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Treatment of Mice with Dextran Sulfate Sodium-Induced Colitis with Human Interleukin 10 Secreted by Transformed *Bifidobacterium longum*

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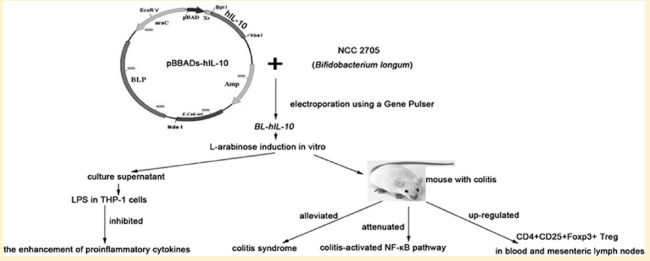
ABSTRACT: Ulcerative colitis (UC) is an inflammatory bowel disease (IBD) the etiology of which has not yet been fully clarified. Cytokine interleukin-10 (IL-10) plays a central role in downregulating inflammatory cascade in UC and is likely a candidate for therapeutic intervention. However, its intravenous administration is costly and inconvenient. Therefore, we established a novel IL-10 delivery system by transforming a hIL-10-containing plasmid into *B. longum* (*BL-hIL-10*) and investigated its effects on 5% dextran sulfate sodium (DSS)-induced ulcerative colitis in mice and the possible underlying mechanism. Our results show that (1) hIL-10 was expressed and secreted into the culture supernatant of *BL-hIL-10* after L-arabinose induction *in vitro* as examined by Western blot, enzyme-linked immunosorbent assay (ELISA) and RT-PCR; (2) addition of *BL-hIL-10* culture supernatant had no cytotoxic effect and morphological alteration, but significantly inhibited the enhancement of proinflammatory cytokines by lipopolysaccharide (LPS) in THP-1 cells; (3) oral administration of *BL-hIL-10* alleviated colitis syndrome of the model mice, attenuated colitis-activated NF-κB pathway measured by DNA-binding assay and colitis-elevated expression of proinflammatory cytokines examined with CCK cytotoxic kits, and upregulated $CD4^+CD25^+Foxp3^+$ Treg in blood and mesenteric lymph nodes measured by flow cytometry. In conclusion, *BL-hIL-10* as a novel oral hIL-10 delivery system has been successfully established and oral administration of *BL-hIL-10* alleviated inflammatory damage of colonic tissue in the model mice by blocking the colitis-activated NF-κB pathway and upregulating $CD4^+CD25^+Foxp3^+$ Treg in blood and mesenteric lymph nodes in mice.



KEYWORDS: ulcerative colitis, *Bifidobacterium longum*, *BL-hIL-10*, interleukin 10

INTRODUCTION

Ulcerative colitis (UC) is a type of inflammatory bowel disease (IBD) characterized by chronic gastrointestinal inflammation. These inflammatory processes are orchestrated by the production of chemokines and proinflammatory cytokines.[1] Interleukin 10 (IL-10) is an immunosuppressive and anti-inflammatory cytokine that inhibits both antigen presentation and subsequent release of proinflammatory cytokines, thereby attenuating mucosal inflammation. IL-10 knockout mice have colitis that has many relevant immunological, pathological and physiological similarities to human UC,[2,3] indicating that IL-10 plays a pivotal role in the mucosal immune system and chronic ileocolitis. As a therapeutic candidate, recombinant human IL-10 plays a central role in downregulating inflammatory cascades.[4] Systemic administration of IL-10 by intravenous injection is sufficient to inhibit proinflammatory Th1 lymphocyte responses and abrogate colitis in colitis mouse models induced by dextran sodium sulfate (DSS) and transfer of $CD4^+CD45RB^{high}$ cells, respectively.[5,6] Clinical trials in UC patients have shown that daily administration of IL-10 by bolus injection into vein for over 1 week is safe and well tolerated but has minimal therapeutic efficacy compared with placebo.[7] However, its short half-life[8] does not allow sufficient mucosal penetration to inhibit the proinflammatory response. Therefore, a more sustained and focused IL-10 delivery to the gastrointestinal mucosa may improve its effectiveness.

The etiology and pathogenesis of UC are unclear currently. However, research evidence supports the hypothesis of an inappropriate response to either endogenous microbial flora within the intestine alone or with some components of

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autoimmunity in UC development and healing.[9] In UC patients, the number of *Bifidobacterium* in colonic mucosa is reduced,[10] while that of *Bacteroides*, in particular *Bacteroides vulgatus*, is elevated.[11] *Bacteroides vulgatus* is able to either penetrate the gut epithelium or proliferate on its epithelial surface, thus evoking the inflammatory response.[12] *Bifidobacterium* spp. are the dominant probiotic bacteria inhabiting the ileum and large intestine of human and other warm-blooded animals. An increased *Bifidobacterium* population in colonic mucosa has many beneficial effects on human health by regulating immunomodulation, preventing infection, reducing serum cholesterol, promoting lactose digestion and protecting from colon cancer.[13−15] The effects of intestinal *Bifidobacterium* species such as *B. longum* (*BL*) on preventing UC occurrence or exacerbation by attenuating intestinal inflammation are currently under investigation in both animal models and UC patients.[16]

The previous study has shown that *Bifidobacterium* is easier to colonize in the colon and more suitable than *Lactobacillus*, therefore it is more effective for controlling intestinal inflammation.[17] In addition, oral administration of *BL-hIL-10* is a more convenient and controllable operation than rectal infusion under sedation with adenoviral vectors encoding murine IL-10[18], and adenoviral vectors[18] as a simple vector have no treatment effect on the colitis. In addition, heterogeneous expression of *hIL-10* in *Bifidobacterium* exerts double beneficial functions from both IL-10 and *Bifidobacterium*, and may increase treatment efficiency in colitis model mice. Here we show that oral administration of *BL-hIL-10* has the ability of rectal hIL-10 to induce colonic IL-10 expression and to prevent mouse from developing colitis with high effectiveness by blocking the NF-κB pathway and reducing proinflammatory cytokines and can recover intestinal immunity function by upregulating $CD4^+CD25^+Foxp3^+$ regulatory T cells (Treg) expression.

MATERIALS AND METHODS

Reagents. *B. longum* strain NCC2705 was kindly provided by the Nestle Research Center (Lausanne, Switzerland). Restriction endonucleases *Bpi*I and *Xba*I, polymerase and T4 DNA ligase were purchased from MBI Fermentas Inc. (Vilnius, Lithuania). cDNA of human interleukin 10 was synthesized by Shanghai Yingjun Biological Engineering Technology and Services Ltd. (China). Protease and phosphatase inhibitor cocktail was purchased from Roche (USA). Human monocyte cell strain THP-1 (TIB-202) was kindly provided by ATCC (USA). Commercial rh-IL-10 was purchased from Shanghai SinoBio Biotech Co., Ltd. (China). Primers used for PCR and qRT-PCR were projected and synthesized by Takara (Japan).

Plasmid Construction, Transformation of *B. longum* and Identification. pBBAD/Xs is a shuttle vector between *Escherichia coli and B. longum*[19] as described elsewhere in detail.[20] Construction of pBBADs-GFP has been described previously.[19] The human IL-10 expression vector pBBADs-hIL-10 was constructed by replacing GFP with human IL-10 gene at the *Bpi*I and *Xba*I sites of plasmid pBBADs-GFP and verified by restriction enzyme digestion and sequencing (Takara, Japan) as shown in Figure 1A. The control no-load vector (pBBADs-0) was constructed by filling in with DNA polymerase Klenow fragment and ligation using DNA ligase. Plasmids pBBADs-hIL-10, pBBADs-GFP and pBBADs-0 were transformed into *B. longum* NCC 2705 by electroporation using a Gene Pulser and Pulse Controller apparatus (Bio-Rad, USA) at 2.0 kV, 25 μF and 200 Ω, as described previously.[19] The transformed bacteria were plated on 24 plates precoated with BYG agar (1.5%, w/v) supplemented with 0.05% L-cysteine and 100 μg/mL ampicillin and incubated anaerobically at 37 °C for 48 h. Full-size colonies were selected and cultured for 48 h in BYG broth containing 100 μg/mL ampicillin. Positive clones of *B. longum* with pBBADs-hIL-10 (*BL-hIL-10*) and pBBADs-0 (*BL0*) were identified using PCR analysis to amplify the target gene hIL-10 (Table 1). Transformed *B. longum* with pBBADs-GFP (*BL-GFP*) and the *B. longum*'s 16S recombinant DNA gene,[19] and their morphologies have been described previously.[20]

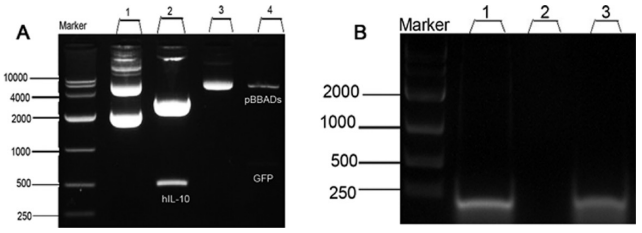

Figure 1. Identification of transformed *BL* and *E. coli* by restriction enzyme digestion and PCR. A: Identification of *E. coli* transformed with plasmid containing the targeted gene. 1: Commercial plasmid with hIL-10 gene. 2: Commercial plasmid with hIL-10 gene digested by *Xba*I and *Bpi*I. 3: pBBADs-GFP plasmid. 4: pBBADs-GFP plasmid digested by *Xba*I and *Bpi*I. B: Identification of hIL-10 gene in *BL-hIL-10* by PCR. 1: pBBADs-hIL-10 plasmid extracted from *E. coli*. 2: pBBADs-0 plasmid extracted from BL0. 3: pBBADs-hIL-10 plasmid extracted from *BL-hIL-10*.

***In Vitro* Induction of Gene Expression.** Transformed bacteria were cultured in BYG broth supplemented with 100 μg/mL ampicillin at 37 °C. Expression of targeted genes were induced by adding 0.2% L-arabinose (Invitrogen Inc., USA) when absorption of bacterium suspension at OD_{695nm} reached ~0.6. Culture supernatants and bacterium pellets were collected after continuous culture for 12, 24, and 36 h, respectively, and stored at − 70 °C for future use.

Monocyte THP-1 Cell Culture and Treatment. THP-1 cells were cultured in RPMI 1640 (PPA, Austria) medium supplemented with 10% heat-inactivated fetal calf serum (Gibco, USA), 1% nonessential amino acids, 1% L-glutamine and 0.2% of zeocin (100 mg/mL)[21] at 37 °C in an incubator supplemented with 5% CO_2. For treatment with IL-10, cells were inoculated at a density of 5×10^3 cells per well into a 96 well plate containing RPMI 1640 supplemented with or without 100 μL of supernatant of *BL-hIL-10* after induction with 0.2% L-arabinose for 24 h, 50 ng/mL and 100 ng/mL commercial rhIL-10, respectively, and cultured at 37 °C for 8 h. The cytotoxic effect of all treatment was examined using CCK cytotoxic kits (Nanjing Kengen Bioteck. Co., Ltd., China) according to the protocol provided by the manufacturer. Each treatment was carried out repeatedly in 8 wells.

For treatment of THP-1 cells with LPS, cells were plated at a density of 6×10^5 cells/well in a 24 well plate containing RPMI 1640 supplemented with (1) 1 μg/mL LPS (Sigma, Austria) and 100 μL of supernatants of *BL-hIL-10* induced with 0.2% L-arabinose; (2) 1 μg/mL LPS (Sigma, Austria) and 100 μL of supernatants of *BL-hIL-10* without induction; (3) 100 μL of supernatants of *BL-hIL-10* induced with 0.2% L-arabinose; (4) 100 μL of supernatants of *BL-hIL-10* without induction; (5) 1 μg/mL LPS (Sigma, Austria) and 50 ng/mL or 100 ng/mL commercial rhIL-10, respectively; (6) 50 ng/mL or 100 ng/mL

Table 1. Nucleotide Sequences of Primers Used for PCR and qRT-PCR

gene	forward sequence	reverse sequence
hIL-10	CTTCAAGGGTTACCTGGGTT	GCGTTCTTCACCTGCTCC
TNF-α	GGCTGCCCTGTCCTTCCTAGATA	CGAGCTAGTGACACTCTGACACTCC
IL-1β	TCCAGGATGAGGACATGAGCAC	GAACGTCACACACCAGCAGGTTA
IL-6	CCACTTCACAAGTCGGAGGCTTA	GCAAGTGCATCATCGTTGTTCATAC
IFN-γ	CGGCACAGTCATTGAAAGCCTA	GTTGCTGATGGCCTGATTGTC
GAPDH	TGTGTCCGTCGTGGATCTGA	TTGCTGTTGAAGTCGCAGGAG

Table 2. Disease Activity Index Scoring System

score	weight loss	stool consistency	occult/bloody stools
0	−	normal	normal
1	1−5%		
2	6−10%	loose	occult +
3	11−15%		
4	>15%	diarrhea	bloody stools

commercial rhIL-10, respectively. Eight hours after culture, cell supernatants were harvested by centrifugation and stored at −20 °C for future measurement. The experiments were performed in duplicate at least three times.

Animals and Experimental Design. Six week old male BALB/c mice weighing 18−20 g from Guangdong Medical Animal Center were kept at room temperature (22 °C) in a room with controlled 12 h light/dark cycle and free access to standard rodent chow. Ten mice without any treatment were assigned to the normal group. The colitis was induced in 40 mice by free access to 5% DSS solution for seven days. After that, these 40 mice were randomly assigned into 4 groups and treated for an additional 7 days as follows: 10 mice used as colitis control were given 0.2 mL PBS once a day through an oral-gastric tube by oral administration; the other three colitis groups of 10 mice were administrated once a day 0.2 mL of PBS suspensions (6×10^8 cells per mL) of *BL-hIL-10*, *BL0* and *BL-GFP* induced with 0.2% L-arabinose for 24 h, respectively. All mice were then fasted for eight hours, then sacrificed by cervical dislocation and examined as described below. All animal experiments were reviewed and approved by the Guangdong Animal Medical Ethics Committee, China.

Colitis Severity Assessment. Five centimeters of intestines from each mouse was clysised with 200 μL of PBS and irrigating solution three times each and stored at −20 °C. Colitis severity was assessed by both disease activity index (DAI) and histological evaluation. DAI was adopted based on the scoring system of Murthy et al.,[22] which scores body weight loss, stool consistency and rectal bleeding, and calculated as average scores of weight loss, stool consistency and bleeding. Occult blood in the feces was evaluated using test slides from a Fecal Occult Blood Test Kit (Si Jia Biotechnology Company, Guangzhou, China). Normal stool means well-formed pellets; loose means pasty stools that do not stick to the anus; diarrhea means liquid stools that stick to the anus. These parameters are comprehensive functional measures analogous to clinical symptoms observed in human IBD, and the scoring method has been validated by repeated studies[23] (Table 2).

The large intestine was immediately removed and its length was measured to evaluate intestinal shortening. Colons were then fixed, paraffin embedded, sectioned, stained with hematoxylin and eosin (HE), examined under microscope with 100× objective and scored based on the method reported by Hirata et al.[23] by a pathologist who was blinded to the treatment protocol. Three sections of each sample were randomly selected, and eight random fields of each section were examined. The mean scores of each section are listed in Table 3.

Measurement of Myeloperoxidase Activity in Colitis Tissue. Myeloperoxidase activity in colonic tissue homogenate was measured by Myeloperoxidase Assay Kit (Jiancheng Biotech, Nanjing, China) according to the protocol provided by the manufacturer. Reaction was performed at 60 °C in a water bath for 10 min, and absorption of the products at 460 nm was measured with a plate reader (Bio-Rad, USA).

Expression of Inflammatory Genes Measured with Two-Step Real-Time PCR. Total RNA of colonic tissues was extracted using an Ezgene Tissue RNA Miniprep Kit (Biomiga, Genman), and its amount was estimated by measuring the absorbance at 260 nm. 2.0−4.0 μg of RNA was reverse transcribed using a PrimeScript RT reagent Kit (Takara, Japan) at 37 °C for 30 min followed by 85 °C for 5 s in a thermocycler. Real-time PCR was performed with the SYBR Premix Ex Tap (Takara, Japan) in a 4.0 μL reaction system containing 0.5 μL cDNA as template and 0.5 μL of specific primer pairs listed in Table 1 under the following conditions: 95 °C for 10 min followed by 45 cycles of denaturing at 95 °C for 5 s, annealing at 60 °C for 20 s and elongation at 72 °C for 20 s. Housekeeping gene GAPDH was used as internal control for each sample. The PCR specificity was confirmed by a melting curve analysis. The PCR products and primer localization of each gene were examined by agarose gel electrophoresis.

Enzyme-Linked Immunosorbent Assay. IL-10 contents in the culture supernatant of *BL-hIL-10* induced with 0.2% L-arabinose, and the colonic irrigating solution of mice, TNF-α and IL-6 contents in the culture supernatants of THP-1 and the mouse blood plasma samples, and concentrations of TNF-α, IL-6, IL-1β and IFN-γ in homogenates of the colonic tissues were measured by ELISA kits (R&D Systems, USA) according to the protocol provided by the manufacturer.

Measurement of NF-κB (p65) DNA-Binding Activity. Nuclear extracts from 30 mg colons were prepared using a Proteo-JET Cytoplasmic and Nuclear Protein Extraction kit (Fermentas, Germany) according to the manufacturer's protocol. NF-κB activation was measured using a nonradioactive NF-κB specific DNA-binding ELISA kit (Cayman Chemical Company, USA) according to the manufacturer's protocol. This quantitative method is at least 10 times more sensitive than the electrophoresis mobility shift assay.[24]

Western Blot Analysis. Proteins were concentrated from the culture supernatants of the transformed *B. longums* as described previously[20] and separated by 15% SDS−PAGE. IL-10 levels were examined by Western blot using a monoclonal antibody

Table 3. Histological Disease Scoring System

score	histological features
0	normal colonic mucosa
1	loss of one-third of crypts
2	loss of two-thirds of crypts
3	the lamina propria is covered with a single layer of epithelium and mild inflammatory cell infiltration is present
4	erosions and marked inflammatory cell infiltration are present

directed against IL-10 (1:1000, Santa Cruz Biotechnology) and quantified by Quantity One (Bio-Rad).

Thirty micrograms of nuclear proteins extracted from colonic tissues were separated by 12% SDS−PAGE and transferred onto nitrocellulose membranes. NF-κB p65 and histone H2 levels were detected by Western blot using primary monoclonal antibodies directed against NF-κB p65 (1:1000, Santa Cruz Biotechnology) and (1:1500, Santa Cruz Biotechnology), respectively. The single band of NF-κB p65 with a molecular weight of 65 kDa was normalized to histone H2 and quantified by Quantity One (Bio-Rad).

Flow Cytometry. Peripheral blood lymphocyte suspension was isolated directly from peripheral blood of mouse orbital vein using mouse lymphocyte isolation kits according to the manufacturer's instruction (Tai Jian Hao Yang Biological Company, China). Mesenteric lymph nodes (MLN) of ileocecal junction were collected by removing tegument and adipose tissues, cut into small pieces (1−2 mm) and mixed with 0.1% collagenase (Sigma, USA) RPMI 1640 solution. After incubation at 37 °C for 20 min, the small pieces were then ground by 200-mesh stainless steel grit, and centrifuged at 3000 rpm for 5 min. After washing twice with RPMI 1640, lymphocytes from MLN were obtained. Cell surface markers of 1×10^6 lymphocytes from MLN and peripheral blood were stained with $CD4^+$, $CD25^+$-conjugated mAb for 30 min at 4 °C, respectively, according to the manufacturer's instructions (eBioscience, USA) and incubated with freshly prepared fixation/permeabilization working solution at 4 °C for 12 h in the dark. The cell surfaces were then blocked with Fc. Samples were divided into two fractions: one was stained with Foxp3 antibody, while the other was stained with IgG2a. All samples were then subjected to flow cytometry analysis using Epics Altra Facs device (Beckman Coulter).

Statistical Analysis. All data were expressed as mean ± SD and analyzed using software SPSS 13.0. Following the assurance of normal distribution of data, one-way analysis of variance (ANOVA) was used for multiple comparisons, and unpaired Student's *t* test was used to evaluate the level of statistical significance between two groups. Difference with a *p* value less than 0.05 was considered statistically significant.

RESULTS

Identification of *BL-hIL-10* by PCR. *hIL-10* gene sequences in *BL-hIL-10* were identified by PCR. A band of 216-bp fragment of rhIL-10 gene was amplified from the plasmid DNA of *BL-hIL-10* (Figure 1B). No fragment was obtained from untransformed bacteria or *BL0*.

Detection of hIL-10 Expression in *BL-hIL-10 in vitro*. The hIL-10 secreted into the supernatant and in the pellets of *BL-hIL-10* was detected by Western blot and ELISA, respectively (Figure 2). Western blot found a strong protein band of 19 kDa

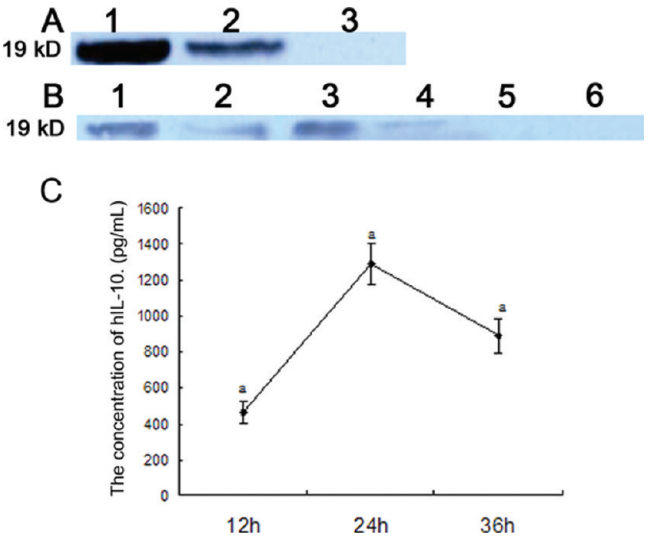

Figure 2. hIL-10 expression in *BL-hIL-10* and *E.coli-hIL-10 in vitro*. A: Western bolt analysis of hIL-10 expression in *E.coli-hIL-10*. 1: *E.coli-hIL-10* induced with 0.2% L-arabinose. 2: *E.coli-hIL-10* without induction with 0.2% L-arabinose. 3: *E.coli-0* induced with 0.2% L-arabinose. B: Western blot analysis of hIL-10 expression in *BL-hIL-10*. 1: commerial hIL-10 protein. 2: *BL-hIL-10* induced with 0.2% L-arabinose for 12 h. 3: *BL-hIL-10* induced with 0.2% L-arabinose for 24 h. 4: *BL-hIL-10* induced with 0.2% L-arabinose for 36 h. 5: *BL0* induced with 0.2% L-arabinose for 24 h. C: ELISA analysis of *BL-hIL-10* protein in *BL-hIL-10* induced with 0.2% L-arabinose for 12 h, 24 and 36 h, respectively. The letter a indicates $p < 0.05$ compared with other groups.

in the cell lysate of *E.coli-hIL-10* after induction and a weak band of 19 kDa in the cell lysate of *E.coli-hIL-10* without induction, but not in the cell lysate of *E.coli-0* with induction (Figure 2A). The molecular weight of the detected bands is concordant with the theoretical molecular weight of mature IL-10 (Figure 2B). ELISA examination indicates that the levels of hIL-10 in both supernatant and cell pellet similarly reached maximum at 24 h of culture (Figure 2C).

Inhibitory Effect of *BL-IL-10* on Inflammatory Cytokines in Culture Supernatant of LPS-Induced THP-1. LPS treatment alone significantly enhanced TNF-α and IL-6 protein levels in culture supernatant of THP-1 cells compared with nontreated cells (Figure 3). This enhancement was inhibited significantly by addition of 100 μL culture supernatant of *BL-IL-10*, 50 ng/mL rIL-10 and 100 ng/mL, respectively ($p < 0.05$). But compared with cells treated with BYG, their levels were still significantly increased ($p < 0.05$). No cytotoxicity was found in all treated cells compared with that of nontreated cells using CCK cytotoxic kits (data not shown).

Effect of *BL-IL-10* on Mouse DAI. No mice died during the experiment. Mice with DSS-induced UC had pasty-to-liquid grossly bloody stools, weight loss, and severe anemia from day

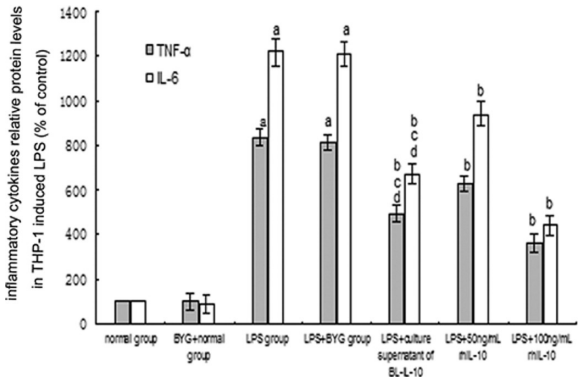

Figure 3. The effects of *BL-hIL-10* culture supernatant on the inflammatory cytokine protein levels in THP-1 induced with LPS. Letters a, b, c and d indicate $p < 0.05$ compared with the normal group, the LPS group, the LPS + 50 ng/mL rIL-10 group and the LPS + 100 ng/mL rIL-10 group, respectively.

5 to day 14. By comparison, mice with DSS-induced UC after treatment with supernatants of *BL*, *BL-hIL-10* and *BL0*, respectively, showed better formed stools with no evident blood and lessened weight loss from day 9 to day 14. DAI score (Table 2), the indicator of the severity of intestinal inflammation, was zero in the normal group. Following exposure to DSS, DAI was significantly increased and reached maximum at day 7 in mice with UC. After treatment with supernatants of *BL-hIL-10*, *BL* and *BL0*, their DAI was significantly decreased compared with control colitis mice from day 8 to day 14 ($p < 0.05$). In particular, the DAI of model mice treated with supernatant of *BL-hIL-10* was significantly lower than that of model mice treated with *BL* ($p < 0.05$) (Figure 4A).

Mice with colitis induced by DSS demonstrated severe intestine shortening due to intestinal inflammation compared to normal mice (Figure 4B). This intestine shortening was significantly suppressed in the DSS-induced colitis model mice treated with supernatants of *BL-hIL-10*, *BL* and *BL0*, respectively ($p < 0.05$). Particularly, the intestine length of mice treated with supernatants of *BL-hIL-10* was longer than that of the mice treated with *BL* ($p < 0.05$) (Figure 4B).

Effect of *BL-IL-10* on Mouse Histological Features. Histological features in the acute phase of UC are mucosal erosions, crypt shortening, edema, and infiltration of neutrophils in the mucosa and lamina propria (Table 3). Compared to the normal group (Figure 4C-1), mice with UC exhibited marked erosion of the lamina propria mucosa, disappearance of glandular epithelium, inflammatory cell infiltration, and other related symptoms (Figure 4C-2). By contrast, after treatment with *BL-hIL-10* (Figure 4C-5), *BL* (Figure 4C-3) and *BL0* (Figure 4C-4), respectively, erosion, disappearance of glandular epithelium and inflammatory cell infiltration tended to be less severe and the histological disease scores were significantly lower ($p < 0.05$). In addition, compared to the *BL*-treated mice, mice treated with *BL-hIL-10* had strikingly lower histological disease score ($p < 0.05$) (Figure 4C).

Effect of *BL-IL-10* on Mouse MPO Activity. The activity of MPO in mice with UC was higher than that of normal mice ($p < 0.05$). When colitis model mice were treated with *BL-hIL-10*, *BL* and *BL0*, respectively, their MPO activities were significantly decreased ($p < 0.05$), and the MPO activity in the *BL-hIL-10*-treated mice was significantly lower than that in the *BL*-treated ones ($p < 0.05$). (Figure 4D).

Effect of *BL-IL-10* on Mouse Cytokine Level. As shown in Figure 5, the activity and protein expression of NF-κB p65, as assessed by Western blotting (Figure 5A) and ELISA (Figure 5B), were markedly increased in mice with UC compared with normal mice ($p < 0.05$). Treatment with *BL-hIL-10*, *BL* and *BL0*, respectively, resulted in a significant reduction of NF-κB p65 activity and protein expression ($p < 0.05$). NF-κB p65 activity and protein expression in the *BL-IL-10*-treated mice were significantly lower than those in *BL*-treated ones ($p < 0.05$) (Figure 5B).

As shown in Figure 6, mRNA expressions (Figure 6A) and protein levels (Figure 6B) of TNF-α, IL-1β, IFN-γ and IL-6 were significantly higher in mice with UC than those in normal mice ($p < 0.05$), as assessed by real-time PCR and ELISA. The protein levels of these inflammatory makers were evidently lower in *BL-hIL-10-*, *BL-* and *BL0*-treated model mice than those in untreated model mice ($p < 0.05$), and significant difference was observed between *BL-hIL-10*-treated and *BL*-treated mice ($p < 0.05$) (Figure 6C).

Effect of *BL-IL-10* on Mouse Immune System. As shown in Figure 7, the percentages of $CD4^+CD25^+Foxp3^+$ Treg in peripheral blood and MLN in mice with UC were lower than those in normal mice, but these decreases were significantly suppressed in *BL-*, *BL0-* and *BL-hIL-10*-treated model mice compared to the untreated model mice. In addition, the percentage of $CD4^+CD25^+Foxp3^+$ Treg was significantly increased in the *BL-IL-10*-treated mice compared with *BL*-treated ones.

All indices examined above were not significantly different between *BL*-treated and *BL0*-treated mice ($P > 0.05$)

DISCUSSION

The results presented here show that oral administration of *BL-hIL-10* attenuated DSS-induced colitis, as demonstrated by a reduction of colitis indices of DAI, intestinal shortening and histological changes at both macroscopic and microscopic levels. This effect was related specifically to transgenic expression of IL-10 and the protection of the vector *BL*, since the empty PBS vehicle (colitis control group) was unable to modify the course of disease. Its underlying mechanism requires further investigation.

In the current study, biologically active hIL-10 secreted by *BL-hIL-10* restrains inflammatory effects *in vivo*. Addition of commercial rhIL-10 and supernatant of *BL-hIL-10* induced with 0.2% L-arabinose diminished production of proinflammatory cytokines TNF-α and IL-6 in LPS-stimulated THP-1 cells, respectively. Previous studies have demonstrated that the expression of endogenous IL-10 was delayed compared to that of IL-1 and TNF-α in the initial period of LPS-stimulated monocytes,[25,26] while proinflammatory cytokines TNF-α and IL-6 were downregulated subsequently with increasing IL-10 expression,[27] suggesting that the hysteresis effect of IL-10 in active monocytes inhibits the persistent synthesis of proinflammatory cytokines by negative feedback regulation. Our results showed that the *BL-hIL-10* supernatant exerted an anti-inflammatory biological activity in accordance with the commercial rhIL-10, indicating that additional supplementation of exogenous IL-10 further suppressed proinflammatory cytokine synthesis in LPS-stimulated THP-1 and persistently inhibited the secretion of proinflammatory cytokines by THP-1 during the progressive inflammation. Meanwhile, no cytotoxic effects of *BL-hIL-10* supernatant at its effective dose on THP-1 were found in the CCK cytotoxic test.

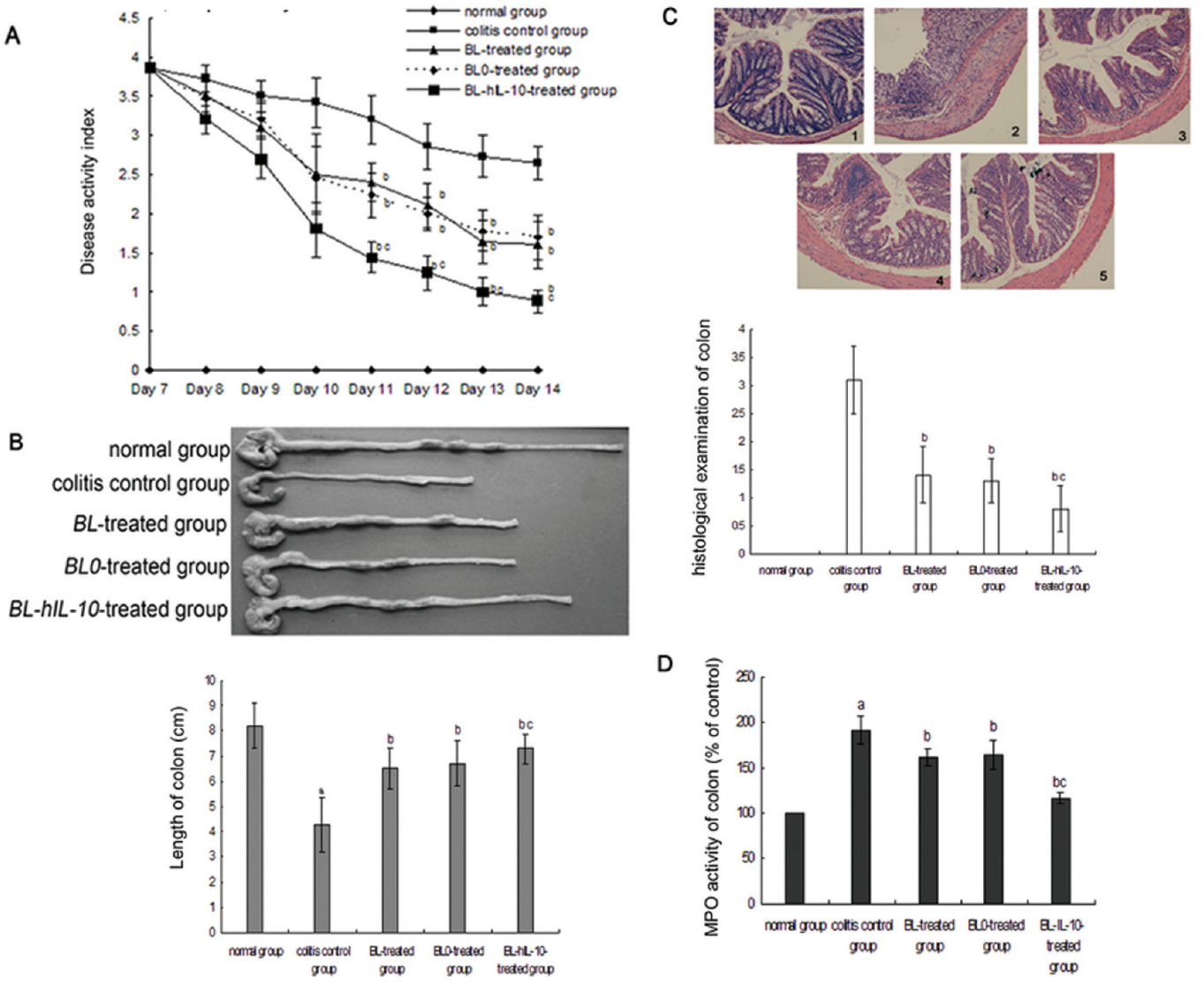

Figure 4. The effects of *BL-hIL-10* on disease activity index, colon length, histological change, histological score and MPO activity of distal colon in mice. A: Disease activity index. B: Length of colon. C: Histological examination of colon in (1) normal mice; (2) mice with colitis; (3) mice with colitis treated with *BL*; (4) mice with colitis treated with *BL0*; (5) mice with colitis treated with *BL-IL-10*. D: MPO activity of colon. Letters a, b and c indicate $p < 0.05$ compared with normal mice, mice with colitis, and mice with colitis treated with *BL*, respectively.

We also explored the potential mechanism that *BL-hIL-10* administration as a therapeutic means for mouse intestinal inflammation inhibits the acute-phase response to NF-κB signaling pathway. NF-κB as a key influential factor is a member of the Rel family of transcription factors and a heterodimer composed of two subunits, p50 and p65. Under basal conditions, NF-κB binds to its inhibitor IκB and presents in the cytoplasm as inactive Rel/NF-κB transcription complexes. When stimulated with certain inflammatory factors, IκB undergoes phosphorylation, ubiquitination and subsequent degradation, thereby enabling translocation of NF-κB into the nucleus[28] and leading to activation of NF-κB, which is a critical mediator in amplification of the inflammatory cascade and plays a pivotal role in the regulation of immune and inflammatory responses by controlling transcription of inflammatory cytokine genes.[29] This study further validated the important role of *BL-hIL-10* in UC pathogenesis and the inflammatory process. Our results showed that NF-κB (p65) DNA-binding activity and protein expression in the nuclear extraction of intestinal mucosa of the DSS-induced colitis model mice treated with supernatant of *BL-hIL-10* were significantly decreased compared to those of the model mice without treatment. Furthermore, *BL-hIL-10* treatment significantly downregulated proinflammatory cytokines (TNF-α, IL-6, IL-1β and IFN-γ) at both mRNA and protein levels in the model mice. First and foremost, we have confirmed that administration of *BL-hIL-10* massively increased hIL-10 abundance in colon and secreted sufficient hIL-10 to inhibit NF-κB (p65) DNA-binding activity and protein expression, thereby blocking inflammation response and preventing the cascade of inflammatory events in mice with UC induced by DSS. It is well-known that there is a positive feedback between proinflammatory cytokines and the NF-κB pathway: on one hand, activated NF-κB pathway can increased expression of certain proinflammatory cytokines, such as IL-1, IL-6, IL-12 and TNF-α in the inflamed mucosa of patients with UC;[30] on the other hand, the released cytokines activate the NF-κB pathway, which continuously increases

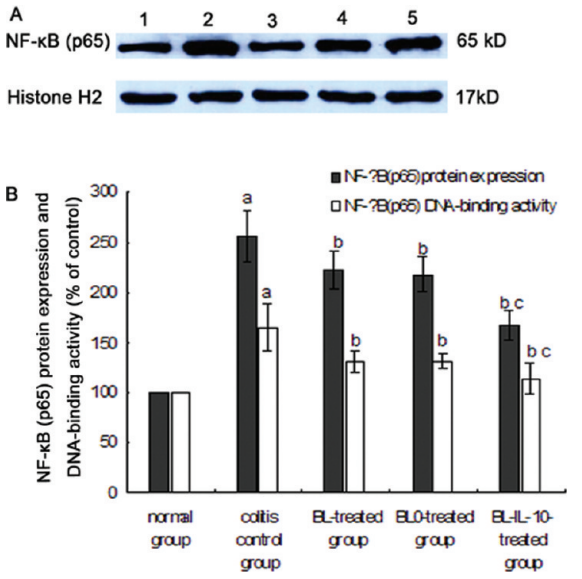

Figure 5. The effects of *BL-IL-10* on NF-κB (p65) protein expression and DNA-binding activity of distal colon in mice. A: NF-κB (p65) protein expression in (1) normal mice; (2) mice with colitis; (3) mice with colitis treated with *BL-IL-10*; (4) mice with colitis treated with *BL*; (5) mice with colitis treated with *BL0*. B: Relative NF-κB (p65) protein expression and DNA-binding activity. Letters a, b and c indicate $p < 0.05$ compared with those in normal mice, mice with colitis and mice with colitis treated with *BL*, respectively.

intestinal inflammation[31] and further promotes proinflammatory cytokine expression. The two processes form a feedback loop.[32] Administration of *BL-hIL-10* diminishes the production of proinflammatory cytokines and consequently blocks the vicious feedback cycle between NF-κB and proinflammatory cytokines *in vivo*. Moreover, IL-10 as an anti-inflammatory cytokine is insufficient in intestinal mucosa of colitis mice.[33] Enhancing intestinal hIL-10 content through administering *BL-hIL-10* to the DSS-induced colitis model mice to exert its anti-inflammatory activity is another way that *BL-hIL-10* inhibits the intestinal inflammation. The effects of IL-10 in stimulating intestinal electrolyte absorption, inhibiting chloride secretion and maintaining epithelial barrier integrity may have beneficial effects in UC patients. Preliminary clinical study has demonstrated that local administration of IL-10 in UC patients also suppresses mucosal inflammation.[34] MPO activity is a useful index for evaluating granulocyte infiltration in colonic tissues following induction of colitis. The decreased MPO activity in the DSS-induced colitis model mice treated with *BL-hIL-10* implied that the formation of such potent cytotoxic oxidants as HOCl from H_2O_2, chloride ions and *N*-chloramines in neutrophils is decreased. By inhibiting MPO activity and proinflammatory cytokines in lamina propria monocytes or macrophages, *BL-hIL-10* interrupted the feedback loop between upregulation of inflammatory mediators and the recruitment of leukocytes. This agrees with our previous observation that MPO activity is increased in the DSS-induced colitis model mice.[35,36] Last but not least, levels of proinflammatory cytokines in blood plasma were decreased in colitis mice treated with *BL-hIL-10,* indicating that administration of *BL-hIL-10* also could control inflammatory state and alleviate inflammatory response of the whole body.

Besides the anti-inflammatory effects, *BL-hIL-10* has immunosuppressive activities. Our data indicated that the percentage

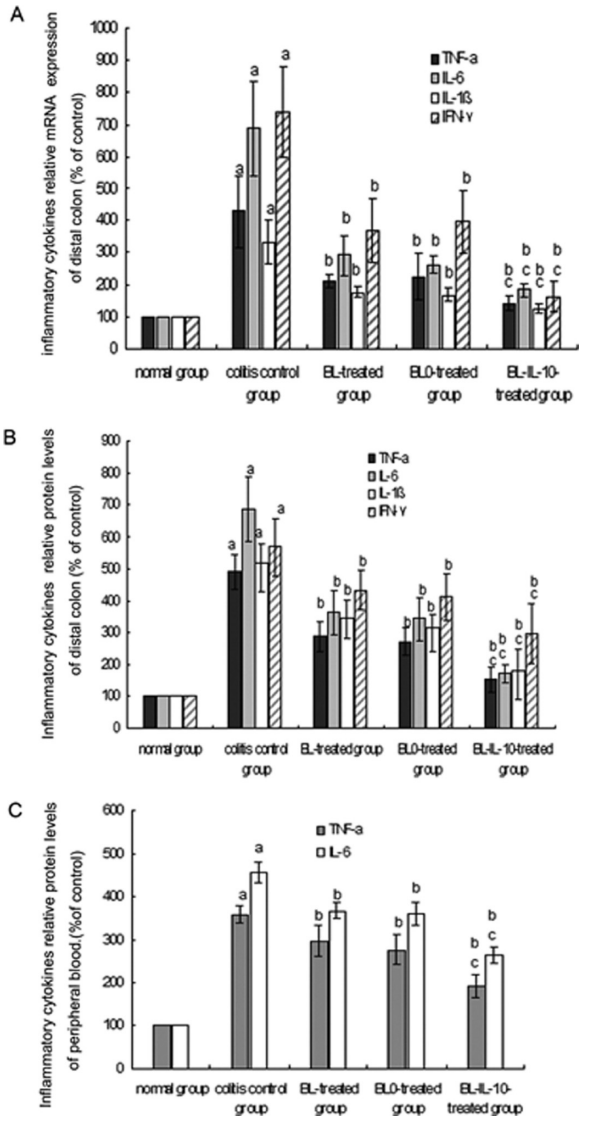

Figure 6. The effects of *BL-IL-10* on relative mRNA and protein levels of inflammatory cytokines in distal colon and peripheral blood. A: Relative mRNA level of inflammatory cytokines. B: Relative protein levels of inflammatory cytokines in distal colon. C: Relative protein levels of inflammatory cytokines in peripheral blood. Letters a, b, and c indicate $p < 0.05$ compared with normal mice, mice with colitis and mice with colitis treated with *BL*, respectively.

of $CD4^+CD25^+Foxp3^+$ Treg in peripheral blood and MLN in the DSS-induced colitis model mice treated with *BL-hIL-10* were higher than those in the untreated model mice, but similar to those in mice without UC. Previous studies have demonstrated that hIL-10 from *BL-hIL-10* can promote regulatory T cell differentiation and activation. $CD4^+CD25^+$ has been considered as the biomarker of naturally occurring Treg that are essential in maintenance of immune tolerance in the gut because they downregulate immune responses to both foreign and self-antigens in active UC.[37−39] Foxp3 is a member of the *forkhead-winged* helix family of transcription factors, which play critical roles in the development and function of $CD4^+CD25^+$ Treg[40,41] and functional integrity of $CD4^+CD25^+Foxp3^+$ in MLN of UC patients with active medically refractory disease.[42] Short-term treatment with hIL-10 secreted from *BL-hIL-10*

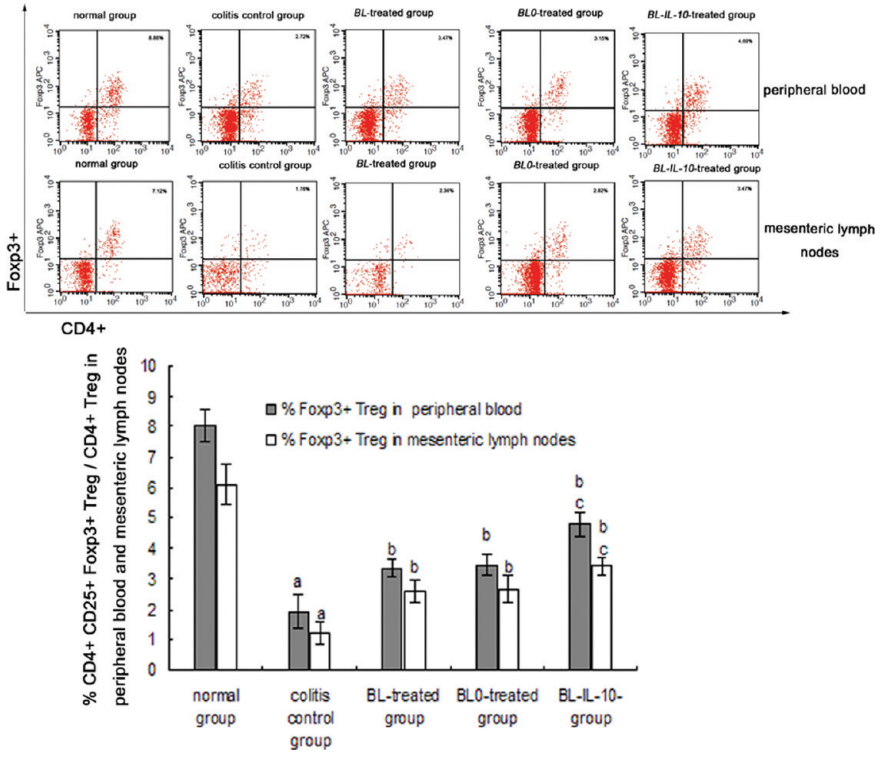

Figure 7. The effects of *BL-IL-10* on percentage of $CD4^+CD25^+Foxp3^+$ Treg in peripheral blood and mesenteric lymph modes of colitis mice. Letters a, b and c indicate $p < 0.05$ compared with the normal group, colitis control group and *BL*-treated group, respectively.

may indeed have prolonged immunomodulatory effects. The addition of hIL-10 exerts effective immunosuppressive activities and enhances expression of Foxp3, resulting in activation of $CD4^+CD25^+$ Treg. Since large numbers of mucosally activated lymphocytes reach the blood circulation after expansion and maturation within MLN and suppress excessively immune response in mouse intestinal tract, manipulation of $CD4^+CD25^+Foxp3^+$ Treg may be beneficial in treatment of established colitis.

BL as the *BL-hIL-10* vector has special and effective regulatory functions in the anti-inflammation immunity in UC. In our experiment, treatment with *BL* and *BL0* inhibited NF-κB signaling pathway in the DSS-induced colitis model mice and recovered $CD4^+CD25^+Foxp3^+$ Treg. This result is in agreement with the previous result that increase of proportion of *BL*, the representative probiotic in the gut of mice with UC, could remodel the microbial population barrier in the gut when used as the carrier for recombinant pediocin PA-1 expression and secretion under the guide of α-amylase signal peptide,[43] suggesting that *BL* may be the most suitable carrier for human gut hormone gene expression and secretion in the intestinal tract for colitis therapy. The repeated inflammation of colonic tissue may lead to cell mutations and subsequently increase the risk of colon cancer in patients with long-standing UC.[44] *BL* has significant beneficial effects that protect UC patients from colon cancer. Our data showed that treatment of the colitis model mice with *BL* and *BL0* could significantly alleviate colitis symptoms. *Bifidobacterium* is easier to colonize in the colon and more suitable than *Lactobacillus*, so administration of *Bifidobacterium* is a better and effective way to control intestinal inflammation.[17] Moreover, oral administration of BL-hIL-10 is more convenient and controllable than rectal infusion under sedation with adenoviral vectors encoding murine interleukin 10,[18] and adenoviral vectors alone have no treatment effect on the colitis.

Carrying a plasmid neither attenuates *BL*'s beneficial characteristics nor gains any epactal therapeutically harmful effects. All these pleiotropic effects may increase its therapeutic value and application prospects.

In conclusion, our experiment provided evidence that *BL-hIL-10*, as a biologically engineered, secretion carrier of active hIL-10, is effective in treatment of mice with UC. Because hIL-10 has a central role in downregulating inflammatory cascade and matrix metalloproteinases, *BL-hIL-10* is a likely candidate for therapeutic intervention.

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Author Contributions

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ABBREVIATIONS USED

UC, ulcerative colitis; DSS, dextran sulfate sodium; *B. longum*, *Bifidobacterium longum*; IL-10, interleukin 10; hIL-10, human interleukin 10; *BL-IL-10*, *B. longum* strain for secretion of

biologically active human IL-10; Treg, regulatory T cells; ELISA, enzyme-linked immunosorbent assay.

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