Clostridium butyricum alleviates dextran sulfate sodium-induced experimental colitis and promotes intestinal lymphatic vessel regeneration in mice

Xing Chen[1,2#], Lin Ma[1#], Xiaolin Liu[1], Jun Wang[1], Yan Li[1], Qi Xie[1], Jing Liang[1]

[1]Department of Oncology, The First Affiliated Hospital of Shandong First Medical University & Shandong Provincial Qianfoshan Hospital, Shandong Key Laboratory of Rheumatic Disease and Translational Medicine, Shandong Lung Cancer Institute, Jinan, China; [2]Department of Oncology, Qingdao Women and Children's Hospital, Qingdao, China

Contributions: (I) Conception and design: Q Xie; (II) Administrative support: J Liang, Y Li; (III) Provision of study materials or patients: X Liu, J Wang; (IV) Collection and assembly of data: L Ma, X Chen; (V) Data analysis and interpretation: Q Xie; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

[#]These authors contributed equally to this work.

Correspondence to: Professor Yan Li; Dr. Qi Xie; Professor Jing Liang. Department of Oncology, The First Affiliated Hospital of Shandong First Medical University & Shandong Provincial Qianfoshan Hospital, Jinan 250014, China. Email: liyan16766@163.com; xieqi2012@163.com; liangjing0531@163.com.

Background: Inflammatory bowel disease (IBD) is the most common precancerous lesion of colitis-associated colon cancer (CAC). Studies have confirmed that pathological changes in intestinal lymphatic vessels (LVs) significantly promoted the development of IBD-associated carcinogenesis. An imbalance in the microecology of the intestinal flora is a key factor in the progression of IBD. As a result, therapeutic techniques that focus on the relationship between LV regeneration and flora management might be a potential treatment strategy.

Methods: We investigated the role of *Clostridium butyricum* (*C butyricum*) in a dextran sulfate sodium (DSS)-induced IBD mouse model. Balb/c mice were given 3% DSS in their drinking water for 8 days to produce acute colitis and simultaneously administrated with *C butyricum* for 12 days. Hematoxylin and eosin (H&E) staining was used to evaluate the degree of colitis tissue damage. Levels of the lymphatic endothelial cell (LEC)-specific marker LYVE-1 and intestinal expressions of pro-lymphatic vascular endothelial growth factor (VEGF)-C and VEGF-D were determined using immunohistochemical assays.

Results: In a DSS-induced IBD mouse model, we found that butyric acid-producing *C butyricum* significantly reduced disease activity index (DAI) scores in mice, reversed the shortening of the colon, weakened the degree of damage to colonic epithelial tissues, inhibited lymphocyte infiltration, and reduced pathological damage to the colon. To our knowledge, this is the first time that tissue expressions of LYVE-1, VEGF-C, and VEGF-D have been seen to increase in IBD-model mice after treatment with *C butyricum*.

Conclusions: Our findings suggest that *C butyricum* might alleviate IBD in DSS-induced IBD-model mice by promoting intestinal LV regeneration.

Keywords: Inflammatory bowel disease (IBD); colorectal cancer; *Clostridium butyricum* (*C butyricum*); lymphangiogenesis; intestinal microenvironment

Submitted Jan 25, 2022. Accepted for publication Mar 21, 2022. This article was updated on September 16, 2022.
The original version was available at: https://dx.doi.org/10.21037/atm-22-1059
doi: 10.21037/atm-22-1059

Introduction

Colon cancer is the third most common cancer globally, and colitis-associated colon cancer (CAC) is an important type of colon cancer. For decades, inflammatory bowel diseases (IBDs) have been regarded as the precancerous condition of CAC (1). IBD is a group of gastrointestinal disorders characterized by chronic and relapsing inflammation and includes Crohn's disease (CD) and ulcerative colitis (UC). The main clinical features of IBD include abdominal pain, diarrhea, acute fever, and stools containing mucus, pus, and blood. Genetic instability, epigenetic variation, a dysregulated immune response induced by inflammatory mediators, oxidative stress, and intestinal microecological changes are critical factors in IBD-associated carcinogenesis (2). Further clarification on the pathogenesis of IBD and a search for new treatment strategies could prevent CAC.

It is generally accepted that classical immune system components and inflammatory factors, such as Th1/2/17 cells, Treg cells, IL-1β, IL-6, and TNF-α, play important roles in the development of IBD (3). Non-immune cells, such as epithelial, endothelial, and vascular cells, are also closely related to the development of IBD. Pathological lymphatic dysfunctions, such as lymphatic obstruction, submucosal edema, and reduced lymphatic drainage, have been observed in a large number of intestinal biopsies of patients with IBD (4), indicating that defects in the intestinal lymphatic regulation are a common characteristic of IBD. Lymphatic vessels (LVs) are an important histological component of the mesentery and the lymphatic system. Intestinal LVs are essential for chylomicron production, cholesterol and lymphatic absorption, the transport of protein molecules and antigens, and immune cell trafficking (5). Functional LVs have recently been found to have a vital role in preventing and alleviating inflammation in the intestine (6). The LV is critical for *in vivo* immune response monitoring because it is both the major transport conduit for cells and inflammatory mediators into lymph nodes and the location of immune response and inflammation (7). In patients with long-term IBD, intestinal LVs become obstructed and dysfunctional, causing inflammatory cells to clump together and be unable to migrate away from inflamed areas, exacerbating the inflammatory response. Meanwhile, the loss of intestinal LVs might hasten the progression and recurrence of IBD-induced colon cancer (8).

Studies have identified a few critical factors participating in intestinal lymphangiogenesis. Under the activation of inflammatory or physicochemical stimuli, lymphatic endothelial cells (LECs) might undergo proliferation or lymphangiogenesis in the course of IBD (9). Prostaglandin E receptor EP4 stimulation aids in mucosal repair from DSS-induced acute colitis by promoting lymphangiogenesis (10). LYVE-1 is the main hyaluronic acid (HA) receptor in afferent LVs and LECs. LYVE-1 is one of the few known receptors limited to LVs and directly involved in lymphangiogenesis, leukocyte migration, and tumor metastasis (11). LECs that express LYVE-1 can differentiate into LVs via signaling pathways involving transcription factors such as VEGF-C/VEGF-R3 (12). VEGF-C and VEGF-D are growth factors, similar in structure and function, that are considered key to inducing lymphangiogenesis via binding to VEGFR3 (13,14). Reports have demonstrated that LV defects lead to the accumulation of inflammatory cells and aggravate intestinal inflammation in IBD (15). By blockading lymphangiogenesis in a DSS-induced colitis mouse model via anti-VEGFR3 antibodies or angiopoietin-$2^{-/-}$, intestinal inflammation has been exacerbated (16), suggesting that dysregulated LVs contribute to IBD development. In contrast, Lee *et al.* demonstrated that enhancing intestinal lymphangiogenesis by delivering VEGF-C significantly promoted lymph flow and suppressed intestinal inflammation, increasing inflammatory cell mobilization from the inflamed intestinal site to the draining lymph nodes (17). Therefore, a treatment strategy promoting lymphangiogenesis in patients with IBD shows promise, and a clinical treatment strategy targeting intestinal LV regeneration or functional recovery is worthy of attention.

An imbalance in the microecology of the intestinal flora is an important mechanism, provoking host immune-system diseases, such as IBDs (18). The study of the intestinal flora of patients with CAC has shown that intestinal flora changes significantly in the early stage of the disease (19). *Clostridium butyricum* (*C butyricum*), a gram-positive anaerobe, exists in the intestines of healthy humans and animals and has been widely used in the repair of the intestinal epithelium and the regulation of gastrointestinal function (20). A metabolic study found that the butyric acid content in fecal extracts from patients with IBD was significantly lower than in those from a healthy control population (21), implying that supplements with butyric acid-producing *C butyricum* might reduce the occurrence and development of IBD. To prevent acute experimental

colitis, *C butyricum* as a probiotic promotes interleukin-10-producing macrophages via the Toll-like receptor 2/myeloid differentiation primary response gene 88 pathway in inflamed mucosa. The immunosuppressive cytokine IL-10, which is released by T_h2 cells and suppresses T_h1 cell activity, has been linked to IBD development. IL-10-deficient mice are used as a genetic model for IBD (22). However, the mechanism by which *C butyricum* suppresses intestinal inflammation is not fully understood, especially regarding whether *C butyricum* alleviates colitis via the regulation of intestinal lymphangiogenesis.

To our knowledge, this study is the first to find that *C butyricum* promoted the regeneration of intestinal LVs in a DSS-induced colitis mouse model and upregulated intestinal LYVE-1, VEGF-C, and VEGF-D expressions. The administration of *C butyricum* to IBD-model mice promoted the recovery of dysfunctional LVs and reduced immune cell infiltration at the inflamed site, which might have alleviated intestinal inflammation by transporting inflammatory cells to draining lymph nodes through the reconstructed LVs. We present the following article in accordance with the ARRIVE reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-1059/rc).

Methods

Animal model

Forty SPF female Balb/c mice (6–8 weeks old, weight 16–19 g) were obtained from Beijing Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China) and raised in the animal experiment center of the First Affiliated Hospital of Shandong First Medical University. Although both males and females suffer DSS-induced colitis robustly, males have a more aggressive and severe disease progression than females. Males, on the other hand, are more prone to hurt each other in cage fights. As a result, the female mice were chosen as the experiment's observation target (23). The mice were housed in cages at a controlled temperature (20±2 ℃) with 60% humidity on a 12-hour light/dark cycle. Forty mice were randomly divided into 4 groups and treated as follows: (I) a control group (n=10); (II) a *C butyricum* group (n=10), in which mice were given an intragastric *C butyricum* solution (200 μL, 1,000 mg/kg) on days 2, 4, and 6 of the experiment; (III) a DSS group (n=10), in which the mice drank water containing 3.0% DSS for 8 days; and (IV), a DSS + *C butyricum* group (n=10), in which mice were given water containing 3.0% DSS and an intragastric *C butyricum* solution (200 μL, 1,000 mg/kg) on days 2, 4, and 6 of the experiment. On the eighth day, the solution in the drinking troughs of the DSS and DSS + *C butyricum* groups was replaced with the same volume of water. On days 8 to 11 of the experiment, the mice in the *C butyricum* and DSS + *C butyricum* groups were given an intragastric solution of 200 μL *C butyricum* with the optimal concentration of 5×10^6 CFU/mL (24). On day 12 of the experiment, the mice were sacrificed, and the length of their colons was measured. Experiments were performed under a project license (No. S300) granted by institutional ethics board of the First Affiliated Hospital of Shandong First Medical University, in compliance with institutional guidelines for the care and use of animals in the First Affiliated Hospital of Shandong First Medical University.

Assessment of the severity of colitis

The mice in the DSS and DSS + *C butyricum* groups were provided with drinking water containing 3% DSS to induce acute experimental colitis. The severity of the colitis was assessed using a disease activity index (DAI) score, graded on a scale of 0–4, and calculated daily from the combined scores for the percentage of weight loss, stool characteristics, and abnormal blood in the stools (detected using a fecal occult blood kit).

Hematoxylin and eosin staining

The colon tissues were fixed with 4% paraformaldehyde, embedded in paraffin, and sliced into 4 μm specimens. All sections were stained with hematoxylin and 0.5% eosin for histological examination. Pathological changes in the tissue structure were observed under a microscope to further evaluate the degree of colitis tissue injury in terms of the degree and range of inflammation and crypt injury. The level of tissue injury was calculated as the sum of the scores for each item (*Table 1*).

Immunohistochemical staining

After dewaxing and hydration, the colon tissue sections were pretreated with citrate buffer for antigen retrieval and incubated with 3% H_2O_2 for endogenous peroxidase activity blocking. After incubation with 10% goat serum, slides were stained overnight with an anti-LYVE-1 antibody solution (cat. no. 51011-1-AP; 1:40 dilution; Proteintech Group, Inc.

Table 1 Criteria for evaluating the degree of colitis tissue injury

Assessed items	Grade	Description
Degree of inflammation	0	None
	1	Mild
	2	Moderate
	3	Severe
Inflammation range	0	None
	1	Limited to the mucous layer
	2	Limited to the submucosa
	3	Infiltrating the whole layer
Crypt injury	0	None
	1	1/3 of basement membrane damaged
	2	2/3 of basement membrane damaged
	3	Crypts disappeared, and surface epithelium present
	4	Crypts and surface epithelium disappeared
Overall percentage of injury	0	0
	1	1–25%
	2	26–50%
	3	51–75%
	4	76–100%

Rosemont, USA) and anti-VEGF-C/D antibody solution (cat. no. 22601-1-AP; cat. no. 26915-1-AP; 1:50 dilution; Proteintech Group, Inc) at 4 ℃. They were then incubated with goat anti-mouse/rabbit IgG polymer (*R&D Systems*, Inc. Minneapolis, USA). Expressions of LYVE-1, VEGF-C, and VEGF-D in colonic tissues from the 4 groups of mice were observed under a microscope.

Statistical analysis

The experimental results were recorded in Microsoft Excel 2003 (Microsoft Corporation, Redmond, USA), and the metrological data were expressed as mean ± standard deviation. SPSS26 software (IBM, Armonk, USA) was used to analyze the data. Two-way analysis of variance (ANOVA) was used to compare each group's body weight and DAI scores. One-way ANOVA was used to compare the colon length data of each group. The least significant difference test (LSD-t) was used to assess significance. GraphPad Prism 6.02 (GraphPad Software, San Diego, USA) was used to create the flow charts and statistical charts.

Results

C butyricum ameliorated DSS-induced experimental colitis in mice

It has been demonstrated that intestinal colonization by *C butyricum* suppresses intestinal inflammation in DSS-induced colitis (25). However, the IBD-model mice in most studies were treated with DSS for 6 to 8 days, with histological changes during the acute inflammation stage under observation (6,23). Few studies have focused on the pathological changes during the recovery phase after DSS stimulation ceases. To determine whether colonization by *C butyricum* had equal treatment efficacy on colitis during the acute inflammation and recovery stages, SPF Balb/c mice were given 3% (weight/volume) DSS dissolved in their drinking water for 8 days to induce colitis. They were simultaneously treated with *C butyricum* by oral gavage for 12 days (*Figure 1A*). In our study, the drinking water containing DSS was replaced with distilled water on day 8, and the body weight, DAI score, and colon length of the mice were evaluated on day 8 and day 12. On day 8, there was no significant difference in body weight between the *C butyricum* group (19.56±0.79 g) and the control group ($P>0.05$), while the body weight of the mice in the DSS group (16.17±1.44 g) and the DSS + *C butyricum* group (18.43±0.91 g) both decreased significantly ($P<0.05$), suggesting that the DSS-induced colitis model was successfully established. In addition, there was a significant difference in body weight between the DSS and DSS + *C butyricum* groups (18.43±0.91 g) ($P<0.0001$) (*Figure 1B*), which indicated that *C butyricum* ameliorated DSS-induced body weight loss. After removing the DSS drinking water on day 8, we explored the role of *C butyricum* during the inflammation recovery period. On day 11, the body weight of the DSS + *C butyricum* group (19.37±0.15 g) was significantly higher than that of the DSS group (16.40±1.56 g) ($P<0.0001$) (*Figure 1B*), suggesting *C butyricum* treatment accelerated recovery from DSS-induced experimental colitis.

In terms of the DAI score on day 8, there was no significant difference between the *C butyricum* group (0.11±0.19) and the control group (0.22±0.19) ($P>0.05$).

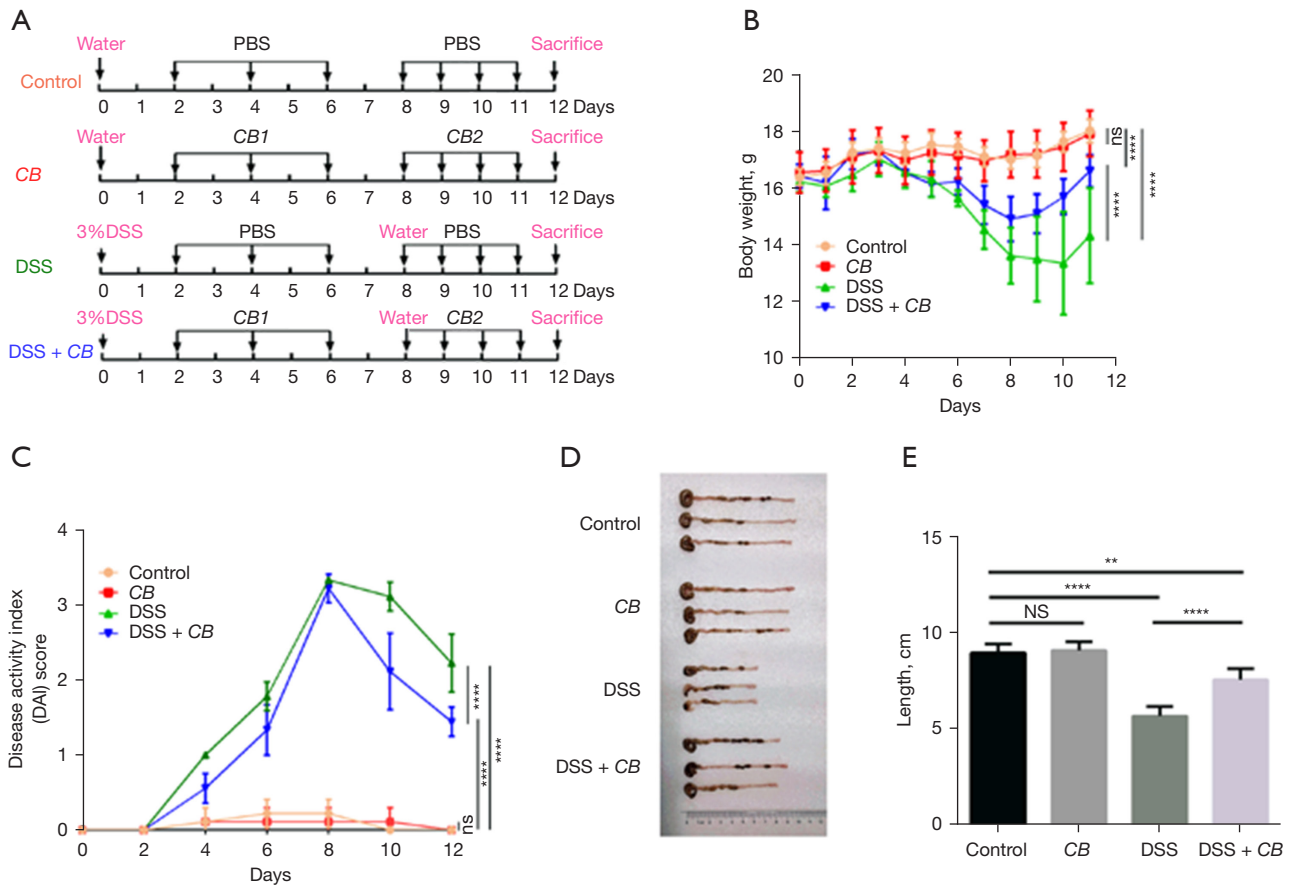

Figure 1 *C butyricum* alleviates dextran sulfate sodium-induced experimental colitis. (A) Flow chart of the experimental design using DSS-induced IBD-model mice. (B) Effect of *C butyricum* on body weight of the DSS-induced IBD-model mice. The body weight of the mice in the DSS group DSS + *C butyricum* group both decreased remarkably, and there was a significant difference in body weight between the DSS and DSS + *C butyricum* groups. (C) Changes in DAI scores of DSS-induced IBD-model mice after treatment with *C butyricum*. DAI scores of mice in the DSS and DSS + *C butyricum* groups were significantly higher than the control group's scores. The DAI score of the DSS group was significantly higher than that of DSS + *C butyricum* group in the recovery stage of inflammation from day 8 to day 12. (D,E) Comparison of the colonic lengths in four groups. The colonic tissue from ileocecal part to anus (3 mice in each group) was photographed and measured. Differences were assessed with ANOVA (ns, not significant; **, P<0.01; ****, P<0.0001). PBS, phosphate balanced solution; *CB*, *Clostridium butyricum*; DSS, dextran sulfate sodium; IBD, inflammatory bowel disease; DAI, disease activity index.

However, the DAI scores of the mice in the DSS group (3.33±0.00) (P<0.0001) and the DSS + *C butyricum* group (3.11±0.38) (P<0.0001) were significantly higher than those of the control group mice (0.22±0.19). The DAI scores between the DSS group (3.33±0.00) and the DSS + *C butyricum* group (3.11±0.38) were significantly different (P<0.0001). In the inflammation recovery stage from day 8 to day 12, the DAI scores of the DSS group (2.89±0.55) were significantly higher than those of the DSS + *C butyricum* group (2.22±0.80) (P<0.0001) (*Figure 1C*). Colon length in the mice was negatively correlated to the severity of colitis (26). We found that colon length in the DSS group (7.07±0.12 cm) (P<0.0001) and the DSS + *C butyricum* group (8.27±0.31 cm) (P<0.0001) was shorter than colon length in the control group (8.53±0.38 cm) (*Figure 1D*). However, there was no significant difference in colon length between the *C butyricum* group (8.73±0.25 cm) and the control group (P>0.05) (*Figure 1D,1E*). A comparison of the colonic lengths in the DSS group (7.07±0.12 cm) and the DSS + *C butyricum* group (8.27±0.31 cm) showed significantly longer colons in the latter than in the former (P<0.0001) (*Figure 1D,1E*). Collectively, *C butyricum* treatments

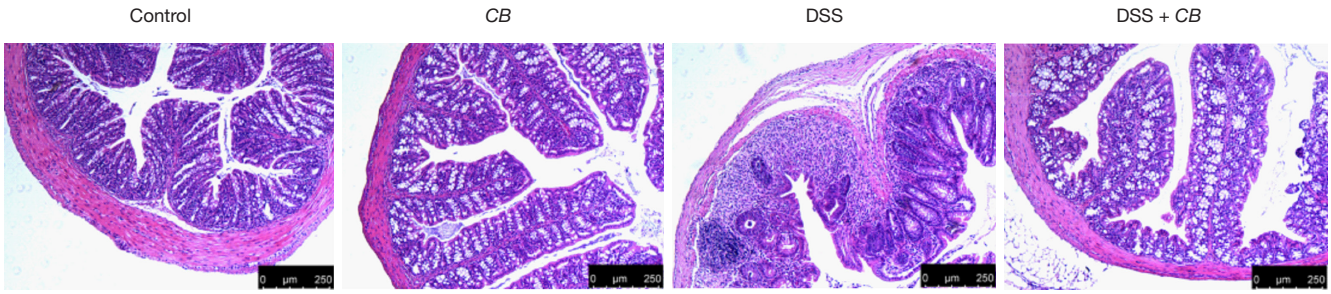

Figure 2 H&E staining was used to reveal the effect of *C butyricum* in the recovery of inflamed tissues in DSS-induced IBD-model mice (magnification: 100×). After treatment with *C butyricum*, the DSS + *C butyricum* group showed less severe colonic pathological damage and less inflammatory cell infiltration than the DSS group. H&E, hematoxylin and eosin; *CB*, *Clostridium butyricum*; DSS, dextran sulfate sodium; IBD, inflammatory bowel disease.

significantly reversed the DSS-induced colonic shortening and alleviated the symptoms of colitis in mice.

C butyricum attenuated colonic pathological damage and reduced inflammatory cell infiltration in the DSS-induced experimental colitis model

We further evaluated colitis severity by analyzing the degree of colitis tissue damage and inflammatory cell infiltration. No significant histopathological damage was observed between the control and *C butyricum* groups (*Figure 2*). Severe colonic tissue damage, such as crypt reduction or disappearance and intestinal epithelial damage, was found in the DSS group. Furthermore, the infiltration of a large number of inflammatory cells into the intestinal mucosa was observed (*Figure 2*). However, when compared to the results of the DSS group, treatment with *C butyricum* was found to restore the colonic pathological injuries induced by DSS significantly and reduce intestinal inflammatory cell infiltration, suggesting that treatment with *C butyricum* could effectively reduce the grade of pathological injury and intestinal inflammatory cell infiltration caused by DSS-induced colitis.

C butyricum significantly promoted intestinal LYVE-1 expression in the DSS-induced experimental colitis model

To explore whether *C butyricum* regulated LV regeneration in DSS-induced experimental colitis, the expression of LYVE-1, an LV-specific marker, was determined using immunohistochemical staining. When compared to LYVE-1 expression in the control group, the expression of LYVE-1 in the DSS group was found to be significantly lower. The intestinal lymphatic vasculature was especially inconsistent in DSS-induced experimental colitis (*Figure 3*). However, *C butyricum* treatments upregulated LYVE-1 expression in the DSS-induced experimental colitis model (*Figure 3*), suggesting that *C butyricum* could promote intestinal LV regeneration of in the colitis model.

C butyricum upregulated intestinal VEGF-C and VEGF-D expression in the DSS-induced experimental colitis model

To explore the mechanism through which *C butyricum* regulated LV regeneration in the DSS-induced experimental colitis model, the expressions of intestinal VEGF-C and VEGF-D-key factors regulating lymphangiogenesis—were compared among the 4 groups using immunohistochemical staining. It was found that the expression of intestinal VEGF-C and VEGF-D in the DSS group was significantly lower than in other groups (*Figure 4*). After treatment with *C butyricum*, the expressions of intestinal VEGF-C and VEGF-D in the colitis model both increased (*Figure 4*), suggesting *C butyricum* may induce lymphangiogenesis by promoting the secretion of VEGF-C and VEGF-D in intestinal tissue.

Discussion

In this study, we successfully established and IBD mouse model using a 3% DSS solution. We simultaneously treated the IBD-model mice with *C butyricum*. We found that *C butyricum* suppressed DSS-induced experimental colitis in SPF Balb/c mice. It also increased body weight and colon length, decreased the DAI score, attenuated colonic pathological damage, and reduced intestinal

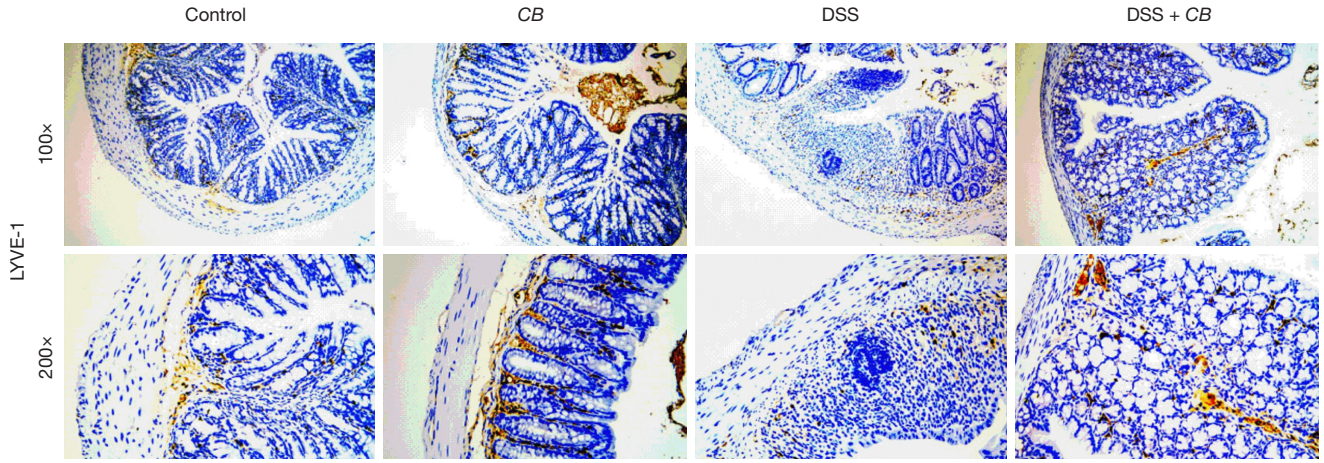

Figure 3 LYVE-1 expression in IBD-model mice was detected by immunohistochemical staining and captured by microscope (magnification: 100× and 200×). LYVE-1 expression was markedly lower in the DSS group than in DSS + *C butyricum* group. LYVE-1, Lymphatic Vessel Endothelial Receptor-1; *CB*, *Clostridium butyricum*; DSS, dextran sulfate sodium; IBD, inflammatory bowel disease.

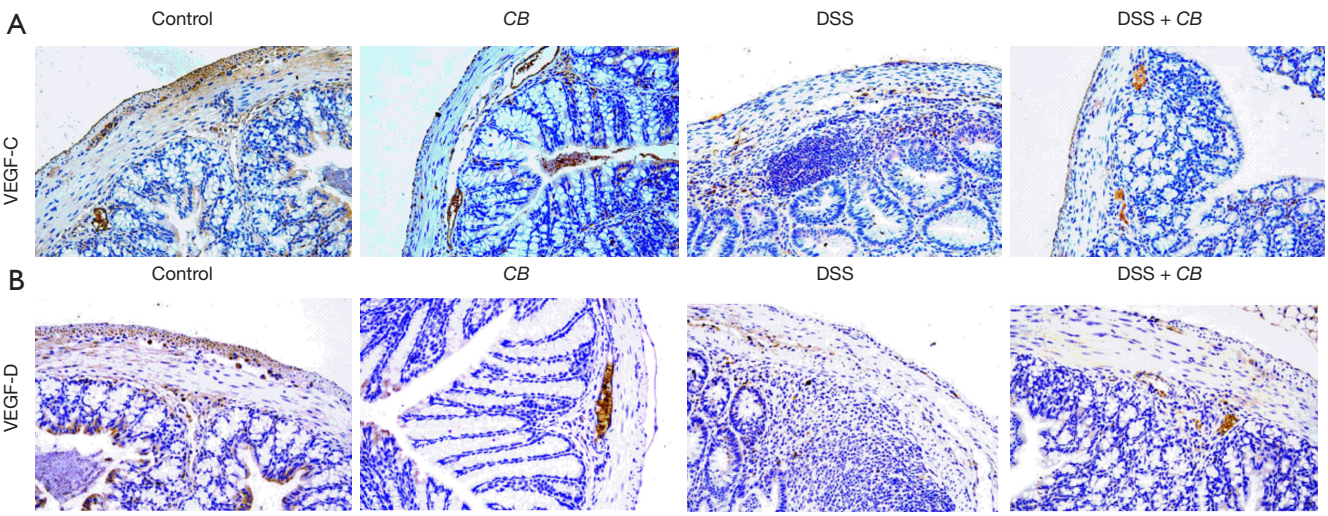

Figure 4 Immunohistochemical assay was used to reveal the effect of *C butyricum* on VEGF-C/VEGF-D protein expression in IBD-model mice (magnification 200×). The expression of VEGF-C and VEGF-D were upregulated in the DSS + *C butyricum* group after treatment with *C butyricum*, in contrast to the DSS group. VEGF, vascular endothelial growth factor; *CB*, *Clostridium butyricum*; DSS, dextran sulfate sodium; IBD, inflammatory bowel disease.

inflammatory cell infiltration (*Figure 5*). Our findings were consistent with other research. Liu *et al.* demonstrated that *C butyricum* inhibited colitis associated with colon cancer in C57BL/6 mice treated with DSS and azoxymethane (AOM) (27). Wang *et al.* found that treatments with combinational probiotics (*Lactobacillus reuteri*, *Bacillus coagulans*, *Bifidobacterium longum*, and *C butyricum*) reduced DSS-induced colitis in SPF C57BL/6N-model mice (28) and that *C butyricum* decreased DAI scores and histological injury in DSS-induced Balb/c mice, repairing the damaged structure of the tight junction (29). Overall, *C butyricum* was found to reduce intestinal inflammation in Grem-free (GF) mice or SPF Balb/c and C57b mice, suggesting the anti-inflammatory efficacy of *C butyricum* treatments in IBD-model mice was not dependent on the mouse species. It is important to note that DSS stimulation for inducing

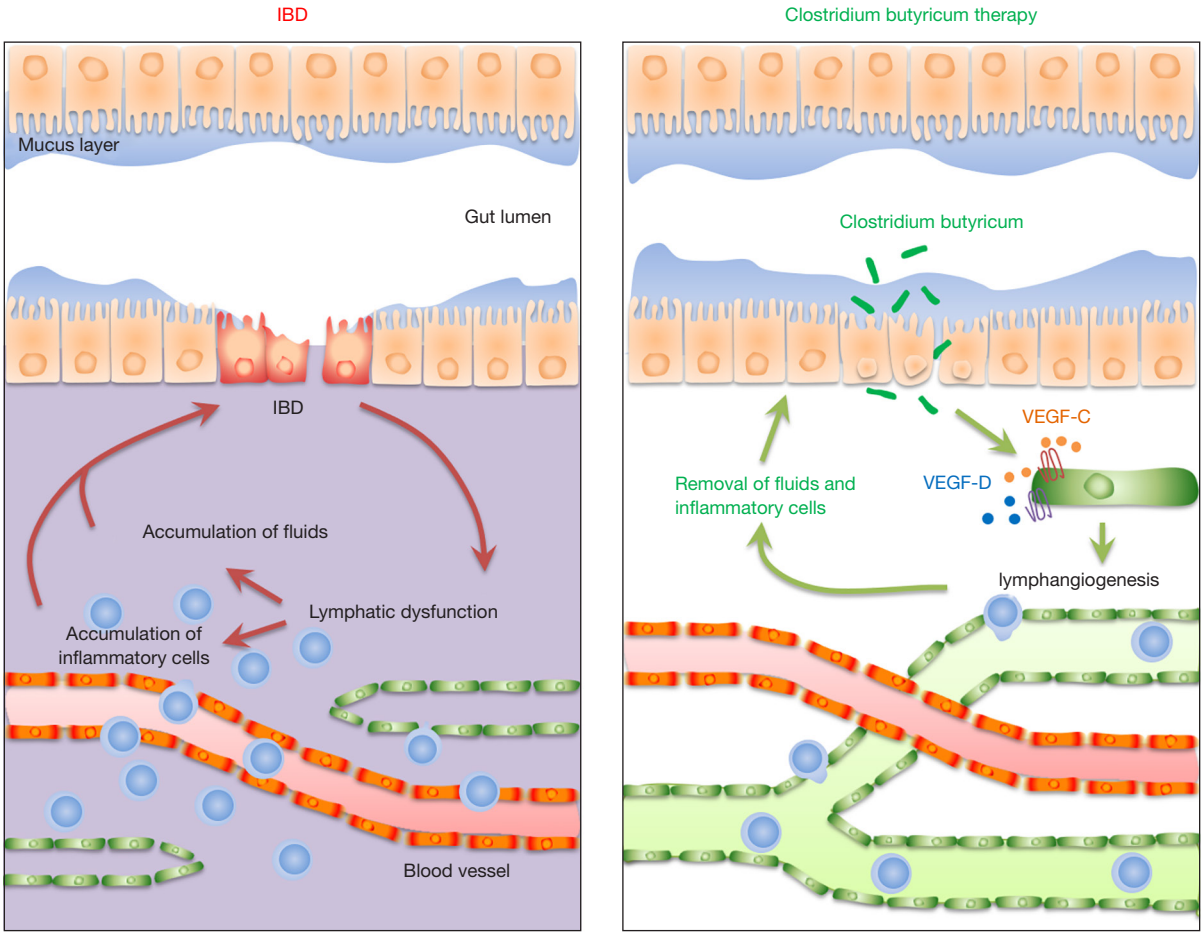

Figure 5 Schematic figure. *C butyricum* promoted the recovery of dysfunctional LVs in a DSS-induced experimental colitis model by upregulating intestinal VEGF-C and VEGF-D expression. Transportation of the inflammatory cells through reconstructed LVs reduced immune cell infiltration to the inflamed tissue. LVs, lymphatic vessels; IBD, inflammatory bowel disease; DSS, dextran sulfate sodium; VEGF-C, vascular endothelial growth factor C; VEGF-D, vascular endothelial growth factor D.

an IBD model in most studies to date has lasted 6 to 8 days, which only allows for observation of the histological changes during the acute inflammation stage (30,31). In our study, we also observed pathological changes in IBD-model mice during the recovery phase after we removed the drinking water containing DSS. We found that *C butyricum* accelerated recovery from colitis in the IBD-model mice.

Compared with other intestinal bacteria, *C butyricum*, which is a beneficial bacterium used to treat intestinal diseases in humans and animals, can ferment and decompose nutrients, producing a large amount of butyric acid. Butyric acid is a short-chain fatty acid and has important physiological functions. For example, butyrate is thought to reduce symptoms in patients with IBDs and can effectively inhibit the proliferation and promote apoptosis of colon cancer cells, thus inhibiting the occurrence and development of colon cancer (20). Many intestinal epithelial cells are necrotic in patients with IBDs, resulting in epithelial barrier defects and increased intestinal permeability. The butyric acid-producing *C butyricum* can alleviate inflammation in patients with IBDs by entering the damaged areas in the intestine (32). To our knowledge, this study is the first to have found that *C butyricum* induced LV regeneration. We observed that in contrast to DSS treatments, after treatments with *C butyricum*, vasculature structure improved, LYVE-1 expression increased and the number of infiltrated inflammatory cells was reduced, suggesting functional LVs might participate in immune

cell trafficking and increased lymph flow. The mechanism by which LVs were regenerated following *C butyricum* treatments was also investigated. Lymphangiogenic VEGF-C and VEGF-D expressions increased in colon tissue of the DSS + C *butyricum* group, indicating that *C butyricum* may stimulate lymphangiogenesis via the VEGF -VEGFR3 pathway.

Lymphangiogenesis is a process involving several factors in which a series of cellular activities, such as the proliferation and differentiation of LECs, participate in the formation of new LVs. In terms of *C butyricum* increasing VEGF-C/D expression, there is no direct evidence to support our findings that *C butyricum* promoted intestinal VEGF-C/D expression. However, macrophages have an important role in inflammatory-induced lymphangiogenesis. Studies have shown that the expression of VEGF-C and VEGF-D and their receptor VEGFR-3 in macrophages has a very significant correlation with the local LV density in tumors (33,34). In one study, the blockade of VEGF-C and VEGF-D signaling decreased obstruction-induced lymphangiogenesis, and the depletion of macrophages decreased lymphangiogenesis in the obstructed kidney (35). Another study found that after mycoplasma infection, tracheal macrophages produced lymphangiogenic VEGF-C and VEGF-D, also indicating that macrophages play a vital role in lymphangiogenesis during acute inflammation (36). In DSS colitis mice models, VEGF-C/D enhanced lymphangiogenesis, whereas inhibiting VEGFR-3 resulted in disease aggravation. In contrast, pharmaceutical suppression of lymphangiogenesis improved disease activity, but lymphangiogenesis stimulation by VEGF-C/D overexpression worsened intestinal inflammation in the same colitis animal (37). Additionally, IL-10 might participate in increased intestinal VEGF-C and VEGF-D expression in the colon after *C butyricum* treatment. Hos *et al.* demonstrated that IL-10 promoted corneal lymphangiogenesis and inhibited inflammation. However, IL-10 did not directly affect the LECs. Thus, they proved that IL-10 indirectly regulates inflammatory corneal lymphangiogenesis via macrophages with macrophage deletion (38). Furthermore, C *butyricum*-induced IL-10-producing Treg cells and macrophages have been found to collaborate to promote VEGF-C production in the colon following C *butyricum* treatments (22).

In summary, our study clarified the effect of *C butyricum* on the recovery of intestinal LVs in IBD-model mice and found that *C butyricum* upregulated intestinal VEGF-C and VEGF-D expression in a DSS-induced experimental colitis model. Aware of the vital role of LVs in IBD pathogenesis, we found that the administration of *C butyricum* in IBD-model mice promoted the recovery of dysfunctional LVs and reduced immune cell infiltration to the inflamed site, which might alleviate intestinal inflammation by transporting inflammatory cells through reconstructed LVs. Our findings provide a new treatment strategy aimed at LV regeneration in patients with IBD that might prevent the occurrence of CAC.

Acknowledgments

Funding: This work was supported by the Major Science and Technology Innovation Project of Shandong Province (No. 2018CXGC1220); Shandong Provincial Key Research and Development Program (No. 2019GSF108188); National Natural Science Foundation of China (No. 81801983); Traditional Chinese Medicine Science and Technology Development Plan of Shandong Province (No. 2019-0377); Qianfoshan Grant (Nos. QYPY2020NSFC1015, QYPY2020NSFC0821).

Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at https://atm.amegroups.com/article/view/10.21037/atm-22-1059/rc

Data Sharing Statement: Available at https://atm.amegroups.com/article/view/10.21037/atm-22-1059/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-22-1059/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Experiments were performed under a project license (No. S300) granted by institutional ethics board of the First Affiliated Hospital of Shandong First Medical University, in compliance with institutional guidelines for the care and use of animals in the First Affiliated Hospital of Shandong First Medical University.

Open Access Statement: This is an Open Access article

References

1. Nambu R, Warner N, Mulder DJ, et al. A Systematic Review of Monogenic Inflammatory Bowel Disease. Clin Gastroenterol Hepatol 2022;20:e653-63.
2. Collij V, Klaassen MAY, Weersma RK, et al. Gut microbiota in inflammatory bowel diseases: moving from basic science to clinical applications. Hum Genet 2021;140:703-8.
3. Chou WC, Rampanelli E, Li X, et al. Impact of intracellular innate immune receptors on immunometabolism. Cell Mol Immunol 2022;19:337-51.
4. Oliver G, Kipnis J, Randolph GJ, et al. The Lymphatic Vasculature in the 21st Century: Novel Functional Roles in Homeostasis and Disease. Cell 2020;182:270-96.
5. Wong BW. Lymphatic vessels in solid organ transplantation and immunobiology. Am J Transplant 2020;20:1992-2000.
6. Daley SK, Witte MH, Washington J, et al. Role of Lymphatic Deficiency in the Pathogenesis and Progression of Inflammatory Bowel Disease to Colorectal Cancer in an Experimental Mouse Model. Inflamm Bowel Dis 2019;25:1919-26.
7. Schwager S, Detmar M. Inflammation and Lymphatic Function. Front Immunol 2019;10:308.
8. Jalkanen S, Salmi M. Lymphatic endothelial cells of the lymph node. Nat Rev Immunol 2020;20:566-78.
9. Alpert L, Yassan L, Poon R, et al. Targeted mutational analysis of inflammatory bowel disease-associated colorectal cancers. Hum Pathol 2019;89:44-50.
10. Hosono K, Kojo K, Narumiya S, et al. Prostaglandin E receptor EP4 stimulates lymphangiogenesis to promote mucosal healing during DSS-induced colitis. Biomed Pharmacother 2020;128:110264.
11. Jackson DG. Hyaluronan in the lymphatics: The key role of the hyaluronan receptor LYVE-1 in leucocyte trafficking. Matrix Biol 2019;78-79:219-35.
12. He M, He Q, Cai X, et al. Role of lymphatic endothelial cells in the tumor microenvironment-a narrative review of recent advances. Transl Lung Cancer Res 2021;10:2252-77.
13. Black LM, Farrell ER, Barwinska D, et al. VEGFR3 tyrosine kinase inhibition aggravates cisplatin nephrotoxicity. Am J Physiol Renal Physiol 2021;321:F675-88.
14. Zhou Y, Zhu X, Cui H, et al. The Role of the VEGF Family in Coronary Heart Disease. Front Cardiovasc Med 2021;8:738325.
15. Nikolakis D, de Voogd FAE, Pruijt MJ, et al. The Role of the Lymphatic System in the Pathogenesis and Treatment of Inflammatory Bowel Disease. Int J Mol Sci 2022;23:1854.
16. Tacconi C, Ungaro F, Correale C, et al. Activation of the VEGFC/VEGFR3 Pathway Induces Tumor Immune Escape in Colorectal Cancer. Cancer Res 2019;79:4196-210.
17. Lee AS, Hur HJ, Sung MJ. The Effect of Artemisinin on Inflammation-Associated Lymphangiogenesis in Experimental Acute Colitis. Int J Mol Sci 2020;21:8068.
18. Chen L, Wang J. Gut microbiota and inflammatory bowel disease. WIREs Mech Dis 2022;14:e1540.
19. Geravand M, Fallah P, Yaghoobi MH, et al. Investigation of enterococcus faecalis population in patients with polyp and colorectal cancer in comparison of healthy individuals. Arq Gastroenterol 2019;56:141-5.
20. Stoeva MK, Garcia-So J, Justice N, et al. Butyrate-producing human gut symbiont, Clostridium butyricum, and its role in health and disease. Gut Microbes 2021;13:1-28.
21. Ferrer-Picón E, Dotti I, Corraliza AM, et al. Intestinal Inflammation Modulates the Epithelial Response to Butyrate in Patients With Inflammatory Bowel Disease. Inflamm Bowel Dis 2020;26:43-55.
22. Shi Y, Xu LZ, Peng K, et al. Specific immunotherapy in combination with Clostridium butyricum inhibits allergic inflammation in the mouse intestine. Sci Rep 2015;5:17651.
23. Yang Y, Zheng X, Wang Y, et al. Human Fecal Microbiota Transplantation Reduces the Susceptibility to Dextran Sulfate Sodium-Induced Germ-Free Mouse Colitis. Front Immunol 2022;13:836542.
24. Li Y, Zhu W, Zuo L, et al. The Role of the Mesentery in Crohn's Disease: The Contributions of Nerves, Vessels, Lymphatics, and Fat to the Pathogenesis and Disease Course. Inflamm Bowel Dis 2016;22:1483-95.
25. Liu M, Xie W, Wan X, et al. Clostridium butyricum protects intestinal barrier function via upregulation of tight junction proteins and activation of the Akt/mTOR signaling pathway in a mouse model of dextran sodium

sulfate-induced colitis. Exp Ther Med 2020;20:10.
26. Wu X, Chen H, Gao X, et al. Natural Herbal Remedy Wumei Decoction Ameliorates Intestinal Mucosal Inflammation by Inhibiting Th1/Th17 Cell Differentiation and Maintaining Microbial Homeostasis. Inflamm Bowel Dis 2022. [Epub ahead of print].
27. Liu M, Xie W, Wan X, et al. Clostridium butyricum modulates gut microbiota and reduces colitis associated colon cancer in mice. Int Immunopharmacol 2020;88:106862.
28. Wang Y, Xie Q, Zhang Y, et al. Combination of probiotics with different functions alleviate DSS-induced colitis by regulating intestinal microbiota, IL-10, and barrier function. Appl Microbiol Biotechnol 2020;104:335-49.
29. Xie Y, Zhou L, Yao X, et al. Protective Effects of Clostridium Butyricum in a Murine Model of Dextran Sodium Sulfate-Induced Colitis That Involve Inhibition of the TLR2 Signaling Pathway and T Helper 17 Cells. Am J Med Sci 2020;360:176-91.
30. Bian X, Wu W, Yang L, et al. Administration of Akkermansia muciniphila Ameliorates Dextran Sulfate Sodium-Induced Ulcerative Colitis in Mice. Front Microbiol 2019;10:2259.
31. Chen T, Wang R, Duan Z, et al. Akkermansia muciniphila Protects Against Psychological Disorder-Induced Gut Microbiota-Mediated Colonic Mucosal Barrier Damage and Aggravation of Colitis. Front Cell Infect Microbiol 2021;11:723856.
32. Li W, Zhang L, Xu Q, et al. Taxifolin Alleviates DSS-Induced Ulcerative Colitis by Acting on Gut Microbiome to Produce Butyric Acid. Nutrients 2022;14:1069.
33. Mielcarska S, Kula A, Dawidowicz M, et al. Assessment of the RANTES Level Correlation and Selected Inflammatory and Pro-Angiogenic Molecules Evaluation of Their Influence on CRC Clinical Features: A Preliminary Observational Study. Medicina (Kaunas) 2022;58:203.
34. Abdulla MH, Shaik AS, Vaali-Mohammed MA, et al. Expression of VEGF, EGF and HGF in early- and late-stage colorectal cancer. Mol Clin Oncol 2021;15:251.
35. Lee AS, Lee JE, Jung YJ, et al. Vascular endothelial growth factor-C and -D are involved in lymphangiogenesis in mouse unilateral ureteral obstruction. Kidney Int 2013;83:50-62.
36. Baluk P, Hogmalm A, Bry M, et al. Transgenic overexpression of interleukin-1beta induces persistent lymphangiogenesis but not angiogenesis in mouse airways. Am J Pathol 2013;182:1434-47.
37. Wang XL, Zhao J, Qin L, et al. Promoting inflammatory lymphangiogenesis by vascular endothelial growth factor-C (VEGF-C) aggravated intestinal inflammation in mice with experimental acute colitis. Braz J Med Biol Res 2016;49:e4738.
38. Hos D, Bucher F, Regenfuss B, et al. IL-10 Indirectly Regulates Corneal Lymphangiogenesis and Resolution of Inflammation via Macrophages. Am J Pathol 2016;186:159-71.

Cite this article as: Chen X, Ma L, Liu X, Wang J, Li Y, Xie Q, Liang J. Clostridium butyricum alleviates dextran sulfate sodium-induced experimental colitis and promotes intestinal lymphatic vessel regeneration in mice. Ann Transl Med 2022;10(6):341. doi: 10.21037/atm-22-1059

