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***Bifidobacterium infantis* attenuates colitis by regulating T cell subsets responses**

Yuan KT *et al*. Bifidobacterium infantis and colitis

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**Abstract**

**AIM:** to investigate the effect of *Bifidobacterium infantis* (*B. infantis*) on the T cell subsets and attenuating the severity of experimental colitis in mice

**METHODS:** Normal BALB/c mice were fed with different doses of *B. infantis* for 3 wk, T cell subsets and related cytokine profiles in mesenteric lymph nodes (MLN) were detected by Flow cytometry and real-time RT-PCR. Colitis was induced by trinitrobenzene sulfonic acid (TNBS) in mice. Before colitis induction, mice were fed with high dose *B. infantis* for 3 wk. Cytokine profiles in MLN and histological changes of colonic tissue were examined 6 d after colitis induction.

**RESULTS:** No significant cytokine profiles changed in normal mice fed with low dose *B. infantis*. But Th1-related cytokines (IL-2, IFN-γ, IL-12p40), Th17-related transcription factor and cytokines (RORγt, IL-21, IL-23), regulatory T cells (Tregs)-related transcription factor and cytokines (Foxp3, IL-10) were increased in normal mice fed with high dose *B. infantis*. Furthermore, flow cytometry assay showed *B. infantis* increased the number of CD4+Foxp3+ Tregs and Th17 cells in MLN. Colitis was induced by TNBS in mice, characterized by colonic inflammation and aberrant Th1 and Th17 responses. Feeding with high dose *B. infantis* for 3 wk before colitis induction decreased the inflammatory cell infiltration and goblet cell depletion and restored the intestinal epithelium. In addition, *B. infantis* feeding reduced Th1-related cytokines (T-bet, IL-2 and IFN-γ) and Th17-related cytokines (IL-12p40, RORγt, IL-17A, IL-21 and IL-23), and increased Tregs-related molecules (Foxp3, IL-10 and TGF-β) in colitis mice.

**CONCLUSION:** *B. infantis* effectively attenuated TNBS-induced colitis by decreasing Th1 and Th17 responses and increasing Foxp3+ Tregs response in colonic mucosa.

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**Key words:** Bifidobacterium; Colitis; Cytokines; Th17; Regulatory T cells

**Core tip:** Inflammatory bowel disease was a common autoimmune disorder characterized by chronic inflammation of the gastrointestinal tract. Abnormal immune cell responses contribute to the pathogenesis of the colitis. Probiotics are found to regulate the intestinal immune system and play a beneficial role in treating colitis. In our study, we showed that single *Bifidobacterium infantis* (*B. infantis*) reduced the intestinal inflammation in TNBS-induced colitis mice though decreasing the Th1 and Th17 responses and promoting the Foxp3+ Tregs response in MLN. This underlying mechanism of regulating immune system of *B. infantis* may have significant clinical implications in treating inflammatory bowel diseases and preventing colorectal cancer.

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**INTRODUCTION**

Inflammatory bowel diseases (IBD) are an autoimmune disorder that is characterized by chronic inflammation of the gastrointestinal tract. IBD comprise mainly Crohn's disease (CD)and ulcerative colitis. The pathogenesis of IBD involves genetic susceptibility, gut microbiota and host immune system[1]. It is widely accepted that the abnormal gut microbiota was a key to the IBD pathogenesis. Several studies showed a striking reduction of commensal microbiota including Firmicutes and Bacteroides and an overgrowth of Proteobacteria in the intestinal tissue from IBD patients[2]. Therefore extensive research is focused on modifying the intestinal flora with probiotic bacteria.

Probiotics are live microorganisms that benefit health when supplied in adequate amounts. Among them, Bifidobacterium infantis (*B. infantis*) is a commensal microbe isolated from the human gastrointestinal mucosa and has been studied for its ability to treat irritable bowel syndrome[3], and to protect against inflammatory conditions including Helicobacter pylori infection[4] and IBD[5-7]. The mechanisms behind the amelioration of IBD are still largely unknown. IBD results from an inadequate Foxp3+ regulatory T cells (Tregs) response in the face of an overly exuberant Th1 and Th17 responses in CD and Th2 response in ulcerative colitis[8]. *B. infantis* feeding reduced the number of Th1 and Th17 cells and increased the proportion of Foxp3+ Tregs within the lamina propria (LP) in DSS-induced colitis which mimics CD[5]. Moreover, *B. infantis* had some influence on some cytokine expressions. For instance, *B. infantis* inhibited the expression of Th17 related IL-17A and induced the expression of IL-10 in DSS-induced colitis mice[7] and decreased Th1 proinflammatory cytokines (TNF-α, IL-12 and IFN-γ) and maintained the level of TGF-β in IL-10 KO mice that is a spontaneous genetic model of CD[6]. However, the effects of single *B. infantis* feeding on trinitrobenzene sulfonic acid (TNBS)–induced colitis which is an animal model of CD and its underlying immune mechanism were poorly understood.

We hypothesized that *B. infantis* may ameliorate the intestinal inflammation in TNBS–induced colitis by affecting the differentiation of CD4+ T subsets and cytokine expressions.

**MATERIALS AND METHODS**

***Animals***

Female BALB/c mice, 6-8-wk old, were purchased from Chongqing Medical University and maintained in standard conditions. All the experimental procedures were approved and performed according to the guidelines of the Institutional Animal Ethics Committee.

***Experiment design***

In the first experiment, normal mice were randomly divided into 3 groups of five animals each: control group, low dose *B. infantis* (6 × 107 cfu/d) group (Low-*B. infantis*) and high dose *B. infantis* (6 × 108 cfu/d) group (High-*B. infantis*). *B. infantis* (6 × 109 cfu/g) was provided by Guangzhou Baoxing Biotechnology Company (Guangzhou, China). Mice received oral administration of low dose or high dose *B. infantis* once daily for 3 wk. The control group received the PBS.

For induction of colitis, 50 μl TNBS [50 mg/kg body weight (BW)] dissolved in 0.9% (w/v) NaCl/ethanol (50:50 v/v) was intrarectally administered to anesthetized mice 4cm proximal to the anal verge through a 3.5F catheter as described[9]. TNBS was purchased from Sigma (St. Louis, MO, United States). In the TNBS colitis model, three groups of five animals each were utilized. The Group 1 was the negative control group, which were administered PBS, instead of TNBS. Group 2 was the TBNB-3 group as these mice were sacrificed by cervical dislocation 3 d after administration of TNBS. Group 3 was TNBS-6 group which were sacrificed 6 d after administration of TNBS.

In the *B. infantis* treated models, two groups of five animals each were used. Group 1 received a daily oral administration of high dose *B. infantis* (6 × 108 cfu/d) for 3 wk before colitis induction (*B. infantis*-TNBS group). The colitis control animals were administered PBS using the same technique before colitis induction (PBS-TNBS group). Animals were sacrificed by cervical dislocation 6 days after TNBS induction. After cervication, MLNs were selected carefully from an inflamed area of the bowel in all animals. The summary of study population is in table 1.

***RNA extraction and real time PCR***

Total RNA was extracted from MLN in mice using TRIzol reagent (Invitrogen) according to the manufacturer’s instruction. 2 μg total RNA was transcribed to cDNA using a reverse-transcription kit (TaKaRa). Primer sequence was as follows: T-bet (forward) 5′-CAACAA CCCCTTTGCCAAAG-3′ and (reverse) 5′-TCCCCCAAGCAGTTGACAGT-3′, IL-2 (forward) 5′-CCTGAGCAGGATGGAGAATTACA-3′ and (reverse) 5′-TCCAGA ACATGCCGCAGAG-3′, IFN-γ (forward) 5′-TCAAGTGGCATAGATGTGGAA, GAA-3′ and (reverse) 5′-TGGCTTGCAGGATTTTCATG-3′, IL-12p4 (forward) 5′-GGAAGCACGGCAGCAGAATA-3′ and (reverse) 5′-AACTTGAGGGAGAAG TAGGAATGG-3′, GATA3 (forward) 5′-CCTACCGGGTTCGGATGTAA-3′ and (reverse) 5′-CACACACTCCCTGCCTTCTGT-3′, IL-4 (forward) 5′-ACAGGAGAA GGGACGCCAT-3′ and (reverse) 5′-GAAGCCCTACAGACGAGCTCA-3′, RORγt (forward) 5′-GCTGTCAAAGTGATCTGGAG-3′ and (reverse) 5′-GGTGGAACT TATGGGAAATC-3′, IL-21 (forward) 5′-GAGGACCCTTGTCTGTCTGG-3′ and (reverse) 5′-TCATCTTTTGAAGGAGCCATTT-3′, IL-23 (forward) 5′-GAGGTG GACTGGACTACCGA-3′ and (reverse) 5′-GGAACTGCTACTGCTCTTGA-3′, Foxp3 (forward) 5′-CCCATCCCCAGGAGTCTTG-3′ and (reverse) 5′-CCATGACTA GGGGCACTGTA-3′, IL-10 (forward) 5′-ATAACTGCACCCACTTCCCA-3′ and (reverse) 5′-TCATTTCCGATAAGGCTTGG-3′, TGF-β (forward) 5′-GAAGGCAGA GTTCAGGGTCTT-3′ and (reverse) 5′-GGTTCCTGTCTTTGTGGTGAA-3′, GAPDH (forward) 5′-CATCACTGCCACCCAGAAGA-3′ and (reverse) 5′-TGAAGT CGCAGGAGACAACC-3′. Real-time reverse transcription polymerase chain reaction (RT-PCR) was performed with ABI 7300. The reaction mixture consisted of 5.0 μl of cDNA, 12.5 μl of SYBR Green qPCR SuperMix-UDG/ROX (Invitrogen) and 6.5 μl of ddH20. All the PCR experiments were performed under the same condition as follows: 95 °C for 10 min, 95 °C for 15 s and 60 °C for 1 min, up to 40 cycles. GAPDH was used as an internal control. Gene expression was calculated relative to GAPDH.

***Flow cytometry analysis***

Single cell suspensions were prepared from MLN cells of each group. In order to identify Tregs in MLN, cells were surface labeled with a FITC labled anti-mouse CD4 (eBioscience) and PE labeled anti-mouse CD25 antibodies (eBioscience). Surface labeling was followed by permeabilization with the Foxp3 fixation/permeabilization buffer and intracellular labeling APC anti-mouse/rat Foxp3 antibody (eBioscience). To measure Th17 cells, cells were pre-stimulated for 4 hours with PMA (50 ng/ml, Sigma) and ionomycin (500 ng/ml, Sigma) in the presence of Brefeldin A (1 mg/ml, eBioscience) at 37 °C and 5% CO2. Then cells were washed in PBS and surface labeled with CD3-FITC and CD4-PE-Cy5. For intracelluar labeling IL-17A, these cells were permeabilized with IL-17A fixation/permeabilization buffer (eBioscience) and stained with anti-IL-17A-PE (eBioscience). Cells were incubated with Affinity Purified anti-mouse CD16/32 to block non-specific staining. IgG isotypes (BD pharmingen) were used as a control in all FACS experiments. Data were acquired on a FACS Calibur flow cytometer with CELLQuest software.

***Histological analysis***

For histological examination, a sample of colonic tissue located precisely 3 cm above the anal canal was obtained from the mice of all groups. The colonic tissues were fixed in 10% neutral buffered formalin and embedded in paraffin for histological analysis. Four-micrometer-sections were deparaffinized with xylene and stained with hematoxylin and eosin using routine techniques.

***Statistical analysis***

Statistical analysis was carried out using GraphPad Prism for Windows (Version 5.0). Significant differences between different doses of *B. infantis* groups were assessed by one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison test. Differences among experimental colitis groups induced by TNBS for 3 d or 6 d were evaluated with one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison test. The difference between PBS-TNBS group and *B. infantis*-TNBS were measured by a paired *t* test. Data are given as means ± SE. Differences were considered significant at *P* < 0.05.

**RESULTS**

***B. infantis augmented the expression of Th1-, Th17- and Tregs-related cytokines in MLN in normal mice***

To investigate the effect of *B. infantis* on T cell cytokine expressions, we studied the relative expression of different cytokines in MLN from normal mice by real-time RT-PCR. Low dose *B. infantis* feeding (6 × 107 cfu/d) caused no significant change in all cytokine expressions, but high dose *B. infantis* feeding (6 × 108 cfu/d) augmented the expression of Th1-, Th17- and Tregs-related cytokines in MLN (Figure 1). After feeding with high dose *B. infantis*, the mRNA of Th1 cytokines IL-2 and IL-12p40 increased significantly in the MLN when compared with PBS control (IL-2, *P <* 0.05; IL-12p40, *P* < 0.005 ) (Figure 1A and C). The mRNA level of proinflammatory cytokine IFN-γ also seemed to elevate but did not reach the significance in comparison with control (Figure 1B). Th17-related cytokine IL-23 and transcription factor RORγ increased more than 2-fold in MLN by *B. infantis* feeding compared with control group (*P <* 0.005) (Figure 1E and G). IL-21 also tended to rise. Moreover, mice receiving *B. infantis* had a significant increase of Foxp3 and IL-10 mRNA in MLN in comparison with PBS control (Foxp3, *P <* 0.005; IL-10, *P <* 0.05) (Figure 1H and I). However, no difference in the mRNA expression of Th2-related cytokine IL-4 was detected after *B. infantis* feeding (Figure 1D).

***B. infantis* *increased the levels of CD4+T cells, Th17 cells and CD4+Foxp3+ Tregs in the MNL in normal mice***

We measured the numbers of CD4+T cells and Th17 cells in the MLN in normal mice by flow cytometry to determine if they were altered after *B. infantis* feeding. In order to identify CD4+T and Th17 cells, we utilized antibodies against CD3, CD4, and IL-17A to detect them. Flow cytometric analyses revealed that high dose *B. infantis* increased slightly the percentage of CD4+T cells in MLN compared with PBS control (Figure 2A and C). *B. infantis* also up-regulated the level of Th17 cells (Figure 2B and C). However, both the increase of CD4+T and Th17 cells did not reach significance (*P* > 0.05). For deciding the change of the population of Tregs after *B. infantis* feeding, CD4, CD25 and Foxp3 were measured by flow cytometry. *B. infantis* feeding slightly increased the percentage of CD4+Foxp3+ Tregs compared with control (9.99% ± 0.26% *vs* 9.40% ± 0.38%) (Figure 3D). The increased CD4+Foxp3+ Tregs by *B. infantis* feeding were also mostly derived from CD4+CD25+T cells, not induced from CD4+CD25-T (Figure 3B and C).

***Colonic damage in TNBS-induced colitis mice and upregulation of Th1-and Th17- responses***

TNBS-induced colitis is a well-characterized animal model of Th1-and Th17-mediated colitis, and it mimics human CD. But the appropriate time when the pathological change of colon exhibited dramatically was not known. Therefore we decided to undergo the histological analysis in colitis control mice on day 3 and day 6 after intratrectal instillation of TNBS. Hematoxylin and eosin staining was used to evaluate the severity of TNBS-induced colitis. Integral colonic epithelium and no inflammatory cell infiltration were observed in the slice from the negative control mice (Figure 4A). Sections of colons from mice on day 3 after instillation of TNBS showed extensive inflammatory cell infiltration (Figure 4B). In contrast, mice on day 6 after the instillation of TNBS, exhibited goblet cell depletion, colonic epithelium loss and more inflammatory cell infiltrated in the colon (Figure 4C). These microscopic evaluations of colons from mice induced by TNBS revealed pathologic correlations with CD and longer duration after colitis induction was linked to more severe inflammation in colons.

TNBS-induced colitis was associated with excessive Th1-and Th17-responses. Therefore we measured the relative mRNA expressions of different T cell cytokines in MLN by real-time RT-PCR at day 3 and 6 after TNBS induction (Figure 5). Compared with the negative control group, the mRNA expressions of Th1 cytokines (IL-2, IFN-γ and IL-12p40) displayed no change in colonic tissues from mice on day 3 after TNBS induction (TNBS-3) (Figure 5A-C). However, IL-2 and IL-12p40 increased strikingly in mice on day 6 after TNBS induction (TNBS-6) compared with PBS control group (IL-2, *P <* 0.05; IL-12p40, *P <* 0.05) (Figure 5A and C), the IFN-γ also increased a little but significantly. Mice in TNBS-6 group had a significant higher expression of Th17 related molecules (RORγt and IL-17A) in colonic tissue than control mice (RORγt, *P <* 0.01; IL-17A, *P <* 0.005) (Figure 5E and H). The expression of other Th17-related cytokines (IL-21 and IL-23) increased dramatically in TNBS-3 group and in TNBS-6 group compared to the control group, though mice in TNBS-3 have higher expressions of both cytokines more than mice in TNBS-6 group (Figure 5F and G). However, TNBS did not induce the change of IL-4 mRNA representing Th2 (Figure 5D). Thus, we established the TNBS colitis model successfully and the colon damage was associated with increased activity of Th1 and Th17 cells. Besides, we determined the appropriate time, on day 6 after colitis induction, to evaluate the histological change and Th1- and Th17-responses.

***Attenuation of experimental colitis by B. infantis is associated with downregulation of Th1- and Th17-responses***

We then investigated whether *B. infantis* reduced the severity of experimental colitis. Mice were daily administrated with high dose of *B. infantis* (6 × 108 cfu/d) for 3 wk before induction of colitis by the instillation of TNBS. On day 6 after TNBS induction, sections of colons from mice in PBS-TNBS group showed extensive goblet cell depletion, epithelial loss as well as dense chronic inflammatory infiltrates in the LP (Figure 6A). In contrast, administration of *B. infantis* drastically diminished goblet cell depletion and the intensity of inflammatory infiltrates and restored the intestinal epithelium in *B. infantis*-TNBS group (Figure 6B). These results revealed that *B. infantis* can attenuate the intestinal inflammation in experimental colitis model.

Next we tested whether Th1\Th17 reponses were downregulated after *B. infantis* feeding in experimental colitis mice. As shown in Figure 7, analyses of cytokine mRNA expressions showed that Th1 cytokines (IFN-γ and IL-12p40) and Th17 cytokines (IL-17A and IL-23 ) were significantly decreased in the colons of mice receiving *B. infantis* before induction of colitis in comparison with mice receiving PBS before induction of colitis (IFN-γ, *P <* 0.05; IL-12p40, *P <* 0.05; IL-17A < 0.05; IL-23, *P <* 0.01) ( Figure 7C, D, I and J). Th1 cytokine IL-2 seemed to fall down but not significantly (Figure 7B and J)). In addition, *B. infantis* feeding led to a lower level of Th1 transcription factor T-bet and Th17 transcription factor RORγt compared with PBS control (T-bet, *P <* 0.05) (Figure 7A and G). No significant difference in mRNA expression of Th2-related GATA3 and IL-4 was measured between PBS-TNBS group and *B. infantis*-TNBS group. However, surprisingly, *B. infantis* feeding increased the mRNA expressions of Foxp3, IL-10 and TGF-β in colitis mice significantly when compared with PBS-TNBS group (Foxp3, *P <* 0.01; IL-10, *P <* 0.05; TGF-β, *P <* 0.05) (Figure 7K-M). Taken together, these results show that *B. infantis* feeding attenuates the development of colonic inflammation in TNBS-induced colitis model in association with downregulation of both Th1- and Th17-responses.

**DISCUSSION**

This study showed that *B. infantis* feeding to mice reduced the severity of TNBS-induced colitis, associated with suppression of Th1 and Th17 responses and promotion of Foxp3+ Tregs response within MLN. This revealed that the attenuation of TNBS-induce colitis by *B. infantis* feeding is mediated by regulating the differentiation of CD4+ T subsets and cytokine expressions.

Firstly, we revealed that *B. infantis* feeding reduced the severity of TNBS-induced colitis. TNBS-instillation resulted in extensive goblet cell depletion, epithelial loss and dense inflammatory cell infiltrations in the LP. However colitis mice with *B. infantis* feeding showed a noticeable resolution of inflammatory infiltrates and goblet cell depletion and restored the intestinal epithelium. Next, the study explored the relation between the attenuation of colitis by *B. infantis* and the change of T cell subsets responses, given that immune cell responses play a crucial role in the pathogenesis of colitis. Mucosal dendritic cells present gut antigens to the adaptive immune system and then direct the polarization of naïve CD4+ T cells towards different effector T-helper cells (Th1, Th2 and Th17) and Tregs[10]. These effector Th cells are important for combating pathogen infections; when aberrant they release enormous proinflammatory cytokines and initiate excessive inflammation, resulting in chronic inflammatory diseases. Nonetheless, the gut immune system has mechanisms of tolerance that avoid uncontrolled Th1, Th2 or Th17 responses. Foxp3+ Tregs displayed immunosuppressive function by suppressing Th cells and secreting anti-inflammatory cytokines IL-10 and TGF-β[11]. It’s well known that aberrant Th1 and Th17 responses and deficient Tregs response contribute to IBD.

Classically, CD has been associated with Th1 response. In our study, levels of Th1 cytokines (IL-2, IL-12p40 and IFN-γ) were up-regulated in the MLN from TNBS-induced colitis mice. Th1 cells secrete a high level of IFN-γ, a proinflammatory cytokine responsible for macrophage activation. IFN-γ is essential for IBD pathogenesis as the administration of anti-IFN-γ antibody to mice can prevent the development of colitis[12] and the treatment with fontolizumab, an anti-IFN-γ antibody, improved clinical response and remission in patients with active CD compared with placebo[13]. Consistent with these results, our colitis mice were detected to have a reduction of IFN-γ mRNA in MLN after *B. infantis* feeding. IL-12 has two subunits, p40 and p35 and is mainly produced by macrophages/monocytes and can be efficiently induced by intracellular bacteria. IL-12 plays a pivotal role in the Th1 differentiation and induces naïve T cells to produce IFN-γ. Administration of antibody against its subunit IL-12p40 attenuated TNBS colitis with decreased levels of Th1 cells and IFN-γ in LP[14]. In patients with active CD, anti-IL-12p40 treatment reduced Th1-mediated inflammatory cytokines and promoted clinical remission[15]. In this experiment, colitis mice down-regulated the mRNA of IL-12p40 following *B. infantis* feeding. Further, the study revealed that Th1 transcription factor T-bet was up-regulated in colitis mice but was down-regulated by *B. infantis* feeding. T-bet is a member of the T-box transcription factor family that is essential for Th1 development as Th1 cells cannot be generated either in vitro or in vivo without it[16]. These outcomes showed *B. infantis* seemed to weaken the Th1 cell response via regulating its differentiation-related factors and cytokine expressions in TNBS-induced colitis mice.

In the recent decade, the discovery of the Th17 subset has rapidly expanded the understanding of IBD. High levels of Th17 and its cytokine IL-17 were detected in inflamed colons of mice and patients with IBD[17]. IL-17A is a potent mediator of inflammatory responses as it induces proinflammatory cytokines such as IL-6, IL-8, and monocyte chemotactic protein-1 (MCP-1)[18]. Increased IL-17A mRNA was measured in our colitis model, though *B. infantis* reduced it. Likely, in DSS-induced colitis, *B. infantis* decreased Th17 cells and IL-17A[5]. Interestingly, IL-17A was also verified to induce production of IL-12 and promote the Th1 response[19], implying that IL-17A downregulation by *B. infantis* may facilitate the inhibition of Th1 response in IBD. IL-21, a CD4+ T cell-derived cytokine produced in excess in IBD was found to promote the differentiation of Th17 cells from naïve CD4+ T cells and prevent the TGF-β-dependent expression of Foxp3from naïve CD4+ T cells[20]. IL-21-deficient mouse have less Th17 cells with reduced severity of colitis after induction by either DSS or TNBS[21]. However, we found that *B. infantis* feeding led to the reduction of IL-21 in colitis mice. Orphan nuclear receptor RORgammat (RORγt) is constitutively expressed by Th17 cells and the key transcription factor to Th17 differentiation[22]. In addition, RORγt can regulate the production of IL-17A and IL-17F to induce chronic colitis[23]. The animal experiment showed a reduction in RORγt mRNA in colitis mice fed with *B. infantis*. Another key cytokine, IL­23 secreted by DCs potently promoted Th17 amplification, sustained and strengthened the Th17 phenotype. Several groups concurrently showed that IL-23 played a crucial role in development of IBD in different colitis models partly because IL-23 induced proinflammatory cytokines such as TNF-α, IFN-γ and IL-6 in the colon[24]. Moreover, other work has shown that IL-23 inhibited Foxp3+ Tregs to induce colitis[25]. It is, therefore, of great importance to find that *B. infantis* feeding reduced the IL-23 mRNA in colitis model. Taken together, *B. infantis* suppressed Th17 responses in TNBS-induced colitis mice.

Foxp3+ Tregs are a specialized class of lymphocytes characterized by the expression of the forkhead transcription factor Foxp3. Foxp3+ Tregs are essential for normal immunohomeostasis by suppressing Th effector cells[11]. The importance of Tregs in the prevention of colitis has been confirmed by previous studies showing that transfer of naïve CD4+ T cells without Tregs into immunodeficient mice resulted in inflammation and colitis, while co-transfer of Tregs ameliorated or reversed pathology[26]. In IBD patients, reduced numbers of Foxp3+ Tregs were observed in the peripheral blood and greater numbers of Foxp3+ Tregs in the intestinal mucosa, although the increase in intestine of IBD was lower compared with non-IBD inflammatory conditions[11]. Given the lower Tregs, the expansion of Tregs seems to be a therapeutic tool for the treatment of IBD. There is some evidence that probiotics administration are able to ameliorate the colitis by increasing Foxp3+ Tregs in the colon tissue, such as probiotic mixtures (lactobacillus, bifidobacterium and streptococcus), (lactobacillus and bifidobacterium) and (bifidobacterium, lactobacillus and enterococcus)[27-29]. In our normal mice, single *B. infantis* feeding increased the Foxp3+ Tregs in the MLN. Similarly, *B. infantis* feeding increased Foxp3mRNA in colitis mice. Consistent with our results, human volunteers taking *B. infantis* had elevated levels of Foxp3 expression in PBMC[30]. The expression of Foxp3 is critical because that it controls the development of Tregs and that the immunosuppressive function of Tregs requires high level of Foxp3 expression[31]. Decreased Foxp3 expression could lead to impaired Tregs function and be causal for immune disorders including human immunodysregulation, polyendocrinopathy, enteropathy, X- linked (IPEX) syndrome[32]. Hence, the increased Foxp3 mRNA induced by *B. infantis* feeding may enhance the immunosuppressive activities in colitis mice. Besides, *B. infantis* increased Tregs-related IL-10 and TGF-β in the colon tissue in our colitis model. IL-10 is a potent anti-inflammatory cytokine, produced by both nonimmune and immune cells including Tregs. IL-10 has a particular significance in IBD because IL-10–deficient mice develop colitis[33]. And Tregs–specific deletion of IL-10 resulted in spontaneous colitis[34]. Apart from IL-10, Tregs produce large amounts of TGF-β. TGF-β is a potent regulatory cytokine that inhibits Th cell proliferation, differentiation and activation, and decreases secretion of harmful cytokines[35]. Application of TGF-β blocking antibody eliminated the ability of Tregs to prevent colitis and TGF-β-deficient Tregs failed to suppress colitis[36]. What’s more, TGF-β is involved in the induction of Foxp3 and drives the differentiation of naïve T cells to a Tregs phenotype[37]. In all, *B. infantis* promoted Foxp3+ Tregs responses in association with increased levels of Foxp3, L-10 and TGF-β in the MLN in TNBS-induced colitis mice.

However, *B. infantis* feeding had some different effects on the immune cells in different gut environment. In normal gut environment a high dose elicited some Th1, Th17 and Tregs responses with a slight increase of Th17 cells and Tregs and their-related cytokines, though this did not cause pathological inflammation. One reason why *B. infantis* feeding resulted in a little high immune response is that *B. infantis*, which is derived from human gut, is regarded as foreign antigen, thereby triggering weak responses. But this immune response did not lead to pathological inflammation, which indicated that there was a proper balance among Th1, Th17 and Tregs in maintaining gut homeostasis. This was confirmed by evidence that a good balance between Tregs and Th17 is essential for both eliminating potential pathogens and suppressing intestine inflammation[38]. In some way, a proper increase in Th1 and Th17 response may enhance the mucosal defense against pathogens and thus be beneficial to the promotion of the host intestinal immunity.

It is of great importance to the potential of *B. infantis* to suppress the IBD because the risk of colorectal cancer is increased in ulcerative Colitis and CD[39]. Animal model revealed that the setting of intestinal inflammation contributed to the tumorigenesis through T cell and TLR-mediated inflammation[40]. And IL-17 immunoreactive cells were significantly expressed in normal mucosa of colon cancer patient[41]. However, a high density of Foxp3+ Tregs in tumor tissue was associated with improved prognosis in colorectal cancer patients in early T stage[42].Thus reducing chronic intestinal inflammation appears to be a good strategy in preventing the incidence of colorectal cancer. The microbial dysbiosis was found in colon cancer patients[41], indicating that improvement of intestinal flora environment may benefit these patients. Studies showed that supplementation of viable Bifidobacteria before surgery can reduce infection complication of surgery in patients with colorectal cancer[43]. More importantly, probiotics can delay or block the development of tumor from inflammation disorders by reducing inflammation. Pretreatment with the probiotic *VS*L#3, a mixture of bacteria (lactobacillus, bifidobacterium and streptococcus) can attenuate the inflammation in TNBS-induced colitis, delaying transition to dysplasia and cancer[44]. Bifidobacterium lactis prevents DSS-induced acute colitis and DSS colitis-associated cancer in mice[45]. *B. infantis* has the potential to attenuate intestinal inflammation through suppressing Th1 and Th17 cell responses as well as promoting Foxp3+ Tregs. So *B. infantis* may reduce risk of the transition from IBD to colorectal cancer, even slow down the existing cancer progression and improve the prognosis.

However, the major drawback of this study is that we failed to measure the frequency of Th17 cells and Foxp3+ Tregs and identify its origin in colitis mice and *B. infantis*-fed colitis mice. The expression of Foxp3 can be induced in naturally thymic-drived CD4+CD25+ Tregs or in mature CD4+ “conventional” T cells *in vitro* and *in* *vivo*[11]. Nevertheless, Foxp3+ Tregs from CD4+CD25+ Tregs or from CD4+CD25- conventional T cells both have similar immunosuppressive functions. In normal mice, we found that increased Foxp3+ Tregs by *B. infantis* were mostly derived from the CD4+CD25+ Tregs in the MLN. But whether the origin of the increased Foxp3 mRNA in MLN in our colitis mice and *B. infantis*-treated colitis mice were also derived from CD4+CD25+ Tregs or induced from peripheral CD4+CD25- T was not known. Previous studies showed that mixed probiotics (bifidobacterium, lactobacillus and enterococcus) or (lactobacillus and bifidobacterium) feeding led to an elevated level of CD4+CD25+Foxp3+ cells in MLN or intraepithelia in TNBS colitis mice[28,29]. However, in the same model, probiotic IRT5 administration increased the CD4+CD25−Foxp3+ cells in MLN[27]. For this phenomenon, one explanation is that the individual component of probiotic mixtures had different function on the population of Tregs in the intestine. In order to make it clear the origin of elevated levels of Foxp3 mRNA in our *B. infantis*-treated colitis mice, further works will be done in the future.

In conclusion, we showed that *B. infantis* attenuated intestinal inflammation in TNBS-induced colitis mice though suppressing the Th1 and Th17 responses and increasing the Foxp3+ Tregs response in MLN. This underlying mechanism of regulating immune system of *B. infantis* may have significant clinical implications in treating IBD and preventing colorectal cancer.

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**COMMENTS**

***Background***

Crohn's disease (CD), one type of inflammatory bowel disease, is characterized by a chronic inflammation of intestinal tract. Abnormal microbiota and mucosal immune cell responses contribute to the pathogenesis of CD.

***Research frontiers***

Alterations of gut microbiota cause immune dysregulation, leading to autoimmune attacks, as a result of serious damage of intestinal mucosal barrier. CD involves aberrant Th1 and Th17 responses and deficient T regulatory cell response. This suggested regulating immune cell responses could potentially attenuate intestinal inflammation.

***Innovations and breakthroughs***

In our study, we found that *Bifidobacterium infantis* (*B. infantis*) had the potential to effectively attenuate the colitis induced by 2,4,6-trinitrobenzene sulfonic acid. These beneficial effects were associated with the roles of *B. infantis* in the suppression of Th1 and Th17 cell responses and the promotion of Foxp3+ T regulatory cell response, restoring the balance of T cell subsets responses.

***Applications***

Understanding the mechanism underlying the effects of *B. infantis* in treating experimental colitis, had significant clinical implications in effectively and safely treating inflammatory bowel disease and futher preventing colorectal cancer associated with intestinal inflammation.

***Peer review***

The authors investigated effects of *B. infantis* on treating experimental colitis which mimics CD and explore its potential mechanism by measuring levels of Th1, Th17 and Foxp3+ T regulaory cells and expressions of their related cytokines and transcription factors in mesenteric lymph nodes. It has revealed that *B. infantis* effectively relieved the colitis, which was associated with reducing the Th1, Th17 responses and increasing the T regulatory cell response. The results provide valuable evidence for understanding the mechanism of probiotics treating inflammatory bowel disease such as CD.

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**Figure 1** **mRNA expression of T cell-related cytokines from mesenteric lymph nodes of normal BALB/c mice fed with different doses of *Bifidobacterium infantis*.** A: IL-2; B: IFN-γ; C: IL-12p40; D: IL-4; E: RORγt; F: IL-21; G: IL-23; H: Foxp3; I: IL-10. Control: Mice from control group; Low: Mice treated with low dose *B. infantis*; High: Mice treated with high dose *B. infantis* real-time RT-PCR was employed to examine the expression of Th1, Th17 and Foxp3+ Tregs related cytokines. Data are from 5 mice per group. Data are expressed as relative expression and the control group was chosen as calibrator. Data represent mean ± SE. a*P <* 0.05 *vs* the control group; b*P <* 0.01 *vs* the control group. IL: Interleukin; IFN: interferon.



**Figure 2** **Representative flow plots and levels of CD4+ Tand CD4+IL-17A+ T cells in mesenteric lymph nodes from normal mice fed with high dose *Bifidobacterium infantis*.** A: Levels of mesenteric lymph nodes (MLN) CD4+T cells in PBS-control and *Bifidobacterium infantis* (*B. infantis*) treated groups; B: Levels of CD4+IL-17A+T cells in PBS-control and *B. infantis* treated groups; C: The percentage of MLN CD4+T cells; D: the percentage of CD4+IL-17A+T cells. Results are either shown as mean ± SE from three independent experiments, *n* = 5 (bar graphs) or as a representative flow cytometry graph.



**Figure 3** **Representative flow plots and levels of Foxp3+ cells in mesenteric lymph nodes from normal mice fed with high dose *Bifidobacterium infantis*.** A: Levels of Foxp3+ cells in mesenteric lymph nodes from PBS-control group and *Bifidobacterium infantis* (*B. infantis*) group; B: Levels of CD4+Foxp3+ Tregs from PBS-control group and *B. infantis* group; C: The percentage of the Foxp3+ cells derived from CD4+CD5+Tcells. Data are mean ± SE with 5 mice per group from three independent experiments (bar graphs) or as a representative flow cytometry graph.



**Figure 4 Representative histological images of the colons samples from trinitrobenzene sulfonic acid - induced colitis mice.** A: colon images from negative control mice; B: colon images from mice on day 3 after trinitrobenzene sulfonic acid (TNBS) induction; C: Colon images from mice on day 6 after TNBS induction. All images are at the same original magnification (× 40). Data are representative of three independent experiments.

 

**Figure 5** **mRNA expression of T cell-related cytokines from mesenteric lymph nodes of trinitrobenzene sulfonic acid - induced colitis mice.** A: IL-2; B: IFN-γ; C: IL-12p40; D: IL-4; E: RORγt; F: IL-21; G: IL-23; H: IL-17A. real-time RT-PCR was employed to examine the expression of Th1, Th17 and Foxp3+ Tregs related cytokines. Control: In the control group, the animals were administered with PBS; trinitrobenzene sulfonic acid (TNBS)-3: In the TNBS-3 group, the animals were sacrificed 3 d after TNBS induction; TNBS-6: In the TNBS-6 group, the animals were sacrificed 6 d after TNBS induction. Data are from 5 mice per group. Data are expressed as relative expression and the control group was chosen as calibrator. Data represent mean ± SE. a*P <* 0.05 *vs* the control group; b*P <* 0.01 *vs* the control group. IL: Interleukin; IFN: interferon.



**Figure 6 Representative histological images the colons samples from trinitrobenzene sulfonic acid - induced colitis mice fed with high dose *Bifidobacterium infantis*. A**: Colon images from trinitrobenzene sulfonic acid (TNBS)-induce colitis mice fed with PBS; B: Colon images from TNBS-induced mice fed with high dose *Bifidobacterium infantis.* All images are at the same original magnification (× 40). Data are representative of three independent experiments.





**Figure 7 mRNA expression of T cell-related cytokines from mesenteric lymph nodes of trinitrobenzene sulfonic acid - induced colitis mice fed with high dose *Bifidobacterium infantis*.** A: T-bet;B: IL-2; C: INF-γ; D: IL-12p40; E: GATA3; F: IL-4; G: RORγt; H: IL-17A; I: IL-21; J: IL-23; K: Foxp3; L: IL-10; M: TGF-β. real-time RT-PCR was employed to examine the expression of Th1, Th17 and Foxp3+ Tregs related cytokines. PBS-TNBS: In the PBS-TNBS group, the animals were fed with PBS before TNBS induction; *Bifidobacterium infantis* (*B. infantis*)-TNBS: In the *B. infantis*-TNBS group, the animals were fed with high dose *B. infantis* before TNBS induction. Data are expressed as relative expression and the control group was chosen as calibrator. Data are mean ± SE (*n* = 5, per group). a*P <* 0.05 *vs* PBS-TNBS. b*P <* 0.01 *vs* the control group. IL: Interleukin; IFN: interferon; TNBS: trinitrobenzene sulfonic acid.