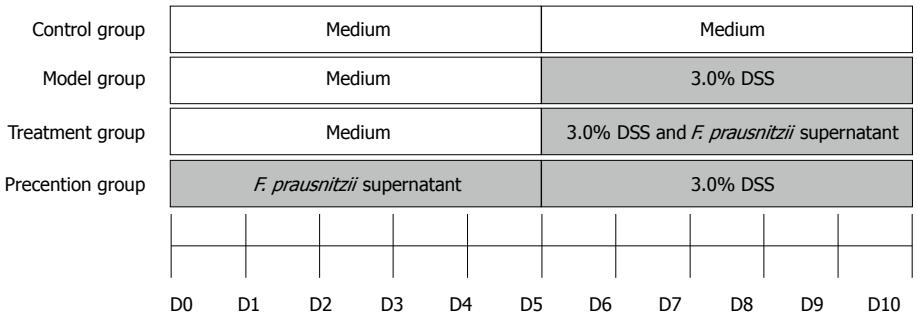


Figure 1 Flow diagram of the study design. DSS: dextran sulfate sodium; *F. prausnitzii*: *Faecalibacterium prausnitzii*.

MATERIALS AND METHODS

Animals

All experiments were approved by the Experimental Animal Ethical Committee of Nanjing Drum Tower Hospital, the Affiliated Hospital of Nanjing University Medical School. Forty male C57BL/6J mice aged 8-10 wk and weighing 18-22 g were obtained from the Animal Center, Nanjing Drum Tower Hospital (Nanjing, China). The mice were allocated equally and randomly to four groups: control group, model group, treatment group, and prevention group. The group divisible design is shown in Figure 1. The period of observation was 10 d. In the first 5 d, the mice in the prevention group were given supernatant of *F. prausnitzii* (five times concentrated, 0.1 mL/10 g) through gavage once a day, while the other groups received the same dosage of medium. For the next 5 d, all groups, except for the control group, were treated with 3.0% DSS in their drinking water *ad libitum*, the treatment group was fed *F. prausnitzii* supernatant by gavage once a day.

Mice were weighed daily and sacrificed by cervical dislocation at day 10. Colons were dissected, and the distance from cecum to anus was measured. The colon tissues were fixed in 4% formalin for later pathological examination and immunohistochemical study. The peripheral blood and spleen were isolated for testing Th17 cells and cytokines.

F. prausnitzii culture

F. prausnitzii (ATCC 27766, Manassas, VA, United States) was cultured anaerobically at 37 ℃ in LYHBHI medium [main component of brain-heart infusion medium (37 g/L, BD, Franklin Lakes, NJ, United States), yeast extract (5 g/L, Oxoid, Basingstoke, United Kingdom), cellobiose (1 g/L, Sigma, St. Louis, MO, United States), maltose (1 g/L, Amresco, Solon, OH, United States), hemin (5 mg/L, Sigma), and cysteine (0.5 g/L, Sigma)]. The number of live bacteria (colony-forming units, CFU) was calculated according to optical density (OD) at 600 nm. The supernatant was collected from cultures with 109-1010 CFU/mL (OD = 1.9). Sterile culture medium acted as placebo. Bacterial supernatant and sterile culture medium were lyophilized and stored at -80 ℃. They were thawed and diluted to five times concentrated solution with phosphate buffered saline (PBS) before administration.

Colon histopathologic grading

The histopathologic grading of colon damage was scored by two blinded pathologists under microscope based on Neurath Scoring criteria as previously described[19]. In short, 4: transmural leukocyte infiltrations, high vascular density, loss of goblet cells, and thickening of the colon wall; 3: high level of leukocyte infiltration, thickening of the colon wall, high vascular density; 2: low level of leukocyte infiltration; 1: very low level of leukocyte infiltration; and 0: no inflammation.

Isolation of splenic mononuclear cells

Splenic mononuclear cells were isolated from spleens through Ficoll–Isopaue density gradient centrifugation[20]. Fresh spleens were placed in Roswell Park Memorial Institute (RPMI)-1640 (Gibco, Carlsbad, NY, United States) and mechanically disrupted by a 2 mL syringe plunger into cell suspensions. Cell suspensions were repeatedly aspirated with a sterile Pasteur pipette and gently filtered through a 200 μm strainer. Splenic single-cell suspensions were layered over an equal volume of Ficoll-Hypaque Solution (Haoyang BioScience Corporation, Tianjin, China) per spleen and centrifuged at 1500 rpm for 20 min. The band of leukocyte enriched fraction at the interface was collected after centrifugation at 1800 rpm for 10 min without brake. The resulting splenic mononuclear cell density was counted in a hemocytometer, and viability was assessed by Trypan blue staining.

Fluorescence activated cell sorter analysis of Th17 in mononuclear cells

Flow cytometry followed routine procedures by using 2 × 10^6 cells per sample. The splenic mononuclear cells were stimulated by phorbol-12-myristate-13-acetate (PMA), ionomycin, and brefeldin A for 5 h at 37 ℃ in a 5% CO2 incubator, then labeled with fluorescein isothiocyanate (FITC) anti-mouse CD4 (eBioscience, San Diego, CA, United States) and APC anti-mouse CD3 (eBioscience). After permeabilization and fixed

Table 1 Polymerase chain reaction primers gene sequences

Target gene	Primer sequence	Product length (bp)
ROR-γt	forward: GACGGCCAACTTACTCTTGG reverse: AGAAACTGGGAATGCAGTGG	109
IL-17A	forward: TCCCTCTGTGATCTGGGAAG reverse: CTCGACCCTGAAAGTGAAGG	154
IL-6	forward: CGGAGAGGAGACTTCACAGAG reverse: CATTTCCACGATTTCCCAGA	105
GAPDH	forward: CATGGCCTTCCGTGTTCCTA reverse: TGTCATCATACTTGGCAGGTTTCT	83

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; IL: Interleukin; ROR-γt: Related orphan receptor-γt.

treatment, cells were labeled with PE anti-mouse IL-17 (eBioscience). The stained cells were tested by flow cytometry (BD, San Jose, CA, United States) and analyzed by the Cell Quest software.

Enzyme-linked immunosorbent assay cytokines in murine plasma

Cytokines (IL-17A, IL-6, IL-4) were measured using a commercially available enzyme-linked immunosorbent assay kit (Yunhan Biological Technology Corporation, Shanghai, China) according to the manufacturers' instructions.

Real-time quantitative polymerase chain reaction

Total RNAs were extracted from mid-colon samples taken from mice in each group using the Trizol reagent (Invitrogen, Carlsbad, CA, United States) with the following procedure. The concentration was determined by NanoDrop TM 1100 (NanoDrop Technologies, Wilmington, DE, United States). Total RNA was reversely transcribed into cDNA using reverse transcription kit. The polymerase chain reaction (PCR) reactions were performed in a 96-well Optical Reaction Plate (Applied Biosystems, Foster City, CA, United States) with the following procedure: degeneration 95 ℃ for 30 s, annealing 95 ℃ for 5 s, 40 cycles of 60 ℃ for 34 s. All primers and probes used in this study are listed in Table 1.

Immunohistochemistry

Paraffin slides of colon were re-hydrated in different concentrations of ethanol and washed in PBS. Sections were microwaved in sodium citrate buffer. After blocking with 10% goat serum for 30 min, sections were incubated with rabbit anti-rat IL-17 antibodies (Abcam, Cambridge, United Kingdom) overnight at 4 ℃. Slides were then incubated with the corresponding secondary antibody (Zsbio, Beijing, China), labelled with horseradish peroxidase, developed using a diaminobenzidine (DAB) reaction, and counterstained with hematoxylin. Cells stained with the antibodies were calculated by random selection of five fields under a microscope at 200 × magnification.

Statistical analysis

The GraphPad Prism version 5.0 (La Jolla, CA, United States) was used for data analysis. Data are presented as mean ± SD and were analyzed using one-way analysis of variance. $P < 0.05$ was considered to be statistically significant.

RESULTS

Symptoms and body weight of mice

Mice became symptomatic (*e.g.*, bloody diarrhea, weight loss, shakes andsloth) by day 3 of drinking 3.0% DSS *ad libitum*. The symptoms worsened with prolonged 3.0% DSS drinking time.

The mice in the model group had obvious weight loss compared to the control group ($P < 0.001$), and the mice from the model group weighed significantly less than those from the treatment and prevention groups. There was no significant difference in weight loss between the treatment group and prevention group (Figure 2).

Colon length and pathological changes

Compared with the control group, the mice in the model group had markedly shorter colon length (7.89 ± 1.536 *vs* 4.92 ± 0.925, $P < 0.001$), more serious colon damage, and higher histopathologic damage scores (0.8 ± 0.632 *vs* 3.7 ± 0.483, $P < 0.01$). Histological examination of model group mice showed that the normal colon mucous membrane structure disappeared, extensive ulceration developed, and a large number of inflammation cells infiltrated. However, culturing supernatant of *F. prausnitzii* in treatment and prevention group mice significantly ameliorated the colon damage by increasing colon length ($P < 0.01$ and $P < 0.05$) and reducing high histopathologic damage scores ($P < 0.05$) as compared with model group (Figure 2).

Th17 cell percentage change in splenic mononuclear cells

The ratio of Th17 cells in splenic mononuclear cells of the model group was significantly higher than that of the control group (4.02 ± 1.111 *vs* 1.34 ± 0.417, $P < 0.001$). It was obviously decreased after preventive and therapeutic application of *F. prausnitzii* supernatant (4.02 ± 1.111 *vs* 2.60 ± 0.839, $P < 0.01$ and 4.02 ± 1.111 *vs* 2.21 ± 1.030, $P < 0.05$), and there was no significant difference between the treatment and prevention groups (Figure 3).

IL-17A, IL–6, and IL-4 levels in peripheral plasma

Plasma IL-17A, IL-6, and IL-4 levels of the control group were significantly different from the model group [15.73 ± 4.382 (pg/mL) *vs* 28.44 ± 4.116 (pg/mL) $P < 0.01$, 81.19 ± 13.609 (pg/mL) *vs* 111.82 ±

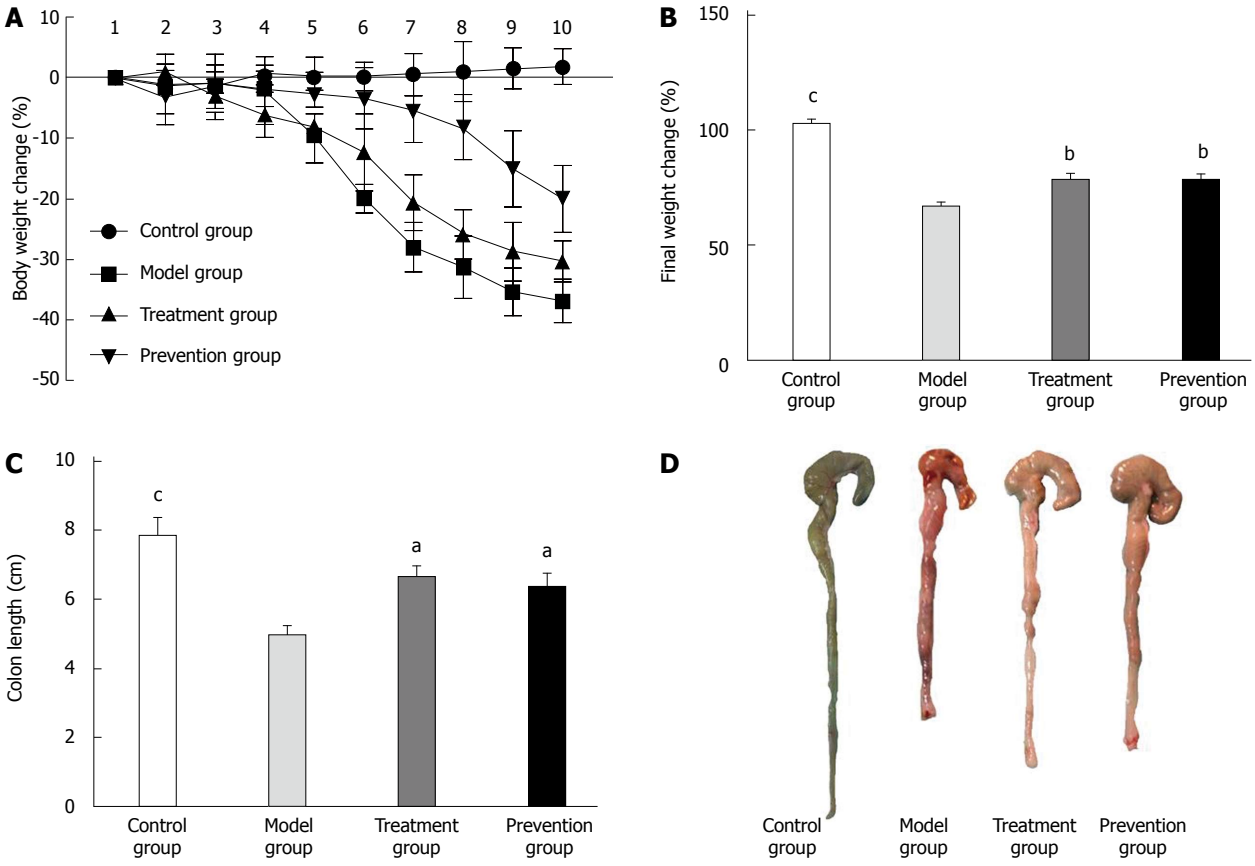


Figure 2 Body weight and colonic length in mice. A, B: Body weight change; C, D: Colon length. Data are the mean ± SD. n = 8-10. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ *vs* model group.

14.369 (pg/mL) $P < 0.05$, 79.91 ± 12.245 (pg/mL) *vs* 38.16 ± 9.507 (pg/mL) $P < 0.001$]. The plasma levels of IL-17A in the treatment and prevention groups were significantly lower than that in the model group ($P < 0.05$). Plasma IL-6 level in the treatment group was also significantly less than that in the model group ($P < 0.05$), but the difference was not statistically significant between the prevention group and model group. On the contrary, level of plasma IL-4 in the prevention group was obviously higher than that in the model group ($P < 0.05$), while no difference was found between the treatment group and the model group (Figure 3).

Expression of cytokines and RORγt mRNA in colon mucosal tissue

The expression of IL-17A, IL-6, and RORγt mRNA in colon tissue of mice in the model group was significantly higher than that in the control ($P < 0.001$) and treatment groups ($P < 0.05$). When compared with the model group, the expression of IL-17A and RORγt mRNA in colon inflammatory tissue of the treatment and prevention groups was significantly decreased ($P < 0.01$ or $P < 0.05$). There was no difference, however, in IL-6 between the model group and prevention group. As shown in Figure 4, the expression of cytokines and RORγt mRNA in colon mucosal tissue did not significantly differ between the treatment and prevention groups.

Immunohistochemistry

To investigate the effects of IL-17A and RORγt on colon tissue, we conducted immunohistochemical staining of proinflammatory cytokines in tissue sections. Consistent with the results of quantitative real time PCR, the expression of IL-17A and RORγt in the colon tissue of model group mice was significantly increased compared to that in the control group ($P < 0.001$) and treatment group ($P < 0.05$). Although the expression of RORγt in colon tissue was declined after protective use of *F. prausnitzii*, there was no difference between the model and prevention groups (Figure 5).

DISCUSSION

In this study, we found that *F. prausnitzii* supernatant ameliorated colitis in mice by regulating Th17 cell differentiation and inhibiting the excretion of relevant inflammatory cytokines. We also found that *F. prausnitzii* supernatant was effective in the treatment and prevention of DSS-induced mice colitis by inhibiting differentiation of Th17 cell.

Both living *F. prausnitzii* and *F. prausnitzii* supernatant, which contains a mixture of secreted products,

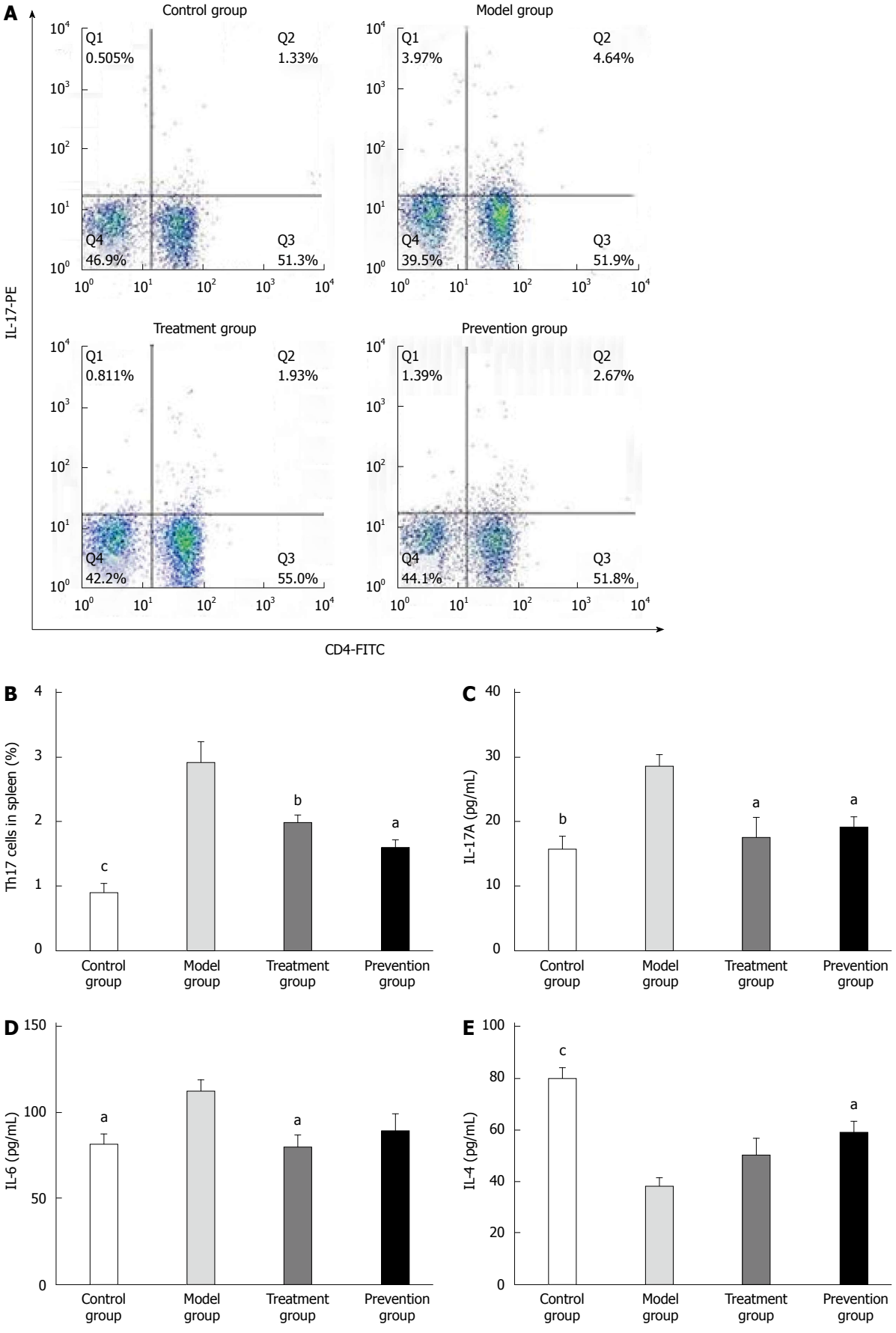


Figure 3 Proportion of Th17 cells in splenic mononuclear cells and plasma cytokines levels. Flow cytometry figures (A) and statistical analysis (B) of Th17 cell in each group of the mice splenic MNC. Plasma IL-17 A (C), IL–6 (D) and IL-4 (E) levels by enzyme-linked immunosorbent assay. Data are the mean ± SD. n = 8-10. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ *vs* model group. IL: Interleukin; MNC: mononuclear cells.

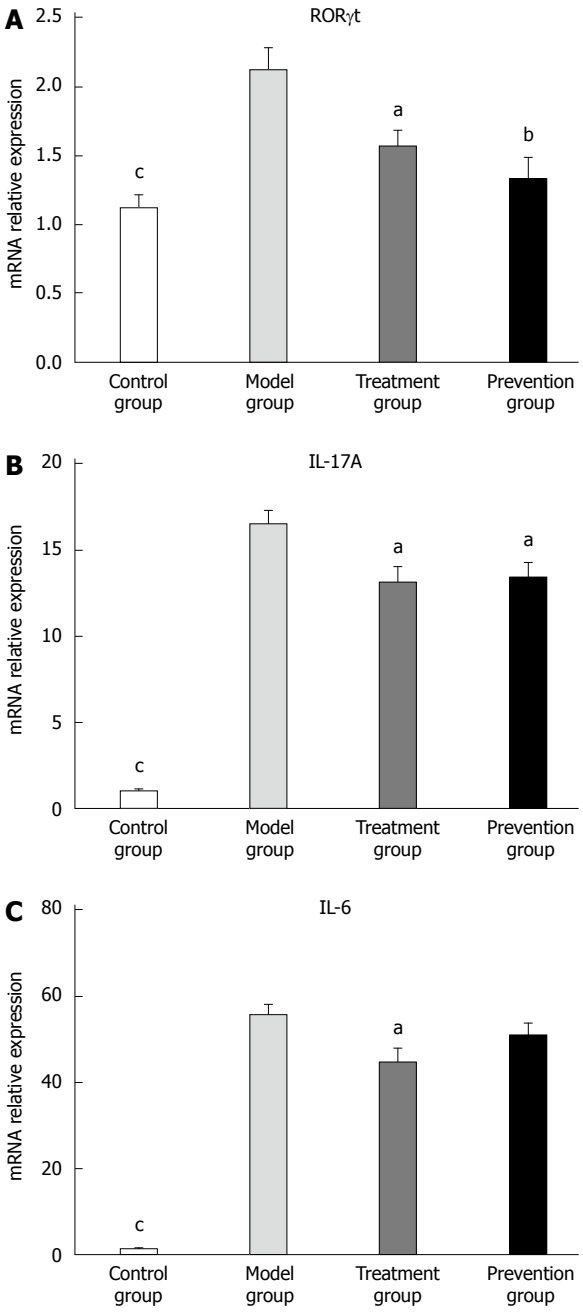


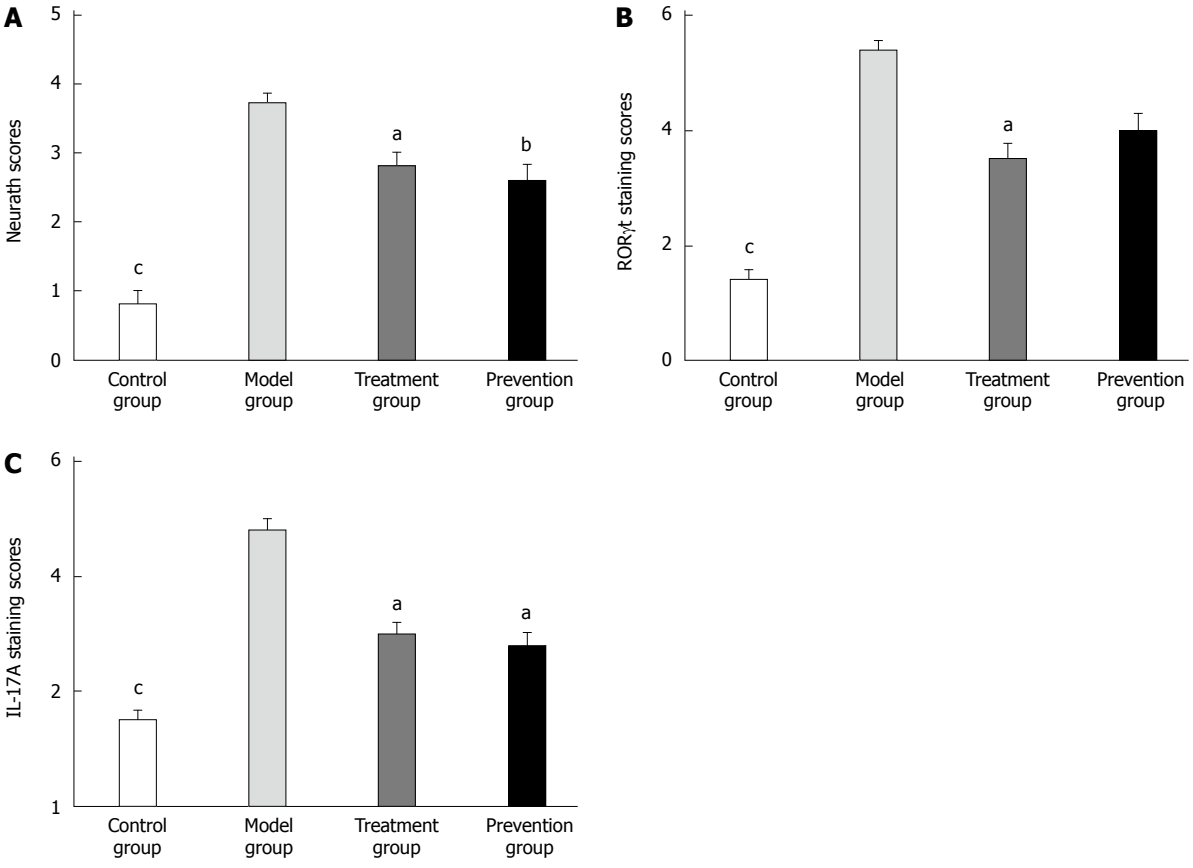
Figure 4 Cytokine mRNA expression in colon mucosal tissue. A: RORγt mRNA; B: IL-17A mRNA; C: IL-6 mRNA. Data are the mean ± SD. n = 8-10. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ *vs* model group. ROR-γt: Related orphan receptor-γt; IL: Interleukin.

have been shown to have an anti-inflammatory effect[21]. Compared to *F. prausnitzii*, its supernatant could be more effective therapeutically, as it may have a longer shelf-life, which would facilitate delivery, handling, and administration[22]. However, the exact composition and the anti-inflammatory mechanism of *F. prausnitzii* supernatant are currently largely unknown. Therefore, we explored the effects and immune mechanisms of *F. prausnitzii* supernatant on DSS-deduced colitis. Our study showed that the plasma levels of IL-17A and IL-6, the protein and mRNA expression of IL-17A and RORγt in intestinal mucosa, and the Th17 cell ratio of spleen cells ($P < 0.01$) in supernatant treatment group were significantly decreased compared to those in the model group. This finding indicated that the therapeutic use of *F. prausnitzii* supernatant could ameliorate DSS-induced colitis through inhibiting Th17 cells. Carlsson *et al*[23] previously demonstrated that the supernatant of *F. prausnitzii* affected the function of the intestinal barrier.

Th17-related gene polymorphisms are associated with IBD susceptibility[24]. Th17-derived cytokines, such as IL-17A, IL-6, and IL-22, have been shown to be upregulated in the inflamed intestine of IBD patients[25,26]. IL-17A is a strong inflammatory cytokine, which can enhance cell permeability and promote the generation of other pro-inflammatory cytokines and chemokines[27]. Animal experiments, however, have found that neither IL-17A knockout nor neutralization of IL-17 could protect DSS-administrated mice from colitis, suggesting that the role of IL-17 in intestinal inflammation may not be entirely pathogenic[14,28]. Adequate expression of IL-17A plays an important role in maintaining intestinal immune function. Consistent with previous studies, we found that IL-17A levels in the plasma, spleen, and colon tissue were significantly increased in mice with colitis and that these levels were remarkably downregulated in mice treated with *F. prausnitzii* culture supernatant. Therefore, *F. prausnitzii* supernatant could attenuate DSS-induced mice colitis, possibly by inhibiting the expression of IL-17A[15,29].

We also found that levels of IL-6 in plasma and colon tissues of colitis mice were significantly reduced after *F. prausnitzii* supernatant treatment. *F. prausnitzii* supernatant could alleviate mice colitis by downregulating IL-6 levels and inhibiting Th17 cell differentiation, thus leading to reduced secretion of inflammatory cytokines (such as IL-17A and IL-6) and attenuation of the local inflammatory response. However, the regulation of IL-6 expression in the treatment and prevention groups was inconsistent, suggesting that there might be other ways of inhibiting Th17 differentiation. Fu *et al*[29] demonstrated that boosting of Th2 associated cytokines (IL-4, IL-13, and IL-10) can reverse Th17-mediated intestinal inflammation. We also found that plasma IL-4 levels in mice of the prevention group were significantly greater than those in the model group.

In conclusion, *F. prausnitzii* supernatant can prevent DSS-deduced colitis in mice by inhibiting the generation of Th17 cells in the spleen and intestinal mucosa, leading to a reduction of IL-17A and IL-6 levels and attenuation of intestinal inflammation. This study provides the theoretical basis for the application of *F. prausnitzii* supernatant in UC treatment and prevention. However, what specific substances in the supernatant of *F. prausnitzii* possess biological activity needs to be elucidated in future studies. The safety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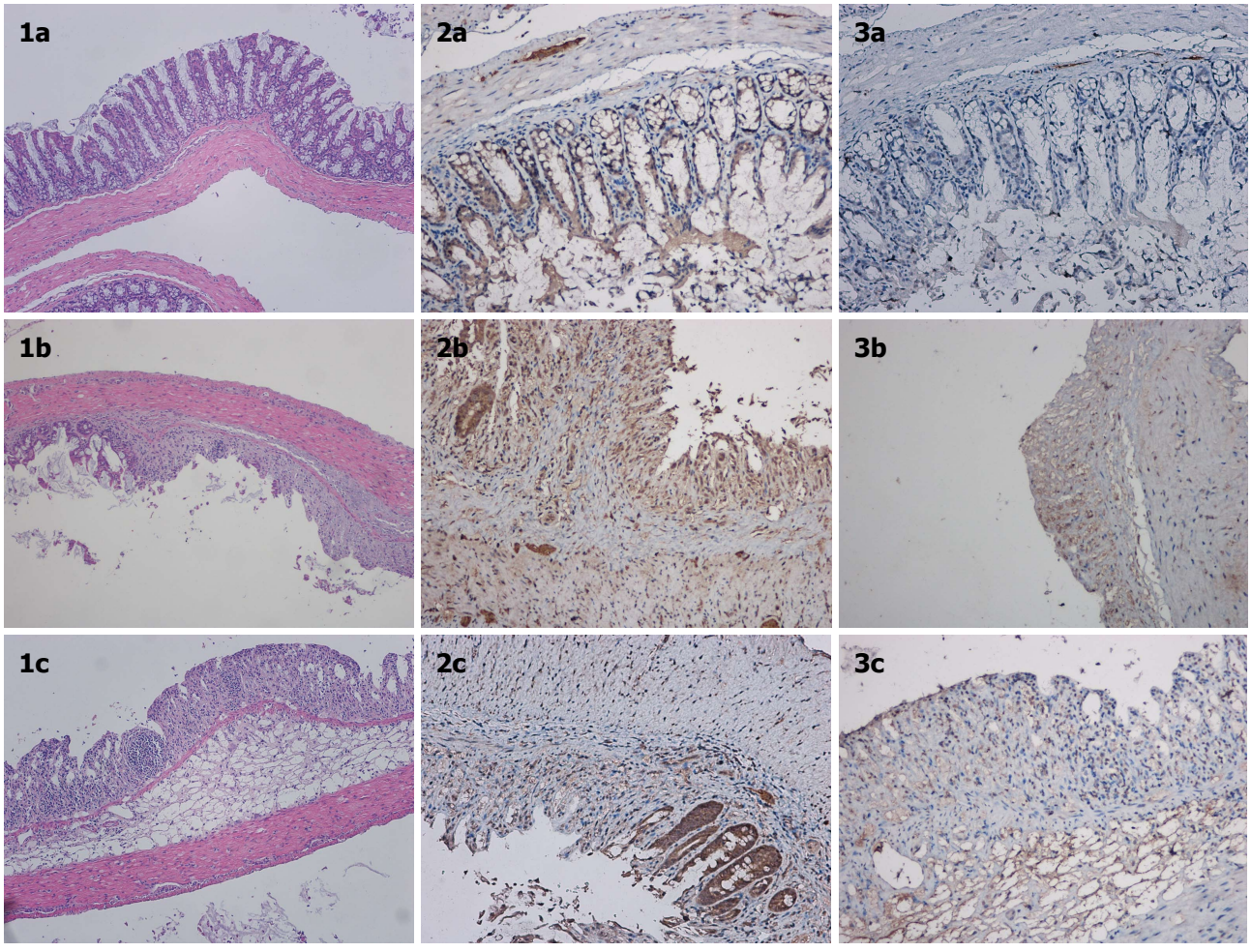
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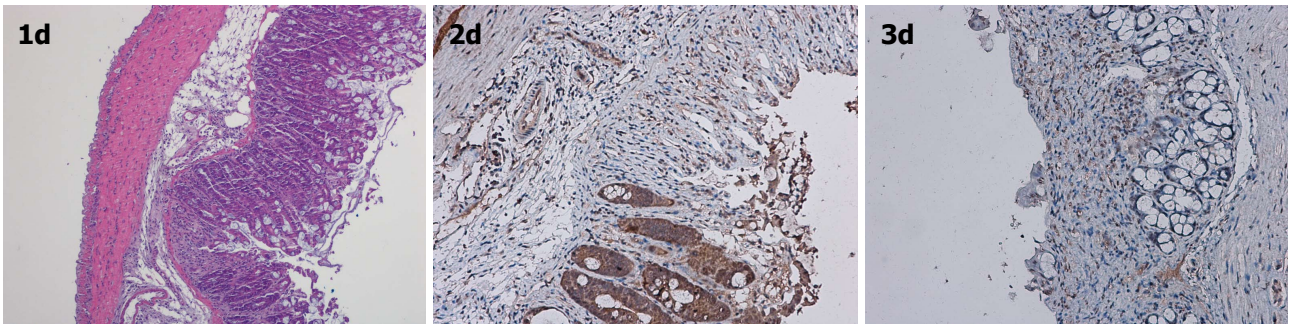

Figure 5 Colon Neurath Scores and related orphan receptor-γt and interleukin-17A protein expression. Colon Neurath Scores (A, 100 magnifications), RORγt (B, 200 magnifications), and IL-17A (C, 200 magnifications) protein expression in mice colon. Representative images of mice colonic mucosa (1a-1d). Representative immunohistochemical staining of RORγt (2a-2d) and IL-17A (3a-3d) in mice colon mucosa. Control group (a); model group (b); treatment group (c); prevention group (d). Data are the mean ± SD. n = 8-10. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ *vs* model group.

and efficacy of *F. prausnitzii* supernatant also warrant further investigation by more large scale clinical trials.

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COMMENTS

Background

Inflammatory bowel disease (IBD) is a multifactorial ailment characterized by intestinal inflammation, and its etiology is complicated and ambiguous. Factors that contribute to IBD include genetic background, environment, intestinal flora imbalance, and immune disorder as well as the interactions between them.

Research frontiers

Faecalibacterium prausnitzii (*F. prausnitzii*) is a common anaerobic bacteria that colonizes the human gut, and it plays a critical role in IBD. *F. prausnitzii* supernatant has anti-inflammatory and immune regulatory activity. Previously, the authors showed in animals that both the bacteria and its supernatant relieved trinitro-benzene-sulfonic acid-induced colitis in rats. However, the specific mechanism is largely unclear.

Innovations and breakthroughs

This study is the first to show that the preventive and therapeutic use of *F. prausnitzii* supernatant could ameliorate dextran sulfate sodium (DSS) induced mice colitis through inhibiting Th17 cells. The molecular mechanism of proliferation and differentiation of Th17 cells was different. *F. prausnitzii* supernatant may treat colitis in mice by downregulating IL-6 and preventing the upregulation of IL-4.

Applications

This study investigated the molecular mechanism of the preventive and therapeutic use of *F. prausnitzii* supernatant for IBD and provided evidence for the prevention and treatment of the disease.

Terminology

F. prausnitzii is the major bacterium of the Clostridium leptum group and is one of the most abundant anaerobic bacteria in human gut.

Peer-review

The study investigates the preventive and therapeutic role of *F. prausnitzii* supernatant in a mouse model of DSS-induced ulcerative colitis. The topic is interesting, and the design and methods have clear scientific values. The data are clear and well presented.

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