Bifidobacterium infantis attenuates colitis by regulating T cell subset responses

Li Zuo, Kai-Tao Yuan, Li Yu, Qing-Hong Meng, Peter Chee-Keung Chung, Ding-Hua Yang

Li Zuo, Qing-Hong Meng, Peter Chee-Keung Chung, Department of Immunology, Guiyang Medical College, Guiyang 550004, Guizhou Province, China
Kai-Tao Yuan, Department of Surgery, the First Affiliated Hospital of Sun Yat-sen University, Guangzhou 510080, Guangdong Province, China
Li Yu, Department of Paediatrics, Guangzhou First Municipal People’s Hospital, Guangzhou 510180, Guangdong Province, China
Ding-Hua Yang, Department of Hepatobiliary Surgery, Nan Fang Hospital, Southern Medical University, Guangzhou 510515, Guangdong Province, China

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Correspondence to: Ding-Hua Yang, MD, PhD, Professor, Department of Hepatobiliary Surgery, Nan Fang Hospital, Southern Medical University, 1838 North Guangzhou Dadao, Guangzhou 510515, Guangdong Province, China. dhyang5810@yahoo.com
Telephone: +86-20-61641706 Fax: +86-20-61641706
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Abstract

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

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

**RESULTS:** No significant change in cytokine profiles was observed in normal mice fed low dose *B. infantis*. However, Th1-related cytokines (IL-2, IFN-γ, IL-12p40), Th17-related transcription factor and cytokines (RORγt, IL-21, IL-23), regulatory T cell (Treg)-related transcription factor and cytokines (Foxp3, IL-10) were increased in normal mice fed high dose *B. infantis*. Furthermore, flow cytometry assay showed *B. infantis* increased the numbers of CD4+ Foxp3+ Tregs and Th17 cells in MLNs. Colitis was successfully induced by TNBS in mice, characterized by colonic inflammation and aberrant Th1 and Th17 responses. Feeding 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 Treg-related molecules (Foxp3, IL-10 and TGF-β) in colitis mice.

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

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

**Core tip:** Inflammatory bowel disease is 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 *Bifidobacterium infantis* (*B. infantis*) reduced the intestinal inflammation in TNBS-induced colitis mice though decreasing the Th1 and Th17 responses and promoting the Foxp3⁺ Treg response in mesenteric lymph nodes. This mechanism underlying the regulatory effect of *B. infantis* on the immune system may have significant clinical implications in treating inflammatory bowel disease and preventing colorectal cancer.

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**INTRODUCTION**

Inflammatory bowel disease (IBD) is an autoimmune disorder that is characterized by chronic inflammation of the gastrointestinal tract. IBD comprises 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 plays a key role in the pathogenesis of IBD. 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 using 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[4,5] and IBD[6-7]. The mechanisms behind the amelioration of IBD by *B. infantis* are still largely unknown. IBD results from an inadequate Foxp3⁺ regulatory T cell (Treg) response in the face of overly exuberant Th1 and Th17 responses in CD and an abnormal Th2 response in ulcerative colitis[8]. *B. infantis* feeding reduced the numbers of Th1 and Th17 cells and increased the proportion of Foxp3⁺ Tregs within the lamina propria (LP) in DSS-induced colitis which mimics CD[9]. Moreover, *B. infantis* had some influence on the expression of some cytokines. For instance, *B. infantis* inhibited the expression of Th17 related IL-17A and induced the expression of IL-10 in DSS-induced colitis mice[10], and decreased Th1 proinflammatory cytokines (TNF-α, IL-12 and IFN-γ) and maintained the level of TGF-β in IL-10 knockout (KO) mice that are a spontaneous genetic model of CD[11]. However, the effects of single *B. infantis* feeding on trinitrobenzene sulfonic acid (TNBS)-induced colitis, which is an animal model of CD, and the underlying immune mechanism were still 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 expression.

**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 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: a control group, a low dose *B. infantis* (*B. infantis*) and a high dose *B. infantis*. *B. infantis* (*B. infantis*; 6 × 10⁹ cfu/d) was provided by Guangzhou Baoxing Biotechnology Company (Guangzhou, China). Mice received oral administration of low or high dose *B. infantis* once daily for 3 wk. The control group received 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 4 cm proximal to the anal verge through a 3.5F catheter as described previously[12]. TNBS was purchased from Sigma (St. Louis, MO, United States). In the TNBS colitis model, three groups of five animals each were utilized. Group 1 was a negative control group, which was administered with PBS, instead of TNBS. Group 2 was designated as the TNB-N group as these mice were sacrificed by cervical dislocation 3 d after administration of TNBS. Group 3 was designated as the TNBS-6 group in which mice were sacrificed 6 d after administration of TNBS.

In the *B. infantis* treated model, two groups of five animals each were used. Group 1 received daily oral administration of high dose *B. infantis* (6 × 10⁹ cfu/d) for 3 wk before colitis induction (*B. infantis*-TNBS group). The colitis control animals were administered with PBS using the same technique before colitis induction (PBS-TNBS group). Animals were sacrificed by cervical dislocation 6 d after TNBS induction, and MLNs were selected carefully from the inflamed area of the bowel in all animals.

**RNA extraction and real-time PCR**

Total RNA was extracted from MLNs using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Total RNA (2 μg) was transcribed to cDNA using a reverse-transcription kit (TaKaRa). Primer sequences were as follows: T-bet (forward) 5'-CAACAA GCCCTTTGCGCAAG-3' and (reverse) 5'-TCCCTCCAAGCAGTTGACAGT-3'; Foxp3 (forward) 5′-TCCCCCAAGCAGTTGACAGT-3′, IL-2 (forward) 5′-CAACAA CCCCTTTGCCAAAG-3′ and (reverse) 5′-TCCCCCAAGCAGTTGACAGT-3′; and (reverse) 5′-TCCCCCAAGCAGTTGACAGT-3′; IL-2 (forward)
5'-CTTGAGCAGATGGAGAATTACA-3' and (reverse) 5'-TCCAGA ATACGGCCGAG-3', IFN-γ (forward) 5'-TCAAGTGCTAGTATGTTGAA, GAA-3' and (reverse) 5'-TGCCCTTGAGATTTTCTAGT-3', IL-12p40 (forward) 5'-GGAAGACGACGACACAATA-3' and (reverse) 5'-AACCTGAGGAGAAG TAGGAATGG-3', GAPDH (forward) 5'-CCCTCCTCGCTTCGTCG-3' and (reverse) 5'-CAACACATCTCCTGGCTTCTG-3', IL-4 (forward) 5'-CAAGGAGAA GGAGGCCAT-3' and (reverse) 5'-GAAGCCCTACAGACAGCTCA-3', ROPr (forward) 5'-GCTGTCAAGGTGACCTGGAG-3' and (reverse) 5'-GGTGAAGCTATGGAAAGAC-3', IL-12p40 (forward) 5'-GGAGACCCTTGTCTGTCGG-3' and (reverse) 5'-TCATCTTTTGAAGAGCCATT-3', IL-23 (forward) 5'-GAAGTG GACCTGAGACCAG-3' and (reverse) 5'-GGAACTGTACTGCTTTGAA-3', FOXP3 (forward) 5'-CCCATCCCAGAGATTCTTG-3' and (reverse) 5'-CCATGACTGAGCAGGTATGA-3', IL-10 (forward) 5'-ATACCTGCACCTCTCCA-3' and (reverse) 5'-TCATTTCGGATAAGGCTTGG-3', TGF-β (forward) 5'-GAAGGCGAAGTTCAGGGTG-3' and (reverse) 5'-GGTTTCGCTTCTTTGGTGAA-3', GAPDH (forward) 5'-CATCAGTCGCAACCAGAGA-3' and (reverse) 5'-TGAAGT GCAGAGGAGACACC-3'.

Real-time reverse transcription polymerase chain reaction (RT-PCR) was performed with the ABI 7300 system. 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 ddH2O. 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 MLNs of each group. In order to identify Tregs in MLNs, cells were first surface labeled with FITC labeled anti-mouse CD4 (eBioscience) and PE labeled anti-mouse CD25 antibodies (eBioscience). After that, cells were fixed, permeated and intracellularly stained with APC labeled antibodies against CD3, CD4, and IL-17A to detect Th17 cells, Th17 cells augmented the expression of Th1-, Th17- and Treg-related cytokines in MLNs in normal mice.

RESULTS

B. infantis augments the expression of Th1-, Th17- and Treg-related cytokines in MLNs in normal mice

To investigate the effect of B. infantis on T cell cytokine expression, we studied the relative expression of different cytokines in MLNs of normal mice by real-time RT-PCR. Low dose B. infantis feeding (6 × 10⁷ cfu/d) caused no significant changes in the expression of all cytokines tested, but high dose B. infantis feeding (6 × 10⁸ cfu/d) augmented the expression of Th1-, Th17- and Treg-related cytokines in MLNs (Figure 1). After high dose B. infantis feeding, the mRNA expression of Th1 cytokines IL-2 and IL-12p40 increased significantly in the MLNs 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 the control group (Figure 1B). Th17-related cytokine IL-23 and transcription factor RORγt increased more than 2-fold in MLNs after B. infantis feeding compared with the control group (P < 0.005) (Figure 1E and G). IL-21 also tended to rise. Moreover, mice receiving B. infantis had a significant increase in Foxp3 and IL-10 mRNA expression in MLNs in comparison with PBS control (Foxp3, P < 0.005; IL-10, P < 0.05) (Figure 1F 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 increases the levels of CD4+ T cells, Th17 cells and CD4+ Foxp3+ Tregs in the MLNs in normal mice

We measured the numbers of CD4+ T cells and Th17 cells in the MLNs 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...
The increased CD4⁺Foxp3⁺ Tregs by B. infantis feeding were also mostly derived from CD4⁺CD25⁺ T cells, not 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. However, the appropriate time when the
pathological change of the colon was dramatic was not known. Therefore we decided to perform the histological analysis in colitis control mice on days 3 and 6 after intrarectal 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 slices from the negative control mice (Figure 4A). Sections of colonic tissues taken 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 epithelial loss and more inflammatory cells infiltrated in the colon (Figure 4C). These microscopic evaluations of colons from mice with colitis induced by TNBS revealed pathologic correlations with CD and longer duration after colitis induction was linked to more severe inflammation in the colon.

TNBS-induced colitis was associated with excessive Th1- and Th17-responses. Therefore we measured the relative mRNA expression of different T cell cytokines in MLNs by real-time RT-PCR on days 3 and 6 after TNBS induction (Figure 5). Compared with the negative control group, the mRNA expression 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 significantly 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), and the IFN-γ also increased a little but insignificantly. Mice in the TNBS-6 group had significantly higher expression of Th17 related molecules (RORγt and IL-17A) in the 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 the TNBS-3 group and in the TNBS-6 group compared to the control group, though mice in the TNBS-3 group had higher expression of both cytokines than mice in the 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
Figure 3  Representative flow plots and levels of Foxp3+ cells in mesenteric lymph nodes from normal mice fed 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; C: Levels of CD4+Foxp3+ Tregs from *B. infantis* group; D: The percentage of the CD4+Foxp3+ Tregs. Data are mean ± SE with 5 mice per group from three independent experiments (bar graphs) or as a representative flow cytometry graph.
administered with high dose B. infantis (6 × 10^8 cfu/d) for 3 wk before induction of colitis by the instillation of TNBS. On day 6 after TNBS induction, sections of colonic tissues from mice in the 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 (Figure 6B). These results revealed that B. infantis can attenuate the intestinal inflammation in the experimental colitis model.

Next we tested whether Th1/Th17 responses were downregulated after B. infantis feeding in experimental colitis mice. As shown in Figure 7, analyses of cytokine mRNA expression showed that Th1 cytokines (IFN-γ and IL-12p40) and Th17 cytokines (IL-17A and IL-23) were significantly decreased in the colon 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 lower levels 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 the PBS-TNBS group and B. infantis-TNBS group. However, surprisingly, B. infantis feeding increased the mRNA expression of Foxp3, IL-10 and TGF-β in colitis mice significantly when compared with the 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, which was associated with suppression of Th1 and Th17 responses and promotion of Foxp3+ Treg response within MLNs. This revealed that the attenuation of TNBS-induced colitis by B. infantis feeding is mediated by regulating the differentiation of CD4+ T subsets and cytokine expression.

We found 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 intestinal epithelium. Next, the study explored the relation between the attenuation of colitis by B. infantis and the change of T cell subset 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 is well known that aberrant Th1 and Th17 responses and deficient Treg 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 MLNs 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 MLNs after *B. infantis* feeding. IL-12 has two subunits, p40 and p35, and it 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 the 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 showed down-regulated 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

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+ Treg 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 the calibrator. Data represent mean ± SE. *P < 0.05 vs the control group; **P < 0.01 vs the control group. IL: Interleukin; IFN: Interferon.
outcomes showed B. infantis seemed to weaken the Th1 cell response via regulating its differentiation-related factors and cytokine expression 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 [22, 23]. 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) [10]. 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 [3]. Interestingly, IL-17A was also verified to induce production of IL-12 and promote the Th1 response [35], 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 naive CD4+ T cells and prevent the TGF-β-dependent expression of Foxp3 by naive CD4+ T cells [29]. IL-21-deficient mice have less Th17 cells with reduced severity of colitis after induction by either DSS or TNBS [25]. However, we found that B. infantis feeding led to the reduction of IL-21 in colitis mice. Orphan nuclear receptor RORγt (RORγt) is constitutively expressed by Th17 cells and is the key transcription factor to Th17 differentiation [25]. In addition, RORγt can regulate the production of IL-17A and IL-17F to induce chronic colitis [29]. The animal experiment showed a reduction in RORγt mRNA in colitis mice fed B. infantis. Another key cytokine, IL-23 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 [31]. Moreover, other work has shown that IL-23 inhibited Foxp3 Tregs to induce colitis [29]. It is, therefore, of great importance to find that B. infantis feeding reduced the IL-23 mRNA in the 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 the intestine of IBD patients was lower compared with non-IBD inflammatory conditions [37]. Given the lower Tregs, the expansion of Tregs seems to be a therapeutic tool for IBD. There is some evidence that probiotic administration is able to ameliorate the colitis by increasing Foxp3+ Tregs in the colonic tissue, such as probiotic mixtures (lactobacillus, bifidobacterium and streptococcus; lactobacillus and bifidobacterium; bifidobacterium, lactobacillus and enterococcus) [27-29]. In our normal mice, single B. infantis feeding increased the Foxp3+ Tregs in the MLNs. Similarly, B. infantis feeding increased Foxp3 mRNA in colitis mice. Consistent with our results, human volunteers taking B. infantis had elevated levels of Foxp3 expression in PBMCs [30]. The expression of Foxp3 is critical because it controls the development of Foxp3+ Tregs and the immunosuppressive function of Tregs requires a high level of Foxp3 expression [21]. Decreased Foxp3 expression could lead to impaired Treg function and be causal for immune disorders including human immunodysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome [31]. Hence, the increased Foxp3 mRNA induced by B. infantis feeding may enhance the immunosuppressive activities in colitis mice. Besides, B. infantis increased Treg-related IL-10 and TGF-β in the colonic 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 particular significance in IBD because IL-10-deficient mice develop colitis [32] and Treg-specific deletion of IL-10 resulted in

Figure 6  Representative histological images of colon samples from trinitrobenzene sulfonic acid-induced colitis mice fed high dose Bifidobacterium infantis. A: Colon image from a trinitrobenzene sulfonic acid (TNBS)-induced colitis mouse fed PBS; B: Colon image from a TNBS-induced mouse fed high dose Bifidobacterium infantis. All images are at the same original magnification (× 40).
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![Graphs showing relative expression levels of various cytokines and transcription factors](image-url)
spontaneous colitis\cite{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\cite{30}. Application of TGF-β blocking antibody eliminated the ability of Tregs to prevent colitis and TGF-β-deficient Tregs failed to suppress colitis\cite{30}. What’s more, TGF-β is involved in the induction of Foxp3 and drives the differentiation of naive T cells to a Treg phenotype\cite{35}. Overall, *B. infantis* promoted Foxp3+ Treg responses in association with increased levels of Foxp3, L-10 and TGF-β in the MLNs in TNBS-induced colitis mice.

However, *B. infantis* feeding had some different effects on the immune cells in different gut environments. In normal gut environment a high dose elicited some Th1, Th17 and Treg responses with slight increases of Th17 and TGF-β in the MLNs in TNBS-induced colitis mice.

It is of great importance for the potential of *B. infantis* to suppress IBD because the risk of colorectal cancer is increased in patients with ulcerative colitis and CD\cite{39}. Animal models revealed that the setting of intestinal inflammation contributed to the tumorigenesis through T cell and TLR-mediated inflammation\cite{40}. IL-17 immunoreactive cells were significantly expressed in the normal mucosa of colon cancer patient\cite{39}. However, a high density of Foxp3+ Tregs in tumor tissue was associated with improved prognosis in colorectal cancer patients in early T stage\cite{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\cite{43}, 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\cite{44}. More importantly, probiotics can delay or block the development of tumor from inflammation disorders by reducing inflammation. Pretreatment with the probiotic *V. brevis*, a mixture of lactobacillus, bifidobacterium and streptococcus, can attenuate the inflammation in TNBS-induced colitis, delaying transition to dysplasia and cancer\cite{45}. *Bifidobacterium lactis* prevents DDS-induced acute colitis and DDS colitis-associated cancer in mice\cite{38}. *B. infantis* has the potential to attenuate intestinal inflammation through suppressing Th1 and Th17 cell responses as well as promoting Foxp3+ Tregs. Therefore, *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 frequencies of Th17 cells and Foxp3+ Tregs and identify their origin in colitis mice and *B. infantis*-fed colitis mice. The expression of Foxp3 can be induced in naturally thymic-driven CD4+CD25+ Tregs or in mature CD4+ “conventional” T cells *in vitro* and *in vivo*\cite{41}. 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

**Figure 7** mRNA expression of T cell-related cytokines from mesenteric lymph nodes of trinitrobenzene sulfonic acid-induced colitis mice fed high dose *Bifidobacterium infantis*. A: T-bet; B: IL-2; C: IFN-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+ Treg related cytokines. PBS-TNBS: In the PBS-TNBS group, the animals were fed PBS before TNBS induction; *Bifidobacterium infantis*-TNBS: In the *B. infantis*-TNBS group, the animals were fed high dose *B. infantis* before TNBS induction. Data are expressed as relative expression and the control group was chosen as the calibrator. Data are mean ± SE (*n* = 5, per group). *P* < 0.05 vs PBS-TNBS; *P* < 0.01 vs the control group. IL: Interleukin; IFN: Interferon; TNBS: Trinitrobenzene sulfonic acid.
MLNs. But whether the origin of the increased Foxp3 mRNA in MLNs in our colitis mice and B. infantis-treated colitis mice was also derived from CD4+CD25+ Tregs or from peripheral CD4+CD25+ T was not known. Previous studies showed that feeding mixed probiotics (bifidobacterium, lactobacillus and enterococcus) or (lactobacillus and bifidobacterium) led to elevated levels of CD4+CD25 Foxp3+ cells in MLNs.[24,29] However, in the same model, probiotic IRT5 administration increased the CD4+CD25 Foxp3+ cells in MLNs.[25] For this discrepancy, it is explained that the individual component of probiotic mixtures had different function on the population of Tregs in the intestine. In order to elucidate the origin of elevated levels of Foxp3 mRNA in our B. infantis-treated colitis mice, further studies will be done in the future.

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

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