

Escherichia coli promotes DSS-induced murine colitis recovery through activation of the TLR4/NF-κB signaling pathway

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Abstract. Increasing evidence suggests that intestinal microbiota have critical function in the pathogenesis of inflammatory bowel disease. This present study investigated the effects of Escherichia coli (E. coli) in mice with dextran sulfate sodium (DSS)-induced colitis. Furthermore, Toll-like receptor 4 (TLR4) and nuclear factor-κB (NF-κB) gene expression was measured by reverse transcription-quantitative polymerase chain reaction. In total, two experiments were performed. In the first experiment, four groups were established in BALB/c mice: i) Group A, control (no treatments); ii) group B, DSS-induced colitis; iii) group C, DSS-induced colitis bacteria depleted (BD) mice; and iv) group D, E. coli-treated group was also significantly higher. These effects were abolished in TLR4−/− mice, suggesting that E. coli may have promoted recovery through the TLR4 pathway. The present study indicated that E. coli promoted recovery from DSS-induced colitis in mice, potentially through activation of the TLR4/NF-κB signaling pathway.

Introduction

Inflammatory bowel disease (IBD) is a chronic, relapsing, non-specific inflammatory intestinal condition, which is characterized by acute exacerbation followed by remission. IBD includes ulcerative colitis (UC) and Crohn's disease (CD). UC is a chronic inflammatory disorder of the colon, which causes bloody diarrhea, abdominal pain and weight loss (1). The underlying pathogenic mechanisms of IBD remain unclear, although they have been attributed partially to dysregulation of immune reactions that respond to intestinal flora, and to genetic and environmental factors (1).

Dextran sulfate sodium (DSS)-induced colitis is a well-established experimental model that presents with various signs and symptoms of human UC, including diarrhea, weight loss, bloody stools, mucosal ulceration, and shortening of the large intestine (2). Previous research has shown that mice with acute DSS-induced colitis display similar expression profiles of cytokines and histological changes to those observed in human IBD, particularly UC (2).

The incidence of IBD in China is increasing annually (3). The role of intestinal microbiota in the pathophysiology of IBD has recently garnered attention. Previous studies have demonstrated the intestinal microbiota serves an important role in gut inflammation, as follows: i) In the cluster of differentiation 45Rb+/high transfer IBD model, the transfer of naive helper T cells to Rag2−/− mice leads to microbiota-dependent intestinal inflammation, whereas in germ-free mice, transfer of naive helper T cells do not develop colitis (4,5). ii) It has been reported that fecal stream diversion can improve intestinal inflammation in CD (6). iii) Antibiotics are effective in the treatment of IBD to some extent (7). iv) Antibiotics, such as ciprofloxacin and metronidazole, are also available for anal lesions and the prevention of recurrence in CD (8). v) Many IBD susceptibility genes are involved in the identification and processing of microbiota (9). However, the role of normal...
intestinal flora in the recovery of colitis has received little attention, and has not been reported in the literature. The present study investigated this topic and aimed to clarify the role of intestinal *E. coli* in the recovery process of colitis. The results indicated that the presence of normal intestinal flora may be a necessary condition for the recovery of colitis, potentially through activation of the Toll-like receptor 4 (TLR4)/nuclear factor-κB (NF-κB) signaling pathway, which provides evidence for the use of intestinal flora to treat IBD.

**Materials and methods**

**Reagents and animals.** DSS solution (3.5%; Sigma Healthcare, Melbourne, Australia) was dissolved in sterile, distilled water and freshly prepared every other day (10). Antibiotic solutions were composed of kanamycin (8 mg/ml), gentamicin (0.7 mg/ml), polymyxin 34,000 U/ml, metronidazole (4.3 mg/ml) and vancomycin (0.9 mg/ml).

A total of 40 adult BALB/c (8-week-old females; 19.20±2 g), 10 C57BL/6 (8-week-old females; 18.8±2 g) and 10 TLR4−/− mice (8-week-old females; 19±2 g) were purchased from the Animal Experimental Center of Guangdong Academy of Medical Sciences (Guangzhou, China). Animals were maintained under standard conditions, and fed rodent food and water, according to the Guide for the Care and Use of Laboratory Animals (11). The mice were maintained as follows: Temperature, 20-22˚C; relative humidity, 55±5%, 12-h light/dark cycle, and *ad libitum* access to food and water. Mice were fed in cages containing an average of six mice/cage.

**DSS-induced colitis model and bacteria-depleted (BD) mice.** Mice were administered 3.5% DSS in drinking water for 5 days, followed by normal drinking water for 14 days, according to a method described previously by Chen et al (10).

Prior to the experiment, 6-week-old adult mice were depleted from intestinal bacteria by administration of antibiotic solution (100 μl/day/mouse) for 2 weeks. Fresh mouse fecal samples were harvested daily and cultured in aerobic and anaerobic conditions. When bacterial growth could not be detected in the culture media, the mice were classified as BD mice.

**Group classification.** The mice were randomly divided into 4 groups as follows: i) Group A, normal drinking water; ii) group B, DSS-induced colitis only; iii) group C, DSS-induced colitis in BD mice; and iv) group D, DSS-induced colitis in BD mice treated with *E. coli* (fed *E. coli* 1x10^6 CFU once every other day feeding). The animals received treatments for 14 days. The experiments were carried out using BALB/c mice, and subsequently repeated with C57BL/6 and TLR4−/− mice.

In the TLR4−/− experiment, the mice were randomly divided into three groups as follows: i) Control group (Group A1); ii) Group B1, DSS-induced colitis mice in BD C57BL/6 mice treated with *E. coli* as described previously in Group D; Group C1, DSS-induced colitis mice in BD TLR4−/− mice treated with *E. coli* as described previously in Group D.

**Outcome measures**

**Clinical indicators.** Animal body weight, stool characteristics and occult blood were recorded and evaluated daily. These parameters were scored by a trained observer blinded to the protocol, in order to calculate disease activity index (DAI), as described in Table I. The mice were sacrificed on day 14 using CO₂, and total colon specimens were collected. The samples were stained with hematoxylin and eosin (H&E), which was used for the pathological score evaluation of the colon tissues. The histological severity of colitis was classified into mucosal damage (D) and extent of disease (E), according to the scoring criteria proposed by Cooper et al (12). The criteria were as follows: D: 0 points, none; 1 point: 1/3 of the crypt near the basement membrane was lost; 2 points, 2/3 of the crypt near the basement membrane was lost; 3 points, all crypts were destroyed, leaving only the surface mucosal epithelium; 4 points: Crypt epithelium was lost. E: 0 points, none; 1 point, focal lesions; 2 points, lesions were present in ~1/3 mucosa; 3 points, lesions were present in 2/3 mucosa; 4 points, lesions were present in all mucosal tissues. The histological score (HS) is equal to the product of D and E, i.e., HS=D x E.

**Histopathology.** The distal ileum, colon and rectum were removed and cleaned with cold sterile saline. The end of the colon (1 cm from anus) was divided into 0.5 cm sections. The histological specimens were fixed in 10% neutral formalin at room temperature for 12 h, paraffin embedded, sliced and stained with hematoxylin (Mayer) for 5 min and 0.5% eosin for 1-3 min at room temperature (H&E staining). A blinded method was used in order to score the pathological sections according to the severity of colitis, as previously described (13).

**Colonic myeloperoxidase (MPO) detection.** Tissue MPO activity was determined using the MPO Peroxidation Assay kit (cat. no. KA1338; Abnova, Taipei, Taiwan), as described previously (14). MPO is an index used to evaluate neutrophil infiltration (15). Colon tissue weights were measured prior to cooling in liquid nitrogen and stored at -80˚C. The tissues were homogenized four times (5 sec each) with 10 sec intervals by ultrasonic pulverization (14 kHz) at 4˚C, and the supernatant was removed by centrifugation at 14,000 x g for 20 min at 4˚C. After 5 min, the absorbance value was measured at 460 nm to determine the MPO activity of each sample.

**Immunohistochemical method for the detection of NF-κB.** The application of the standard three-step antibody method was used to detect NF-κB expression levels (16,17). Antibody I [NF-κB p65 (F-6), cat. no. sc-8008; Santa Cruz Biotechnology, Inc., Dallas, TX, USA] was a monoclonal antibody specific for the NF-κB p65 subunit. Antibody II [goat anti-mouse immunoglobulin G (IgG)-B: sc-2039, Santa Cruz Biotechnology, Inc.] was a biotin-conjugated mouse IgG antibody specific for the goat anti-mouse epitope. Antibody I (10 mg/ml) was incubated with the sections for 1 h in a humid chamber at room temperature. Subsequently, Antibody II (2 mg/ml) was incubated with the sections for 1 h in a humid chamber at room temperature. Finally, the sections were incubated with avidin-biotin complex reagent containing horseradish peroxidase (cat. no. 554058; BD Biosciences, Franklin Lakes, NJ,
Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) for the detection of TLR4 expression. RNA was extracted using MiniBEST Universal RNA Extraction kit (cat. no. 9767; Takara Biotechnology Co., Ltd., Dalian, China), SuperScript™ IV First Chain Synthesis system (cat. no. 18091050; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used for RT, and TaqMan™ Fast Advanced Master Mix (cat. no. 4444557; Thermo Fisher Scientific, Inc.) was used for PCR. Total RNA was isolated from colon tissues and qPCR was performed as previously described (18,19). Gene expression was normalized to GAPDH using the 2ΔΔCt method (20). The primers used were as follows: GAPDH (253 bp), 5'-ACAGCAAACAGGTGGTTGACAC-3' (forward) and 5'-TCTGAGGTGGTACACGCGACACTT-3' (reverse); TLR4 (239 bp), 5'-CCAGAGCCGTGGTGTATCT-3' (forward) and 5'-TCAAGGGTTTCCCCATCCACAC-3' (reverse); and NF-κB p65 (251 bp), 5'-GGCAGACTCCTTATCAACC-3' (forward) and 5'-GAGGTGTCTGCCCATCGT-3' (reverse). The data were representative of at least three independent experiments.

Statistical analysis. The results were analyzed using GraphPad statistical software (v.5.0; GraphPad Software, Inc., La Jolla, CA, USA). Experiments were repeated three times. The results were presented as the mean ± standard deviation. One-way analysis of variance followed by Bonferroni’s correction was used for comparisons between groups. Kruskal-Wallis test and Steel-Dwass test were performed to compare histological scores among the test groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Clinical findings. The body weight of mice decreased following ingestion of DSS. When DSS was ceased after 5 days, the weight of the mice slightly decreased until day 6. In the BALB/c experiment, the non-<i>E. coli</i> BD treatment group mice continued to decrease in weight after DSS treatment was ceased. The body weight of the mice in the <i>E. coli</i> treatment group declined slightly (Fig. 1A). In the experiment using TLR4<sup>−/−</sup> mice, the <i>E. coli</i> treatment BD TLR4<sup>−/−</sup> group retained the weight decrease. The body weight of the <i>E. coli</i>-treated DSS-induced BD C57BL/6 mice declined slightly, but increased following the cessation of DSS treatment (Fig. 1B).

In BALB/c mice, the survival rate at the end of the experiment was 100% for groups A, B and D. Group C exhibited a survival rate of 60% (Fig. 2A), suggesting that bacterial depletion followed by DSS-induced colitis resulted in increased mortality. In the experiment using C57BL/6 and TLR4<sup>−/−</sup> mice, Groups A1 and B1 had a survival rate of 100%, whereas C1 had a survival rate of 60%, suggesting that treatment with <i>E. coli</i> could promote recovery in BD wild-type mice, but not in TLR4<sup>−/−</sup> mice. This finding demonstrated that <i>E. coli</i> may have promoted recovery through the TLR4 pathway (Fig. 2B).

DAI was used to assess the severity of colitis in the DSS-induced colitis mice. All mice with DSS-induced colitis underwent fecal occult blood tests from day 3 of DSS ingestion. In BALB/c mice, Groups B and D had significantly lower DAI scores compared with Group C (P<0.05; Fig. 3A). In the TLR4<sup>−/−</sup> experiment, the DAI score of groups A1 and B1 declined slowly from day 10 of the experiment. However, the clinical score of the TLR4<sup>−/−</sup> group exhibited a continuous increase (Fig. 3B).

Histopathology. In BALB/c mice with DSS-induced colitis (Group B), the colon was shorter than that noted in the mice of group A, and showed extensive mucosal and glandular defects with crypt destruction and a large amount of inflammatory cell infiltration (Fig. 4A and B). In Group D, the colonic mucosa defects were partially repaired, compared with group C (Fig. 4C and D). Concomitantly, the crypt damage decreased and inflammatory cell infiltration and depth of inflammation were reduced. There was a significant reduction in tissue damage in group D compared with group C (P=0.03; Fig. 4E).

In the TLR4<sup>−/−</sup> experiments (Fig. 5), no signs of inflammatory response were noted in Group A1 (Fig. 5A). Group C1 indicated extensive mucosal and glandular defects, crypt destruction and inflammatory cell infiltration (Fig. 5C). In the <i>E. coli</i>-treated BD C57BL/6 mice with DSS-induced colitis (Group B1), crypt damage decreased, and inflammatory cell infiltration and depth of inflammation were reduced, thus indicating that the colonic mucosal defects were partially repaired compared with in group C1 (Fig. 5B). The differences noted between TLR4<sup>−/−</sup> and wild type C57BL/6 group were significant (P<0.05; Fig. 5D).

<i>MPO</i> activity. In wild-type BALB/c mice, the intestinal mucosal MPO activity in Group C was significantly reduced compared with that in Groups B and D (P<0.05). Notably, the intestinal mucosal MPO activity in the <i>E. coli</i>-treated BD TLR4<sup>−/−</sup> group was significantly reduced compared with that noted in the <i>E. coli</i>-treated BD C57BL/6 group (P<0.05; Fig. 6).

| Score | Stool | Hematochezia | Weight loss (%) |
|-------|-------|--------------|-----------------|
| 0     | Normal form | Occult blood negative | 0 |
| 1     | Loose and not forming | Occult | 1-5 |
| 2     | Loose and not forming | Occult blood positive | 5-10 |
| 3     | Watery stool | Eye blood | 10-15 |
| 4     | Watery stool | Eye blood | >15 |
NF-κB activation. In BALB/c mice, activated NF-κB was mainly localized in the lamina propria, as demonstrated by brown nuclear staining under high magnification (data not shown). Groups B and D had higher NF-κB expression.
whereas group C had a significantly lower expression level of NF-κB, compared with these groups (P<0.05). In addition, the results indicated that the TLR4−/− group exhibited less NF-κB expression compared with the E. coli-treated BD C57BL/6 group (P<0.05; Fig. 7), suggesting that TLR4 knockout decreased NF-κB cell activation by E. coli.

**Discussion**

IBD is thought to be caused by an imbalance of the immune response and the intestinal flora, although the mechanism by which the intestinal flora affects the development of this disease remains unclear (21,22). The intestinal microbiome has been shown to have an important role in the development of IBD (13,23). The mutual communication between intestinal microbes and the intestinal mucosa regulates the expression of intestinal genes (23).

Several studies in various animal colitis models have demonstrated the importance of the resident luminal flora in the initiation and perpetuation of intestinal inflammation (24). Non-pathogenic microorganisms in the intestinal mucosa may alter the immune response in the intestinal epithelial barrier function in vitro (24). Feces within the intestinal lumen can

**TLR4 and NF-κB mRNA expression.** In control and DSS-induced BD BALB/c mice, the mRNA expression levels of TLR4 and NF-κB was relatively low. Following administration of E. coli, TLR4 and NF-κB mRNA expression levels increased significantly (P<0.05; Fig. 8A).

In the experiment using TLR4−/− mice, Group C1 exhibited significantly decreased mRNA levels of TLR4 and NF-κB compared with group B1 (P<0.05; Fig. 8B).
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Figure 6. *Escherichia coli* treatment decreases MPO activity in DSS-induced BD mice, but not DSS-induced BD TLR4-/- mice. MPO was measured in order to evaluate neutrophil infiltration. *P<0.05. Group A, control; Group B, DSS; Group C, DSS + BD; Group D, DSS + BD + *E. coli*; Group A1, control (C57); Group B1, DSS + BD + *E. coli* (C57); Group C1, DSS + BD + *E. coli* (TLR4-/-). MPO, myeloperoxidase; DSS, dextran sulfate sodium; BD, bacteria-depleted; TLR4, Toll-like receptor 4; *E. coli, Escherichia coli*.

Figure 7. *Escherichia coli* treatment increases NF-κB activity in DSS-induced BD mice (group D), but not DSS-induced BD TLR4-/- mice (group C1). Group D vs. group C (*P<0.05); NF-κB activity in TLR4-/- mice experiment: Group C1 vs. group B1 (*P<0.05). Group A, control; Group B, DSS; Group C, DSS + BD; Group D, DSS + BD + *E. coli*; Group A1, control (C57); Group B1, DSS + BD + *E. coli* (C57); Group C1, DSS + BD + *E. coli* (TLR4-/-). DSS, dextran sulfate sodium; BD, bacteria-depleted; NF-κB, nuclear factor-κB; TLR4, Toll-like receptor 4.

Figure 5. Histological analysis of mice colon sections in the TLR4-/- mice experiment. (A) Hematoxylin and eosin staining of the mouse colon section in control C57BL/6 mice (group A1), (B) *E. coli*-treated DSS-induced BD C57BL/6 mice (group B1) and (C) *E. coli*-treated DSS-induced BD TLR4-/- mice (group C1). TLR4-/- mice had extensive mucosal and glandular defects, crypt destruction and inflammatory cell infiltration. In *E. coli*-treated DSS-induced mice, the colon mucosa defects were partially repaired, the crypt damage decreased, inflammatory cell infiltration and depth of inflammation was reduced. (D) Colon sections were histologically scored. *P=0.04. DSS, dextran sulfate sodium; BD, bacteria-depleted; TLR4, Toll-like receptor 4; *E. coli, Escherichia coli*.
contain up to $1 \times 10^{11}$ bacteria per gram, with *E. coli* as the predominant species (25). *E. coli* is the most populous bacteria in the gut of humans and various animals (25). To the best of our knowledge, no relevant reports exist in regards to the involvement of the normal intestinal *E. coli* in experimental colitis recovery.

In general, mouse colitis caused by DSS induction heals itself (26). In contrast to this outcome, BD mice that were orally administered antibiotics for 2 weeks in the present study were unable to recover from damage caused by DSS induction, indicating that host intestinal microbial interactions were necessary for recovery. The results demonstrated that commensal *E. coli* reduced mortality in BD mice with DSS-induced colitis, and promoted colitis recovery. This suggested that the presence of normal intestinal *E. coli* is necessary for recovery.

Inflammation and necrosis of intestinal mucosa leads to colon shortening and these alterations reflect the degree of damage to the intestinal tissue. In the current study, the colon length of DSS-induced mice was shorter than that of normal mice. *E. coli* treatment could prevent colon shortening, which may be associated with the ability of *E. coli* to reduce intestinal inflammation and scarring (27). With regard to the histopathological findings, the *E. coli* treatment groups had restored intestinal mucosal integrity, reduced intestinal hemorrhage and inflammatory cell infiltration, and had a lower histological score than DSS-induced BD mice without *E. coli* treatment. However, in TLR4$^{-/-}$ mice, *E. coli* treatment could not reduce the colon tissue damage. These results indicated that *E. coli* promoted recovery of DSS-induced colonic inflammation and that this effect was potentially mediated, at least partially, through the TLR4 receptor.

NF-κB is a transcription factor that regulates the expression of a series of inflammatory factors (28). In the present study, *E. coli*-treated DSS-induced BD mice expressed more NF-κB compared with untreated DSS-induced BD mice, which suggested that *E. coli* promoted NF-κB activation and accelerated inflammation recovery. By contrast, TLR4$^{-/-}$ mice showed no significant recovery from colitis, suggesting that *E. coli* promoted DSS-induced colitis recovery through activation of the TLR4/NF-κB signaling pathway.

In conclusion, the presence of normal intestinal flora may be a necessary condition for the recovery of colitis, potentially through activation of the TLR4/NF-κB signaling pathway. This indicated that host intestinal microbial interactions were critical to colitis recovery. This study provided evidence for the use of intestinal flora to treat IBD. In the future, more studies are required to identify the mechanisms and confirm its efficacy.

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**Availability of data and materials**

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

**Authors’ contributions**

WL and JC were responsible for experimental design. JL and BD performed the experiments. AC, FH, BP and ZW were responsible for statistical analysis. JL and BD wrote the manuscript. All authors were involved in manuscript revision.

**Ethics approval and consent to participate**

The protocol was approved by The Review Board of the Institute of Medical Animal Laboratory at the Guangzhou Medical University (Guangzhou, China).
Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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