

Roseburia intestinalis inhibits interleukin-17 excretion and promotes regulatory T cells differentiation in colitis

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Abstract. *Roseburia intestinalis* (*R. intestinalis*) is one of the dominant intestinal bacterial microbiota and is decreased in patients with inflammatory bowel disease (IBD). It helps protect colonic mucosa against the development of inflammation and subsequent IBD, however its underlying mechanisms are unclear. The aim of the present study was to evaluate the anti-inflammatory properties of *R. intestinalis* *in vitro* and in an animal model of IBD. The effects of *R. intestinalis* on disease activity index (DAI) scores, intestinal pathology, the expression of interleukin (IL)-17 and the frequency of CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Treg) were evaluated *in vivo* in a model of 2,4,6-trinitrobenzenesulfonic acid solution (TNBS)-induced colitis. Compared with the control group, TNBS-treated mice had significantly higher secretion of IL-17, higher DAI scores, a lower ratio of Treg, reduced colon lengths and higher histological scores for colon inflammation. The administration of *R. intestinalis* significantly downregulated the expression of IL-17, increased the ratio of Treg and ameliorated the high DAI scores and the pathological signs of inflammation in the colon compared with mice treated with TNBS alone. Gene expression profiling was also used to detect the expression of IL-17 in human IBD and healthy control specimens. To extend these findings to an *in vitro* model of inflammation the human colon epithelial cell line NCM460 was stimulated with lipopolysaccharide (LPS) to induce inflammation and co-cultured with *R. intestinalis* and changes in IL-17 expression were evaluated. *R. intestinalis* inhibited the LPS-induced secretion of IL-17 by NCM460 cells. In conclusion, these results demonstrate that *R. intestinalis* inhibits IL-17 secretion and promotes Treg differentiation

in colitis, suggesting that *R. intestinalis* could be of potential use in the treatment of IBD.

Introduction

Inflammatory bowel diseases (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), are chronic relapsing and non-resolving inflammatory disorders that are characterized pathologically by gastrointestinal inflammation and epithelial injury (1,2). The pathogenesis and etiology of IBD are still unclear and widely thought to involve genetic factors, the intestinal microbiota, immune dysfunction, and environmental factors (3). IBD has become a global disease with an increasing incidence worldwide (4), and it has a significant effect on morbidity and quality of life (5). Since currently available treatments for IBD are unsatisfactory, new therapeutic strategies are desirable (6,7).

The gastrointestinal tract is the primary site of interaction between the host immune system and microorganisms, both symbiotic and pathogenic. The balance in the community structure of gut bacteria may be intimately associated with the proper function of the immune system (8-11). Numerous studies have revealed the close relationship between the composition of the gut microbiota and IBD (12,13). We previously used 16S-rRNA genome sequencing to detect differences in the intestinal microbiota between CD patients and healthy controls (HCs), and found that the species *R. intestinalis* (*R.I.*) was significantly decreased in CD patients. In agreement with our findings, a number of other studies have also shown that the abundance of *R. intestinalis* was decreased to varying degrees in IBD patients (14,15), indicating that this species is closely related to the development of IBD.

Cytokines also have a crucial role in the pathogenesis of IBD, as they regulate multiple aspects of the inflammatory response. In particular, the imbalance between pro-inflammatory and anti-inflammatory cytokines that occurs in IBD impedes the resolution of inflammation and instead leads to disease perpetuation and tissue destruction (16). On the other hand, regulatory T cells (Treg), a suppressive T cell population, can restrain the progression of inflammation (17,18). Based on our previous findings and other reports, we hypothesize that *R. intestinalis* protects

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the intestinal mucosa from inflammation by regulating the secretion of cytokines and the differentiation of Treg. To investigate this, we evaluated the potential therapeutic effects of *R. intestinalis* on intestinal inflammation both *in vivo* and *in vitro*. *R. intestinalis* increased the level of interleukin (IL)-17 secretion and Treg differentiation and protected colon epithelial cells from pathological damage in an animal model of chemically induced inflammation. These findings suggest that *R. intestinalis* could be a potential treatment for IBD.

Materials and methods

Ethics approval. All animal experiments were approved by the Ethical Committee of Medical Research, Third Xiangya Hospital, Affiliated Hospital of Central South University.

***R. intestinalis* culture and preparation.** *R. intestinalis* (DSMZ-14610) was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany) and grown anaerobically at 37°C in Lytic/10 Anaerobic/F Medium (BD Biosciences, Franklin Lakes, NJ, USA). The number of live bacteria (colony-forming units; CFU) was determined according to the absorbance at 600 nm (A600). For *in vitro* studies, bacterial cells were washed and resuspended at 1×10^9 cells/ml in medium. For animal experiments, the bacterial suspension (1×10^9 CFU in 100 μ l) was administered to mice by intragastric gavage.

Animals and 2,4,6-trinitrobenzenesulfonic acid solution (TNBS)-induced colitis. Mice (BALB/c, 6 weeks old, male) were obtained from the Animal Center, Xiangya School of Medicine (Hunan, China), and animal experiments were performed at the same facility. The mice were maintained under specific pathogen-free conditions according to the Animal Regulations of Hunan Province, China. Mice were acclimatized to the facility before experiments were initiated. The mice were then randomly assigned to four groups (n=6): A control group without colitis, a group in which mice were preconditioned with R.I. prior to the induction of colitis with TNBS (R.I. Pre), a group in which colitis was induced but which did not receive R.I. (TNBS), and a group in which R.I. was administered after the induction of colitis with TNBS (R.I. Treat). Starting on day 1, the preconditioned group received *R. intestinalis* intragastrically for 2 days. On day 3, the mice in the groups in which colitis was induced were given 100 μ l of TNBS (a 1:1 mixture by volume of 5% TNBS and absolute ethanol) intrarectally, while the control group received normal saline. Bacteria were administered to the R.I. Pre and R.I. Treat groups by intragastric gavage on days 5 and 7. Mice were observed and weighed, and fecal occult blood was measured daily and used to calculate the disease activity index (DAI) using a previously published grading system (19) (Table I). On day 9, the mice were weighed and sacrificed. Serum was collected and colon tissues were removed, washed and opened, fixed in 10% neutral buffered formalin solution, embedded in paraffin, cut into tissue sections, and stained with hematoxylin and eosin (H&E). Inflammation grading was carried out by two independent blinded observers, and lesions were analyzed using histological scoring criteria, as previously described (20) (Table II).

Table I. Criteria for diseases activity index scores in mice.

Weight loss (%)	Stool characters	Hematochezia	Score
0	Normal	OB negative	0
1-5			1
5-10	Loose	OB positive	2
10-15			3
>15	Sloppy stools	Bloody stools	4

OB, occult blood.

Immunohistochemistry. The paraffin-embedded samples were cut into 4- μ m-thick sections, which were boiled in sodium citrate solution (pH 6.0; Goodbio Technology, Wuhan, China) for 18 min and then cooled at room temperature. The sections were incubated with an IL-17 antibody (Boosen, Beijing, China) at 4°C overnight and then with the corresponding secondary antibody (Goodbio Technology) for 30 min, followed by staining with 3,3'-diaminobenzidine (DAB; Mai New Biotechnology Development Company, Fuzhou, China). The samples were observed under a microscope by two independent blinded observers.

Flow cytometric analysis of Treg in murine peripheral blood. Mononuclear cells were isolated from murine peripheral blood by Ficoll-Isopaque density gradient centrifugation (Ficoll-Paque; GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The cells (2×10^6 cells/sample) were labeled with FITC anti-mouse CD4 (BD Bioscience), APC anti-mouse CD25 (BD Bioscience), and PE anti-mouse Foxp3 (BD Bioscience). The stained cells were analyzed by flow cytometry (BD Bioscience) using Cell Quest software (BD Bioscience).

Experiments on NCM460 cells. The human colon epithelial cell line NCM460 was obtained from the Cancer Research Institute of Central South University (Changsha, China). NCM460 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin at 37°C with 5% CO₂ and grown to 70-80% confluence. Cells were stimulated with lipopolysaccharide (LPS) (1 μ g/ml) and then co-cultured with *R. intestinalis* (1×10^9 CFU/ml in 30 μ l) for 24 h.

Quantitative polymerase chain reaction (qPCR). Total RNA was extracted and reverse transcribed into cDNA, which was then amplified by qPCR for detecting the mRNA levels of targeted genes. The primers used are shown in Table III. The amplified PCR products were identified by agarose gel electrophoresis. The results were quantitated using the $2^{-\Delta\Delta Cq}$ method, with expression of GAPDH mRNA as an internal reference.

Protein extraction and western blotting. Total protein was extracted with radioimmunoprecipitation assay (RIPA) buffer containing phosphatase and protease inhibitors. The protein concentration was determined using the BCA Protein Assay

Table II. Criteria for assessment of microscopic colonic damage.

Score	Criteria
0	No inflammation
1	Low level of lymphocyte infiltration with infiltration seen in a <10% hpf, no structural changes observed
2	Moderate lymphocyte infiltration with infiltration seen in 10-25% hpf, crypt elongation, bowel wall thickening which does not extend beyond mucosal layer, no evidence of ulceration
3	High level of lymphocyte infiltration with infiltration seen in 25-50% hpf, high vascular density, thickening of bowel wall which extends beyond mucosal layer
4	Marked degree of lymphocyte infiltration with infiltration seen in >50% hpf, high vascular density, crypt elongation with distortion, transmural bowel wall-thickening with ulceration

Hpf, high powered field.

Table III. List of quantitative polymerase chain reaction primers.

Primer	Forward (3'-5')	Reverse (5'-3')
IL-17	TACAACCGATCCACCTCACCTT	AGCCCACGGACACCAGTATCT
GAPDH	GGAAGCTTGTCAATGGAATC	TGATGACCCTTTTGGCTCCC

IL, interleukin.

kit (Beyotime, Shanghai, China). After quantification, the proteins were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Merck KGaA, Darmstadt, Germany). Membranes were blocked with 5% nonfat dried milk, immunoblotted with a GAPDH polyclonal Ab (1:1,000) and an IL-17 rabbit polyclonal Ab (1:1,000) at 4°C overnight, incubated with secondary antibodies for 1 h at 37°C, and then developed with an enhanced chemiluminescence (ECL) detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Cytokine detection in serum and cell supernatants. Cytokine concentrations in mouse serum and cell culture supernatants were quantified using IL-17 ELISA kits according to the manufacturers recommendations.

Statistical analyses. Data were expressed as the standard deviation of the mean and analyzed by one-way ANOVA (SPSS 18.0; SPSS, Inc., Chicago, IL, USA) with an SNK post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference. All reported results are the average of three independent experiments.

Results

R. intestinalis exerts anti-inflammatory effects in mice. Male, 6-week-old BALB/c mice were randomly divided into four groups (n=6): A control group without colitis, a group in which colitis was induced (TNBS), a group with colitis that was treated with *R. intestinalis* (R.I. Treat), and a group that was preconditioned with R.I. prior to the induction of colitis with TNBS (R.I. Pre). In the R.I. Pre group, *R. intestinalis*

was administered intragastrically daily for 2 days before colitis was induced with TNBS, while the R.I. Treat group was given *R. intestinalis* after the induction of colitis with TNBS, on days 5 and 7 (Fig. 1A). At the end of the experiment, the TNBS group mice had significantly higher DAI scores (Fig. 1B), shorter colon lengths (Fig. 1C and D), and higher histological scores (Fig. 1E) than the control group. These symptoms were significantly ameliorated by the administration of *R. intestinalis*. In addition, the R.I. Pre group showed improvement of inflammatory symptoms earlier (from day 6) and a greater anti-inflammatory effect overall than the R.I. Treat group (Fig. 1B-F), suggesting that early administration of *R. intestinalis* preparations could lead to better anti-inflammatory effects. Moreover, histological examination showed that the TNBS mice developed extensive ulceration in the colon, with large numbers of infiltrating neutrophils and some infiltrating mononuclear cells, while *R. intestinalis*-treated mice displayed only mild mucosal inflammation with a relatively a low level of neutrophil infiltration (Fig. 1F).

IL-17 is upregulated in human IBD specimens, and *R. intestinalis* inhibits the expression of IL-17 in mice with TNBS-induced colitis. IL-17 is mainly produced by Th17 cells, macrophages, and neutrophils (21). A number of recent studies have suggested that IL-17 plays an important role in the pathogenesis of IBD (22,23). Therefore, we evaluated IL-17 gene expression in a large cohort of HC, UC, and CD tissues (colon tissue and human peripheral blood mononuclear cells) using data deposited into the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database [no. GSE59071 (24) and no. GSE9452 (25)]. This analysis revealed that IL-17 mRNA

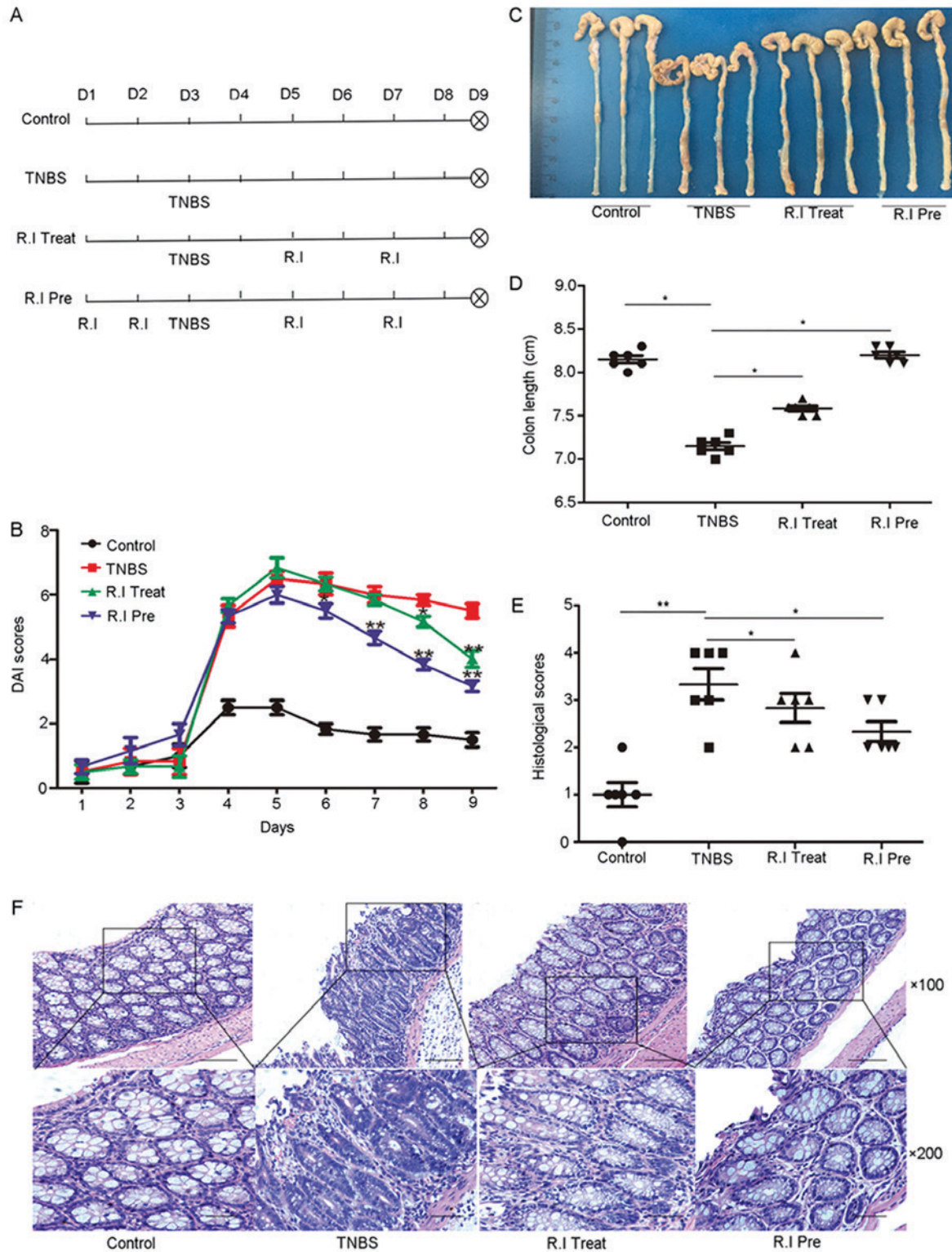


Figure 1. Growth conditions and colon histology. (A) Experimental design. (B) The DAI scores of each group. * $P < 0.05$, ** $P < 0.01$ vs. the TNBS group. (C) Image showing differences in colon length across the groups. (D) The mean colon lengths were plotted and analyzed statistically. (E) The colon histological scores. (F) Representative images of mouse colonic mucosa. The upper and lower panels are at magnification, $\times 100$ and $\times 200$, respectively. * $P < 0.05$, ** $P < 0.01$. TNBS, 2,4,6-trinitrobenzenesulfonic acid solution; DAI, disease activity index.

levels were significantly upregulated in UC and CD compared to the HC (Fig. 2A and B). This finding was confirmed in our animal experiment, which revealed higher IL-17 levels in both the serum and colon tissue of mice with TNBS-induced colitis. Furthermore, the elevated IL-17 was decreased by treatment with *R. intestinalis* (Fig. 2C and D).

R. intestinalis inhibits the expression of IL-17 in the human colon epithelial cell line NCM460. To verify the function of *R. intestinalis in vitro*, the human colon epithelial cell line NCM460 was stimulated with LPS to create a model of cellular inflammation. NCM460 cells were divided into four groups: A control group, an LPS group, a group treated with

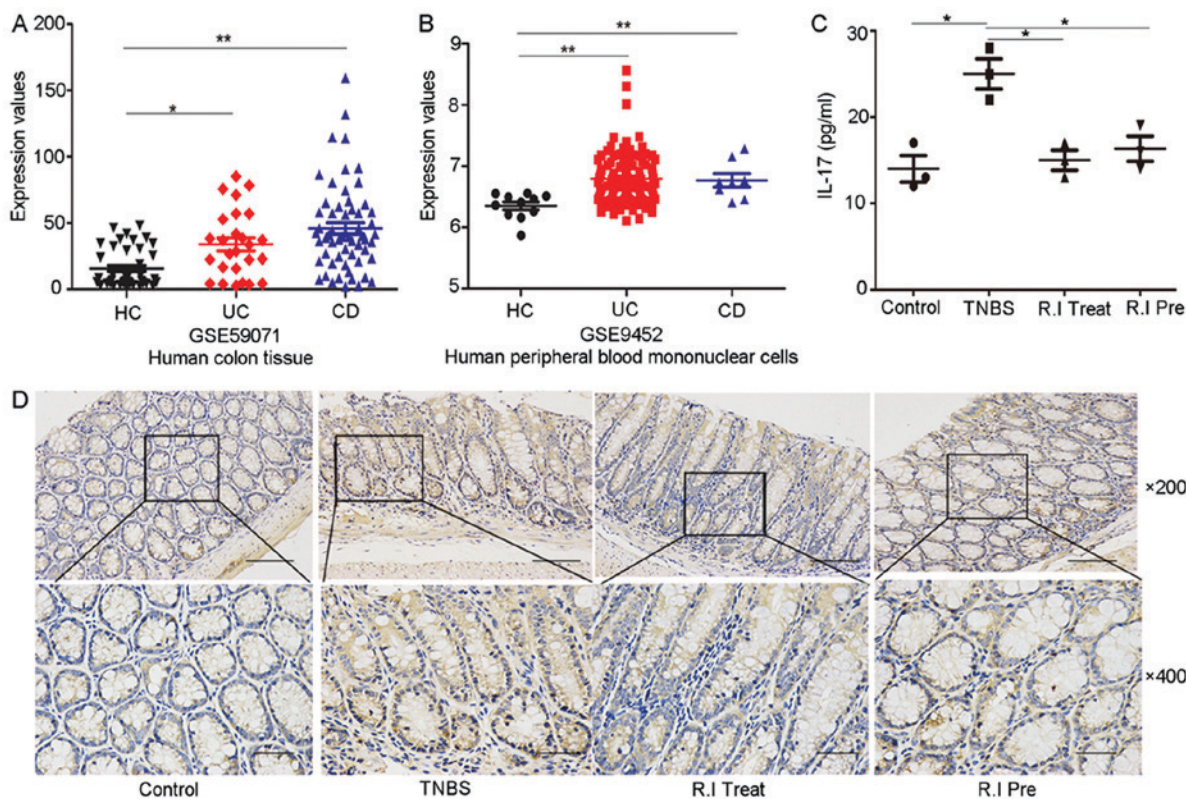


Figure 2. IL-17 expression in human specimens, and in mouse serum and colon tissue. Relative expression of IL-17 mRNA in HC, UC and CD tissues, based on data obtained from the NCBI's GEO database (A) GSE59071 and (B) GSE9452. (C) IL-17 concentrations in mouse serum. (D) Representative immunohistochemical staining of IL-17 in mouse colon mucosa. The upper and lower panels are magnification, x200 and x400, respectively. * $P < 0.05$, ** $P < 0.01$. HC, healthy control; UC, ulcerative colitis; CD, Crohn's disease; IL, interleukin.

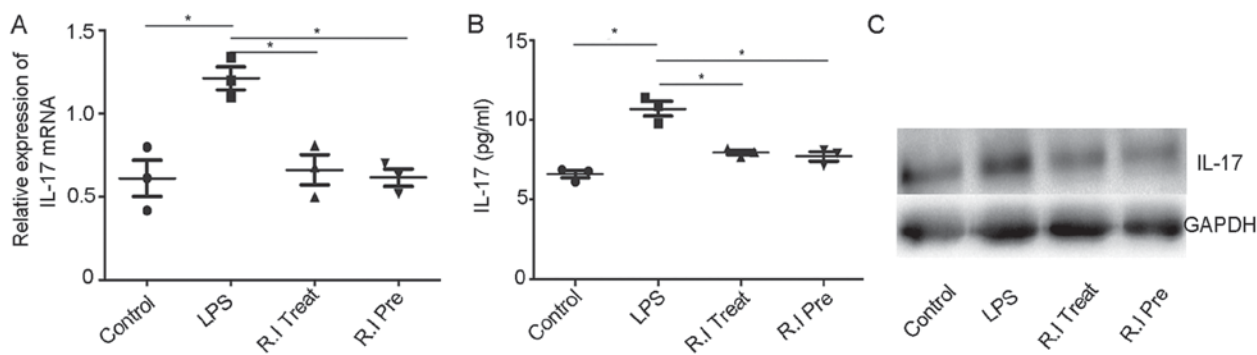


Figure 3. The effects of *R. intestinalis* on LPS-induced inflammation in NCM460 cells. In each group, the expression of IL-17 were assessed by (A) reverse transcription-quantitative polymerase chain reaction, (B) ELISA and (C) western blot analysis. * $P < 0.05$. IL, interleukin; LPS, lipopolysaccharide.

LPS and *R. intestinalis* (R.I. Treat), and a R.I. preconditioned group (R.I. Pre), in which NCM460 cells were co-cultured with *R. intestinalis* 12 h before inflammation was induced with LPS. The expression of IL-17 was detected by RT-qPCR, ELISA, and western blotting. In agreement with our *in vivo* results, the IL-17 mRNA levels were upregulated in the LPS-stimulated cells, and the induction of IL-17 could be decreased by either preconditioning or co-culturing with *R. intestinalis* (Fig. 3A). The real-time PCR results were confirmed by ELISA (Fig. 3B) and western blotting (Fig. 3C).

R. intestinalis promotes regulatory T cell differentiation in the mouse peripheral blood. In the *in vivo* studies, the numbers of CD25⁺Foxp3⁺ regulatory T cells (Treg) in the

peripheral blood of the TNBS group mice were statistically lower than in the control mice without colitis. After treatment with *R. intestinalis*, the numbers of CD4⁺CD25⁺Foxp3⁺ Treg cells in the peripheral blood cells increased compared with the TNBS group. Furthermore, the R.I. Pre group showed a greater increase in the frequency of CD4⁺CD25⁺Foxp3⁺ Treg than the R.I. Treat group (Fig. 4).

Discussion

The causes of IBD are multifactorial, but it is well recognized that disturbed intestinal bacterial homeostasis may contribute to the onset and recurrence of IBD (26). Although more and

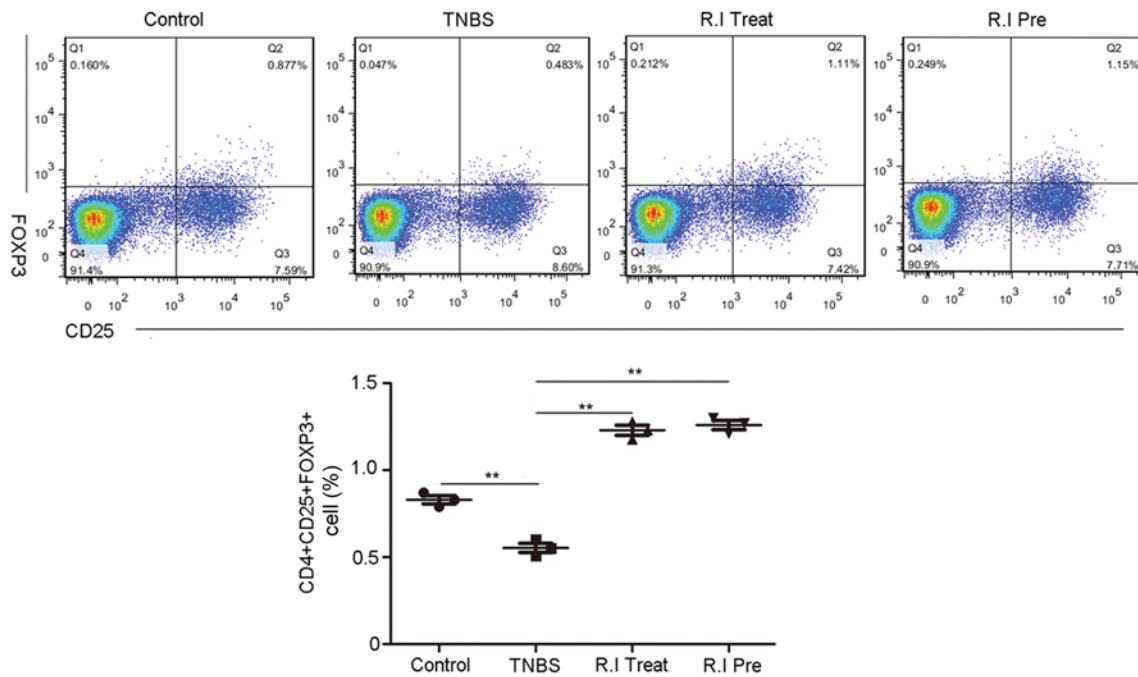


Figure 4. Induction of CD4⁺CD25⁺Foxp3⁺ regulatory T cells in the mouse peripheral blood. **P<0.01. CD, cluster of differentiation; R.I., *Roseburia intestinalis*.

more bacterial species have been shown to be associated with IBD and tested in animal models and clinical trials, the molecular mechanisms of the protective effects of probiotics are largely unknown. Recently, a growing number of studies have shown that probiotics play a protective role against colitis by effectively regulating the secretion of cytokines (upregulating the secretion of anti-inflammatory cytokines and inhibiting the secretion of pro-inflammatory cytokines) (27,28) and promoting the differentiation of Treg (29).

R. intestinalis is composed of Gram-positive to Gram-variable rods (30) and belongs to the family *Clostridium* cluster XIVa, which has a strong regulatory effect on the polarization of Treg cells (29). A number of studies have demonstrated that the abundance of *R. intestinalis* decreased to varying degrees in IBD patients. In agreement with these findings, our previous research using 16S-rRNA genome sequencing revealed that *R. intestinalis* decreased significantly in CD patients, leading to the hypothesis that the presence of *R. intestinalis* protects the intestine from inflammatory damage.

IL-17 is a pro-inflammatory cytokine that is reported to be closely related to IBD development (31). Consistent with these reports, we evaluated IL-17 gene expression in data sets deposited into the GEO database and found that it was significantly upregulated in UC and CD patients compared to HCs, confirming the association between IL-17 secretion and colon inflammation. To evaluate whether R.I. could inhibit colon inflammation, we measured the IL-17 levels in an animal model of chemically induced colitis and in an *in vitro* model of cellular inflammation in which LPS-treated NCM460 colon cells were co-cultured with *R. intestinalis*. Treg, a suppressive subset of CD4⁺ T cells, also play a critical role in the maintenance of intestinal homeostasis and self-tolerance (32). Our *in vivo* and *in vitro* results demonstrate that *R. intestinalis* can inhibit the secretion of IL-17 and promote the differentiation

of Treg in colorectal colitis. IL-10, which is the major effector cytokine secreted by Treg cells, plays crucial role during the resolution phase of infection (33). Some probiotics, including *Bacteroides fragilis* and *Parabacteroides distasonis*, reduce intestinal inflammation through the production of IL-10 (34,35), which suggests a potential mechanism through which *R. intestinalis* could act as a probiotic in the treatment of IBD.

Interestingly, in our study, when *R. intestinalis* was administered to the animals 2 days before the induction of colitis with TNBS, the protective effect was stronger and was apparent earlier than in the mice in which R.I. was administered after the induction of colitis, demonstrating that early feeding of *R. intestinalis* preparations could lead to better anti-inflammatory effects. This finding may be explained by data obtained with the Kaede transgenic mice, which revealed a constant trafficking of immune cells between the intestine and other parts of the body (36). Therefore, early intake of the probiotic may have anti-inflammatory effects on the immune cells trafficking through the intestine even before the inflammatory stimulus is administered. Jun Li and colleagues also found that a novel probiotic mixture effectively reduced hepatocellular carcinoma (HCC) growth in mice, especially when the probiotics were administered before the implantation of the tumor. This probiotic mixture, when given 1 week in advance of tumor implantation, resulted in a strong antitumor effect that was associated with reduced secretion of IL-17 and other anti-inflammatory factors (37).

In this study, *R. intestinalis* exerted significant anti-inflammatory effects in colorectal colitis *in vivo* and *in vitro* by inhibiting the secretion of IL-17. Furthermore, R.I. promoted the differentiation of Treg in the peripheral blood in a mouse model of TNBS-induced colitis. The detailed mechanisms through which *R. intestinalis* regulates cytokine

secretion and T cell differentiation are being investigated in our ongoing studies. In conclusion, *R. intestinalis* could be a candidate probiotic for the treatment or prevention of IBD, and further research will be necessary to elucidate the safety, efficacy, optimum dose, and mechanism of this bacterium in the clinical practice.

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Availability of data and materials

The datasets analyzed during the current study are available in the National Center for Biotechnology Information Gene Expression Omnibus database (nos. GSE59071 and GSE9452; ncbi.nlm.nih.gov/geo/). The rest of the data used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CZ performed experiments and wrote the article. KS contributed to the design of the study and revised the manuscript. ZS and YQ performed the data analysis and revised the manuscript. BT, WL, SW, KT and ZY performed the western blot analysis and immunohistochemistry experiments and revised the manuscript. XW contributed to the conception of the study and gave final approval for publication. All the authors read and approved the final version.

Ethics approval and consent to participate

All animal experiments were approved by the Ethical Committee of Medical Research, Third Xiangya Hospital, Affiliated Hospital of Central South University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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