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***Basic Study***

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

Huang XL *et al*. *F.prausnitzii* supernatant ameliorates mice colitis

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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 distributed into 4 groups, which named control group, model group, treatment group, and prevention group, respectively. Mice were weighed daily. In day 10, the colon length was measured, the colorectal histopathologic damage score (HDS) was assessed, 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 (ROR-γt) and IL-17A in colon inflammatory mucosa tissue were determined by immunohistochemical assay, and the expression levels of ROR-γt mRNA, IL-17A mRNA and IL-6 mRNA were also detected by real-time quantitative PCR. The proportion of Th17 in mononuclear cells in spleen was assayed by fluorescence activated cell sorter.

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

**CONCLUSION:** *F. prausnitzii* supernatant exerts protective and therapeutic effects on DSS-induced colitis in mice, probably mediated by inhibiting Th17 differentiation and IL-17A secretion in plasma and colon mucosa tissues. It can also treat colitis in mice by down-regulating IL-6 and prevent them by up-regulating IL-4.

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

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**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 DSS-induced colitis in mice through inhibiting Th17 cell differentiation and inflammatory cytokines release.

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**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 is not fully elucidated, IBD is associated with genetic background, environmental factors, intestinal flora imbalance and immune disorder[[1-4](#_ENREF_1)]. It has been hypothesized that an undesired intestinal mucosal immune response to intestinal flora imbalance contributes to the onset of IBD in genetically susceptible individual.

Th17 cell is defined as cell producing the cytokine IL-17A, but it also can secrete many other cytokines such as IL-17F, IL-6 and IL-23 in an inflammatory response[[5](#_ENREF_5)]. Th17 cell is characterized by the expression of transcription factor retinoic acid-related orphan receptor (RORγt) and is increasingly recognized as paramount in the development of human autoimmune diseases, including IBD[[6-8](#_ENREF_6)]. In the intestine of IBD patients, elevated numbers of Th17 cell and increased RORγt and IL-17 levels are found[[9](#_ENREF_9)]. The differentiation of Th17 cell from naive CD4+ T cells is known to be affected by multiple cytokines, such as TGF-β, IL-6, IL-4 and IL-23[[10](#_ENREF_10),[11](#_ENREF_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 human gut[[12](#_ENREF_12)]. *F. prausnitzii* plays an important role in maintaining the intestinal health and providing energy to the colonocytes[[13](#_ENREF_13)]. A recent study indicated that the abundance of *F. prausnitzii* was decreased in IBD patients compared with healthy controls[[14](#_ENREF_14)]. Our previous animal experiment also confirmed that both the bacteria and its supernatant relieved trinitro-benzene-sulfonic acid induced colitis in rats[[15](#_ENREF_15)]. Nevertheless, the specific mechanism is largely unclear.

Dextran sulfate sodium (DSS) induced colitis is a well-established animal model used for IBD pathogenesis and preclinical studies for over two decades[[16](#_ENREF_16),[17](#_ENREF_17)]. Furthermore, it has been proved that the clinical features and pathological changes of DSS-induced colitis in mice were similar to human UC[[18](#_ENREF_18)]. Here in this research we mainly observed whether the *F. prausnitzii* supernatant could relieve DSS-induced colitis in mice by reducing Th17 cells and inflammatory cytokines.

**MATERIALS AND METHODS**

***Animals***

All experiments have been 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 randomly into 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 ten days. In the first five days, the mice of prevention group was given supernatant of *F. prausnitzii* (5 times concentrated, 0.1 ml/10 g) through gavage once a day, while the other groups received the same dosage of medium. For the latter five days, all groups were treated with 3.0% DSS in their drinking water ad libitum except for control group, and the treatment group were feed with *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 cell and cytokines.

***F. prausnitzii culture***

*F. prausnitzii* (ATCC 27766, Manassas, VA, United States) was cultured anaerobically at 37 °C in LYHBHI medium [main component of brain-heart infusion medium(37 g/L, BD, United States), yeast extract (5 g/L, Oxoid, United Kingdom), cellobiose ( 1 g/L, Sigma, United States), maltose (1 g/L, Amresco, United States), hemin (5 mg/L, Sigma, USA) and cysteine(0.5 g/L, Sigma, United States)]. 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. Then the bacterial supernatant and sterile culture medium were lyophilized and stored at -80 ℃. They were thawed and diluted to 5 times concentrated solution with 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](#_ENREF_19)]. (In short, 4: transmural leucocyte infiltrations, high vascular density, loss of goblet cells, and thickening of the colon wall; 3: high level of leucocyte infiltration, thickening of the colon wall, high vascular density; 2: low level of leucocyte infiltration; 1: very low level of leucocyte infiltration and 0: no inflammation).

***Isolation of*** ***splenic*** ***mononuclear cells***

Splenic mononuclear cells were isolated from spleens through Ficoll–Isopaue density gradient centrifugation[[20](#_ENREF_20)]. Fresh spleens were placed in RPMI-1640 (Gibco, New York, United States), 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 haemocytometer 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 × 106 cells per sample. The splenic mononuclear cells were stimulated by phorbol-12-myristate-13-acetate (PMA), ionomycin and brefeldin A for 5 h in 37 ℃, 5% CO2 incubator, then labeled with FITC anti-mouse CD4 (eBioscience, United States), APC anti-mouse CD3 (eBioscience, United States). After permeabilized and fixed treatment, cells were labeled with PE anti-mouse IL-17 (eBioscience, United States). The stained cells were tested by flow cytometry (Becton Dickinson, United States) and analyzed by the Cell Quest software.

***enzyme-linked immunosorbent assay 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 PCR***

Total RNAs were extracted from mid-colon samples taken from mouse 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 transcripted into cDNA using reverse transcription kit. The 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 concentration of ethanol and washed in PBS. Sections were microwaved in sodium citrate buffer. After being blocked with 10% goat serum for 30 min, they were incubated with rabbit anti-rat IL-17 antibodies (Abcam, 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 microscope at 200 × magnification.

***Statistical analysis***

The GraphPad.prism version 5.0 was used for data analysis. Data were presented as mean±SD and were analyzed using one-way ANOVA. *P <* 0.05 was considered to be significant.

**RESULTS**

***symptoms and body weight of mice***

Mice had symptoms (such as bloody diarrhea, weight loss and depression) at the third day after drinking 3.0% DSS ad libitum. The symptoms aggravated along with the prolonging of 3.0% DSS drinking time.

The mice in the model group had an obviously weight loss compared to the control group (*P <* 0.001). And the mice from the model group were significantly lighter than those from the treatment group and the prevention group. But there was no significant difference of weight loss between treatment group and prevention group (Figure 2).

***colon length and pathological changes***

Compared with the control group, the mice in 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 the mice in the model group 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 mice from treatment and prevention group can significantly ameliorate the colon damage by increasing colon length (*P <* 0.01 and *P <* 0.05) and reducing high histopathologic damage scores (*P <* 0.05), compared with model group (Figure 2).

***Th17 cell percentage change in splenic mononuclear cells***
The ratio of Th17 cell 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). But there was no significant difference between treatment group and prevention group (Figure 3).

***IL-17 A, IL–6 and IL-4 levels in peripheral plasma***Plasma IL-17A , IL-6 and IL-4 levels of the control group had significantly difference with 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 ± 14.369 (pg/ml) *P <* 0.05, 79.91 ± 12.245 (pg/ml) *vs* 38.16 ± 9.507 (pg/ml) *P <* 0.001]. The IL-17A levels of plasma in the treatment and prevention groups were significantly lower than those in the model group (*P <* 0.05). Plasma IL-6 level in the treatment group also significantly decreased than that in the model group (*P <* 0.05), but there was no statistical significance 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 group (*P <* 0.001) and the treatment group (*P <* 0.05). Comparison with the model group, the expression of IL-17A and RORγt mRNA of colon inflammatory tissue of treatment and prevention groups had significantly declined (*P <* 0.01 or *P <* 0.05), but there was no difference of 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 qRT-PCR, the expression of IL-17A and RORγt in colon tissue in model group mice significantly increased than that in the control group (*P <* 0.001) and treatment group (*P <* 0.05). Though the expression of RORγt in colon tissue is declined after protective use of *F. prausnitzii*, there was no difference between the model group and prevention group (Figure 5).

**DISCUSSION**

In this study, we found that *F. prausnitzii* supernatant ameliorated the mice colitis by regulating Th17 cell differentiation and inhibiting the relevant inflammatory cytokines excretion. We also found that *F. prausnitzii* supernatant had the same effect in treating and preventing DSS-induced mice colitis, with different mechanisms in inhibiting differentiation of Th17 cell.

*F. prausnitzii* supernatant containing a mixture of secreted products, has been proved to have an anti-inflammatory effect as well as living *F. prausnitzii*[[21](#_ENREF_21)]. Compare to *F. prausnitzii*, supernatant could be more effective therapeutics, as they may have a longer shelf-life and facilitates to be delivered, handled and administrated[[22](#_ENREF_22)]. However, the exact composition and the anti-inflammatory mechanism of *F. prausnitzii* supernatant is currently largely unexplored. So we chose to use *F. prausnitzii* supernatant in our experiments to explore the effects and immune mechanisms 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 ROR-γt in intestinal mucosa and the Th17 cell ratio of spleen cells (*P <* 0.01) in supernatant treatment group were significantly decreased than those in colitis group. It indicated that the therapeutic use of *F. prausnitzii* supernatant could ameliorate DSS-induced colitis through inhibiting Th17 cell. Anders *et al*[[23](#_ENREF_23)] also demonstrated that the supernatant of *F. prausnitzii* affected the function of intestinal barrier.

Th17-related gene polymorphisms are associated with IBD susceptibility[[24](#_ENREF_24)]. Th17-derived cytokines, such as IL-17A, IL-6 and IL-22, have been shown to be upregulated in inflamed intestinal of IBD patients[[25](#_ENREF_25),[26](#_ENREF_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](#_ENREF_27)]. Nonetheless, animal experiments have implicated 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](#_ENREF_14),[28](#_ENREF_28)]. Adequate expression of IL-17A plays an important role in maintaining intestinal immune function. Consistent with previous studies, we found IL-17A levels in the plasma, spleen and colon tissue were significantly increased in mice with colitis, and were remarkably downregulated in the mice prevented and treated by *F. prausnitzii* culture supernatant, indicating that *F. prausnitzii* supernatant could attenuate DSS-induced mice colitis probably due to the inhibition of IL-17A expression[[15](#_ENREF_15),[29](#_ENREF_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, which indicated that *F. prausnitzii* supernatant could alleviate mice colitis by down regulating IL-6 levels and inhibiting Th17 cell differentiation, and thus reduced the secretion of inflammatory cytokines (such as IL-17A and IL-6) and then attenuated local inflammatory response. However, inconsistence of the regulation of IL-6 expression between treatment group and prevention group suggested that there might be other ways of inhibiting Th17 differentiation. Fu SH et al demonstrated that boosting of Th2 associated cytokines (IL-4, IL-13 and IL-10) can reverse Th17-mediated intestinal inflammatory[[29](#_ENREF_29)]. We also found that the plasma IL-4 levels in mice of prevention group was significantly increased than those in 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 reduction of IL-17A and IL-6 and attenuation of the intestinal inflammation. This study provides theory basis for further application of *F. prausnitzii* supernatant in UC treatment and prevention in practice. However, what specific substances in supernatant of *F. prausnitzii* and what kind of materials possessing biological activity need to be elucidated in future studies? The safety and effectiveness 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. Genetic background, environmental factors, intestinal flora imbalance, immune disorder and interactions between them contributed to it.

***Research frontiers***

*Faecalibacterium prausnitzii* (*F. prausnitzii*) is one common anaerobic bacteria colonized in human gut, and plays a critical role in IBD. *F. prausnitzii* supernatant has anti-inflammatory and immune regulatory activity. The authors’ previous animal experiment also confirmed that both the bacteria and its supernatant relieved trinitro-benzene-sulfonic acid-induced colitis in rats. But the specific mechanism is largely unclear.

***Innovations and breakthroughs***

This study firstly showed that the preventive and therapeutic use of *F. prausnitzii* supernatant could ameliorate dextran sulfate sodium (DSS) induced mice colitis through inhibiting Th17 cells, whereas the molecular mechanism of proliferation and differentiation of Th17 cells was different. It may treat colitis in mice by down-regulating IL-6 and prevent them by up-regulating IL-4.

***Applications***

This study investigated the molecular mechanism of the preventive and therapeutic use of *F. prausnitzii* supernatant for IBD and provided the 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 UC induced by DSS. The paper is interesting. The design and methods have clear scientific values. The data are clear and well presented.

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**Table 1 PCR primers gene sequences**

|  |  |  |
| --- | --- | --- |
| **Target gene** | **Primer sequence** | **Product length（bp）** |
| *ROR-γt* |

|  |
| --- |
| forward:GACGGCCAACTTACTCTTGG |

 | 109 |
|  | reverse:AGAAACTGGGAATGCAGTGG |  |
| *IL-17A* | forward:TCCCTCTGTGATCTGGGAAG | 154 |
|  | reverse:CTCGACCCTGAAAGTGAAGG |  |
| *IL-6* |

|  |
| --- |
|  forward:CGGAGAGGAGACTTCACAGAG |

 | 105 |
|  | reverse:CATTTCCACGATTTCCCAGA |  |
| *GAPDH* | forward:CATGGCCTTCCGTGTTCCTA | 83 |
|  | reverse:TGTCATCATACTTGGCAGGTTTCT |  |

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**Figure 1** **Flow diagram of the study design.**



**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.



**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.



**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.



**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.