Probiotic Mixture VSL#3 Alleviates Dextran Sulfate Sodium-induced Colitis in Mice by Downregulating T Follicular Helper Cells*

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Summary: Clinical trials have shown beneficial effects of probiotics on inflammatory bowel diseases (IBD), although the exact mechanism remains unknown. VSL#3, a mixture of 8 probiotic bacteria, has been confirmed to have adjunctive therapeutic effects on colitis. T follicular helper (Tfh) cells, a new separate subset of CD4+ T helper cells, have been proved to play a vital role in autoimmunity. The present study aimed to identify the beneficial effect of the probiotic mixture VSL#3 on the mouse model of colitis by regulating Tfh cells. Dextran sulfate sodium (DSS) was used to induce chronic colitis in C57BL/6 mice. VSL#3 (3×10^9 live bacteria) was given to C57BL/6 mice every other day for 60 days by gavage. The disease activity index (DAI), histological activity index (HAI), colon length and myeloperoxidase (MPO) activity were detected. Immunofluorescence was used to visualize the location of Tfh cells. Immunoglobulins, Tfh cells and plasma cells were quantified by enzyme-linked immunosorbent assay (ELISA), flow cytometry, real-time PCR or Western blotting. The results showed that after DSS treatment, the humoral immunity was disordered in C57BL/6 mice, with increased IgM, IgG and IgA levels in colonic mucus and increased Tfh cells in mesenteric lymph nodes (MLN). VSL#3 treatment showed anti-inflammatory effects as evidenced by reduced DAI score, HAI score and MPO activity. IgM, IgG and IgA levels were significantly reduced in colon mucus, and the number of Tfh cells was markedly decreased in MLN after VSL#3 treatment. It was concluded that VSL#3 alleviates DSS-induced colitis by downregulating Tfh cells, and Tfh cells may become a potential therapeutic target for IBD.
Key words: T follicular helper cells; probiotics; VSL#3; humoral immunity; inflammatory bowel disease

Inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), is a chronic relapsing inflammatory disorder of the bowel that affects social and psychological well-being of people if poorly controlled[1]. Although the etiology of the disease is elusive, dysbiosis in the intestinal flora and aberrant activation of immune system are thought to play key roles[2].

Probiotics are defined as nonpathogenic microorganisms that have beneficial effects on the host if ingested in sufficient numbers and have been thought as an alternative approach to maintain or re-establish intestinal homeostasis in IBD patients[3]. VSL#3, a mixture of 8 bacteria, has been suggested to have clinical potential in the treatment of UC patients in mild-to-moderate active period and maintenance of remission of the condition[4, 5]. The action mechanisms of VSL#3 have been extensively reported, such as change in microbiota composition[6], immunomodulatory function by interacting with immune cells (eg. Th1, Th17, dendritic cells and macrophage)[7–9] and regulation of intestinal barrier integrity[10].

T follicular helper (Tfh) cells, a new separate subset of CD4+ T helper cells, have been proved to play critical roles in B cell differentiation and antibody secretion[11]. Tfh cells are characterized by excretion of IL-21 and high expression of surface markers CXC chemokine receptor type 5 (CXCR5), inducible co-stimulator (ICOS), programmed cell death 1 (PD1) and transcription factor B-cell lymphoma 6 (Bcl6). Similar to other CD4+ T cell subsets, Tfh cells response needs to be controlled strictly because aberrant Tfh cells response causes autoimmunity diseases such as rheumatoid arthritis (RA), systemic lupus

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erythematosus (SLE) and diabetes[12]. In the intestine, it has been demonstrated that excessive Tfh cells can impact the production of IgA, generate antibodies with auto-reactive properties and lead the bacteria to go beyond the mucosal barrier[13, 14]. The plasticity of Tfh cells in intestinal disorders is not fully understood[15]. The present study aimed to examine whether the probiotic, VSL#3, could alleviate mouse colitis by regulating Tfh cells.

1 MATERIALS AND METHODS

1.1 VSL#3 Preparation

VSL#3 (3×10^{11} live total bacteria/g) was kindly provided by Janssen Pharmaceutical Companies of Johnson & Johnson (USA). Dissolved in normal saline, approximately 3×10^{9} live bacteria were given to C57BL/6 mice.

1.2 Induction of Chronic Experimental Colitis

Male C57BL/6 mice, 9 weeks old with an initial mean weight of 23±0.7 g, were randomly assigned to control group, dextran sodium sulfate (DSS, MP Biomedicals, USA) group and DSS+VSL#3 group (n=7, 15, 15 at the beginning of the experiment, respectively; n=7, 7, 9 at the end of the experiment, respectively, after removal of the dead mice) under controlled temperature and photoperiods. Care and experimentation of mice were performed in accordance with institutional guidelines at the animal care unit of Huazhong University of Science and Technology.

Chronic colitis was induced by feeding mice with 2.5% (wt/vol) DSS dissolved in drinking water. In DSS group, mice were treated with 4 cycles of DSS for 6 days/cycle and 9 days of normal drinking water between each cycle. Besides the same treatment as those in DSS group, mice in DSS+VSL#3 group were administered with VSL#3 by gavage every other day from the day mice were exposed to DSS to the end of the experiment. Animals in control group received the same drinking water in the absence of DSS and were gavaged with normal saline every other day (fig. 1A). On day 60, mice were sacrificed to collect serum, mesenteric lymph nodes (MLN) and colon tissues.

1.3 Assessment of Colitis

Mice were observed every other day for body weight, stool consistency, presence of blood in stool. Disease activity index (DAI)[16] ranging from 0 (unaffected) to 12 (severe colitis) and histological activity index (HAI)[17] ranging from 0 (unaffected) to 8 (severe colitis) were used to evaluate the severity of colitis. Myeloperoxidase (MPO) activity in colonic mucosa was detected according the manufacturer's protocol (Nanjing Jiancheng, China).

1.4 ELISA

The ELISA plates (eBioscience, USA) were coated with captured antibody and blocked with blocking buffer according to the manufacturer's protocol. Two-fold serial dilutions of the standards were used to make the standard curve. The dilutions for serum IgG, IgA and IgM were at 1:10000, 1:100 and 1:1000, respectively, and for scraped intestinal mucus, the dilutions were at 1:10, 1:1000 and 1:10, respectively. After samples, detection antibody, substrate solution and stop solution were added to each well, the plates were read at 450 nm.

1.5 Immunofluorescence

Colon and MLN were fixed with 4% normal buffered formalin, embedded in paraffin and sectioned at 5 μm thickness with a paraffin microtome. After dewaxing and hydration, sections were stained with anti-mouse Bcl6 antibody (Santa Cruz, USA) and anti-mouse CXCR5 antibody (Santa Cruz, USA), then soaked in a cocktail containing secondary goat anti-rabbit-488 and goat anti-mouse-594 antibodies and finally rinsed and mounted with the fluorescent dye 4′-6-Diamidino-2-phenylindole (DAPI) under a coverslip. Images were acquired by Nikon A1-Si confocal microscopy.

1.6 Flow Cytometry

After separation, MLN were triturated and filtrated by a 70-μm nylon cell strainer to obtain single-cell suspensions. Different anti-mouse antibodies and isotype-matched controls were added to the cell suspension: FITC conjugated anti-mouse CD93 antibody, APC conjugated anti-mouse CD138 antibody, FITC conjugated anti-mouse CD4 antibody, PE-Cy5 conjugated anti-mouse CXCR5 antibody and PE conjugated anti-mouse Bcl6 (R&D, USA). Flow cytometry analyses were performed on BD FACSCanto II (Becton, Dickinson and Company, USA).

1.7 Real-time Quantitative PCR

Total RNA from mouse colon and MLN was extracted with TRIzol reagent (Takara Bio Inc., Japan), and reversely transcribed by PrimeScriptTM RT Reagent Kit (Takara Bio Inc., Japan) according to the manufacturer's protocol. Real-time PCR reactions were performed using a SYBR Premix Ex Taq Kit (Takara Bio Inc., Japan) in Roche Light Cycler 480 System. The relative mRNA levels were normalized against housekeeping gene GAPDH and the results were analyzed by the $2^{-\Delta\Delta CT}$ method. Primers for real-time qRT-PCR were shown as follows: mouse IL-21, forward 5′-GCAGCACAGGCTAAGAGCTTGTA-3′, reverse 5′-TGGCTAGTGGAGAAGCCTTCA-3′; mouse Bcl6, forward 5′-GCACTGGGCAAACACAACAT-3′, reverse 5′-AGCGTGCCGGGTAAACTG-3′; mouse Blimp-1, forward 5′-TCTGTTCAAGCCGAGGCAT-CC-3′, reverse 5′-TCTTGGGAACTGTGTCATTAG-3′; mouse GAPDH, forward 5′-TTCACCACCATGGAG-AAGGC-3′, reverse 5′-GGCATGGACTGTGGTCAT-GA-3′.

1.8 Western Blot Analysis

Proteins were extracted from the mouse MLN and quantified using protein assay. Immunoblot analysis was conducted using Bcl6 and Blimp-1 antibody (Santa Cruz, USA) and GAPDH. The results were assessed via a chemiluminescent detection system according to the manufacturer's protocol and exposure to autoradiography film.

1.9 Statistical Analysis

Data were shown as the mean±standard deviation (SD). The DAI score and body weight were analyzed using 2-way ANOVA. HAI score, colon length, results of ELISA, flow cytometry, real-time quantitative PCR and Western blotting were analyzed by one-way ANOVA. $P<0.05$ was considered statistically significant.

2 RESULTS

2.1 VSL#3 Alleviates DSS-induced Mouse Colitis

Clinicopathologic study showed a moderate to severe colitis, with loss of weight, diarrhea/loose feces and visible fecal blood, which suggested the successful establishment of the DSS-induced mouse colitis model. DAI score was significantly decreased in DSS+VSL#3 group when compared with DSS group (fig. 1C, $P<0.05$). Additionally, DSS treatment significantly decreased the length of colon, aggravated histological damage in the colon and increased the level of MPO activity (fig. 1D–1F, $P<0.01$), which were significantly alleviated by VSL#3 supplementation (fig. 1D–1F, $P<0.05$).

2.2 VSL#3 Treatment Decreases Immunoglobulins in Colonic Mucus of Mice with DSS-induced Colitis

No significant changes were observed in the levels of serum IgM, IgG and IgA between DSS and DSS+VSL#3 groups (data not shown). As shown in fig. 2, DSS induced the increases in levels of IgM, IgG and IgA in colonic mucus (fig. 2A–2C, $P<0.01$). After treatment with VSL#3, the levels of immunoglobulins IgM, IgG and IgA were significantly decreased (fig. 2A–2C, $P<0.05$), indicating a profound effect of

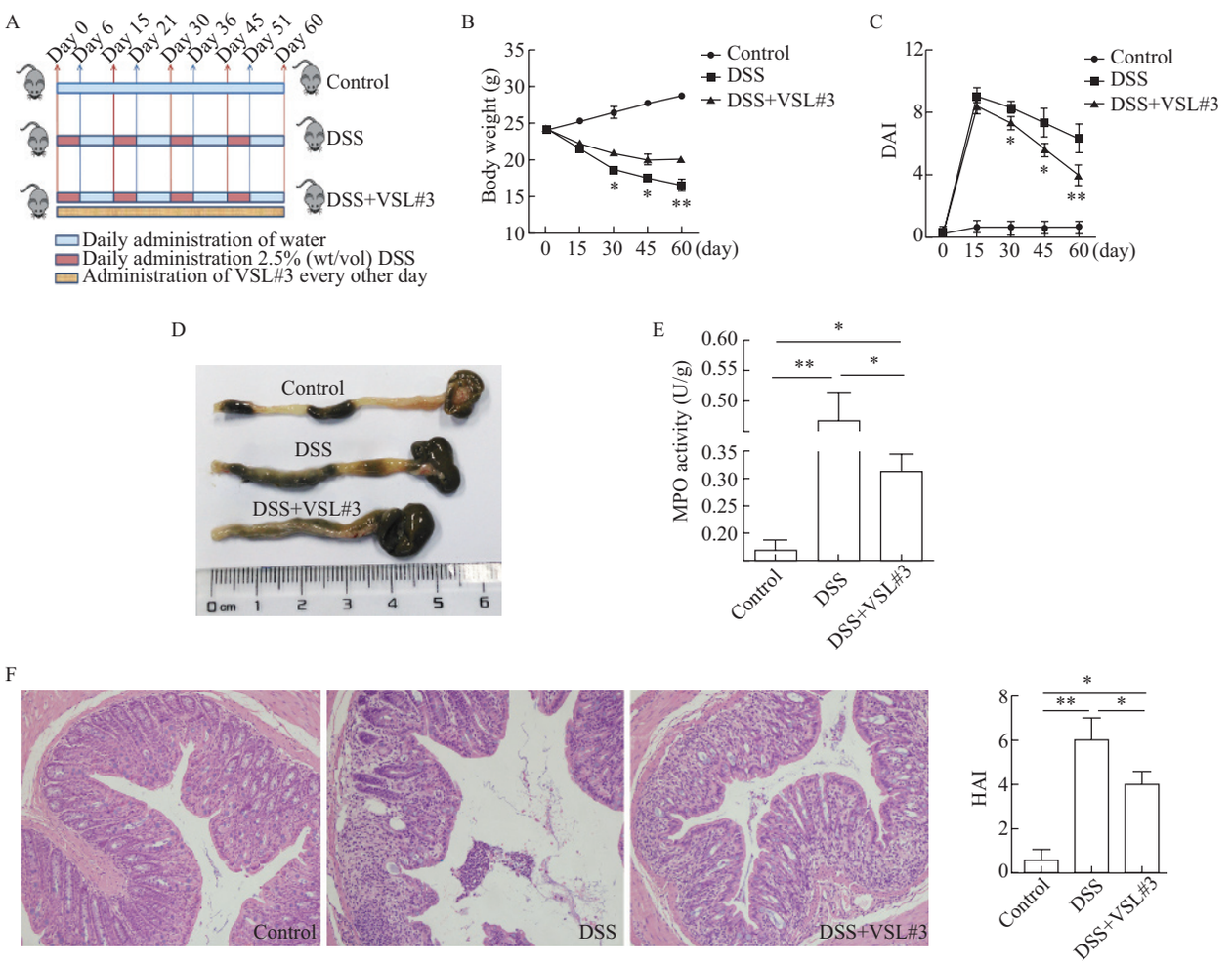

Fig. 1 Assessment of DSS-induced colitis in the control, DSS, and DSS+VSL#3 groups

A: schematic diagrams for establishment of the chronic colitis mouse model and VSL#3 treatment; B–E: comparison of body weight (B), DAI score (C), colon length (D) and MPO (E) among the 3 groups; F: hematoxylin and eosin staining of colons (×100) and HAI score in the 3 groups. Data were expressed as mean± SD. $^{*}P<0.05$, $^{**}P<0.01$

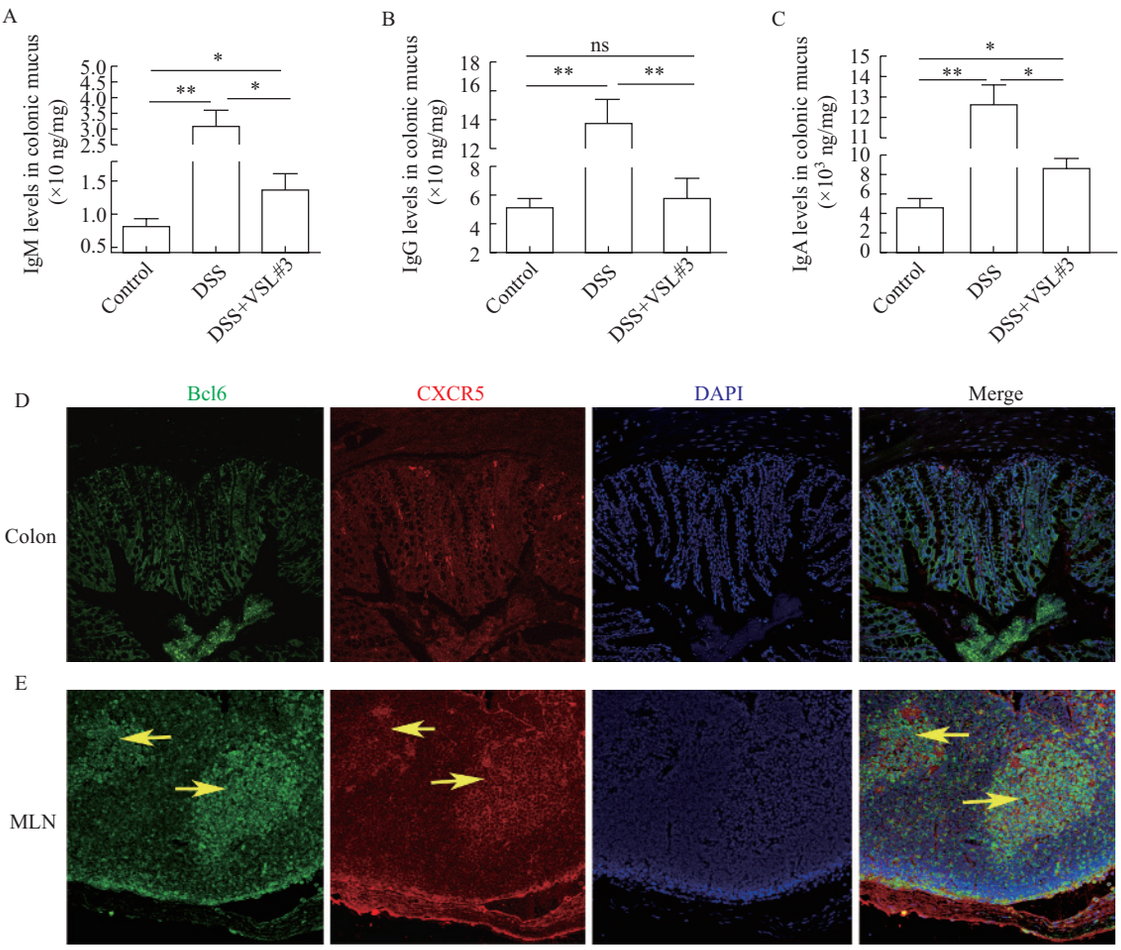

Fig. 2 Levels of s IgM (A), IgG (B), and IgA (C) in colonic mucus in the 3 groups and location of Tfh cells in colon tissues (D, ×200) or MLN (E, as indicated by arrows, ×200) in DSS-induced colitis on day 60
Slides were stained for Bcl6 (green), CXCR5 (red), and DAPI (blue). Data were expressed as mean±SD. $^{*}P<0.05$, $^{**}P<0.01$

VSL#3 on humoral immune response, besides their effects on the immunity profile of Th1, Th17 and Treg as reported previously[18, 19].

2.3 Tfh Cells Are Located in Germinal Centers (GCs) of MLN

As shown in fig. 2D and 2E, in mice with DSS-induced colitis, the Bcl6+CXCR5+ Tfh cells were hardly detectable in the colon by immunofluorescence, but high density of fluorescence was noted in MLN, indicating that there was a scarce of Tfh cells in the colon and Tfh cells were mainly located in MLN.

2.4 Tfh Cells in MLN Increase after DSS Exposure and Decrease after VSL#3 Treatment

After confirming the location of Tfh cells, we quantified Tfh cells with flow cytometry. Firstly, we detected the number of CD93+CD138+ mature plasma cells which produced antibodies and were regulated by Tfh cells. As expected, the number of CD93+CD138+ mature plasma cells was significantly increased after DSS administration and decreased after VSL#3 supplement, which was consistent with the changes of immunoglobulins in colonic mucus (fig. 3A). Although there was no significant difference in total CD4+ cells among the three groups (fig. 3B), the number of Bcl6+CXCR5+ cells in CD4+ cells was remarkably greater in DSS group than in both control and DSS+VSL#3 groups (fig. 3C), indicating that VSL#3 supplement decreased the differentiation of Tfh cells in DSS-induced colitis.

2.5 VSL#3 Decreases Tfh Cells by Affecting the Balance between Bcl6 and Blimp-1 Expression in MLN

Bcl6 is the master transcription factor that regulates the differentiation of Tfh cells, whereas Blimp-1 is an antagonist[20]. To further investigate the mechanism of VSL#3 treatment in the differentiation of Tfh cells, Bcl6 and Blimp-1 mRNA levels in both MLN and colon were detected. Remarkable increase in Bcl6 mRNA and decrease in Blimp-1 mRNA in MLN were found in DSS group (fig. 4A, $P<0.05$), which were significantly reversed by VSL#3 treatment (fig. 4A, $P<0.05$). High expression of cytokine IL-21 is another hallmark of Tfh cells. IL-21 mRNA was found to be overexpressed in MLN from colitis mice, which

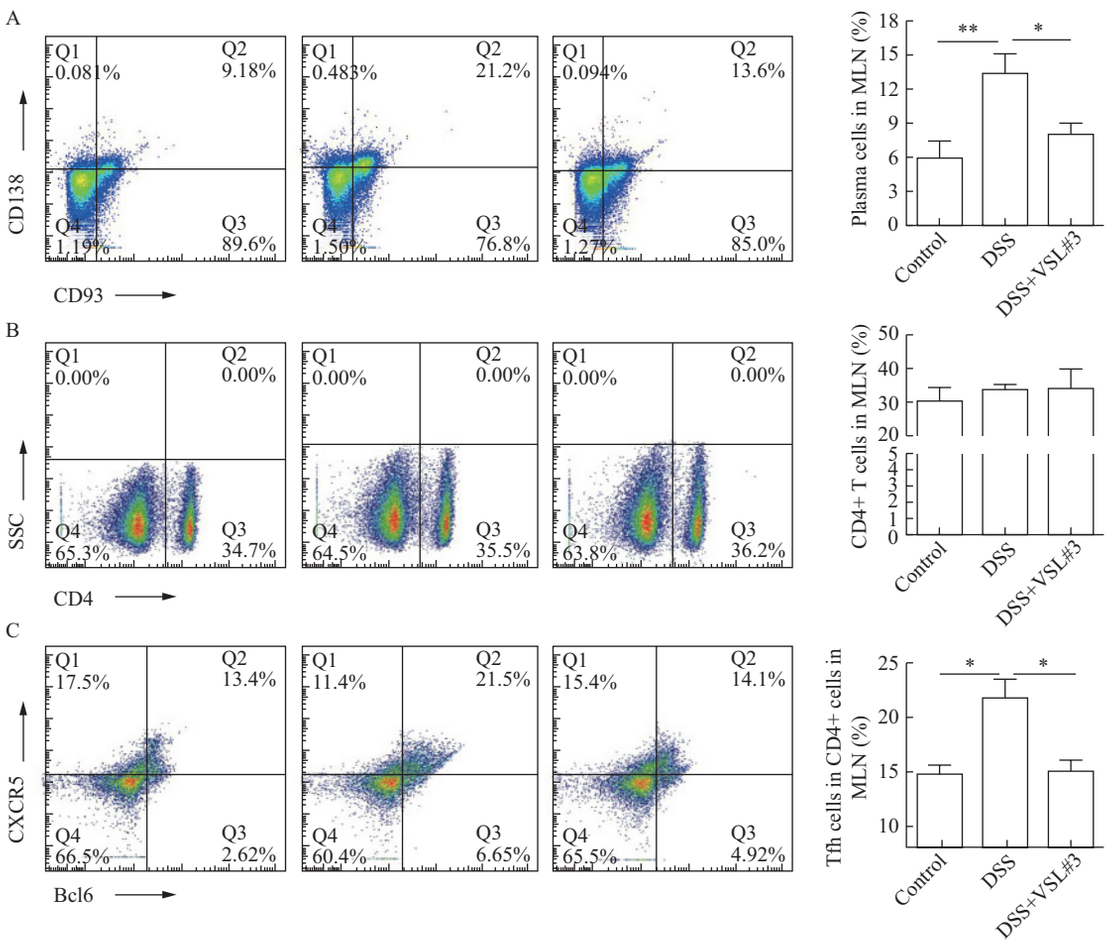

Fig. 3 Quantitative analysis of mature plasma cells and Tfh cells in MLN of the 3 groups by flow cytometry on day 60
A: CD93+CD138+ mature plasma cells in MLN; B: CD4+ T cells in MLN; C: CXCR5+Bcl6+ in CD4+ cells in MLN. Data were expressed as mean±SD. $^{*}P<0.05$, $^{**}P<0.01$

was significantly down-regulated in VSL#3 treated mice (fig. 4A, $P<0.05$). Additionally, VSL#3 treatment could significantly decrease the Bcl6 protein levels increased in the MLN of DSS-induced colitis (fig. 4B). Interestingly, in the colon tissues, the detected mRNA expression profiles of Bcl6 and Blimp-1 were quite different from those in MLN, with no significant difference in Bcl6 and Blimp-1 expression noted after VSL#3 treatment in the colon tissues (fig. 4C).

3 DISCUSSION

The precise mechanism underlying the extensive use of probiotics for IBD remains unknown[21–23]. Understanding the action mechanism of probiotics may provide the rationale for use of probiotics in the treatment of IBD. In this study, we observed the aberrant Tfh cells response in DSS-induced colitis and found that supplementation of VSL#3 could regulate Tfh cells, rendering the mice resistant to the development of colitis.

A number of clinical and basic researches have indicated the therapeutic efficacy of VSL#3 for IBD[24]. VSL#3 has clinical potential in the treatment of UC patients in mild-to-moderate active period and contributes to maintenance of remission[25]. *In vivo* or *in vitro* administration of VSL#3 was effective in reducing pro-inflammatory cytokine levels, changing microbiota composition[6], influencing immune cells[9] and improving epithelial barrier function[10]. Our present study further provided evidence that VSL#3 could alleviate colitis by regulating Tfh cells in DSS-induced colitis.

Usually, human UC or CD is a long-course disease and often recurs. Therefore, we established a chronic colitis model[26] with a developing pathology in our study. In the study, firstly, we observed that VSL#3 could alleviate DSS-induced colitis as evidenced by decreased DAI score, HAI score and MPO activity, and increased body weight and colon length in the DSS+VSL#3 group. Then we found that VSL#3 treatment could significantly reduce the levels of IgM, IgG and IgA in colon mucus in mice with DSS-induced colitis. As Tfh cells are recently believed to be responsible for immunoglobulin production, we speculated that the effect of VSL#3 on DSS-induced

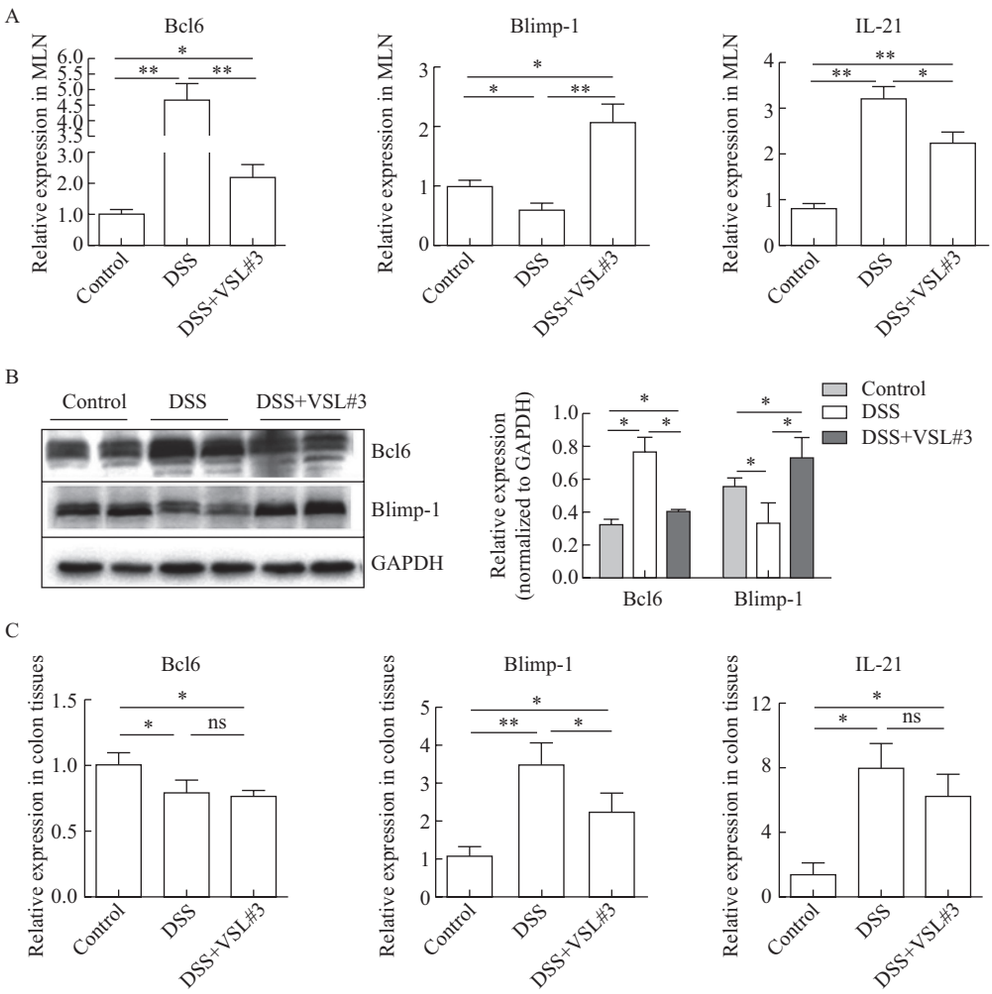

Fig. 4 Effects of VSL#3 on the balance of Bcl6 and Blimp-1 levels in MLNs on day 60
A: Bcl6, Blimp-1 and IL-21 mRNA levels in the 3 groups in MLN; B: Bcl6 and Blimp-1 protein levels in MLN (the left panel shows Western blots and the right panel is the results of statistical analysis); C: Bcl6, Blimp-1 and IL-21 mRNA levels in the 3 groups in colon tissues. Data were expressed as mean±SD. ns: no significant difference; $^{*}P<0.05$, $^{**}P<0.01$

colitis might be through regulating Tfh cell expression. As expected, we detected an aberrant increase in Tfh cells located in MLN in DSS-induced colitis, and VSL#3 treatment could significantly reduce the number of Tfh cells in MLN, which suggested that VSL#3 may alleviate colitis by down-regulating the number of Tfh cells.

A great body of evidence suggests that Tfh cells are involved in the pathogenesis of autoimmune response[27]. They are reported to be expanded in the peripheral blood of patients with RA, SLE and Sjögren syndrome, and correlated with disease activity in these autoimmune diseases[28–30]. In recent years, some researches unveil the secret role of Tfh cells in gastrointestinal tract. It has been demonstrated that excessive Tfh cells affect the selection of IgA plasma cells and generate antibodies with auto-reactive properties, leading the gut bacteria to go beyond the mucosal barrier[13, 14]. The cytokine, IL-21, secreted by Tfh cells, modulates the activity of several immune cell types that are involved in tissue damage in IBD[31], and blockade of IL-21 signaling attenuates the inflammation in experimental models of IBD[32, 33]. With the increased recognition of functional plasticity and diversity of Tfh cells, it is believed that better understanding of the extrinsic and intrinsic mechanisms that control plasticity of Tfh cells will have important therapeutic applications to control autoimmunity, like IBD. Up to now, the influence of use of microbiota, especially probiotics, on Tfh cells remains unknown.

Moreover, RT-PCR analysis in our study showed that the mRNA levels of Bcl6 were profoundly increased and those of Blimp-1 obviously decreased in DSS-induced colitis, which was greatly reversed by VSL#3 treatment. Given that Bcl6 is the master transcription factor of Tfh cells and its expression is repressed by Blimp-1, we speculated that the decrease of Tfh cells might be attributed to the negative action

of VSL#3 on Bcl6, which, thereby, promoted the expression of Blimp-1. And then the decreased Tfh cells after VSL#3 treatment led to the less secretion of IL-21 that influenced the expression of Tfh cells in turn[34]. We also examined the expression of Bcl6, Blimp-1 and IL-21 in colon tissues. The results were quite different from those in MLNs, which showed no significant difference in Bcl6 and Blimp-1 expression in colon tissues after VSL#3 treatment. As a transcriptional regulation factor, Bcl6 is found to be important in not only regulating B cell growth but also influencing epithelial apoptosis[35], and Blimp-1 was reported to promote colonic inflammation by down-regulating NLRP12[36]. Whether Bcl6 and Blimp-1 have functions in intestinal epithelium is not clear. Possibly, in the colon tissues, the decreased expression of Bcl6 and Blimp-1 was mainly a feedback for apoptosis in the intestinal epithelium, which needs a further study.

In summary, in this study, we provided evidence that VSL#3 could alleviate DSS-induced colitis by regulating Tfh cells, and Tfh cells may be a potential therapeutic target for IBD.

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Conflict of Interest Statement

The authors declare no conflict of interest in this study.

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