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Lactobacillus plantarum CBT LP3 ameliorates colitis via modulating T cells in mice

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ABSTRACT

Lactobacillus plantarum has been identified as a probiotic bacterium owing to its role in immune regulation and maintenance of intestinal permeability. Here, we investigated the anti-colitic effects and mechanism of *L. plantarum* CBT LP3 (LP3). This *in vivo* study was performed using dextran sodium sulfate (DSS) to induce colitis in mice. Mice were randomly divided into three groups: a control supplied with normal drinking water, a DSS-treated group followed by oral administration of vehicle, and a DSS-treated group gavaged with LP3 daily for 7 days following DSS administration. An analysis of macrophages and T cell subsets harvesting from peritonium cavity cells and splenocytes was performed using a flow cytometric assay. Gene expression and cytokine profiles were measured using quantitative reverse transcriptase polymerase chain reaction. The administration of LP3 significantly attenuated disease activity and histolopathology compared to control. LP3 had anti-inflammatory effects, with increased induction of regulatory T cells and type 2 helper T cells in splenocytes and restoration of goblet cells accompanied by suppression of proinflammatory cytokine expressions. These findings suggest that *L. plantarum* CBT LP3 can be used as a potent immunomodulator, which has significant implications for IBD treatment.

1. Introduction

Inflammatory bowel diseases (IBDs), including ulcerative colitis (UC) and Crohn's disease (CD), are chronic relapsing inflammatory disorders of the gastrointestinal tract (Abraham and Cho, 2009). The pathogenesis of IBD is still largely unexplained, although it is known that genetic, environmental, and immunological factors are involved. Chronic intestinal inflammation in IBD involves an immunologic imbalance in the macrophages and T cell subsets as well as aberrant inflammatory cytokine productions (Fonseca-Camarillo and Yamamoto-Furusho, 2015). In addition, the intestine's diverse microbial ecosystem can affect the differentiation of innate and adaptive immunities by microbial interaction with the immune system (Hold et al., 2014). Accumulating evidence indicates that IBDs involve disrupted homeostatic interactions between the microbiota and the mucosal immune system, along with subsequent immune dysregulation (Park et al., 2018; Sartor, 2008).

Despite the various therapeutic options for IBD, current drug-based treatments have shown limited clinical efficacy and a high incidence of side effects, including increased infectious complications and drug resistance during treatment (Chande et al., 2015; Lee et al., 2017). Because of the evidence implicating microbial dysbiosis in IBD, it has been suggested that probiotics improve dysbiosis and reduce intestinal inflammation. Probiotics may be an alternative and safe therapeutic approach in IBD treatment (Ganji-Arjenaki and Rafieian-Kopaei, 2018). Probiotics are live microorganisms, which could confer a health benefit

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to the host (Ganji-Arjenaki and Rafieian-Kopaei, 2018). Indeed, recent studies suggest that probiotics have the ability to ameliorate abnormal immune responses and have potential benefits in IBD, although conflicting results have been observed in clinical use (Dignass et al., 2012; Ganji-Arjenaki and Rafieian-Kopaei, 2018; Tursi et al., 2010). Probiotics can regulate gut barrier function and cytokine production by affecting immune cells such as regulatory T cells, macrophages, and dendritic cells (Ganji-Arjenaki and Rafieian-Kopaei, 2018). Nevertheless, the mechanisms underlying the effects of most probiotics on IBD are not well understood.

Lactobacillus plantarum is a lactic acid bacterium found in dairy products and gastrointestinal, vaginal, and urogenital tracts and used in the fermentation of dairy products (Seddik et al., 2017). It is known to inhibit gas-producing bacteria in the intestine and have a positive effect in some irritable bowel syndrome patients. It is also able to deliver vehicles for therapeutic compounds or proteins *in vivo* due to its ability to survive in the human gastrointestinal tract (Seddik et al., 2017). Previous studies have shown that *L. plantarum* has an anti-inflammatory effect owing to its suppression of proinflammatory cytokines and enhancement of the intestinal epithelial barrier (Seddik et al., 2017). However, despite its safety and promising health benefits, the particular mechanisms underlying the role of *L. plantarum* in colitis remain less understood.

The aim of this study was to explore the role of *L. plantarum* CBT LP3 in ameliorating murine colitis and to investigate new options for IBD treatment. We induced colitis by administration of dextran sulfate sodium (DSS) in mice to determine the impact of *L. plantarum* CBT LP3 on gut inflammation and investigate its role in the immune response and goblet cells.

2. Materials and methods

2.1. Probiotics preparation

Lactobacillus plantarum CBT LP3 (KCTC 10782BP) were kindly provided by Cell Biotech (Gimpo, Korea). *L. plantarum* CBT LP3 were cultured in Luria-Bertani broth in a 37 ˚C incubator and optimally resuspended in sterile phosphate-buffered saline (PBS).

2.2. DSS-induced colitis mouse model and assessment

All animals were kept under standard conditions at 21–22 ˚C under a 12-h light/dark cycle and were acclimatized for 1 week. Six- or eight-week-old male C57BL/6 mice (n = 6 / group) were administered drinking water containing 2.5 % (w/v) DSS (MP Biomedicals, Solon, OH, USA) for induction of colitis for 7 (day 0–6) or 16 (in survival experiment) days, and normal, untreated drinking water was administered for 9 days as a healing model. Probiotic-treated mice were administered 1×10^8 bacteria in 0.1 mL PBS by gavage daily for 7 days (day 7–15). Peritoneal cavity cells (PCCs), spleen, and colons were collected. Mice were checked daily for body weight loss, stool consistency, presence of gross blood in the stool or at the anus, and overall mortality. Disease activity index (DAI), which incorporates body weight loss, stool consistency, and gross bleeding, was calculated as described in the previous study (Kim et al., 2019). The entire colon from the cecum to the anus was removed after sacrificing the mouse and colon length was measured between the ileocecal junction and the proximal rectum. The colon was opened and gently cleared of stool using PBS. The distal colon was cut into 2–3 pieces for periodic acid-Schiff (PAS) staining and RNA isolation. All animal experiments were performed in accordance with all applicable Korean laws and were reviewed and approved by the Institutional Animal Care and Use Committee of Yonsei University Severance Hospital (IACUC Approval No: 2015-0407).

2.3. Periodic acid-Schiff staining and histomorphological scoring

Colon tissues were fixed with 10 % formalin solution (pH 7.4) overnight, embedded in paraffin on a slide, and sectioned using standard protocols. Sections were then deparaffinized and subjected to PAS staining to evaluate the degree of inflammation. Images were obtained using a light microscope (Olympus BX41; Olympus Optical, Tokyo, Japan). The histopathologic evaluation and goblet cell scoring were evaluated as previously described (Kim et al., 2019).

2.4. Isolation of peritoneal cavity cells from the peritoneum and splenocytes from the spleen and flow cytometric analysis

PCCs and splenocytes were isolated from peritoneum and spleen of mice, respectively, as previously described (Seo et al., 2017). After depletion of red blood cells with RBC lysis buffer, the cells were analyzed using flow cytometry.

For flow cytometric analysis, single cell suspensions (10^6 cells) were blocked with 2.5 % normal mouse and rat serum in FACS buffer (PBS containing 2 % FBS) and stained for 30 min at 4 ˚C with the appropriate antibodies. The antibodies used included: monoclonal anti-Foxp3 (150D/E4), anti-TREM-1 (TR3MBL1), anti-F4/80 (BM8), anti-TLR4 (UT41), and anti-GATA3 (TWAJ) antibodies purchased from eBioscience (San Diego, CA, USA); monoclonal anti-CD3 (145-2C11) and anti-CD4 (GK1.5) antibodies purchased from BD Biosciences (San Jose, CA, USA); and monoclonal anti-CD206 (C068C2) antibody purchased from Biolegend (San Diego, CA, USA). For intracellular staining, a Foxp3/Transcription factor staining buffer set was purchased from eBioscience. Data were acquired using a FACSVerse flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, San Carlos, CA, USA).

2.5. Extraction of RNA and quantitative real-time reverse-transcription polymerase chain reaction

Total RNA was extracted using TRIzol Reagent (Life Technologies, Carlsbad, CA, USA) and reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Pittsburgh, PA, USA) according to the manufacturer's protocol. Amplification was carried out using SYBR Green Master Mix (Thermo Fisher Scientific) on a StepOne Plus real-time PCR system (Thermo Fisher Scientific) for 45 cycles using the following thermocycling steps: 95 ˚C for 30 s, 60–63 ˚C for 30 s, and 72 ˚C for 40 s. All real-time reverse-transcription polymerase chain reactions (RT-PCRs) were run in triplicate and quantitative analysis was performed using the relative standard curve method as described in the previous study (Seo et al., 2017). Results are presented as fold change compared with the control sample after normalization to the level of transcription of the endogenous control, *β-actin*. Real-time qRT-PCR primers are summarized in Table 1.

Table 1
List of primers used for qRT-PCR.

Gene	Sequence (5′–3′)
Mouse	
Tnfa	F: CAA AGG GAG AGT GGT CAG GT
	R: ATT GCA CCT CAG GGA AGA GT
Il1b	F: GCA ACT GTT CCT GAA CTC AAC T
	R: ATC TTT TGG GGT CCG TCA ACT
IL10	F: CCC ATT CCT CGT CAC GAT CTC
	R: TCA GAC TGG TTT GGG ATA GGT TT
Il17	F: CAG CAG CGA TCA TCC CTC AAA G
	R: CAG GAC CAG GAT CTC TTG CTG
Inos	F: GGC AGC CTG TGA GAC CTT TG
	R: GCA TTG GAA GTG AAG CGT TTC

F: forward primer, R: reverse primer.

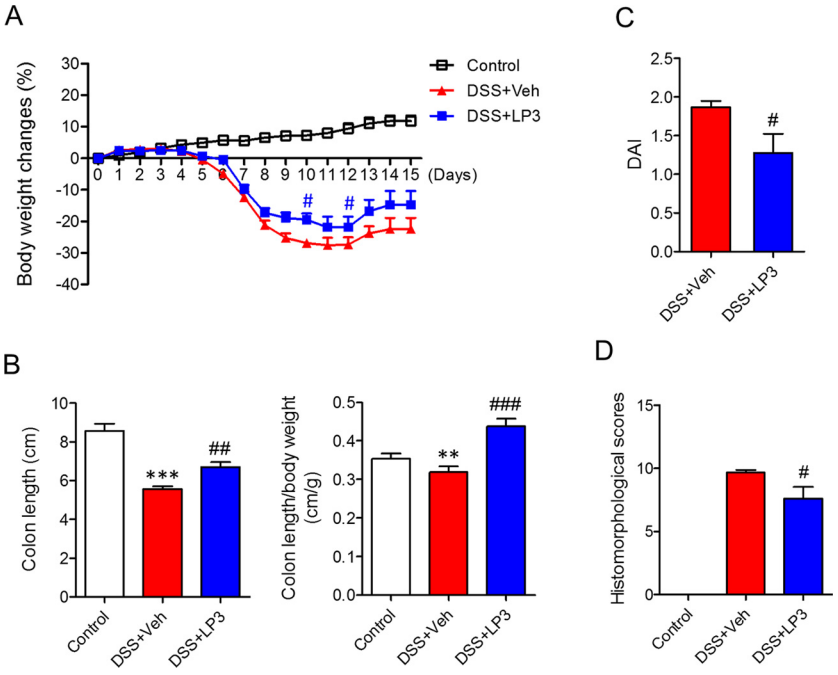

Fig. 1. Effect of *L. plantarum* CBT LP3 on DSS-induced mouse colitis. We administered 2.5 % dextran sodium sulfate (DSS) to 8-week-old male C57BL/6 mice starting from day 0 for 7 days and changed to pure water for recovery before sacrifice (day 15). The DSS-treated mice were administrated *L. plantarum* CBT LP3 (1×10^8) daily by gavage for 7 days (day 7–15). Body weight (A), colon length (B), disease activity index (C), and histomorphological score (D) were evaluated.
Data represent mean ± SEM (n = 6 / group). $^{***}P < 0.005$ vs. Control, $^{\#}P < 0.05$ vs. DSS + Veh, $^{\#\#}P < 0.01$ vs. DSS + Veh. Control, supplied with normal drinking water; DSS + Veh, DSS-treated plus PBS; DSS + LP3, DSS-treated plus *L. plantarum* CBT LP3.

2.6. Statistical analysis

All results were expressed as means of ± standard errors of the means (SEMs). GraphPad Software (La Jolla, CA, USA) was used for all analysis. The significance of differences between conditions was assessed using Student's *t*-test and one-way ANOVA. *P* values < 0.05 were considered significant.

3. Results

3.1. Effects of L. Plantarum CBT LP3 on mouse colitis

To induce acute colitis in mice, we administered 2.5 % DSS, which is widely used in IBD research. The mice then received *L. plantarum* CBT LP3 (LP3) or vehicle (PBS) by oral gavage to determine the therapeutic effects on colitis. DSS treatment induced severe colitis as assessed by weight loss and DAI scores accompanied by microscopic damage. In contrast, the LP3-treated group showed a quick recovery from body weight loss, colon length, and DAI compared to mice in the vehicle-treated group when DSS was substituted with drinking water (Fig. 1A–D). Furthermore, the LP3-treated group exhibited a trend of higher survival compared to the vehicle-treated group with continuing DSS treatment, although there was no statistical significance (Fig. 2). Histologic examination consistently revealed an improvement in inflammatory signs with reduced inflammatory infiltrates and restored intestinal epithelia in the LP3-treated group compared to the vehicle-treated group (Figs. 1D and 3 A), demonstrating that LP3 alleviates mouse colitis.

3.2. Effects of L. Plantarum CBT LP3 on goblet cell hypoplasia and proinflammatory cytokine expression in colitis

Goblet cells, known as mucin secretory intestinal epithelial cells (Kinoshita et al., 2000), are less numerous and more hypotrophic in DSS-administered colons in mice as well as in intestinal mucosa from IBD patients compared to healthy intestinal mucosa. To identify the effects of LP3 on goblet cells, we microscopically assessed goblet cell loss using PAS-stained colon sections. The vehicle-treated group exhibited severe goblet cell loss, whereas the LP3-treated group showed significantly increased goblet cells containing high levels of mucus

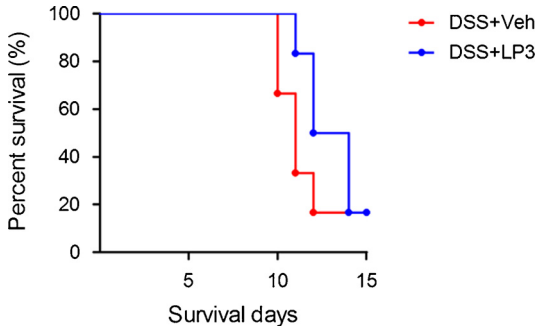

Fig. 2. Effect of *L. plantarum* CBT LP3 on the life span of DSS-treated mice. Six-week-old male C57BL/6 mice were administrated 2.5 % dextran sodium sulfate (DSS) from day 0 to day 15. The DSS-treated mice were administrated *L. plantarum* CBT LP3 (1×10^8) daily by gavage for 7 days (day 0–6). Mouse mortality was analyzed using Kaplan–Meier plots (n = 6 / group). Survival was compared using a log-rank test. Control, supplied with normal drinking water; DSS + Veh, DSS-treated plus PBS; DSS + LP3, DSS-treated plus *L. plantarum* CBT LP3.

(Fig. 3A, and B). The proinflammatory cytokines, which play a central role in the immune response of IBD, are produced by lymphocytes, monocytes, granulocytes, epithelial cells, and fibroblasts (Fonseca-Camarillo and Yamamoto-Furusho, 2015). Thus, to assess the expression levels of proinflammatory cytokines, we performed qRT-PCR for the major cytokines, such as tumor necrosis factor alpha (*Tnfa*), interleukin 17 (*Il17*), and interleukin 1 beta (*Il1b*), and a mediator of reactive nitrogen species such as inducible NO synthase (*Inos*), in the colon tissues. DSS treatment markedly increased expressions of proinflammatory cytokines and *Inos* compared to the control group, in particular *Il17* and *Il1b*. In contrast, LP3 treatment significantly suppressed expression of proinflammatory cytokines and *Inos* (Fig. 3C).

3.3. Effects of L. Plantarum CBT LP3 on macrophage and T helper cell polarization

Immune cell responses play a crucial role in the pathogenesis of IBD. In particular, an imbalance of $CD4^+$ T cells is known to be one of the major drivers in the disease process. Additionally, M1 macrophages

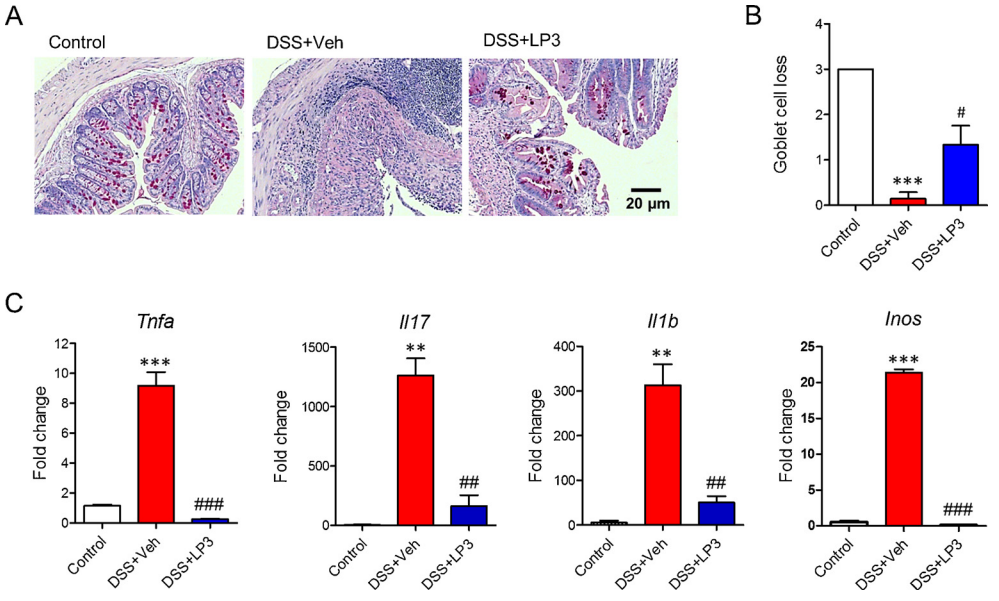

Fig. 3. Effect of *L. plantarum* CBT LP3 on goblet cell and cytokine expressions in DSS-treated mice. We administrated 2.5 % dextran sodium sulfate (DSS) to 8-week-old male C57BL/6 mice from day 0 for 7 days and changed to pure water before sacrifice (day 15). The DSS-treated mice were administrated *L. plantarum* CBT LP3 (1×10^8) daily by gavage for 7 days (day 7–15). (A, B) Representative images of periodic acid-Schiff (PAS)-stained goblet cells in the colon (A) and goblet cell evaluation (B). Scale bar, 20 μm. (C) Transcript level of proinflammatory cytokines (*Tnfa*, *Il17*, *Il1b*) and *Inos* in the colons. Gene expression analysis was determined in duplicate using qRT-PCR analysis.

Data represent mean ± SEM (n = 6 / group). $^{**}P < 0.01$ vs. Control, $^{***}P < 0.005$ vs. Control, $^{\#}P < 0.05$ vs. DSS + Veh, $^{\#\#}P < 0.01$ vs. DSS + Veh, $^{\#\#\#}P < 0.005$ vs. DSS + Veh. Control, supplied with normal drinking water; DSS + Veh, DSS-treated plus PBS; DSS + LP3, DSS-treated plus *L. plantarum* CBT LP3.

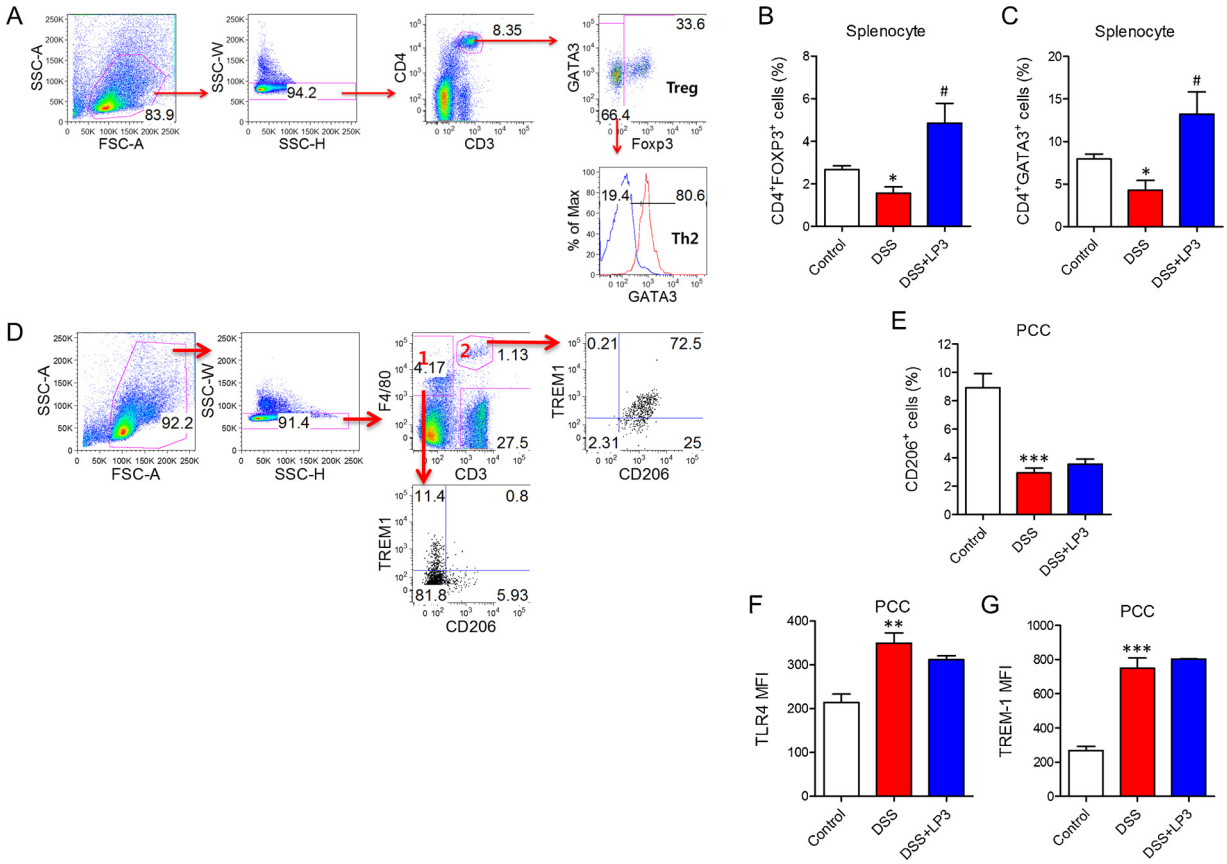

Fig. 4. Effect of *L. plantarum* CBT LP3 on macrophage and T helper cell subset polarization. (A–C) Flow-cytometric analysis of the Treg ($CD4^+FOXP3^+$) and Th2 ($GATA3^+$) populations in the spleens of mice. (A) Representative flow cytometry gating strategies to identify Treg and Th2 populations. (B) Treg cell populations in splenocytes. (C) Th2 cell populations in splenocytes. (D–G) Flow-cytometric analysis of M1/M2 macrophage populations in peritoneal cavity cells (PCCs) from mice. (D) Representative flow cytometry gating strategy to identify M1/M2 macrophage populations. (E) $CD206^+$ populations (M2 marker) in PCCs. (F) Mean fluorescence intensity of TLR4 and TREM-1 (M1 markers) in PCCs.

Data represent mean ± SEM (n = 6 / group). $^{*}P < 0.05$ vs. Control, $^{**}P < 0.01$ vs. Control, $^{***}P < 0.005$ vs. Control, $^{\#}P < 0.05$ vs. DSS + Veh. Control, supplied with normal drinking water; DSS + Veh, DSS-treated plus PBS; DSS + LP3, DSS-treated plus *L. plantarum* CBT LP3.

(classically activated macrophages) have a proinflammatory property and produce high levels of TNF-α, IL-1β, and iNOS, whereas M2 macrophages (alternatively activated macrophages) produce low levels of proinflammatory cytokines and high levels of IL-10 or TGF-β, resolve inflammation, and facilitate tissue repair (Benoit et al., 2008). Therefore, to identify the systemic effects of LP3 on T cell and macrophage subset responses, we next examined those immune cells from the peritoneum and spleen of mice using flow cytometry. As shown in Fig. 4, the LP3-treated group showed drastically increased Treg ($CD4^+FOXP3^+$) and Th2 cell ($CD4^+GATA3^+$) populations in the isolated splenocytes compared with those from the vehicle-treated group, while the DSS-treated group showed significantly reduced Treg and Th2 cell populations. DSS treatment significantly decreased the number of M2 ($CD206^+$) but increased the number of M1 (TLR4, TREM-1) macrophages in PCCs. However, the number of M2 ($CD206^+$) was only slightly increased, and the number of M1 (TLR4, TREM-1) macrophages in LP3-treated mice did not exhibit any significant changes compared with those from vehicle-treated mice. This finding indicates that LP3 modulates T cells but not macrophages, resulting in protective effects on colitis, which could explain the decreased *Tnfa* and *Il17* expressions in the colon.

4. Discussion

Microbial dysbiosis in IBD has been shown to be associated with increased number of anaerobic bacteria of the genus *Bacteroides* but decreased number of the genus *Lactobacillus*, as well as *Bifidobacterium* and *Firmicutes*, suggesting that an imbalance in intestinal microflora contributes to IBD pathogenesis (Trier, 2002). *Lactobacilli*, predominantly present in the intestine, display a wide range of diversity at their genetic and physiological levels (Seddik et al., 2017). It has been reported that *Lactobacilli* modulate inflammatory cytokine profiles in a species-dependent manner (Christensen et al., 2002). For example, soluble proteins produced by *Lactobacillus rhamnosus* GG can regulate intestinal epithelial cell proliferation and prevent cytokine-induced apoptosis (Yan et al., 2007), although a *Lactobacillus rhamnosus* GG clinical trial failed to improve clinical conditions for Crohn's disease (CD) (Bousvaros et al., 2005). *Lactobacillus casei* can induce IL-10 from macrophages (Shida et al., 2006), and *L. paracasei* induces an increase in the population of Treg cells (Seddik et al., 2017) by interaction with the mucosal immune system. Considering these results, not all probiotics are equally beneficial due to different mechanisms of action among them. Therefore, it is necessary to investigate the mechanisms acting on different targets in the inflammatory response according to the specific probiotics.

IBD manifests as mucosal and systemic inflammation occurring primarily in the intestine, in which goblet cell hypoplasia becomes prominent. Goblet cells maintain mucous layers and play a critical role in intestinal barrier function and tissue remodeling (Gordon, 2003; Van der Sluis et al., 2006). Our knowledge of the beneficial roles of *L. plantarum* in gastrointestinal disorders has grown to include the suppression of inflammation and microbial dysbiosis and maintenance of the intestinal barrier (Seddik et al., 2017). In this study, we provided new evidence that *L. plantarum* CBT LP3, a *L. plantarum* strain, has an anti-colitic effect. The LP3-treated group improved body weight loss and shortening of colon length, and DAI as well as histopathology. Intriguingly, we also found that *L. plantarum* CBT LP3 effectively restored goblet cells in the colon.

Increased Th1 and Th17 cells, as well as increased levels of IL-17, are well-known findings in IBD pathogenesis (Park et al., 2005). Moreover, defects in Treg cell function and the imbalance of Th cells resulting in cytokine dysregulation are involved in IBD development (Fonseca-Camarillo and Yamamoto-Furusho, 2015; Mayne and Williams, 2013). Probiotics can modulate T cell maturation through local and systemic interactions with the mucosal immune system (Ganji-Arjenaki and Rafieian-Kopaei, 2018; Spasova and Surh, 2014). In our experiment, the DSS-induced colitic mice showed increased expression of *Tnfa* (Th1 cytokine) and *Il17*, whereas LP3-treated mice displayed decreased *Il17* and *Tnfa* expression in the colon. This elucidates that *L. plantarum* CBT LP3 inhibits Th17 and Th1 responses in intestinal inflammation. Furthermore, administration of *L. plantarum* CBT LP3 increased Treg and Th2 cell populations in the mouse spleen. Treg cells, characterized by expression of forkhead transcription factor Foxp3, are lymphocytes that play a critical role in the pathogenesis of IBD (Fonseca-Camarillo and Yamamoto-Furusho, 2015). Treg cells suppress effector T cells and secrete high levels of IL-10 and TGF-—β, the major anti-inflammatory cytokines in IBD that suppress the production of proinflammatory cytokines (Mayne and Williams, 2013; von der Weid et al., 2001). In addition, Th2 cells, as well as Treg cells, can change the balance of Th1 and Th17 cells. Furthermore, Th2 and Treg cells can contribute to goblet cell hyperplasia both *in vitro* and *in vivo* (Liew et al., 2010; Maizels et al., 2009). It was also reported that Th2 cell expansion skews M1 to M2 macrophage phenotype and induces goblet cell hyperplasia (Liew et al., 2010; Maizels et al., 2009; Miller et al., 2008; Mohrs et al., 2005; Urban et al., 2007). However, *L. plantarum* CBT LP3 did not affect M1 and M2 macrophages in the peritoneum, suggesting the systemic anti-inflammatory effects and important immunomodulatory roles occur mainly in lymphocytes. Our data support, in particular, the notion that gut microbiota can directly affect T cell maturation (Spasova and Surh, 2014). Given the reduced Treg cells in IBD patients, the probiotics modulating Treg cells, such as *Bifidobacterium*, *Streptococcus*, and *Enterococcus* including *Lactobacillus*, appear to effectively treat IBD (Konieczna et al., 2012; Kwon et al., 2010; Roselli et al., 2009; Zhao et al., 2013). *L. plantarum* CBT LP3 has a qualified presumption of safety status and is generally recognized as safe in Europe and North America, and most studies support its safety (Ganji-Arjenaki and Rafieian-Kopaei, 2018; Seddik et al., 2017). However, further studies are needed to unravel the exact mechanism underlying the interactions between probiotics and the host immune system.

5. Conclusion

In conclusion, for the first time, we demonstrated that *L. plantarum* CBT LP3 has anti-colitic effects through modulation of T helper cell subsets and restoration of goblet cells as well as suppression of inflammatory cytokines. This regulatory effect of *L. plantarum* CBT LP3 on immune modulation holds promise for treatment and prevention of IBD.

Declarations of Competing Interest

None.

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