Regulation of the programmed cell death 1/ programmed cell death ligand pathway and immune response by Bifidobacterium infantis in mice with inflammatory bowel disease

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Basic Study

Regulation of the programmed cell death 1/ programmed cell death ligand pathway and immune response by *Bifidobacterium* infantis in mice with inflammatory bowel disease

Lin-Yan Zhou, Ying Xie, Yan Li

Abstract

BACKGROUND

Inflammatory bowel disease (IBD) is caused by an abnormal immune response. Programmed cell death 1 (PD-1) is an immunostimulatory molecule, which interacts with PD ligand (PD-L1) playing a prime important role among autoimmune diseases. *Bifidobacterium infantis* (*B. infantis*) can promote the differentiation of CD (cluster of differentiation) 4^+ T cells into regulatory T cells (Tregs). Tregs participate in the development of IBD and may be related to disease activity. *B. infantis* amplify the expression level of PD-1, PD-L1 and Tregs' nuclear transcription factor forkhead box protein 3 (Foxp3). But the mechanism of *B. infantis* on PD-1/PD-L1 signaling remains unclear.

AIM

Our team attempt to explore the mechanism of *B. infantis* regulating the immune response in IBD.

METHODS

Forty-eight-week-old BALB/c mice were randomly divided into five groups: The control group, dextran sulphate sodium (DSS) model group, DSS + *B. infantis* group, DSS + *B. infantis* + anti-PD-L1 group, and DSS + anti-PD-L1 group. The control group mice were given drinking water freely, the other four groups were given drinking water containing 5% DSS freely. The control group, DSS model group, and DSS + anti-PD-L1 group were given normal saline (NS) 400 μL daily by gastric lavage, and the DSS + *B. infantis* group and DSS + *B. infantis* + anti-PD-L1 group were given NS and 1×10^9 colony-forming unit of *B. infantis* daily by gastric lavage. The DSS + *B. infantis* + anti-PD-L1 group and DSS + anti-PD-L1 group were given 200 μg of PD-L1 blocker intraperitoneally at days 0, 3, 5 and 7; the control group, DSS + anti-PD-L1 group, and DSS + *B. infantis* group were given an intraperitoneal injection of an equal volume of PBS. Changes in PD-L1, PD-1, Foxp3, interleukin (IL)-10, and transforming growth

factor β (TGF-β) 1 protein and gene expression were observed. Flow cytometry was used to observe changes in $CD4^+$, $CD25^+$, $Foxp3^+$ cell numbers in the blood and spleen.

RESULTS

Compared to the control group, the expression of PD-1, Foxp3, IL-10, and TGF-β1 was significantly decreased in the intestinal tract of the DSS mice ($P < 0.05$). Compared to the control group, the proportion of $CD4^+$, $CD25^+$, $Foxp3^+$ cells in spleen and blood of DSS group was visibly katabatic ($P < 0.05$). *B. infantis* upgraded the express of PD-L1, PD-1, Foxp3, IL-10, and TGF-β1 ($P < 0.05$) and increased the proportion of $CD4^+$, $CD25^+$, $Foxp3^+$ cells both in spleen and blood ($P < 0.05$). After blocking PD-L1, the increase in Foxp3, IL-10, and TGF-β1 protein and gene by *B. infantis* was inhibited ($P < 0.05$), and the proliferation of $CD4^+$, $CD25^+$, $Foxp3^+$ cells in the spleen and blood was also inhibited ($P < 0.05$). After blocking PD-L1, the messenger ribonucleic acid and protein expression of PD-1 were invariant.

CONCLUSION

It is potential that *B. infantis* boost the proliferation of $CD4^+$, $CD25^+$, $Foxp3^+$ T cells in both spleen and blood, as well as the expression of Foxp3 in the intestinal tract by activating the PD-1/PD-L1 pathway.

Key Words: Bifidobacterium infantis; Enteritis; Programmed cell death ligand; T-Lymphocytes

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Core Tip: *Bifidobacterium infantis (B. infantis)* can treat inflammatory bowel disease by regulating the intestinal microbiota, alleviating inflammation, and regulating the

immune response. We found that *B. infantis* might have a facilitating effect that accelerate the expression of forkhead box protein 3 (Foxp3) activating the programmed cell death 1 (PD-1)/ PD ligand 1 pathway, as well as the proliferation of Foxp3$^+$ T cells.

INTRODUCTION

Inflammatory bowel disease (IBD) is often described as the cancer that doesn't die, which is arise by the aberrant immune response or the disruption of immune tolerance toward intestinal antigens. Several factors, including the immune system itself, infections, environmental factors, and genetics may make terrible contribution to the development of IBD[1-4]. To maintain tolerance toward the intestinal environment, the intestinal immune system monitors changes in the bacterial microbiota and expression of antigens on the surface of the intestinal mucosa[5-6]. Antigen presenting cells, including dendritic cells and intestinal epithelial cells, present intestinal antigens to CD (cluster of differentiation) 4$^+$ T cells and induce their differentiation into regulatory T cells (Tregs), which maintain tolerance toward the intestinal microbiota. It has been shown that hyperactive T cell responses toward the intestinal microbiota contribute to the inflammatory response seen in IBD[7].

Programmed cell death 1 (PD-1) and PD ligand (PD-L1), are members of CD28/B7 superfamily. This superfamily primarily functions in T cell-mediated immune responses is closely related to several diseases, including autoimmune diseases, tumors, chronic virus infection, and chronic inflammation[8-10]. The role of the PD-1/PD-L1 signaling pathway in glomerulonephritis, systemic lupus erythematosus, rheumatoid arthritis, dilated cardiomyopathy, autoimmune diabetes, and other autoimmune diseases have been widely studied[11,12]. Nevertheless, few researches have examined the role of the PD-1/PD-L1 signaling pathway in IBD[13].

Tregs are a subpopulation of T lymphocyte with immunoregulatory functions[14]. Tregs can inhibit the activation and proliferation of autoreactive T cells by secreting cytokines such as interleukin (IL)-10 and transforming growth factor β (TGF-β), downregulating the function of auxiliary T cells, maintaining intestinal homeostasis,

and maintaining immune tolerance[15,16]. Treg cells are divided into two categories: Natural regulatory T cells (nTregs) and induced regulatory T cells (iTregs). nTregs mature in thymus and are $CD4^+$, $CD25^+$, $Foxp3^+$. iTregs are induced from $CD4^+$ T cells by specific antigen stimulation in the presence of IL-2 and TGF-β1 in the intestine, spleen, and other peripheral sites[17]. Studies in animals have shown that injecting T cells without $CD4^+$, $CD25^+$, Tregs into T cell-deficient mice can induce the development of autoimmune colitis, while injecting T cells along with $CD4^+$, $CD25^+$ Tregs can inhibit the development of colitis. This suggests that $CD4^+$, $CD25^+$ Tregs are vital for inhibition of the intestinal immune response [18]. Maul *et al*[19] found that the percentage of Tregs in the peripheral blood of IBD patients decreased in the active phase of the disease and increased in the remission phase of the disease. Meanwhile, the number of Tregs in the intestinal epithelium increased in the active phase of the disease, but was still significantly lower than the number of Tregs in diverticulitis patients. This suggests that the Tregs participated in the development of IBD and may be related to disease activity.

Animal studies have also shown that the adoptive transfer of immature $CD4^+$ T cells to wild-type $rag^{-/-}$ mice and PD-$L1^{-/-}$$rag^{-/-}$ mice significantly decreased the number of Tregs in PD-$L1^{-/-}$$rag^{-/-}$ mice, suggesting that PD-L1 has a dominating role to play in Treg differentiation[20]. PD-L1 can enhance Treg function and promote the production of IL-10 by Tregs[21]. The differentiation of Tregs is dependent upon the PD-L1 signaling pathway. Higher levels of PD-L1 expression in hepatodendritic cells result in greater induction of Tregs, which maintain tolerance toward transplanted organs.

We found that *Bifidobacterium infantis* (*B. infantis*) can alleviate intestinal epithelial injury and maintain intestinal immune tolerance in a mouse model of IBD, may have underlying therapeutic worth for immunologic injure of IBD. *B. infantis* elevated the expression level of PD-L1 and PD-1 in the intestine by a wide margin, promoted the expression of Tregs' nuclear transcription factor, anti-inflammatory factor IL-10 and TGFβ1. Based on the above information and our previous study[22], we explored the mechanism of *B. infantis* on PD-1/PD-L1 signaling pathway and the enhancement of differentiation and function of Tregs.

MATERIALS AND METHODS

Reagents and antibodies

Dextran sulphate sodium (DSS), molecular weight 36000-50000, was purchased from MP biomedicals (Solon, OH, United States). *B. infantis* freeze-dried powder containing 1.6 × 10^{11} colony-forming unit (CFU)/g was provided by Shandong Kexing Biological Products Co., Ltd. (batch No. 2017012, Shandong Province, China). Invivomab anti mouse PD-L1 was purchased from BIOX cell (NH, United States). BALB/c mice were purchased from Huafukang Biotechnology (Beijing, China). APC rat anti mousecd4, Bb515 rat anti mouse, PE rat anti mouse Foxp3, and Transcription factor buffer set were purchased from BD company (NJ, United States). PD-L1 antibody, PD-1 antibody, and Foxp3 antibody were purchased from Proteintech Group (Chicago, IL, United States). Antibody IL-10 and TGF-β1 were purchased from Abcam (Cambridge, United Kingdom). Real time q polymerase chain reaction (PCR) need flowing reagents: Trizol (Invitrogen, Thermo Fisher, IA, United States), Primescripttm RT Regent kit with g deoxyribonucleic acid (DNA) eraser quick response training (qRT) PCR kit SYBR® premier ex taqtm II (TLI RNaseH Plus, Takara, Japan), Primer (Biotechnology Co., Ltd., China).

Animals

Forty-eight-week-old BALB/c mice, half male and half female, weighing 20 ± 2 g, were raised in pathogen-free conditions in the standalone animal experimental centre affiliated with the Shengjing Hospital of China Medical University. The mice were kept at 20 °C-26 °C, and the relative humidity was 40%-70%, 12 h light/dark cycle. Sterilized water and standard feed were provided for free consumption by the animals. The experimental protocol was approved by the ethics committee of the hospital (No. 2017PS353K). The experimental operators ensured that suitable measures were taken to reduce malaise and injure to the experimental animals.

Experimental grouping and modeling

Experimental grouping: 40 mice were randomly divided into 5 groups: Control group, DSS group, DSS + *B. infantis* group, DSS + *B. infantis* + anti-PD-L1 group, and DSS + anti-PD-L1 group, and marked.

Modeling method: Mice in control group were given free drinking water for 7 d. The other 4 groups were given sterilized water containing 5% DSS for 7 d. The drinking water was changed daily.

Drug intervention: The control group, DSS group, and DSS + anti-PD-L1 group were given a 400 μL normal saline (NS) gavage daily; and the DSS + *B. infantis* group and DSS + *B. infantis* + anti-PD-L1 group were given a 400 μL NS gavage and a *B. infantis* (1×10^9 CFU) gavage daily. The DSS + *B. infantis* + anti-PD-L1 group and DSS + anti-PD-L1 group were given an intraperitoneal injection of PD-L1 blocker (200 μg), and the control group, DSS model group, and DSS + *B. infantis* group were given an intraperitoneal injection of PBS on days 0, 3, 5 and 7.

Specimen collection

General condition of the mice: During the experimental period, the temperament, reactivity, activity, hair color, weight, eating, and defecation of teach mouse were observed seriatim and recorded in detail daily.

Peripheral blood collection: On the 8th day, all animals were anesthetized *via* isoflurane inhalation and beards were removed. Blood was collected through retro-orbital bleeding and placed in blood collection vessels containing ethylene diamine tetra acetic acid (EDTA). The blood and EDTA were fully mixed and stored on ice.

Extraction of single cells from mouse spleen: After blood collection, the mice were sacrificed *via* isoflurane inhalation, dissected along the midline, and the spleen was

fully exposed. After blunt dissection, the spleen was taken out completely, placed in PBS, and transported on ice. The spleen was then transferred to a glass dish containing 1640 medium and mashed with ground glass. Next, the cells were transferred to a centrifuge tube. The samples were simultaneously centrifuged at 1200 revolutions per min for 5 min, and the supernatant was then discarded. Next, 2 mL of red blood cell (RBC) lysate was added to each sample. PBS (3 mL) was added to dilute and stop the lytic reaction, then the samples were centrifuged once more but at 400 × g for 5 min at 4 °C. The supernate was then discarded, 3 mL of PBS was added. The cells were filtered, then centrifuged for 10 min. The supernate was discarded, and cell PBS was instilled to obtain a single cell suspension. All procedures are carried out at 4 °C to ensure cell viability.

Acquisition of mouse colon: After splenectomy, the colon was exposed, and the colon from the blind part to the anus was taken back. After the colon was washed with precooled NS, it was divided into 4 parts. The samples were then transferred to a -80 °C ultra-low temperature refrigerator in liquid nitrogen for long-term preservation.

Detection of CD4⁺, CD25⁺, Foxp3⁺ T cells by flow cytometry

Spleen: Splenic CD4⁺, CD25⁺, Foxp3⁺ T cells were detected by flow cytometry. 100 μL of the prepared single cell spleen suspension was aliquoted into labeled flow tubes. Anti-CD4⁺ and anti-CD25⁺ antibodies were added to the tubes and incubated in a 4 °C cold without light room for 30 min. Next, 1 mL of 1X fix/perm working solution was added to each sample and incubated at 4 °C in the dark for 40 min to permeabilize the nucleus. Anti-Foxp3 antibody was then added and incubated at 4 °C without light for 40 min. Excess antibody was then removed, and the samples were run on a flow cytometer (Facscalibur, BD company, NJ, United States).

Peripheral blood: After RBC lysis, flow cytometry was performed on peripheral blood samples using the same protocol detailed above.

Western blotting

Total protein from the colon was extracted, and the protein concentration was determined. The samples were subjected to electrophoresis at 60V. After marker separation, the voltage was adjusted to 80V. After 30 min, the voltage was adjusted to 100V. The electrophoresis was stopped when the target protein with the lowest molecular weight reached the end of the gel. 100 V was used to transfer the proteins to a membrane. Proteins with a molecular weight less than 25 kiln-dried (kd) were transferred for 25 min, and proteins weighing 26-70 kd were transferred for 70 mi. The membrane was blocked with 2.5% BSA at room temperature for 1.5 h. Antibodies [PD-L1 (1:750), PD-1 (1:500), Foxp3 (1:1000), IL-10 (1:800), TGF-β1 (1:500), GAPDH (1:10000)] were added and allowed to incubate at 4 °C overnight. Goat anti-rabbit immunoglobulin G secondary antibody labeled with horseradish peroxidase was added to the membrane and incubated at room temperature for 1.5 h. In the dark room, a chemiluminescence imaging analysis system was used to image the membrane. Gelpro software was used to analyze the image and carry out quantitative analysis. Protein content = gray value of the target protein of the sample/gray value of the same sample.

Real time qPCR

Ribonucleic acid (RNA) purification: DSS can reduce the purity of RNA, so extra purification of the colon RNA was necessary. RNA purification was performed as follows: 30 μL lithium chloride (8 mol/L) + 270 μL ddH_2O was added to 10 μL RNA and placed on ice for 2 h. The samples were then centrifuged at 14000 × g for 30 min. The supernatant was then discarded, and the RNA was dissolved in 90 μL ddH_2O. Next, 10 μL sodium acetate (3 mol/L) + 200 μL anhydrous ethanol was added to the RNA and incubated at -20 °C for 30 min to precipitate the RNA. The samples were then centrifuged at 14000 × g for 30 min at 4 °C. The supernatant was then discarded, 500 μL 75% ethanol was added, and the samples were gently blown with a pipette to clean the RNA. Next, the samples were centrifuged at 800 × g for 10 min at 4 °C. The supernatant

was discarded, and the RNA was dissolved with 10 μL ddH2O. Finally, the samples were transferred to -80 °C on ice for preservation.

Detection of RNA concentration: The ratio of A260/A280 was eligible for all of the samples, which indicates that the purity of the RNA was high and suitable for further experiments.

Preparation of cDNA by RNA reverse transcription: The gDNA was removed, and the specimens were heated to 42 °C for 2 min. For reverse transcription, the reaction solution was prepared according to Table 1. The samples were heated at 37 °C for 15 min and 85 °C for 5 s. The reaction was then stopped and cooled down to 4 °C.

Concentration and purity of cDNA: XXXX

qRT-PCR: qRT-PCR was carried out as follows: PCR amplification reaction, denaturation at 95 °C for 5 min, PCR reaction at 95 °C for 10 s, and 60 °C for 30 s for 45 cycles.

Statistical analysis

The data are expressed as mean ± standard deviation. The difference among groups were analyzed by using Analysis of Variance. SPSS 23.0 (IBM, NY, United States) and Graphpad 7.0 (Software, CA, United States) statistical software were used to perform the statistical analyses. Two-tailed *P* value were calculated and $P < 0.05$ were considered statistically significant.

RESULTS

The effect of B. infantis on the expression of PD-1 after PD-L1 blockade

Western blot results: Compared to the DSS + *B. infantis* group, PD-1 protein in the DSS + *B. infantis* + anti-PD-L1 group decreased, but the difference was not statistically

significant (P = 0.07). Compared to DSS model group, the expression of PD-1 was no significant distinction in DSS + anti-PD-L1 group (P = 0.62) (Figure 1).

qRT-PCR results: In contrast to control group, PD-1 messenger ribonucleic acid (mRNA) in DSS group decreased significantly ($P < 0.05$). In constrast to DSS group, the expression of DSS + *B. infantis* group increased, but the difference was not statistically significant (P = 0.36). Compared to the *B. infantis* group, PD-1 RNA decreased significantly in the DSS + *B. infantis* + anti-PD-L1 group ($P < 0.05$) (Figure 1).

Effect of B. infantis on Tregs and Foxp3 expression after PD-L1 blockade

Western blot results: Compared to the DSS + *B. infantis* group, Foxp3 protein decreased in DSS + *B. infantis* + anti-PD-L1 group, and the difference was statistically significant ($P < 0.05$). There was no significant difference in Foxp3 protein expression between the DSS model group and DSS + anti-PD-L1 group (P = 0.99) (Figure 2).

RT-qPCR results: In contrast to control group, Foxp3 mRNA in DSS model group decreased significantly ($P < 0.05$); Foxp3 mRNA in DSS + *B. infantis* group was significantly higher than that in DSS model group ($P < 0.05$). Compared to the *B. infantis* group, the expression of Foxp3 mRNA decreased significantly in the DSS + *B. infantis* + anti-PD-L1 group ($P < 0.05$). In comparison with DSS model group, Foxp3 mRNA in DSS + anti-PD-L1 group was also visible distinction ($P < 0.05$) (Figure 2).

Flow cytometry results

Flow cytometry of peripheral blood: Compared to control group, the proportion of peripheral $CD4^+$, $CD25^+$, $Foxp3^+$ cells decreased visibly ($P < 0.05$) of DSS group and increased visibly in the blood of the DSS + *B. infantis* group ($P < 0.05$). Compared to the DSS + *B. infantis* group, the proportion of $CD4^+$, $CD25^+$, $Foxp3^+$ cells in the peripheral blood of DSS + *B. infantis* + anti-PD-L1 group was significantly lower ($P < 0.05$). The

proportion of CD4⁺, CD25⁺, Foxp3⁺ cells in the blood of the DSS + anti-PD-L1 group was also distinctly lower compared to the DSS group ($P < 0.05$) (Figure 3).

Flow cytometry of splenocytes: The ratio of splenic CD4⁺, CD25⁺, Foxp3⁺ cells in DSS model group was significantly lower ($P < 0.05$), comparing to control group. The ratio of CD4⁺, CD25⁺, Foxp3⁺ cells in the DSS + *B. infantis* group was significantly higher than that in the DSS model group ($P < 0.05$). Compared to the DSS + *B. infantis* group, the proportion of CD4⁺, CD25⁺, Foxp3⁺ cells in the DSS + *B. infantis*+ anti-PD-L1 group decreased significantly ($P < 0.05$). The proportion of CD4⁺, CD25⁺, Foxp3⁺ cells in the DSS + anti-PD-L1 group also decreased significantly compared to the DSS group ($P < 0.05$) (Figure 4).

The effect of B. infantis on the expression of IL-10 and TGF-β1 after PD-L1 blockade

Western blot results: The expression of IL-10 and TGF-β1 protein in the DSS + *B. infantis* group was lower than that in DSS + *B. infantis* + anti-PD-L1 group ($P < 0.05$). Compared to the DSS model group, there was no apparent distincton in the express of IL-10 ($P = 0.99$) or TGF-β1 in the DSS + anti-PD-L1 group ($P < 0.05$) (Figure 5).

Real time PCR results: In comparison with control group, mRNA of IL-10 and TGF-β1 in the DSS model group decreased ($P < 0.05$), and mRNA of IL-10 and TGF-β1 in the DSS + *B. infantis* group increased clearly ($P < 0.05$). IL-10 and TGF-β1 mRNA expression in DSS + *B. infantis* + anti-PD-L1 group decreased clearly ($P < 0.05$) contrsating to DSS + *B. infantis* group. Compared to the DSS model group, IL-10 and TGF-β1 mRNA in DSS + anti-PD-L1 group were also statistically distinction ($P < 0.05$) (Figure 5).

DISCUSSION

The specific pathogenesis of IBD has yet to be completely elucidated, and further study is still needed[23]. Currently, abnormal inflammatory responses and continuous inflammatory damage of the intestine are recognized as the basic mechanisms of IBD

pathogenesis[24,25]. Normal intestinal immunity includes intestinal mucosal immunity, T cell immunity, cytokines, and intestinal microecology[26]. It is a complex and interactive process involving several different factors of the immune system. The intestinal mucosal immune system is responsible for monitoring the intestinal microbiota and surface antigens[27], presenting the antigens to original $CD4^+$ T cells, promoting interaction between PD-1 and PD-L1, forming immune tolerance, and preventing the occurrence of autoimmunity. Inflammatory mediators are involved in the pathogenesis of DSS colitis, including interferon γ, tumor necrosis factor α, IL-10, and other cytokines, suggesting that inflammatory immune responses act a critical part in the pathogenesis of IBD[28-30].

PD-1 is a recently discovered immunostimulatory molecule, which interacts with its ligand PD-L1 to regulate T cell-mediated immunity and induce immune tolerance, thus acting a critical part in autoimmune diseases such as IBD, tumor immunity, and acceptance of transplanted organs. Some studies have shown that PD-1 knockout animals were attacked by autoimmune diseases[31-33]. Activation of the PD-1/PD-L1 signaling pathway can induce the differentiation of Tregs[34] and the release cytokines such as IL-10 and TGF-β1 to inhibit the activation and proliferation of reactive T cells, thus maintaining intestinal immune tolerance. Additionally, inhibition of the PD-1/PD-L1 pathway may reduce Tregs[35]. These findings indicate that PD-1/PD-L1 signaling plays a critical role in immune tolerance[36]. We found that after blocking PD-L1, there was no distinction both in protein and mRNA of PD-1 in the intestines of DSS-induced mice, suggesting that PD-L1 itself did not transform the gene or protein express of PD-1 in the intestines of IBD model. However, the expression of PD-1 mRNA in the intestinal tract of the DSS mice was significantly decreased, but the expression of PD-1 protein did not changed after the intervention of *B. infantis*. We speculate that blocking PD-L1 may indirectly inhibit the promotion of PD-1 gene transcription by *B. infantis*, but it does not affect the process of post transcription and translation of PD-1; it may also indirectly inhibit the transcription level of PD-1, but it does not regulate the protein level because of the decrease of PD-L1 Ligand. Additionally, blocking PD-L1 may also

reverse inhibit the transcription of the PD-1 gene because of the high level of PD-1 protein expression. Further study is needed to determine explore how PD-L1 blockade inmacts the transcription and translation of PD-1, and how *B. infantis* can affect PD-1 after PD-L1 blockade.

B. infantis can treat IBD by regulating the intestinal microbiota, alleviating inflammation, and regulating the immune response. Animal studies have shown that *B. infantis* can reduce intestinal wall permeability and edema in IBD mice, reduce neutrophil infiltration, and alleviate the intestinal inflammatory response[37]. Our team found that[38] *B. infantis*, when combined with *Clostridium butyricum*, can increase the number of probiotic bacteria such as *Bifidobacterium* and *Lactobacillus* in the intestinal microbiota of IBD patients, reduce the number of enterococci, improve clinical symptoms, and promote the healing of colonic mucosa. In recent years, it has been found that *B. infantis* also acts an important part in immune regulation, which can promote the proliferation of Tregs[39] and increase the expression of IL-10 and TGF-β1. *In vivo* and *in vitro* experiments have revealed that *B. infantis* can sharply accelerate the differentiation of $CD4^+$ T cells into Tregs by inducing the maturation of resistant dendritic cells and further inhibit the inflammatory response induced by reactive T cell activation. Furthermore, our team found that compared to normal mice, the number of $CD4^+$, $CD25^+$, $Foxp3^+$ T cells in the blood and spleen of the DSS mice decreased and the expression of Foxp3 mRNA in the intestine decreased, suggesting that the differentiation and proliferation of Tregs may be involved into the pathogenesis of IBD[40]. The number of Tregs in the colon is related to the intestinal microbiota: Treg numbers in the colons of sterile mice are significantly reduced. After feeding feces from specific pathogen-free mice to sterile mice, the number of Tregs in the colon significantly increases[41], which shows that Treg numbers are dependent upon the intestinal microbiota. In IBD patients, the numbers of normal intestinal bacteria are decreased, resulting in intestinal microbiota disorder[42]. Therefore, improvements in the intestinal microbiota are helpful to increase the numbers of Tregs, and this mechanism needs further study. We found that *B. infantis* can promote the proliferation of $CD4^+$,

CD25⁺, Foxp3⁺ T cells in the blood and spleen, and it can promote Foxp3 mRNA in the intestine. After blocking PD-L1, the number of CD4⁺, CD25⁺, Foxp3⁺ T cells in the blood and spleen decreased significantly, and the expression of Foxp3 protein and mRNA in the intestine decreased. Therefore, we believe that *B. infantis* can promote the proliferation of Tregs by activating the PD-L1/PD-1 pathway.

B. infantis also promoted the mRNA expression of IL-10 and TGF-β1 in the intestine. After blocking PD-L1, the expression of IL-10 and TGF-β1 protein and mRNA in the intestine decreased significantly. In order to verify whether the changes in IL-10 and TGF-β1 induced by *B. infantis* are regulated by the PD-1/PD-L1 pathway, we blocked PD-L1. After blocking, the TGF-β1 and IL-10mRNA and protein levels were sharply downregulated, indicating that *B. infantis* affected IL-10 and TGF-β1 through the PD-1/PD-L1 pathway. Tregs mainly secrete TGF-β1 and IL-10 to inhibit the inflammatory response. Further, we still need to further confirm the immunosuppressive effect of *B. infantis* in human experiments.

Francisco *et al*[20] found that PD-L1 can downregulate Akt, mTOR, S6, and ERK2, and upregulate PTEN in Tregs. Whether *B. infantis* can accelerate the differentiation and proliferation of Tregs by activating the PD-1/PD-L1 pathway and regulating Akt, mTOR, or PTEN expression needs further study.

CONCLUSION

In conclusion, *B. infantis* may accelerate the proliferation of CD4⁺, CD25⁺, Foxp3⁺ T cells in the spleen and peripheral blood and the expression of Foxp3 in the intestine by activating the PD-1/PD-L1 signal channel. It can also promote the expression of IL-10 and TGF-β1 to reduce the intestinal inflammatory response, which has a therapeutic effect on IBD mice. We will continue to study the value of PD-1 / PD-L1 pathway in IBD and explore the role of *B. infantis* in IBD patients.

ARTICLE HIGHLIGHTS

Research background

Immune inflammatory response plays an extremely important role in the pathogenesis and development of inflammatory bowel disease (IBD). *Bifidobacterium infantis* (*B. infantis*) can repair acute intestinal mucosal injury and maintain autoimmune tolerance in mice with IBD.

Research motivation

The specific mechanism of *B. infantis* in the treatment of IBD is not clear. Solving this problem will provide new fields and orientations for the treatment of IBD.

Research objectives

This study pursued to explore whether *B. infantis* can promote regulatory T cells (Tregs) differentiation through programmed cell death 1 (PD-1) /PD ligand (PD-L1) pathway, so as to promote the expression of Forkhead box protein 3 (Foxp3), interleukin (IL)-10 and transforming growth factor β (TGF-β) 1, and finally achieve the effect of immunosuppression.

Research methods

We first blocked the expression of PD-L1 in the intestine. Western blot and Real time qPCR were used to observe whether the effects of *B. infantis* on PD-1, Foxp3, IL-10 and TGF-β1 changed after blocking PD-L1. We adopted flow cytometry to observe whether there were changes in the differentiation of $CD4^+$, $CD25^+$, $Foxp3^+$ cells in blood and spleen after blocking PD-L1.

Research results

After blocking PD-L1, the promoting effects of *B. infantis* on intestinal PD-1, Foxp3, IL-10 and TGF-β1 were weakened. Meanwhile, the promoting effect of *B. infantis* on $CD4^+$, $CD25^+$, $Foxp3^+$ cells differentiation is also limited.

Research conclusions

B. infantis mediated Foxp3 expression through PD-1/PD-L1 pathway, which promotes Tregs differentiation and improves IL-10 and TGF-β1 expression, so as to reduce the immune and inflammatory response of IBD and play a therapeutic role in IBD.

Research perspectives

To explore the mechanism of *B. infantis* in the treatment of IBD from the cellular level. Human experiments show whether *B. infantis* inhibits immune response through PD-1/PD-L1 pathway.

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