Interleukin-10 gene-carrying bifidobacteria ameliorate murine ulcerative colitis by regulating regulatory T cell/T helper 17 cell pathway

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Abstract

Ulcerative colitis (UC) is a chronic inflammatory bowel disease suggested to be closely related to the imbalance of regulatory T cell/T helper 17 cell (Treg/Th17) signaling. Previously, we constructed an interleukin-10 (IL-10) expression vector, BL-hIL-10, and proved that it ameliorates dextran sulfate sodium-induced intestinal inflammation in mice. In this study, we further explored the mechanisms underlying BL-hIL-10 treatment from the Treg/Th17 imbalance perspective. Our results showed that the oral administration of BL-hIL-10 reduced the UC inflammation in mice significantly, which was assessed by disease activity index, spleen index, and pathological changes in colon tissue. Moreover, the mice after BL-hIL-10 treatment had increased proportion of Treg cells while Th17 cells decreased greatly, leading to the reconstruction of Treg/Th17 balance. Furthermore, the Th17 cell-secreted factors, such as IL-6, IL-17, and IL-23, were reduced, but the Treg-related factors, IL-10 and Transforming growth factor- β 1 (TGF- β 1), were elevated accordingly. Finally, Western blot confirmed the inhibition of nuclear hypoxia-inducible factor-1 α (HIF-1 α) and cytoplasmic mechanistic target of rapamycin (mTOR) and signal transducer and activator of transcription 3 (STAT3) in intestinal tissues. In conclusion, oral administration of BL-hIL-10 can alleviate the inflammation responses of UC in murine model through the restoration of Treg/Th17 imbalance, which might be at least partially due to the inhibition of hypoxia-mTOR-HIF-1 α -Th17 axis as well as IL-6-STAT3-HIF-1 α -Th17 pathway.

Keywords: Ulcerative colitis, bifidobacteria, interleukin 10, T helper 17 cells, regulatory T cells

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Introduction

Ulcerative colitis (UC) is a chronic inflammatory bowel disease (IBD) associated with multiple colonic and extraintestinal complications. Although the etiology of UC is not completely elucidated up to date, autoimmunity has been emphasized in the pathogenesis of specific damage in target organs. It is believed that the abnormal inflammatory process caused by imbalanced mucosal immune system plays an important role in the intestinal lesion of UC.^{1,2} Currently, studies have indicated that the transformation balance of the regulatory T (Treg) cells and T helper 17 (Th17) cells is a key factor in maintaining intestinal immune homeostasis.^{3–6} The abnormal activation of Th17 cells can promote the occurrence of intestinal inflammation and induce autoimmune diseases, while Treg cells may inhibit inflammatory responses in intestines. Consequently, Treg/Th17 imbalance may participate in the pathogenesis of IBD,

which is immunologically characterized by the elevated secretion of interleukin (IL)-17 and other pro-inflammatory cytokines. Actually, the amelioration of intestinal inflammation could be achieved by correcting the imbalance of Treg/Th17,^{7,8} further supporting the hypothesis that targeting Treg/Th17 pathway is one of the therapeutic strategies.

IL-10 plays a negative regulatory role in cell immune system. It may down-regulate the transcription and secretion of IL-1 β , IL-6, and tumor necrosis factor- α and other pro-inflammatory cytokines that are synthesized by T cells and macrophage.^{9,10} Thereby, IL-10 inhibits antigen presentation, and ultimately suppresses T cell-mediated immune response and improves the intestinal inflammation in UC.¹¹ Moreover, IL-10 gene knockout in mice could induce colitis, in which the pathogenesis is very similar to that in human UC in aspects of immunity and pathophysiology, suggesting that IL-10 plays a very important role in the development of UC.^{11,12}

Ecological imbalance of the intestinal tract is also one of the main causes for IBD.¹³ Studies have shown that the population of butyric acid bacteria and bifidobacteria are significantly reduced in the intestinal flora of IBD patients compared with that of healthy individuals. Thus, correspondingly supplementing probiotics may improve intestinal inflammation.^{14,15}

Previously, we had constructed a novel efficient IL-10 delivery system which was named BL-hIL-10, by using *Bifidobacterium longum* (BL) of NCC 2705 strain. It was confirmed that the hIL-10 protein produced by these bacteria is biologically active and has therapeutic functions.¹⁶ In addition, it could inhibit nuclear factor- κ B (NF- κ B) activated signaling pathways in intestine tract and increase CD4⁺CD25⁺Foxp3⁺Treg expression level in peripheral blood and mesenteric lymph nodes in the murine model of UC, which both alleviate inflammatory responses in the intestinal tracts.¹⁶ In this study, we aimed to further explore the mechanism underlying BL-hIL-10 treatment from the aspects of transforming Treg/Th17 balance and related signal pathways.

Materials and methods

Reagents

pBBAD/X was the shuttle vector between *B. longum* and *Escherichia coli*. pBBADs-hIL-10 was the IL-10 expression vector, and transformed into *B. longum* using electroporation method.¹⁶ Radioimmunoprecipitation assay (RIPA) lysis buffer and the protease and phosphatase inhibitors were purchased from Thermo Scientific (Shanghai, China). Mouse Treg/Th17 flow cytometry detection kit (IL-17 A and CD4 + CD25 + Foxp3 as the markers for Th17 and Treg cells, respectively) were purchased from BD Biosciences (San Jose, CA). The enzyme-linked immunosorbent assay (ELISA) kits of transforming growth factor- β 1 (TGF- β 1), IL-6, IL-10, IL-17, and IL-23 were purchased from R&D Systems (Minneapolis, MN). Antibodies targeting signal transducer and activator of transcription 3 (STAT3) and hypoxia-inducible factor-1 α (HIF-1 α) were from Cell Signaling (Danvers, MA). Mechanistic target of rapamycin (mTOR) antibody was from Santa Cruz (Dallas, TX). Dextran sulfate sodium (DSS; MW = 5 kDa) was from Wako Chemical Company (Tokyo, Japan).

Animals and experimental design

A total of 50 male BALB/c mice at age of 6–7 weeks old and weighing 22–26 g were provided by the Experimental Animal Center of Southern Medical University and housed in standard animal facility with constant temperature and humidity and light/dark cycle of 12 h/12 h. They were randomly assigned into five groups with 10 mice in each group. Except the normal control (NC) group, the other mice were orally administrated with DSS (3.5% in drinking water) to induce UC (for 3 days).¹⁶ After additional 24-h pre-induction with 0.2% L-Arb, the mice in four UC groups were treated with bifidobacteria (UC-bacteria), pBBAD/X plasmid containing bifidobacteria (UC-pBBAD/X-bacteria), BL-hIL-10 plasmid containing

bifidobacteria (UC-BL-hIL-10-bacteria), or as blank control (UC-blank), respectively. All mice were given 0.2 mL phosphate-buffered saline (PBS) daily, to which none or different bifidobacteria (5×10^8) as above were added according to the grouping. Following the seven-day treatment, mice were observed for another seven days before the blood, spleens, and colons were harvested for further experiments.

Assessment of inflammation severity

The degree of inflammation in mice was comprehensively assessed based on the daily disease activity index (DAI), spleen index (SI), and hematoxylin & eosin (H&E) staining of colon tissues. DAI was scored according to Murthy scoring system.¹⁷ At sacrifice, the spleens were harvested and weighed. SI was calculated as the ratios of weight (mg) to body weight (g).¹⁸ Colon tissues were routinely fixed in formalin solution and processed for paraffin sections that were afterwards stained with H&E.¹⁹

ELISA

Colon tissues (25 mg) were fully homogenized on ice with RIPA lysis buffer (2 mL), to which both protease and phosphatase inhibitors were added. Following centrifuge at 3000 rpm, the supernatants were collected and the protein concentrations were measured using Bradford method.²⁰ The levels of cytokines in both sera and tissue supernatants were determined using relevant ELISA kits according to the instructions.²¹

Detection of Treg/Th17 cells

Lymphocytes in single cell suspension were prepared from mouse spleens with commercial separation media (Dakewe Biotech, Shenzhen, China). Cells were resuspended in RPMI 1640 media that were supplemented with 10% serum, 50 ng/mL phorbol 12-myristate 13-acetate (Sigma Aldrich, St. Louis, MO), 1 μ g/mL ionomycin (Sigma Aldrich), and proper amount of monensin (BD Biosciences) according to the instruction. After 5-h culture at 37°C, cells were adjusted to 2×10^6 /mL, and then fixed and permeabilized. A mouse Treg/Th17 detection kit (BD Biosciences) was used for the detection of cell phenotypes. Briefly, cells were incubated with the phenotype antibodies or isotype controls at room temperature for 30 min. After washing, cells were resuspended in PBS, and then analyzed by flow cytometry.

Western blot analysis

Nuclear and cytoplasmic proteins were extracted from the lysates of colon tissues as described previously.²² Samples were denatured and then separated with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. The proteins were transferred to nitrocellular membranes, which were then blocked with 5% non-fat milk in PBS. The STAT3 and mTOR in cytoplasmic and HIF-1 α in nuclei were detected using the specific antibodies above.^{23,24}

Statistical analysis

Data were expressed as mean $\pm$ standard error of the mean and statistically analyzed using SPSS13.0 software. Tukey's test was used to compare the differences between two groups after significant effects were observed in a two-way analysis of variance (ANOVA) for three or more groups. $P < 0.05$ was considered statistically significant.

Results

BL-hIL-10 treatment ameliorates inflammation severity of UC in mice

Classic manifestations of UC appeared in all mice in the UC groups before the bacterial treatment, including persistent liquid or bloody stool, weight loss, and decreased activity. After bacterial treatment, as compared to UC-blank group, the other three UC groups showed less liquid or bloody stool and weight loss (data not shown), and had apparently increased activity but decreased DAI. In addition, the mice in UC-BL-hIL-10-bacteria group displayed significantly decreased DAI as compared with those in UC-bacteria or UC-pBBAD/X-bacteria groups, the latter of which showed no difference from each other ($P > 0.05$) (see Figure 1).

All UC mice developed splenomegaly (Figure 2a). The highest SI was seen in the UC-BL-hIL-10-bacteria group as there was no difference between the other three UC groups ($P > 0.05$) (Figure 2b). Meanwhile, the histological evaluation of colon tissues showed that acute inflammation of the intestinal tracts in UC-BL-hIL-10-bacteria group ameliorated greatly but the mice in other UC groups exhibited severe inflammatory responses, including mucosal edema and erosion, reduced crypt, and intense infiltration of lymphocytes (Figure 2c). There was no obvious inflammation in mice from NC group.

BL-hIL-10 treatment regulates the proportion of Treg/Th17 cells

The absolute number of splenocytes is highest in the UC-blank group but lowest in the NC group. There was no difference between the three UC-bacteria groups ($P > 0.05$) although which all were lower than UC-blank group (data not shown). The subpopulations of T cells in spleens were assessed for the effect of BL-hIL-10 on immune system in UC mice. Compared to the mice in NC group, UC-blank mice had significantly reduced proportion of Treg cells but enhanced proportion of Th17 cells in total splenocytes, both of which, however, were relatively reversed upon the treatment with bifidobacteria, pBBAD/X-bifidobacteria, or BL-hIL-10-bifidobacteria. Among the three groups receiving bifidobacteria, BL-hIL-10 group had the highest Treg proportion and lowest Th17 proportion that were similar to the NC group (see Figure 3).

BL-hIL-10 treatment affects the cytokine levels in plasma and colon tissues

The cytokine levels in plasma were determined, showing that the mice in UC-blank group had significantly reduced TGF- β 1 and IL-10 but increased IL-6 and IL-17 as compared with NC group (Figure 4a). However, the treatment of

Figure 1 The treatment with BL-hIL-10-bacteria ameliorated DAI in UC mice. Seven days after the induction of dextran sulfate sodium, the mice in different groups were monitored for DAI. The DAI of normal mice was zero at any time point. DAI, disease activity index; UC, ulcerative colitis. By ANOVA analysis, the UC-BL-hIL-10-bacteria group differed from the others significantly. * $P < 0.05$, compared with UC-blank group at the same time points; $\Delta P < 0.05$, compared with pBBAD/X-bacteria group at the same time points

bifidobacteria especially BL-hIL-10-bifidobacteria lessened the changes induced by UC (Figure 4a). Meanwhile, the levels of IL-23 were assessed in both plasma and tissue supernatants of colons. Interestingly, an increase in IL-23 level was seen in the colon tissues from all UC groups although BL-hIL-10 treatment partially inhibited the magnitude of elevation (Figure 4b). Nevertheless, the IL-23 levels in plasma remained unfluctuated in all groups (Figure 4b).

BL-hIL-10 treatment increases cytoplasmic mTOR and STAT3 and nuclear HIF-1 α in colon tissues

By Western blot, the protein expression levels of cytoplasmic mTOR and STAT3 and nuclear HIF-1 α increased significantly in the UC-blank mice as compared to NC mice. However, the bifidobacterial treatment reduced the expression of these proteins with the highest efficiency seen in the BL-hIL-10-bifidobacteria group (see Figure 5).

Discussions

In the present study, the results showed that the typical symptoms of UC in murine model were ameliorated by the oral administration of BL-hIL-10 bifidobacteria. Moreover, BL-hIL-10 treatment reduced splenomegaly and histological changes of colon tissues in UC mice. Meanwhile, the recovery of Treg cell proportion in splenocytes was seen in BL-hIL-10-treated group, which was also reflected by the correction of cytokine levels in both plasma and colon tissues. Furthermore, the BL-hIL-10 treatment promoted the expression of cytoplasmic mTOR and STAT3 and nuclear HIF-1 α in colon tissues, indicating the inhibition of hypoxia-mTOR-HIF-1 α -Th17 axis and IL-6-STAT3-HIF-1 α -Th17 pathway.

Our findings are consistent to the previous report that the BL-hIL-10 treatment significantly affects not only intestinal but also systemic immune responses in UC mice.¹⁶

Figure 2 BL-hIL-10-bacteria reduced spleen index and histological changes of colon tissues. (a) The representative images of spleens in each groups are shown. The number 1 to 5 indicated the groups of normal control, UC-blank, UC-bacteria, UC-pBBAD/X-bacteria, and UC-BL-hIL-10-bacteria, respectively. (b) The UC-blank group had higher spleen indexes than normal controls, which was reduced upon the bacterial treatments. (c) Similarly, the bacterial treatments decreased the histological changes of colon tissues in UC mice. UC, ulcerative colitis. * $P < 0.05$, compared with UC-blank group; $\Delta P < 0.05$, compared with UC-bacteria group; # $P < 0.05$, compared with UC-pBBAD/X-bacteria group. In (b), P values were from Tukey's test that was performed after ANOVA revealed significant difference between the five groups. (A color version of this figure is available in the online journal.)

Actually, IL-10 plays an important role in the pathogenesis of UC,^{25,26} and the treatment that induces the rise in circulating IL-10 may significantly ameliorate the manifestations of UC.^{16,27} Moreover, the enhancement of circulating IL-10 in UC usually correlates with the changes of systemic immune responses, such as the alteration of cell subpopulations in spleens and lymph nodes or the modulation of other inflammatory factors.^{28,29} Therefore, our results added to these conclusions by providing multiple evidences that exogenous IL-10 also has effect on the systemic immune responses during the treatment of UC in mice.

Recent studies have demonstrated increasing attentions to the roles of Th17 cells and Treg cells in IBD.^{28,30,31} Th17 cells are a new subtype of CD4⁺ T helper cells distinct from the traditional subtypes of Th1 and Th2 cells. The differentiation Th17 cells is under the control of retinoic acid-related orphan receptor, which regulates the expression of IL-17 A and IL-17 F.^{28,32} Consequently, CD4⁺ T helper cells differentiate into Th17 cells under the influence of IL-6 and TGF- β .³³ It had been proven that Th17 cells are involved in the pathogenesis of multiple and autoimmune diseases mainly through secreting inflammatory effectors such as IL-17 A,

IL-17 F, and IL-22.^{3,32} Differentially, Treg cells which are CD4⁺ CD25⁺ Foxp3⁺,³⁴ inhibit the inflammatory responses in intestinal mucosa, leading to the maintenance of local immune balance by primarily secreting cytokines such as IL-10 and TGF- β .^{4,35,36} Therefore, the balance of Treg/Th17 cells is instrumental in the pathogenesis of UC. In this study, the BL-hIL-10 treatment corrected the imbalance of Treg/Th17 cells, strongly verifying that exogenous IL-10 reversely regulates the number of Treg and Th17 cells.

IL-23 is instrumental in the pathogenesis of T cell-mediated colitis and exerts proinflammatory function by promoting CD4⁺ cells to secrete inflammatory cytokines such as IL-17.³⁷ Although it is not involved in the early differentiation of Th17 cells, IL-23 plays an important role in maintaining the phenotype stability, survival, proliferation, and functions of Th17 cells.^{6,38-40} In this study, the level of IL-23 in plasma did not change between all groups, but the IL-23 in colon tissues increased in UC-blank mice and fluctuated with the efficiency of bifidobacterial treatment. The discrepancy between the IL-23 levels in sera and colon tissues may be explained by the differential effect of IL-10 treatment on them, which mainly

Figure 3 The BL-hIL-10 treatment affected the proportions of Treg and Th17 cells in spleens. (a) The representative images of flow cytometry for splenocytes were shown while positive cells were counted from the quadrants of Q3 and Q4. (b) BL-hIL-10 bacteria increased CD4 + CD25 + Foxp3 cells (Treg cells) but reduced IL-17 A cells (Th17 cells) in the spleens from UC mice, which had lower proportion of Treg cells and higher proportion of Th17 cells as compared to normal controls. UC, ulcerative colitis. * $P < 0.05$, compared with UC-blank group; $\Delta P < 0.05$, compared with UC-bacteria group; # $P < 0.05$, compared with UC-pBBAD/X-bacteria group. In (b), P values were from Tukey's test that was performed after ANOVA revealed significant difference between the five groups. (A color version of this figure is available in the online journal.)

suppresses the dominant elevation of IL-23 in local colon tissues but not that in circulating plasma.

The therapeutic effect was more dramatic in mice treated with BL-hIL-10-bacteria than the mice treated with bifidobacteria alone or pBBAD/X-bacteria. This might result from the facts that as normal flora, bifidobacteria are important in the maintenance of natural flora and immune system in colon, and the improvement of bifidobacteria leads to amelioration of UC.⁴¹ There were no significant differences between the bifidobacteria alone and pBBAD/X-bacteria

groups since the plasmid itself has no effect on normal flora. Therefore, the IL-10 expressing bifidobacteria exhibit dual benefits of bifidobacteria plus IL-10 on UC in mice, the latter of which functions through inhibiting the activity of Th17 cells and reducing the secretion of IL-6, IL-17, and IL-23.

HIF-1 is important transcription factor to modulate body's oxygen balance and plays a key role in both physiological and pathological processes. HIF-1 is a heterodimer of subunits HIF-1 α and HIF-1 β , wherein HIF-1 α is the

Figure 4 The BL-HIL-10 treatment changed the levels of inflammatory cytokines. (a). Compared to UC-blank control, the levels of IL-6, IL-10, IL-17, and TGF-β1 in plasma altered upon the bacterial treatments, among which the BL-HIL-10-bacteria had the highest potential of affection. (b) The levels of IL-23 were detected in plasma and colon tissue supernatant, respectively, showing that BL-HIL-10-bacteria reduced IL-23 in the supernatant from UC mice although the IL-23 in plasma kept stable in all groups ($P > 0.05$). UC, ulcerative colitis. * $P < 0.05$, compared with UC-blank group; Δ $P < 0.05$, compared with UC-bacteria group; # $P < 0.05$, compared with UC-pBBAD/X-bacteria group. In (a) and (b), P values were from Tukey's test that was performed after ANOVA revealed significant difference between the five groups accordingly

Figure 5 The BL-HIL-10 treatment inhibited the expression of mTOR, STAT3, and HIF-1α. (a) By Western blot, the cytoplasmic proteins of mTOR and STAT3 and nuclear HIF-1α showed an increase in colon tissues from UC-blank mice, which was partially inhibited by the BL-HIL-10 treatment. (b) The intensities of Western blot bands were measured quantitatively by ImageJ, confirming the enhancement of these proteins in UC mice and relative amelioration after BL-HIL-10 treatment. UC, ulcerative colitis. * $P < 0.05$, compared with UC-blank group; Δ $P < 0.05$, compared with UC-bacteria group; # $P < 0.05$, compared with UC-pBBAD/X-bacteria group. In (b), P values were from Tukey's test that was performed after ANOVA revealed significant difference between the five groups

regulatory subunit.⁴² HIF-1α binds to Foxp3, leading to accelerated degradation of Foxp3 and downstreamly the prohibition of Treg development and function.^{22,23,43,44} Moreover, both Hypoxia-mTOR-HIF-1α-Th17 and IL-6-STAT3-HIF-1α-Th17 pathways are pivotal in the development and activation of Th17 cells that produce inflammatory cytokines such as IL-17. In the present study, we found that nuclear HIF-1α and cytoplasmic mTOR and STAT3 increased in the colon tissues from UC mice as compared to normal mice, but decreased after the treatment with BL-HIL-10-bacteria. This meant that BL-HIL-10 rebalances Treg/Th17 through inhibiting hypoxia-mTOR-HIF-1α-Th17 and IL-6-STAT3-HIF-1α-Th17 pathways.

In conclusion, our results revealed the mechanisms underlying BL-HIL-10 treatment of UC mice and proved that the oral administration of BL-HIL-10 induces an improvement of Th17/Treg balance, which is related to the elevation of TGF-β1 and IL-10 levels and the reduction of IL-6, IL-17, and IL-23 expression in colon tissues. Moreover, the therapeutic effect of BL-HIL-10 producing bifidobacteria involves the inhibition of hypoxia-mTOR-HIF-1α-Th17

and IL-6-STAT3-HIF-1α-Th17 pathways. Therefore, the approach of BL-HIL-10-bifidobacterial treatment may be modified for better efficiency or even expanded to other inflammatory diseases from the aspect of Th17/Treg balance.

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