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**Lactobacillus plantarum** ameliorates colonic epithelial barrier dysfunction by modulating the apical junctional complex and PepT1 in IL-10 knockout mice

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Although the etiology of inflammatory bowel disease (IBD), consisting of ulcerative colitis and Crohn’s disease (CD), is still not fully understood, it is now generally accepted that gut flora provide the continued antigenic stimulation that continuously activates immune cells to cause intestinal injury in genetically susceptible individuals (17, 48). There is persuasive evidence implicating gut flora as a requisite and perhaps a central factor in the development of IBD (51, 52). And there is mounting evidence suggesting that the improvement of the intestinal epithelial barrier function may contribute (38).

It has been speculated that antigens derived from resident gut flora promote inflammation in the presence of an impaired intestinal epithelial barrier (4). Normally, the intestinal epithelium provides an effective barrier against the passage of bacteria and luminal antigens into the underlying tissue compartment through the paracellular and transcellular pathways (35). However, in IBD, both the paracellular and transcellular pathways are impaired, and epithelial barrier dysfunction subsequently occurs. Paracellular permeability is dynamically regulated by an apical intercellular junctional protein complex, referred to as the apical junctional complex (AJC), which consists of tight junctions (TJs) and subjacent adherens junctions (AJs). TJs include transmembrane proteins, such as occludin, claudin family and junction adhesion molecule-A, and scaffolding proteins, such as the zonula occludens protein (ZO). β-Catenin is an important cytoplasmic protein that associate transmembrane proteins with the apical perijunctional F-actin ring in AJs (4, 18). Increased paracellular permeability has been observed in IBD, along with disrupted TJ structure and decreased expression or redistribution of TJ proteins or AJ proteins (1, 27). Several recent studies have shown that probiotics enhance the paracellular permeability by increasing the expression and phosphorylation of TJ proteins, such as ZO-1, claudin-1, or occludin (30, 34, 45). However, the underlying molecular mechanisms by which probiotics decrease the paracellular permeability remain unclear.

Oligopeptide transporter 1 (PepT1) is an important transporter of di-/tripeptides in brush border membranes of enterocytes. Normally, it is mainly expressed in the jejunum and responsible for the absorption of nutrient-related peptides, as well as peptide-derived drugs such as cephalixin, which is recognized as a specific substrate of PepT1 used to evaluate the transport capacity of PepT1 (3). In contrast, PepT1 expression is low and even undetectable in the colon (13). However, in the presence of chronic inflammation, PepT1 expression is aberrantly increased in the colon where bacteria-derived peptides, such as N-formyl-methionyl-leucyl-phenylalanine (fMLP) and muramyl dipeptide, are also increased (31). It has been confirmed that PepT1 transports these bacteria-derived peptides,
resulting in an exaggerated inflammatory response (8). Thus PepT1 plays an important role in the innate immune response to bacteria and intestinal inflammation, by mediating the trans-epithelial transport of bacterial antigens (9). An in vitro study showed that probiotic Lactobacillus casei significantly increased the uptake of glycy1-sarcosine, which is also a specific substrate of PepT1, through the enhancement of PepT1 activity in Caco-2 cells (37). Therefore, we hypothesized that the modulation of PepT1-mediated transepithelial transport also contributes to the enhancement of intestinal epithelial barrier function.

It has been demonstrated that IL-10 knockout (IL-10−/−) mice do not develop colitis when kept in a germ-free environment, but rapidly develop a TH1-predominant intestinal inflammation in the presence of the gut flora (2, 22). Manipulation of the gut flora with probiotics and antibiotics attenuates intestinal inflammation (24, 26). These findings indicate that the inflammatory phenotype of IL-10−/− mice originates from the gut flora. It has also been reported that there is colonic epithelial barrier dysfunction in IL-10−/− mice (27). Over the past decade, IL-10−/− mice have been used as a model of spontaneous colitis for the investigation of the therapeutic efficacy of probiotics and its possible mechanisms (25).

In a previous study, we found that Lactobacillus plantarum (LP) attenuated the upregulation of adhesion molecules such as MadCAM-1 and ICAM-1 was well as colonic inflammation in IL-10−/− mice in which the inflammation was developing, suggesting that LP prevents the development of colitis in IL-10−/− mice (10). However, the therapeutic role of LP in IL-10−/− mice in which colonic inflammation has been established and the underlying mechanisms are still unknown. Therefore, the aims of this study were 1) to confirm whether there is a colonic epithelial barrier dysfunction in IL-10−/− mice in which colonic inflammation has been established; 2) to determine whether LP ameliorates the colonic epithelial barrier dysfunction; and particularly 3) to explore whether the modulation of AJC and PepT1-mediated transepithelial transport contribute to the effect, if LP ameliorates the colonic epithelial barrier dysfunction.

**MATERIALS AND METHODS**

**Probiotic strain.** Lactobacillus plantarum CGMCC 1258 (LP) was obtained from the Institute of Bio-medicine, Shanghai Jiao Tong University, China, and stored in 20% (wt/vol) skim milk at −70°C. LP was routinely cultured in de Man, Rogosa, Sharpe (MRS) plates (Oxoid, Cambridge, UK) at 37°C in anaerobic environment.

**Reagents.** Rabbit polyclonal antibody against PepT1 (H-235) and goat polyclonal antibody against ZO-1 (C-19) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies against occludin and β-catenin and mouse monoclonal antibody against claudin-1 were obtained from Zymed Laboratories (San Francisco, CA). All other antibodies and reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated.

**Animals.** Homozygous IL-10 knockout (IL-10−/−) and wild-type (WT) 129/SvEv mice (Jackson Laboratory, Bar Harbor, ME) were used throughout the study. The mice were housed under specific pathogen-free conditions in Shanghai Jiao Tong University Medical School and consumed a standard sterile diet and filtered water ad libitum under a 12-h light-dark cycle. The genotype of mice was confirmed by PCR procedures performed as previously described (24).

**General experimental protocol.** Eight-week-old female WT and IL-10−/− mice (a total of 80) were equally randomized into four groups, namely WT (i.e., WT mice without LP treatment), WT+LP (i.e., WT mice with LP treatment), IL-10−/− (i.e., IL-10−/− mice without LP treatment), and IL-10−/−+LP (i.e., IL-10−/− mice with LP treatment) groups for all experiments (Fig. 1). Mice in the probiotic groups received a once-daily oral gavage of LP dissolved in 0.5 ml saline at 1 × 10^9 colony-forming unit (cfu), whereas the other two nonprobiotic groups consumed the same volume of saline only for 4 consecutive wk. At the end of the treatment, the animals were euthanized by cervical dislocation.

Fecal samples were collected prior to the treatment (week 0) and at weekly intervals over the treatment period (weeks 1, 2, 3, and 4) for LP analysis. Mesenteric lymph nodes and the spleen were aseptically collected for bacterial translocation analysis. Moreover, colonic tissues were harvested for histological examinations, assays for colonic TNF-α and IFN-γ levels, Western blotting, real-time RT-PCR, immunofluorescence analysis of TJ proteins, ZO-1, occludin, claudin-1, an AJ protein (β-catenin), and PepT1 as described below.

The experimental protocol was approved by the Animal Care and Use Committee and the Ethics Committee of Shanghai Jiao Tong University.

**Histological examinations.** The colon tissues were fixed in 10% neutral phosphate-buffered formalin, routinely processed, sectioned at 6 μm, and stained with hematoxylin and eosin for light microscopic examination. The colons were reviewed in a blind fashion all by the same pathologist. Each segment of the colon (cecum, ascending, transverse, and descending colon) was assigned a histological score ranging from 0 to 4 based on the criteria adapted from Berg et al. (2). The summation of these scores provided a total colon inflammatory score ranging from 0 (no change in any segment) to a maximum of 16 (grade 4 lesions in all four segments) per mouse.

**Microbiological determinations.** Fecal samples were aseptically collected, weighed, dispersed in 2.5 ml Ringer’s buffer supplemented with 0.05% (wt/vol) cysteine hydrochloride, and then serially diluted 10-fold. The dilution (100 μl) was spread onto MRS agar (Oxoid) for LP and then incubated anaerobically for 48 h at 37°C. Anaerobic environments were created by using CO2-generating kits (Anaerocult A, Merck, Darmstadt, Germany) in sealed gas jars (BBL Becton Dickson, Cockeysville, MD). Colonies were picked and identified by morphological appearance, Gram staining, and biochemistry. Bacterial counts are reported as log10 cfu per gram feces (log10 cfu/g).

For bacterial translocation analysis, the aseptically collected mesenteric lymph nodes and spleen were processed as previously described (47). Briefly, 500 μl of the serially dilutions were spread onto blood agar (BBL Becton Dickson) for total anaerobic bacteria and onto the standard tryptic soy agar (BBL Becton Dickson) for total aerobic bacteria. The plates were incubated for 72 h at 37°C under aerobic or anaerobic conditions accordingly. Bacterial counts are reported as log10 cfu per gram tissue.

**Enzyme-linked immunosorbent assay.** The colonic levels of the cytokines TNF-α and IFN-γ were determined by using ELISA kits (BD Pharmingen, Oxford, UK). Briefly, the colon tissues were thawed, cut, and homogenized by using an Ultra-Turrax homogenizer (IKA-WERKE, Germany) in 800 μl of chilled homogenization buffer containing phosphate-buffered saline (PBS), 2% (vol/vol) fetal calf serum, and 0.5% (wt/vol) cetyltrimethylammonium bromide. The homogenate was microcentrifuged at 17,940g for 15 min at 4°C. The protein content of the supernatant was determined by a BCA protein estimation kit (Pierce, Rockford, IL). TNF-α and IFN-γ levels in colonic supernatants were measured according to the manufacturer’s instruction and are expressed as picograms per milligram tissue.

**Transmission electron microscopy.** To observe the ultrastructural changes of tight junctions, colonic segments were prepared for transmission electron microscopy. Briefly, colonic segments were washed and cut into 10 × 5 mm2, fixed in 2% glutaraldehyde, incubated at 4°C for 2 h, and then washed twice with cold PBS. The tissues were...
subsequently fixed in 1% osmium tetroxide for 2 h, washed and dehydrated in a graded ethanol solution, and embedded in epoxy resin. Ultrathin sections (80 nm) were cut with a LKB-V (Philip, Eindhoven, Netherlands). The samples were viewed with a CM-120 (Philip) transmission electron microscope operated at 80 kV and digital images were acquired with a charge-coupled device camera attached to the microscope.

Assessment for the barrier function of tight junctions. The barrier function of tight junctions was determined by measuring transepithelial electrical resistance (TER) and paracellular permeability of non-ionic mannitol, according to the method adapted from Madsen et al. (24). For the evaluation of TER, the freshly isolated and striped colonic mucosae were mounted in a modified Ussing chamber (Easy-Mount Chamber; Physiologic Instruments, San Diego, CA) with an exposed area of 0.3 cm². The tissues were bathed on both sides with 3 ml of oxygenated Krebs buffer, which was maintained at 37°C by a heated water jacket and gassed with 95% O₂-5% CO₂. The tissues were voltage clamped at 0 mV to monitor short-circuit current (Isc) by using a Dual Voltage Clamp amplifier (VCC MC2; Physiologic Instruments) connected via a PowerLab 8SP (AD Instruments, Castle Hill, Australia) to a computer. The current deflection (ΔIsc) was caused by applying a 1-mV pulse for 0.5 s at 60-s intervals under the short-circuit condition. The TER was calculated according to Ohm’s Law (i.e., TER = PD/ΔIsc), where PD is expressed as millivolts (mV), Isc as microamperes per square centimeter (µA/cm²), and TER as ohms times centimeters squared (Ω·cm²).

For the evaluation of paracellular permeability, 3% (wt/vol) mannitol was added to the mucosal side of the isolated colon after a 30-min equilibration period. Then 200 µl of fluid was collected from the serosal side every 30 min for 120 min. The mannitol concentration was determined by high-performance liquid chromatography (HPLC) (Waters, Milford, MA) with a SK-801 shodex (8.0 × 300 mm², 5 µm; Showa Denko, Tokyo, Japan) attached to a refractive index detector (Waters). The mobile phase was HPLC grade water, the flow rate was 1 ml/min, and the column temperature was maintained at 80°C.

Intestinal perfusion technique for PepT1-mediated transepithelial transport. To evaluate the transport capacity of PepT1 in the colon, an uptake experiment of cephalexin was performed by using the intestinal perfusion technique as previously described (5). Briefly, the mice were anesthetized with intraperitoneal ketamine. A laparotomy was performed, an inflow cannula made of Silastic tube (1.65 mm outside diameter, 0.76 mm inside diameter, 10 cm length) was inserted in the colon 2 cm below the cecum, and an outflow cannula was set up at a distance of 1 cm above the rectum. The bile duct was ligated to prevent possible enterohepatic recycling of cephalexin. The colonic segment was then flushed with saline solution (prewarmed to 37°C) to remove residual intestinal contents. Krebs buffer was delivered to colon with a perfusor compact at a flow rate of 2 ml/h (B. Braun Melsungen, Melsungen, Germany) through an inlet tube water jacketed at 37°C before its entry into the intestinal segment. For the peptide transport experiments, 1 mmol/l cephalexin, with or without 50 mmol/l glycine-sarcosine (Gly-Sar), was added in Krebs buffer and delivered to colon for 2 h after a 20-min stabilization period. Gly-Sar, which is also a specific substrate of PepT1, was used as a competitor of cephalexin to determine whether cephalexin is mainly transported by PepT1. Blood samples were collected through the caval vein, and plasma was stored at −70°C until analysis of cephalexin concentration.
Western blotting. The colonic tissues were homogenized, sonicated, and transferred into ice-cold lysis buffer, containing a protease inhibitor cocktail [1 μM/l buffer, 50 mol/l Tris-HCl pH 7.4, 150 mmol/l NaCl, 1% NP-40 (Igepal CA-630), 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, and 5 mol/l EDTA], for 60 min as previously described. The extracted proteins were separated in SDS-polyacrylamide gels (5% stacking gel for all proteins, 8% resolving gel for ZO-1, 10% resolving gel for occludin and β-catenin, and 15% resolving gel for claudin-1) and immunoblotted with the respective primary antibodies against ZO-1, occludin, β-catenin, and claudin-1, respectively. The blots were then incubated with IRDye 800-conjugated goat anti-rabbit IgG or IRDye 700-conjugated goat anti-mouse IgG (LI-COR Biosciences, Lincoln, NE), accordingly. Fluorescence images were captured using the Odyssey Infrared Imaging System (LI-COR) and β-tubulin was used as an internal control.

Real-time RT-PCR. The primer sequences and the expected sizes of PCR products were as follows: PepT1 (sense) 5’-GAG AAA GGG GAG AAC AGA A-3’ and (antisense) 5’-CCG TGC CAA AGT CAA GGT-3’ (214 bp); β-actin (sense) 5’-ACG GCC AGG TCA TCA CTG TTG-3’ and (antisense) 5’-ATG CCA CAG GAT TTC ATA CCC-3’ (91 bp). The total RNA was extracted by using Trizol reagent (Invitrogen, Carlsbad, CA), and reverse transcription was then conducted with 200 U of M-MLV RT RNase H-Deletion Mutant (Promega) at 42°C for 60 min. according to the manufacturer’s recommendations. The mRNA levels of PepT1 were determined by SYBR green real-time PCR by use of the LightCycler Real-time PCR System (Roche Molecular Biochemicals, Mannheim, Germany). RT-PCR was performed in a total volume of 25 μl containing 2X SYBR Premix Ex Taq (TaKaRa), 0.2 μM of each sense and antisense primers with the following program: 95°C for 30 s, followed by 40 cycles consisting of 5 s at 95°C, 5 s at 60°C, 30 s at 72°C for extension. At each cycle, a fluorescence reading was conducted at 72°C. At the end of the program, a melting curve analysis was performed at 72°C for 30 s, followed by a cooling step at 37°C for 30 s. The data were standardized to β-actin for each sample, and the PCR products were electrophoresed on 1% agarose gels and stained by ethidium bromide.

Immunofluorescence. The localization of ZO-1, occludin, claudin-1, β-catenin, and PepT1 proteins was determined by immunofluorescence confocal microscopy. The colon segments were immediately removed, washed with PBS, mounted in the embedding medium (Tissue-Tek, Sakura), and stored at −80°C until use. Frozen sections were cut at 10 μm, and mounted on the slides. The slides were incubated in prewarmed (95°C) 10 mM sodium citrate buffer for 5 min and then cooled to room temperature. The nonspecific background was blocked by incubation with 5% bovine serum albumin plus 5% newborn bovine serum in PBS for 30 min at room temperature. The sections were incubated with goat polyclonal antibody against ZO-1, rabbit polyclonal antibodies against occludin and β-catenin, mouse monoclonal antibody against claudin-1, and rabbit polyclonal antibody against PepT1 at 4°C overnight and afterward with phallolidin-fluorescin isothiocyanate at room temperature for 1 h. The sections were probed with respective Cy3-conjugated secondary IgG antibodies (Jackson ImmunoResearch Laboratories). The nuclei were counterstained with 4’-6-diamidino-2-phenylindole. Next, the slides were washed and mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Slides incubated without any primary antibody were used as negative controls. Confocal analysis was performed with an LSM510 laser scanning confocal microscope (Zeiss, Thornwood, NY).

Statistical analysis. All statistical analyses were performed by use of SPSS 13.0 (SPSS, Chicago, IL). Numerical data with a normal distribution were expressed as means ± SD, and multiple groups were compared by one-way analysis of variance followed by least significant difference post hoc test. Comparisons between two groups were done by Student’s t-tests. Spearman’s rank correlation coefficients were computed for correlation analyses. The χ2 test was used for the comparison of categorical data among the groups. A P value of less than 0.05 was considered statistically significant.

RESULTS

LP treatment reduces colitis and improves clinical manifestation in IL-10−/− mice. Histological analysis showed that colitis developed in all IL-10−/− mice. The inflammatory score was significantly greater in IL-10−/− mice than in WT mice (6.79 ± 1.25 vs. 0.21 ± 0.43; P < 0.001). However, the score in IL-10−/− mice was significantly reduced with LP treatment (from 6.79 ± 1.25 to 3.36 ± 1.01; P < 0.001) (Fig. 2A).

Mucosal ulceration, erosion, and a large number of lymphocyte and neutrophil infiltration in the lamina propria were seen in IL-10−/− mice [28.6% (4/14), 42.9% (6/14) and 100% (14/14), respectively], but in none of the WT mice (Fig. 2, B1–B3). In all IL-10−/− mice treated with LP, only mild inflammatory cell infiltration was seen, without ulceration and erosion (Fig. 2B4).

Diarrhea was more frequently observed in IL-10−/− mice than LP-treated IL-10−/− mice during the 4-wk experiment [73.1% (19/26) vs. 30.8% (8/26), P = 0.002], but not in WT. The body weight was decreased significantly in IL-10−/− mice, compared with WT mice (20.26 ± 1.70 vs. 23.74 ± 1.53 g; P < 0.001). However, treatment with LP led to a weight gain in IL-10−/− mice (22.08 ± 1.78 vs. 20.26 ± 1.70 g; P < 0.001). Rectal prolapse was found in 19.2% (5/26) of IL-10−/− mice, but in none of WT mice and LP-treated IL-10−/− mice throughout the experiment.

LP treatment reduces paracellular permeability and restores paracellular ultrastructure and the expression and distribution of ZO-1, occludin, claudin-1, and β-catenin proteins in IL-10−/− mice. IL-10−/− mice exhibited a significant increase in the cumulative permeation of mannitol through the colonic mucosa, compared with WT mice (2.17 ± 0.37 vs. 0.76 ± 0.14; P < 0.001; Fig. 3A). In accordance with the increase in mannitol permeability, a significant decrease of TER was observed in IL-10−/− mice (38.31 ± 12.70 Ω·cm² vs. 73.34 ± 3.91 Ω·cm²; P < 0.001; Fig. 3B). However, these changes in IL-10−/− mice were completely prevented by the 4-wk treatment with LP (both P > 0.05). Moreover, the paracellular permeability to mannitol significantly correlated with the colonic inflammatory score (r = 0.88; P < 0.001).

Since paracellular permeability is controlled by AJs, especially the TJs, the ultrastructural changes of intercellular TJS were examined by transmission electron microscopy. Compared with WT and WT+LP mice (Fig. 3, C1 and C2), significant ultrastructural changes, such as disrupted TJs and microvilli, a marked increased intercellular gap, vacuolization, chromatin condensation, and even the epithelial apoptosis, were observed in the colonic epithelium of IL-10−/− mice (Fig. 3C). However, LP therapy almost restored these ultrastructural changes (Fig. 3C4).

To identify whether there are changes in the apical junction protein expression and distribution, immunofluorescence assay
whereas the level of ZO-1, occludin, and claudin-1 were significantly reduced in IL-10 decreased expression of ZO-1, occludin, and claudin-1 proteins and mRNA was upregulated in IL-10 WT mice (0.05 prevented by LP treatment (Fig. 4A).

Western blotting showed that the total protein levels of ZO-1, occludin, and claudin-1 were significantly reduced whereas the level of β-catenin was unchanged in IL-10−/− mice, compared with WT mice (P < 0.001, Fig. 4B). The decreased expression of ZO-1, occludin, and claudin-1 proteins in IL-10−/− mice was prevented by the 4-wk LP treatment (Fig. 4B).

LP treatment reduces the expression of PepT1 protein and mRNA and PepT1-mediated transepithelial transport of cephalaxin in IL-10−/− mice. The expression of both PepT1 protein and mRNA was upregulated in IL-10−/− mice, compared with WT mice (0.05 ± 0.01 vs. 0, P < 0.001; 9.33 ± 2.27 vs. 1.42 ± 0.20, P < 0.001, respectively) (Fig. 5, A and B). The expression of PepT1 protein and mRNA in IL-10−/− mice with LP treatment was significantly reduced, compared with IL-10−/− mice without LP treatment (0.02 ± 0.01 vs. 0.06 ± 0.01, P = 0.009 and 3.40 ± 1.28 vs. 9.33 ± 2.27, P = 0.001, respectively) (Fig. 5, A and B).

Immunofluorescence analysis showed a broad distribution and redistribution of PepT1 in the colon of IL-10−/− mice, but not in the colon of WT mice or LP-treated IL-10−/− mice (Fig. 5C).

The plasma cephalaxin concentration was significantly higher in IL-10−/− mice than in WT mice (0.39 ± 0.07 vs. 0.15 ± 0.03 mg/l; P < 0.001). However, in IL-10−/− mice with 4-wk LP treatment, the plasma cephalaxin concentration was significantly lower than that in IL-10−/− mice without LP treatment (0.23 ± 0.05 vs. 0.39 ± 0.07 mg/l; P < 0.001) (Fig. 5D).

The plasma cephalaxin concentrations were significantly lower in WT and IL-10−/− mice with Gly-Sar treatment than in those without Gly-Sar treatment (0.11 ± 0.03 vs. 0.15 ± 0.03 mg/l; P = 0.046, and 0.13 ± 0.04 vs. 0.39 ± 0.04 mg/l, P < 0.001, respectively; Fig. 5E). In addition, the plasma cephalaxin concentration was correlated with the inflammatory score in all type of mice (r = 0.84; P < 0.001).
LP treatment reduces bacterial translocation and expression of proinflammatory cytokines in IL-10\(^{-/-}\) mice. The bacterial count in the mesenteric lymph nodes and spleens was significantly increased in IL-10\(^{-/-}\) mice (4.60 ± 0.67 log\(_{10}\) cfu/g vs. 0 log\(_{10}\) cfu/g and 5.72 ± 0.89 log\(_{10}\) cfu/g, respectively, both \(P < 0.001\)). However, the bacterial count was decreased in IL-10\(^{-/-}\) mice with the 4-wk LP treatment (2.74 ± 1.37 log\(_{10}\) cfu/g vs. 4.60 ± 0.67 log\(_{10}\) cfu/g, \(P = 0.014\) and 3.24 ± 1.81 log\(_{10}\) cfu/g vs. 5.72 ± 0.89 log\(_{10}\) cfu/g, \(P = 0.013\), respectively), although it was still greater than that in WT mice (2.74 ± 1.37 log\(_{10}\) cfu/g vs. 0 log\(_{10}\) cfu/g and 3.24 ± 1.81 log\(_{10}\) cfu/g vs. 0 log\(_{10}\) cfu/g, respectively, both \(P < 0.05\)).

The expression of TNF-\(\alpha\) and IFN-\(\gamma\) in the colonic mucosa was also significantly increased in IL-10\(^{-/-}\) mice, when compared with the levels in WT mice (375.10 ± 78.87 pg/mg vs. 142.52 ± 38.72 pg/mg, \(P < 0.001\), and 603.27 ± 109.78 pg/mg vs. 248.29 ± 56.07 pg/mg, \(P < 0.001\), respectively) (Fig. 6, A and B). However, the expression levels of TNF-\(\alpha\) (205.04 ± 68.85 pg/mg) and IFN-\(\gamma\) (446.37 ± 116.29 pg/mg) in IL-10\(^{-/-}\) mice with the 4-wk LP treatment were significantly reduced, compared with the levels in IL-10\(^{-/-}\) mice (both \(P < 0.001\)), although they were still greater than those in WT mice (\(P = 0.041\) and \(P < 0.001\), respectively). Although there was also a decrease in the expression of TNF-\(\alpha\) (94.17 ± 33.13 pg/mg) and IFN-\(\gamma\) (174.11 ± 45.75 pg/mg) in LP-treated WT mice, the decrease did not reach the statistically significant level (\(P = 0.108\) and \(P = 0.102\), respectively) (Fig. 6, A and B).

DISCUSSION

The present study clearly demonstrated that spontaneous colitis was established in IL-10\(^{-/-}\) mice, with increased paracellular permeability and bacterial translocation. Moreover, PepT1 protein was aberrantly expressed in the colon of IL-10\(^{-/-}\) mice. These findings all indicate colonic epithelial barrier dysfunction in IL-10\(^{-/-}\) mice. On the other hand, treatment with LP effectively attenuated established colitis in IL-10\(^{-/-}\) mice, in conjunction with the improvement in clinical manifestation, reduction of intestinal paracellular permeability, restoration of the paracellular ultrastructure, and expression and distribution of intercellular apical junction complex proteins, including ZO-1, occludin, claudin-1, and \(\beta\)-catenin, and decrease of PepT1 protein. These observations clearly show that LP ameliorates colonic epithelial barrier dysfunction in IL-10\(^{-/-}\) mice. It should be emphasized that LP was detected only in feces of LP fed mice in the present study, suggesting that LP is not a natural component of the gut flora in all mice as Schultz et al. (49) reported. Therefore, the effects we observed in LP-fed IL-10\(^{-/-}\) mice can be fully attributed to the therapeutic administration of LP.

Several elements have been implicated in the pathogenesis of IBD, including genetic, environmental, and immune factors (16). Among these, the breakdown of epithelial barrier function has been reported as a key factor implicated in the development of IBD (57). It has been demonstrated that increased intestinal....
permeability occurs not only in CD, but also in 10–20% of their first degree healthy relatives, a subgroup with an increased risk of developing the disease (36, 40). The increased permeability is due to the reduced expression and redistribution of intercellular junctional proteins (58, 41). Bacterial translocation is also frequently reported in CD (14, 28), suggesting that the increased permeability to luminal antigens and disrupted intercellular junctions are functionally significant.

There is a growing body of evidence that the improvement of intestinal epithelial barrier function may be a key effect of probiotics in the therapy of IBD, although the mechanism of action of probiotics is still not completely clarified (55).

Several in vitro studies have demonstrated that pretreatment of intestinal epithelial cells with probiotics prevents the increase of epithelial paracellular permeability and the decrease in TER induced by pathogenic Escherichia coli or cytokines, resulting in the repair of the intestinal epithelial barrier (20, 46). This effect correlates with the dynamical regulation of AJC, particularly TJs consisting of TJ proteins. Studies have also shown that probiotic bacteria prevent the decline in phosphorylation and the redistribution of TJ proteins caused by...
pathogenic infection in intestinal epithelial cells (20, 45). Furthermore, our previous study showed that LP prevented the abnormal expression and distribution of TJ proteins, including claudin-1, occludin, JAMA-1, and ZO-1 proteins induced by enteroinvasive Escherichia coli in Caco-2 cells (42). As in human IBD, downregulation and redistribution of several TJ proteins have also been identified as the molecular basis of impaired intestinal epithelial barrier function in animal models of IBD (7, 43, 54). Moreover, studies in vivo have demonstrated that probiotic therapy results in changes in the expression of TJ proteins (30, 54). A recent study showed that treatment with VSL#3 (a probiotic compound) completely prevented the decreased expression of ZO-1, occludin, claudin-1, and β-catenin in the colon of IL-10-/- mice. We found that treatment of IL-10-/- mice with LP prevented the decreased expression of ZO-1, occludin, and claudin-1, and the redistribution of ZO-1, occludin, and β-catenin, resulting in a significantly increased TER and decreased paracellular permeability. In addition, the positive correlation between paracellular permeability and inflammatory score further suggests that alterations in the intercellular junction may play an important role in probiotic therapy for intestinal inflammation.

However, although dramatically improving paracellular permeability, probiotics do not completely prevent intestinal inflammation, as demonstrated by a recent study in the IL-10-/- mice (15). Indeed, in the present study, whereas paracellular mannitol fluxes were completely normalized to the level of the

Fig. 5. Effects of probiotic Lactobacillus plantarum on protein and mRNA expression of PepT1 and its location and transport capacity in the colon. A: Western blot analysis for PepT1 protein expression in the colons of WT, WT+LP, IL-10-/-, and IL-10-/-+LP mice (left). Western blots of PepT1 protein with tubulin as an internal control; right, the expression ratio of PepT1 protein over tubulin). Images are representatives for 3 mice in each group, and the values represent means ± SD of 3 mice. B: RT-PCR analysis for PepT1 mRNA expression in the colons of WT, WT+LP, IL-10-/-, and IL-10-/-+LP mice (left, PCR products of PepT1 with β-actin as an internal control; right, the expression ratio of PepT1 over β-actin). Images are representatives for 3 mice in each group, and the values represent means ± SD of 3 mice. C: representatives of immunofluorescence analysis for PepT1 location in the colons of WT, WT+LP, IL-10-/-, and IL-10-/-+LP mice. PepT1 proteins are stained red, nuclei are counterstained blue, and the F-actin is stained green. Images are representatives for 3 mice in each group. D: plasma cephalaxin concentrations in WT, WT+LP, IL-10-/-, and IL-10-/-+LP mice (n = 8). E: competitive inhibition of PepT1 in the colon of WT and IL-10-/- mice (n = 6). Gly-Sar was used as competitive substrate of PepT1. +P < 0.05 compared with WT; *P < 0.05 compared with IL-10-/- mice.
that PepT1-mediated fMLP transport induced intestinal inflammation in the rat small intestine (6). Moreover, it has been shown that muramyl dipeptide, a specific activating ligand for the production of NOD2/CARD15 gene, can also be taken up by PepT1 in colonic epithelial cells, where it activates the NF-κB pathway with the production of downstream proinflammatory cytokines and monocyte chemoattractant protein-1 (56). These findings indicate that PepT1-mediated transepithelial transport of bacterial antigens is involved in the pathogenesis of intestinal inflammation.

In the present study, PepT1 expression was significantly increased both at protein and mRNA levels in the colon of IL-10−/− mice, and LP treatment prevented the increase in PepT1 expression. Moreover, the present study showed that the plasma concentrations of cephalixin, which is widely used for the assessment of PepT1-mediated transepithelial transport pathway, were significantly increased in IL-10−/− mice, but the increase was partially prevented with the LP treatment. Moreover, there was a positive correlation between the plasma cephalixin concentrations and inflammatory scores. Therefore, the present study, for the first time, clearly demonstrates the correlation between the PepT1-mediated transepithelial transport pathway and the pathogenesis of intestinal inflammation.

In the present study, bacterial translocation and proinflammatory cytokine production were both increased in IL-10−/− mice, whereas LP treatment prevented the increase in the bacterial translocation and proinflammatory cytokine production. It is noted that 4-wk LP treatment did not affect the expression of proinflammatory cytokines (e.g., TNF-α and IFN-γ) in our previous study in which IL-10−/− mice were treated at 4-wk old when the colonic inflammation is just developing (10). However, in the present study, IL-10−/− mice were treated at 8 wk old when the intestinal inflammation has already been established, as confirmed by Madsen et al. (24, 26). In addition, the dose of LP used in the present study was twice that in the previous study. Thus the different stage of colitis and different dose of LP may contribute to the different effects of LP on the expression of the proinflammatory cytokines; however, further investigation is needed to clarify this issue.

Taking all data together, we hypothesize that two major pathways, the paracellular pathway (mainly the AJC) and the PepT1-mediated transepithelial pathway, are both impaired in the colon of IL-10−/− mice, resulting in the diffusion of bacteria or bacteria-derived antigens across the intestinal epithelium, and thus both pathways contribute to the epithelial barrier dysfunction and subsequent spontaneous colitis. This epithelial barrier dysfunction may further increase the paracellular permeability to luminal bacteria and antigens, which in turn leads to an exaggerated immune response. This hypothesis is further supported by the observations in the present study that bacterial translocation and proinflammatory cytokine production were both increased in IL-10−/− mice, whereas LP treatment prevented the increase in the bacterial translocation and proinflammatory cytokine production. Obviously, the intestinal epithelial barrier function was enhanced by both the restoration of TJ protein expression and the decrease in PepT1-mediated transport function that attenuated the continued antigenic stimulation to the intestinal immune cells, and thus decreased the proinflammatory cytokine production (e.g., TNF-α and IFN-γ). On the basis of the above mentioned points...
of view and our observations, we postulate that the effects of LP on PepT1, TJ proteins, TNF-α, etc. are a primary driver of the probiotics effects although more extensive mechanistic investigation is required to confirm our speculation. Although, etc. are a primary driver of our view and our observations, we postulate that the effects of probiotics ameliorate gut barrier dysfunction in colitis.

In conclusion, treatment with probiotic LP ameliorates colonic epithelial barrier dysfunction in IL-10−/− mice, by modulating the AJC and PepT1-mediated transepithelial transport pathway. These findings may help better understand the pathogenesis of IBD and develop new therapies for the disease.

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DISCLOSURES

The authors declare that they have no potential conflict of interest.

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