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Lactiplantibacillus plantarum 22A-3-induced TGF-β1 secretion from intestinal epithelial cells stimulated CD103$^+$ DC and Foxp3$^+$ Treg differentiation and amelioration of colitis in mice

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In the present study, we evaluated the anti-inflammatory properties of *Lactiplantibacillus plantarum* 22A-3 (LP22A3) and attempted to elucidate the underlying molecular mechanism. The oral administration of LP22A3 significantly inhibited body weight reduction and decreased colon shortening and colitis score in mice with dextran sulfate sodium (DSS)-induced colitis. It was demonstrated that the production of the active-form of TGF-β tended to increase in both the intestinal epithelial cells (IECs) of the ileum and serum but not in the colon of non-DSS-treated mice by LP22A3. IL-10 level in serum was also elevated by LP22A3-treatment. The mRNA expression of TGF-β, IL-10 and Foxp3 increased only in the small intestines of LP22A3-treated mice. Both the aldehyde dehydrogenase 1 family member A2 (Aldh1a2) mRNA expression and population of CD103$^+$ dendritic cells (DCs) in the small intestine significantly increased in the LP22A3-treated group. LP22A3 induced TGF-β secretion from the IECs of the small intestine with retinoic acid production probably through TLR2, resulting in an increase in CD103$^+$ DCs and the Foxp3$^+$ Treg population. Both cells secrete a high level of anti-inflammatory cytokines, TGF-β and IL-10 contributing to the protective condition in the intestine and thus making it less susceptible to inflammation. This suggested that oral administration of LP22A-3 may be an alternative therapeutic strategy for IBD.

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1. Introduction

Inflammatory bowel disease (IBD) includes two major disorders, ulcerative colitis (UC) and Crohn's disease (CD), and is characterized by chronic relapsing inflammation of the gastrointestinal tract and epithelial injury. UC and CD share several conditions, but they are essentially pathologically distinct disorders. In UC, inflammation is confined to the mucosa and submucosa of the colon with clear demarcations. On the other hand, CD may involve the entire gastrointestinal tract, but is most common in the terminal ileum and inflammation can extend through the intestinal wall from mucosa to serosa.[1,2] Both UC and CD display severe inflammation of the intestine, which is caused by aberrant and excessive cytokine responses by lymphocytes and antigen-presentation cells. In the colonic tissue of patients with UC and CD, activated macrophages, dendritic cells and differentiated T-cells secrete elevated levels of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-6, IL-12, and IL-23.[2,3] These cytokines lead to the destruction of intestinal mucosa and the progression of IBD which is associated with symptoms such as intestinal stenosis, rectal bleeding, abscesses and fistula formation.[2,3] In recent years, the molecular pathogenesis of IBD has advanced substantially, revealing IBD as a complex multifactorial disorder. The development of IBD and the host's susceptibility to intestinal inflammation are affected by several factors, including genetic predisposition, the host's immune system and the intestinal microbiota.[2,4] Among these, strong evidence suggests that interaction of the gut microbiota with the mucosal immune system plays a central role in the initiation and maintenance of intestinal inflammation in IBD.[4–6]

The intestinal bacterial flora contributes significantly to the pathogenesis of IBD along with mucosal immune dysregulation and genetic factors.[4] Dysbiosis in the intestinal microflora is associated with the development of IBD.[5–7] Extensive research has focused on modifying the intestinal microbiota with probiotic bacteria to reduce excessive inflammatory activity and prevent relapses in UC, CD, and pouchitis.[5] Although both *Lactobacillus* and *Bifidobacterium* species are frequently used, it is widely known that within each species,

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specific strains display distinct activities.[8] Thus the use of probiotics requires a greater understanding of their specific effects on the immune system.

It has been reported that pickle-derived *Lactiplantibacillus plantarum* 22A-3 (LP22A3) possesses an anti-inflammatory property in the passive cutaneous anaphylaxis (PCA) reaction.[9] In this present study, the anti-inflammatory effects of LP22A3 were evaluated and an attempt was made to elucidate the underlying molecular mechanism by which LP22A3 exert its anti-inflammatory effects. An *in vitro* gut inflammatory model was used to explore the anti-inflammatory properties of LP22A3. The recognition of LP22A3 by epithelial cells was also discovered using this model. The effect of LP22A3 *via* oral administration on intestinal inflammation was investigated using a dextran sulfate sodium (DSS)-induced colitis mouse model. In addition, the effect of LP22A3 on lamina propria suppressive lymphocyte subsets, including $Foxp3^+$ regulatory T-cells and $CD103^+$ dendritic cells, was investigated by orally administering LP22A3 for one week followed by flow cytometry assessment.

2. Materials and methods

2.1. Reagents

Dulbecco's Modified Eagle's Medium (DMEM), streptomycin, recombinant murine TNF-α and lipopolysaccharide (LPS) from *Escherichia coli* O127 were purchased from Wako Pure Chemical Industries (Osaka, Japan). MEM (Eagle's Minimum Essential Medium) was purchased from Nissui Pharmaceutical Co. Ltd, (Tokyo, Japan). RPMI 1640 medium and MEM non-essential amino acids (NEAA) were purchased from Gibco BRL (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from Biological Industries (Beit, Israel). Penicillin and DSS (M.W. 36 000–50 000) was purchased from MP Biomedicals (Aurora, OH, USA).

2.2. Mice

Female C57BL/6 mice (6 weeks old) were purchased from Japan SLC, Inc. (Shizuoka, Japan). The mice were maintained in filter-top cages under specific pathogen-free conditions in Kobe University Life-Science Laboratory with free access to laboratory chow and water *ad libitum*. All animal experiments were approved and carried out in accordance with the Animal Experiment Ethnics Committee of Kobe University (Permission number: 22-05-06).

2.3. Preparation of UV-inactivated bacteria

LP22A3 was cultured in Mann Rogosa Sharp broth and incubated overnight at 30 °C in an anaerobic chamber. After incubation, bacterial cells were collected by centrifugation (4 °C, 10 000*g*, 5 min) and were resuspended in PBS and washed three times in order to remove the culture medium. Since viable LP22A3 could cause contamination in the culture system, LP22A3 were killed by UV irradiation. The bacterial suspension was irradiated with a UV germicidal lamp to inactivate it before use in *in vitro* experiments. After UV-treatment, viable counts below 10^2 cfu mL^{-1} indicated more than 9-log reduction in viability. Samples were stored at −80 °C until use.

2.4. Cell culture and co-culture system

The human intestinal epithelial cell line, Caco-2 cells were cultured in DMEM with glutamine and 4.5 g of glucose per liter, supplemented with 1% MEM-NEAA, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, and 10% decomplemented FBS (56 °C, 30 min). Murine macrophage cell line, RAW264.7 cells were cultured in DMEM with glutamine and 1.0 g of glucose per liter, supplemented with 10% FBS, 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin. Murine fibrosarcoma cell line, L929 cells were cultured in MEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. Cell cultures were incubated in a humidified 5% CO_2 incubator at 37 °C. A co-culture experiment composed of Caco-2/RAW264.7 cells was performed as described previously.[10] LP22A3 (1×10^8 cfu mL^{-1}) was applied to Caco-2 cells (apical) for 3 h, followed by LPS-stimulation (7 ng ml^{-1}) on RAW265.7 cells (basolateral). After an additional 3 h of incubation, culture supernatants from the basolateral side were collected for TNF-α measurement. The cultured cells were harvested for total RNA isolation and RT-PCR subsequently applied. To assess the effect of TLR2 and TGF-β1 neutralization on LP22A3 activity, mouse anti-TGF-β1 Ab (R&D system, Minneapolis, MN) and mouse anti-TLR2 Ab (Hycult Biotech, Plymouth Meeting, PA) were used. Anti-TGF-β1 Ab and anti-TLR2 Ab were used to treat basolateral and apical sides, respectively, prior to LP22A3 treatment.

2.5. Intestinal ligated loop assay

C57BL/6 mice (7 weeks old, female) were put under anesthesia by injecting them intraperitoneally with 150 μL of Avertin solution. An intestinal loop assay was then performed by ligating three independent 2 cm ileum loops avoiding Peyer's patch, and 0.2 mL of isotype control or anti-TGF-β1 Ab was injected into each loop. After 30 min of incubation, vehicle or LP22A3 sample (1×10^8 cfu) was injected into each loop that had been pretreated with antibodies. After an additional 2 h of incubation, the mice were sacrificed by cervical dislocation and the intestinal loops were harvested. The intestinal loops were cut open longitudinally, and washed using 1× Hank's balanced salt solution (HBSS). The intestinal loops were then treated with 30 mM EDTA/HBSS for 12 min and the epithelial cells were isolated. Total RNA was extracted and measured for TGF-β1 mRNA expression.

2.6. Induction of acute DSS colitis

C57BL/6 mice (7 weeks old, female) were divided into four groups (n = 8): normal and DSS-induced colitis groups each with or without *L. plantarum* 22A-3 treatment. To induce colitis, the mice were challenged with 2% DSS supplied in the drinking water. DSS-treatment was terminated after 7 days and normal drinking water was supplied for the subsequent 3 days. *L. plantarum* 22A-3 (1×10^8 cfu per mouse per day) and

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vehicle treatment were administrated orally using a ball tip gavage needle 7 days prior to DSS-treatment and continuing until sacrifice. All mice were weighed daily and were euthanized on day 10. Their colons were excised, and their lengths were documented. Histological assessment was performed in a blind manner. The degree of inflammation and epithelial damage on microscopic hematoxylin and eosin (HE) staining sections (8 μm) of the distal colon were graded according to the scoring system of Hudert *et al.*[11]

2.7. RNA isolation and quantitative RT-PCR

Total RNA was extracted from colon tissue using a combination of an RNAqueous kit and Plant RNA Isolation Aid (Ambion, Austin, TX). Total RNA from primary cells or culture cells was isolated by Sepasol RNA I super (Nacalai Tesque, Kyoto, Japan). cDNA synthesis was performed with a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster city, CA) according to the manufacturer's protocol. Quantitative PCR assays were analyzed using Applied Biosystem 7500 Fast Real-Time PCR System with TaqMan gene expression assay. The TaqMan gene expression assays were purchased from Applied Biosystems; their IDs are as follows: β-actin (Mm00607939_s1), GAPDH (Hs99999905_m1), IL-8 (Hs00174103_m1), human TGF-β1 (Hs00998133_m1), mouse TGF-β1 (Mm01178820_m1), IL-10 (Mm00439614_m1), Foxp3 (Mm00475162_m1), Aldh1a2 (Mm00501306_m1).

2.8. Preparation and culture of IECs

After 1 week of vehicle or LP22A3 oral administration to 7-week old C57BL/6 mice, blood was collected by cardiac puncture under anesthesia. The ileum was then isolated, opened longitudinally and rinsed with PBS. The intestinal tissue was treated with 1 mM EDTA for 30 min at 37 °C on a shaker, followed by vortexing for 1 min. The liberated IECs were collected by centrifugation (300*g*, 5 min) and washed thrice with RPMI1640. IECs were then resuspended in RPMI1640 containing 10% FBS and cultured in 24-well plates at 1×10^5 cells for 24 hours. Culture supernatants were collected and measured for active-form TGF-β1 with an ELISA kit (Promega, Madison, WI).

2.9. Cytokine quantification in blood serum

The whole blood sample was left undisturbed for 30 min at room temperature, followed by centrifugation at 4 °C, 1000*g*, for 10 min. The supernatant was collected as blood serum and was measured for TGF-β1 and IL-10 levels using an ELISA kit (eBioscience, San Diego, CA). TNF-α contents were quantified with a cytotoxicity assay with L929 cells (actinomycin D-treated murine fibroblast cell line) using recombinant murine TNF-α as the standard, as described by Takada *et al.*[12]

2.10. Isolation of lamina propria lymphocytes and flow cytometry

Isolation of lamina propria (LP) lymphocytes was performed according to adapted protocols of Weigmann *et al.*[13] and Geem *et al.*[14] Briefly, the ileum and colon were harvested and opened longitudinally, washed to remove fecal content, and shaken in HBSS containing 5 mM EDTA and 5% FBS for 20 min at 37 °C twice. The epithelial cell suspension was discarded and the remaining intestinal sections were cut into small pieces and incubated in HBSS containing 5% FBS, 0.5 mg mL^{-1} collagenase VIII (Sigma-Aldrich, St Louis, MO), and 40 μg mL^{-1} DNase I (Roche Diagnostics, Basel, Switzerland) for 20 min at 37 °C followed by vortexing. The digested tissues were washed twice with HBSS. The collected cells were then resuspended in staining buffer containing PBS, 2% FBS and 0.1% NaN_3 and stained for surface CD4-PE-Cy7 (clone: RM4–5, Tonbo Bioscience, San Diego, CA) and CD25-FITC (7D4, BD Bioscience, San Jose, CA). Intracellular staining of Foxp3-PE (FJK-16s, eBioscience) was performed using a Foxp3 Staining Buffer Set (eBioscience) according to the manufacturer's protocol. Separately, LP lymphocytes were stained for surface CD11c-PE (N418, Tonbo Bioscience) and CD103-PE-Cy7 (2E7, Biolegend) for $CD103^+$ DCs analysis. Samples were analyzed by flow cytometry using BD FACSVerse and BD FACSSuite software (BD Bioscience).

2.11. Statistical analysis

The results were represented as the mean ± SE. Statistical significances between any two groups were analyzed by a Student's *t*-test. One-way ANOVA and Tukey–Kramer tests were used to determine the significances between more than two groups. A *P*-value of <0.05 was considered significant.

3. Results

3.1. LP22A3 acted indirectly through Caco-2 monolayer to suppress LPS-stimulated RAW264.7 cells

To evaluate the anti-inflammatory property of LP22A3, we used the previously described intestinal inflammatory co-culture model consisting of a Caco-2 monolayer and RAW264.7 cells on the apical and basolateral sides, respectively.[10] Treatment by UV-inactivated LP22A3 on the Caco-2 cell monolayer inhibited TNF-α production from LPS-stimulated RAW264.7 cells (Fig. 1A), and downregulated IL-8 mRNA expression in Caco-2 cells (Fig. 1B). The suppressive effect of LP22A3 was comparable to budesonide (NF-κB inhibitor) treatment, an IBD therapeutic drug which was used as a positive control. Adjacent intestinal epithelial cells are known to be held together by tight junction complexes, which prevents transepithelial diffusion of microorganisms and other antigens across the epithelium. This led us to speculate that rather than act directly on RAW264.7 cells, LP22A3 must act through the Caco-2 monolayer inducing substances such as TGF-β1, thymic stromal lymphopoietin (TSLP) and retinoic acid. In particular, TGF-β is a crucial pleiotropic cytokine known to have a suppressive effect on tissue macrophages and contributing to the abrogation of the inflammatory response.[15,16] Therefore, we next determined whether LP22A3 was able to induce TGF-β1 secretion from Caco-2 cells. Indeed, treating the Caco-2 monolayer with LP22A3 significantly promoted the secretion of active-form

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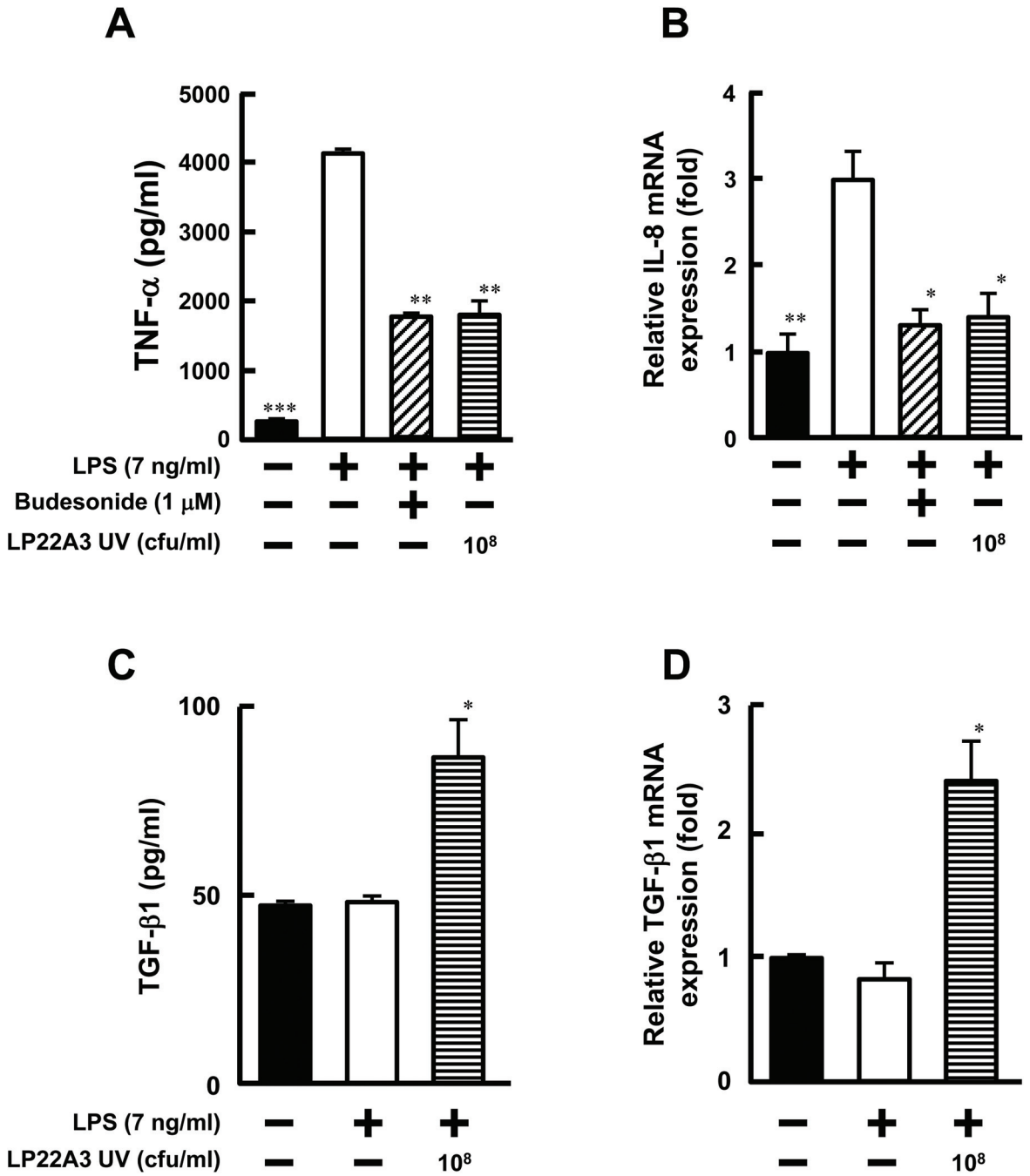

Fig. 1 LP22A3 attenuated TNF-α and TGF-β production, and IL-8 and TGF-β mRNA expression in the Caco-2/RAW264.7 co-culture model. LP22A3 (1×10^8 cfu mL^{-1}) was added into the apical compartment in a Caco-2/RAW264.7 co-culture model for 3 h. Subsequently, LPS was added to the basolateral compartment at a final concentration of 7 ng mL^{-1} and incubated for an additional 3 h. (A) TNF-α production in the basolateral side was determined by a L929 cytotoxicity assay. (B) IL-8 mRNA expression in Caco-2 cells was detected by RT-PCR. (C) TGF-β production from Caco-2 cells was measured by ELISA. (D) TGF-β mRNA expression in Caco-2 cells was detected by Realtime-PCR, where the values represent the means ± SE (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 (*vs.* LPS).

TGF-β1 on the basolateral side (Fig. 1C) and TGF-β1 mRNA expression (Fig. 1D). On the other hand, direct pretreatment of RAW264.7 cells with LP22A3 prior to LPS stimulation did not inhibit TNF-α production (data not shown), which further emphasized that LP22A3 did not act directly on RAW267.4 cells but indirectly *via* the Caco-2 monolayer.

3.2. LP22A3 inhibitory properties depended on its ability to induce TGF-β from Caco-2 cells through recognition by TLR2

To further confirm that TGF-β production induced by LP22A3 treatment actually led to its inhibitory effect, as observed in the co-culture model, we blocked the effect of TGF-β1 by a using TGF-β1 neutralizing antibody. As shown in Fig. 2A and

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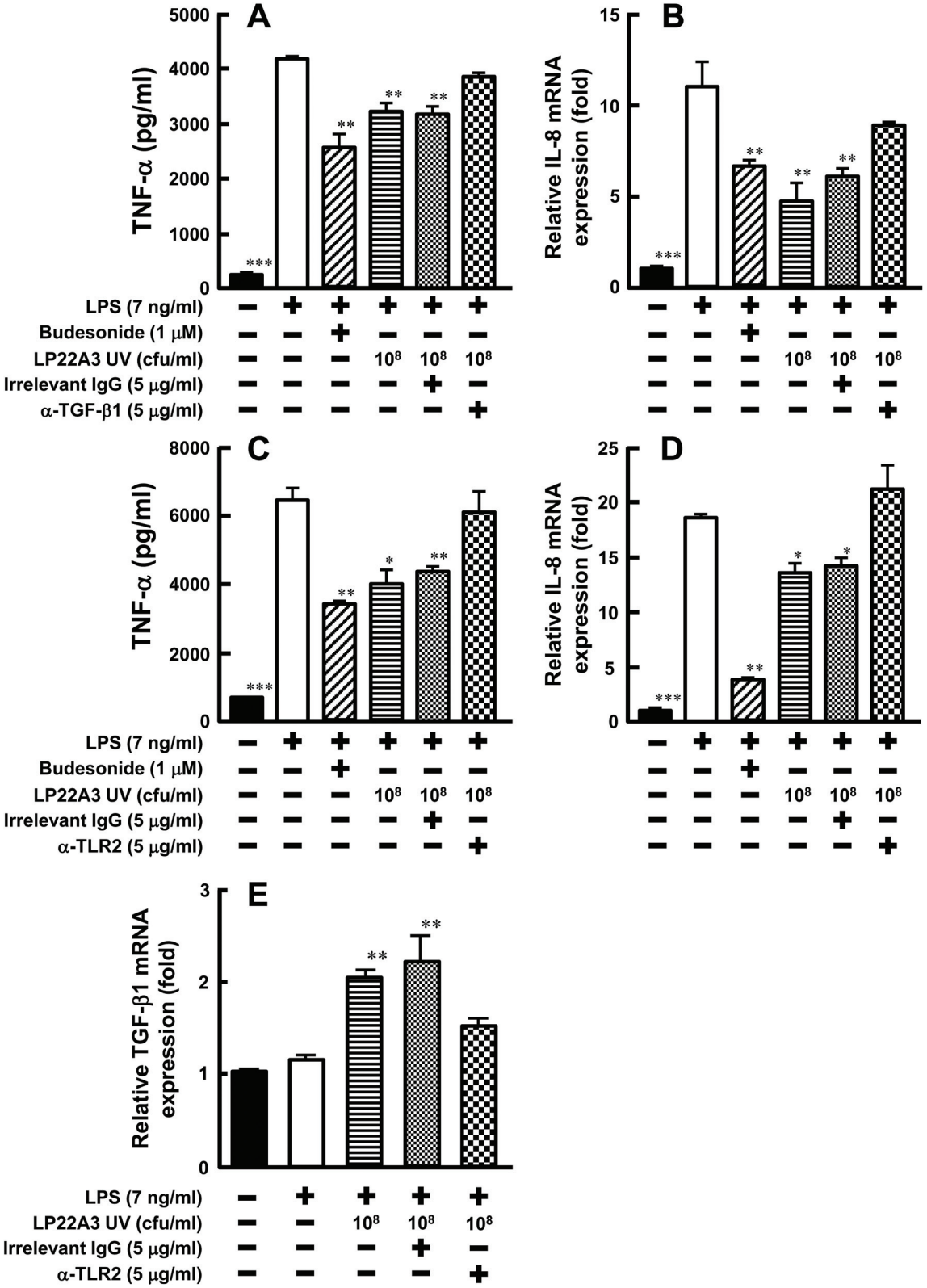

Fig. 2 Effect of TGF-β1 and TLR2 on LP22A3 inhibitory properties. Anti-TGF-β1 or anti-TLR2 neutralizing antibodies were applied to the basolateral compartment of the co-culture model prior to LP22A3 treatment. (A and C) TNF-α production on the basolateral side was determined by a L929 cytotoxicity assay. (B and D) IL-8 mRNA expression in Caco-2 cells was detected by Realtime-PCR. (E) TGF-β1 mRNA expression in Caco-2 cells was detected by Realtime-PCR. Values represent the means ± SE (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 (*vs.* LPS).

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B, anti-TGF-β1 antibody treatment on the basolateral side canceled LP22A3's ability to suppress TNF-α production and IL-8 mRNA expression. Toll-like receptor 2 (TLR2) is widely reported to be expressed on IECs with confirmed functions in both mice and humans and also in the Caco-2 cell line.[17] Therefore we determined whether LP22A3 exerted its inhibitory effects *via* TLR-2 expressed on Caco-2 cells. We blocked TLR2 located on the apical side of the Caco-2 monolayer using an anti-TLR2 antibody. Pretreatment with anti-TLR2 antibody abolished the suppression of TNF-α production and IL-8 mRNA expression by LP22A3 (Fig. 2C and D) and also the upregulation of TGF-β1 mRNA expression in Caco-2 cells (Fig. 2E). Moreover, to confirm whether LP22A3 can be recognized by TLR2 expressed on mouse small intestinal epithelial cells and induce TGF-β1 upregulation, a ligated loop assay technique was adopted. LP22A3 treatment applied to the ligated loop of the ileum resulted in a significant increase in TGF-β1 mRNA expression in IECs compared to the vehicle. However, pretreatment with TLR2 neutralizing antibody to the ligated ileum loop canceled the elevation in TGF-β1 expression (Fig. 3). Collectively, these results revealed that LP22A3's inhibitory properties depended on its ability to induce TGF-β1 through TLR2 expressed on the apical side of IECs.

3.3. Oral administration of LP22A3 ameliorated DSS-induced colitis through the promotion of anti-inflammatory cytokines

It was demonstrated that LP22A3 possessed anti-inflammatory properties *in vitro*. To confirm whether LP22A3 also exhibited this activity *in vivo*, we investigated the ability of LP22A3 to inhibit DSS-induced colitis in mice. Oral administration of viable LP22A3 to DSS-treated mice resulted in body weight recovery on day 10 and approximately 10% less weight loss compared to the DSS control (Fig. 4A). LP22A3 administration also significantly inhibited the shortening of the colon, the colitis score and histological damage to the colon section, all which are signs of inflammation (Fig. 4B and C). Several pieces of evidence suggested that the therapeutic effect of probiotics may be mediated by both IL-10/TGF-β-dependent and independent mechanisms.[18,19] As shown in Fig. 2, LP22A3 was able to upregulate TGF-β1 expression and secretion from IECs. Therefore, mRNA expression of anti-inflammatory cytokines in mouse colonic tissue, including TGF-β1 and IL-10, was also measured. LP22A3 treatment significantly increased TGF-β1 and IL-10 mRNA expression and also the marker of regulatory T-cells, Foxp3, that is known to be upregulated by these regulatory cytokines (Fig. 5). This suggested that administration of LP22A3 ameliorated DSS-induced colitis in mice through the promotion of anti-inflammatory cytokines in the intestine.

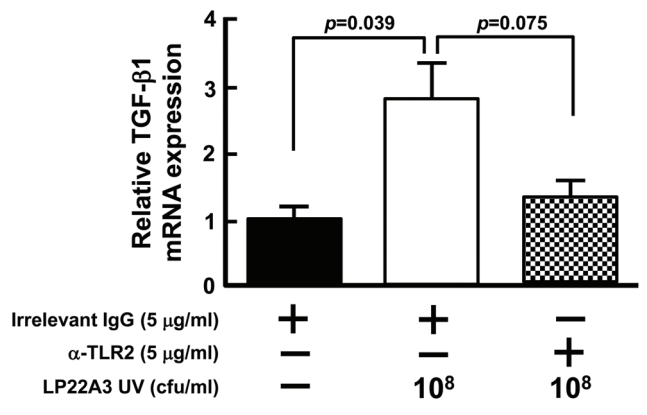

Fig. 3 LP22A3 induced TGF-β1 expression in mouse ileum epithelial cells through TLR2. Three independent ligated intestinal loops were pretreated with an isotype control or anti-TGF-β1 Ab for 30 min. A vehicle or LP22A3 sample was then injected into each loop and incubated for 2 hours. Epithelial cells were isolated, extracted for RNA, and TGF-β1 mRNA expression was detected by Realtime-PCR. Values represent the means ± SE (n = 3).

3.4. LP22A3 induced TGF-β1 secretion from the IECs of the small intestine providing a TGF-β1-rich condition

TGF-β is widely known to be one of the crucial regulators of Foxp3$^+$ regulatory T-cell (Treg) differentiation.[20] Therefore, we speculated that administration of viable LP22A3 prior to DSS-treatment may be sufficient for LP22A3 to modulate the intestinal immune system by providing a TGF-β-rich condition in the intestine. To investigate this aspect, LP22A3 was orally administrated to healthy mice for one week and the IECs of the ileum and colon were cultured to measure TGF-β1 content in addition to serum. As a result, a significant increase in the production of active-form TGF-β1 from IECs of the ileum and serum was observed but not in the colon (Fig. 6A and C). The serum IL-10 level was also elevated in LP22A3-administrated mice (Fig. 6D). This indicated that LP22A3 stimulated TGF-β production from IECs providing an overall TGF-β rich condition that possibly promoted Foxp3$^+$ Treg differentiation. Since Foxp3$^+$ Treg is known to secrete both TGF-β1 and IL-10, an increase in Treg population may also contribute to the elevated level of both cytokines in the serum. These results also suggested that one week of LP22A3 pretreatment is adequate to modulate the intestinal immune system toward a regulatory system that is less susceptible to inflammation.

3.5. Administration of *L. plantarum* 22A-3 promotes Foxp3$^+$ Treg and CD103$^+$ DC development in the small intestine

We further determined whether oral administration of LP22A3 actually led to an increase in the Foxp3$^+$ Treg population. LP22A3 was orally administrated to healthy mice for 1 week and then lamina propria lymphocytes were isolated from the ileum and colon, and assessed for gene expression and frequency of CD25$^+$ Foxp3$^+$ T cell population. Interestingly, mRNA expression of TGF-β1, IL-10 and Foxp3 increased only in the ileum of LP22A3-treated mice and no change was observed in the colon (Fig. 7). Accordingly, a significant increase in the CD25$^+$ Foxp3$^+$ Treg population was observed only in the ileum of LP22A3-treated mice compared to the vehicle (Fig. 8A). Despite LP22A3's ability to ameliorate colitis, these results indicated that LP22A3 acted solely on the ileum to promote TGF-β1 production and Foxp3$^+$ Treg differentiation and not on the colon.

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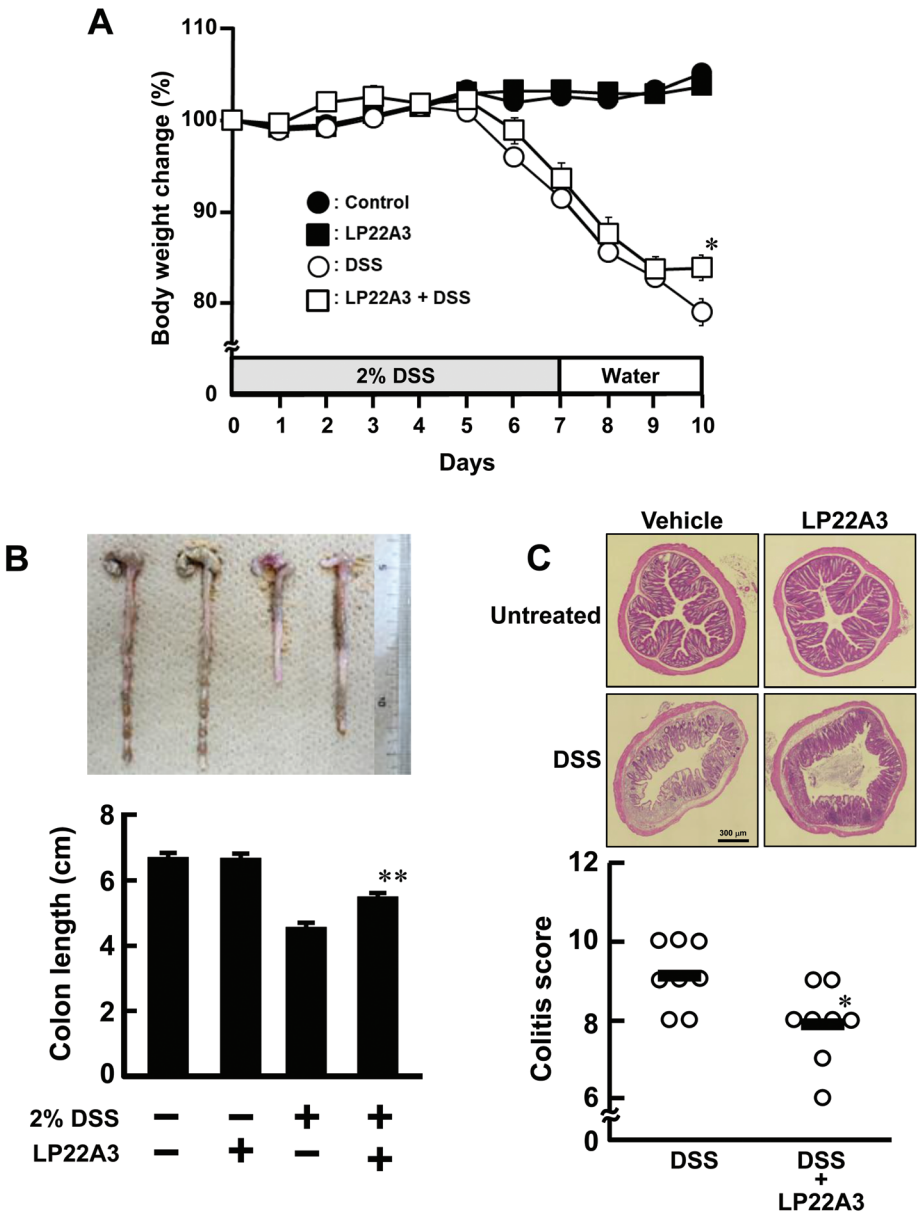

Fig. 4 Administration of LP22A3-ameliorated DSS-induced colitis in mice. (A) Percent of body weight change and (B) colon lengths of each group. (C) Photograph (×40) of HE-stained paraffin sections of a representative colon from each group. The colitis score of the colon sections of DSS-treated mice with or without administration of LP22A3. Values represent the means ± SE (n = 8). *P < 0.05 **P < 0.01 (*vs.* DSS).

Recent studies have shown that specialized populations of CD103$^+$ mucosal tolerogenic DCs can promote Treg differentiation *via* a TGF-β and retinoic acid (RA)-dependent mechanism.[20] We therefore determined whether there was any influence on the CD103$^+$ DC population and RA production. The CD103$^+$ DC population was analyzed by flow cytometry and RA production was evaluated through mRNA expression of aldehyde dehydrogenase-1A2 (*Aldh1a2*), which is exclusively expressed by intestinal CD103$^+$ DCs. Both *Aldh1a2* expression and the frequency of CD103$^+$ DC population in the ileum significantly increased in the LP22A3-treated group (Fig. 8B and C). This suggested that together with TGF-β1 upregulated by LP22A3, the increase in CD103$^+$ DC population possibly led to the promotion of Foxp3$^+$ Treg development. These factors evidently contributed to the anti-inflammatory properties of LP22A3 and its ability to ameliorate colitis in mice.

4. Discussion

In the present study, we demonstrated that LP22A3 possessed inhibitory properties against intestinal inflammation through the promotion of anti-inflammatory cytokine, such as TGF-β1 production. In an *in vitro* co-culture model consisting of

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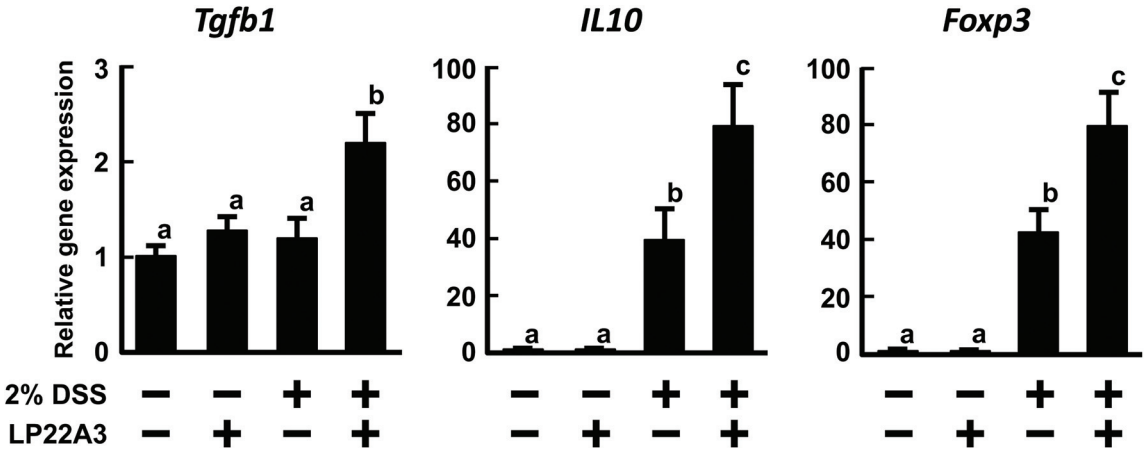

Fig. 5 Administration of LP22A3 promoted anti-inflammatory cytokine expression. The mRNA expression of cytokines and Foxp3 in the colon tissue of untreated or DSS-treated mice with either LP22A3-treated or untreated mice. Values represent the means ± SE (n = 8). Different letters indicate significant differences (P < 0.05).

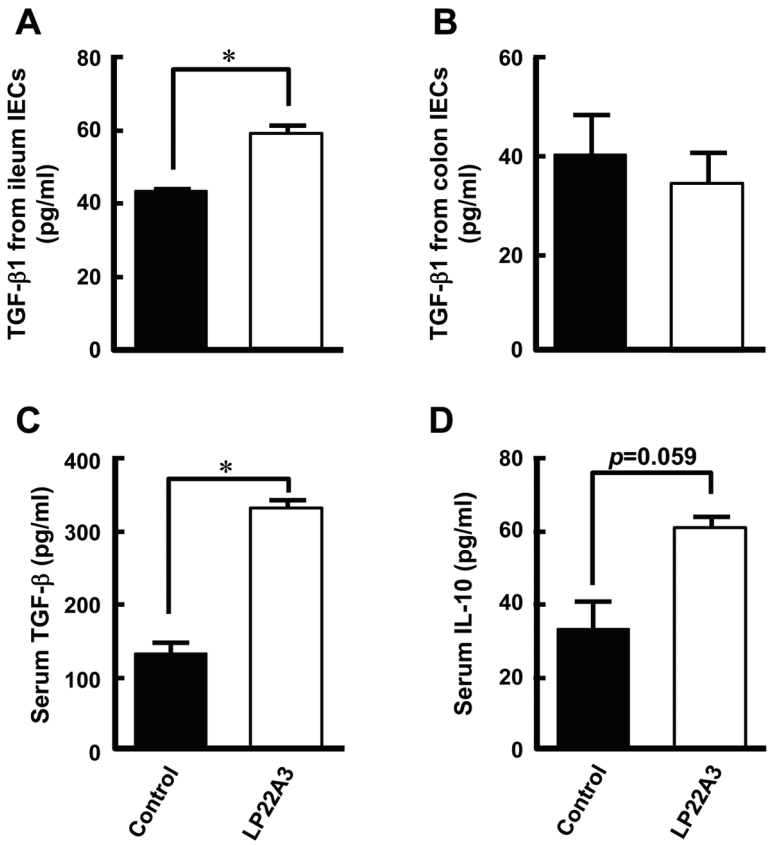

Fig. 6 Administration of LP22A3 induced TGF-β1 secretion from the IECs of the small intestine. LP22A3 was orally administrated for one week and IECs of the ileum (A) and colon (B) were cultured and measured for TGF-β1 production. Serum from untreated and LP22A3-treated mice was measured for (C) TGF-β1 and (D) IL-10 level. Values represent the means ± SE (n = 3). *P < 0.05.

RAW264.7 and Caco-2 cells, UV-inactivated LP22A3 significantly suppressed TNF-α production from LPS-stimulated RAW264.7 cells and IL-8 mRNA expression from Caco-2 cells (Fig. 1A and B). We also revealed that LP22A3 treatment stimulated active-form TGF-β1 secretion and TGF-β1 mRNA expression from a Caco-2 cell monolayer (Fig. 1C and D). Neutralization of TGF-β1 in this setting significantly abolished LP22A3's ability to suppress TNF-α and IL-8 expression (Fig. 2A and B). Collectively, these outcomes indicated that LP22A3 exerted its inhibitory properties through its unique ability to

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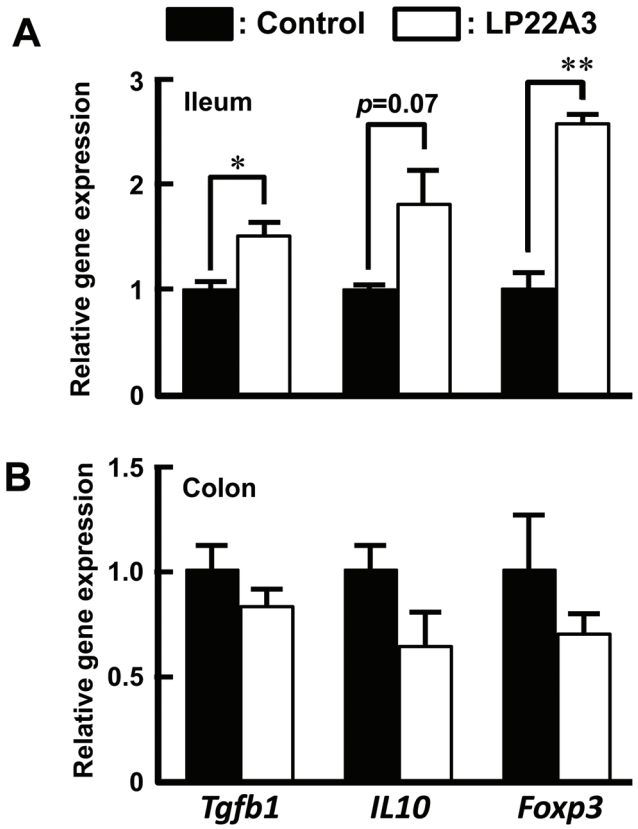

Fig. 7 Administration of LP22A3 induced anti-inflammatory cytokines expression in lymphocytes of lamina propria. LP22A3 was orally administrated for one week. Isolated lamina propria lymphocytes from (A) ileum and (B) colon were measured for TGF-β1, IL-10 and Foxp3 mRNA expression. Values represent the means ± SE (n = 3). *P < 0.05, **P < 0.01.

promote TGF-β1 production from $Caco_2$ cells which have a suppressive effect on macrophage activation. Since it has been reported that TGF-β is thought to block the signaling of TNF-α by inhibiting the binding of TRAF2/TAB2 and TAB3 by Smad7,[21] IL-8 mRNA expression may have been reduced because TNF-α production from RAW was suppressed.

Additionally, we demonstrated that LP22A3 interacted with Caco-2 cells *via* TLR2 because LP22A3 lost its inhibitory effects and upregulation of TGF-β1 mRNA expression when TLR2 on the Caco-2 cell-surface was blocked by anti-TLR2 antibodies (Fig. 2C, D and F). An intestinal ligated loop assay experiment revealed a similar outcome where LP22A3's ability to induce TGF-β1 mRNA expression on mouse small intestinal epithelial cells was canceled by pretreatment with anti-TLR2 antibodies (Fig. 3). The TLR2 signaling pathway is usually associated with an NF-κB-mediated pro-inflammatory response. However, emerging evidence has also revealed that there are cell-type specific differences and plasticity in TLR2 signaling.[22] A recent study reported that TLR2 is capable of triggering an anti-inflammatory response in several settings. Although it is highly possible that LP22A3 exploits a similar TLR2 signaling pathway that leads to TGF-β1 upregulation in intestinal epithelial cells, further analysis is required to elucidate the downstream signaling pathway from TLR2.

Oral administration of viable LP22A3 amelioration of DSS-induced colitis in mice, was evidenced by its inhibition of body weight loss, colon shortening and colitis score (Fig. 4). In addition, the colon tissue of LP22A3-treated mice showed an elevated level of TGF-β1 and IL-10 mRNA expression and also the marker of regulatory T-cell, Foxp3 compared to the non-LP22A3 group (Fig. 5). LP22A3 administration to healthy mice also resulted in an increase in serum TGF-β1 level and TGF-β1 production from IECs in the ileum (Fig. 6). The mRNA expression of TGF-β1, IL-10 and Foxp3 was also elevated in the ileum of LP22A3-treated mice but was not observed in the colon (Fig. 7). Together with the increase in $Foxp3^+$ regulatory T-cells and $CD103^+$ DC population in the small intestine (Fig. 8), these results suggested that the protective effects of LP22A3 against colitis were initiated in the small intestine and involved TGF-β, $Foxp3^+$ Treg and $CD103^+$ DCs. Zhang *et al.* have shown that oral administration of *Lactobacillus rhamnosus* increases intestinal $CD103^+$ DCs and accumulates mucocal Treg.[23] In addition, Jeon *et al.* showed that $CD103^+$ DCs are activated *via* the TLR2/MyD88 pathway, which promotes IL-10 and IL-27 production.[24] Taken together, LP22A3 may be increased *via* the TLR2/MyD88 pathway. The underlying mechanism of LP22A3 activities on the intestinal immune system can be proposed as follow. Oral administration of LP22A3 stimulated TGF-β1 secretion from the IECs of the small intestine and together with retinoic acid produced by $CD103^+$ DCs led to the promotion of $Foxp3^+$ Treg differentiation, possibly in the mesenteric lymph nodes. Tregs promoted by $CD103^+$ DCs would migrate back to the small intestine, resulting in an increase in $Foxp3^+$ Treg population. Since both $CD103^+$ DCs and $Foxp3^+$ Tregs are reported to be crucial in regulating inflammation in the intestine,[25,26] such cell populations may be responsible for LP22A3's therapeutic effects.

Interestingly, although LP22A3 was capable of ameliorating colitis, LP22A3 initiated its protective effects in the small intestine. The reason why LP22A3 acted solely on the small intestine may be the fact that TLR2 is not expressed uniformly throughout the intestine. Immunohistochemical studies have revealed that TLR2 is expressed predominantly in the epithelial cells of the small intestine and at low levels in the colon.[27] It has been demonstrated that induction of intestinal Treg is dependent on TGF-β1 from LP dendric cells, and TGF-β1 is induced through cooperation between TLR2-AP-1 and TGF-β-Smad signaling pathways.[28] Thus, LP22A3 may be recognized more effectively by the IECs of the small intestine than the colon. However, it is still unclear how the effects initiated by LP22A3 in the small intestine eventually extend to the colon. It is possible that pre-administration of LP22A3 prior to DSS-treatment was sufficient to skew the intestinal immune system toward the suppressive side, characterized by an increase in anti-inflammatory cytokines, regulatory T-cells and tolerogenic DCs. At the time of inflammation, lymphocytes and monocytes including T-cells are recruited to the inflamed

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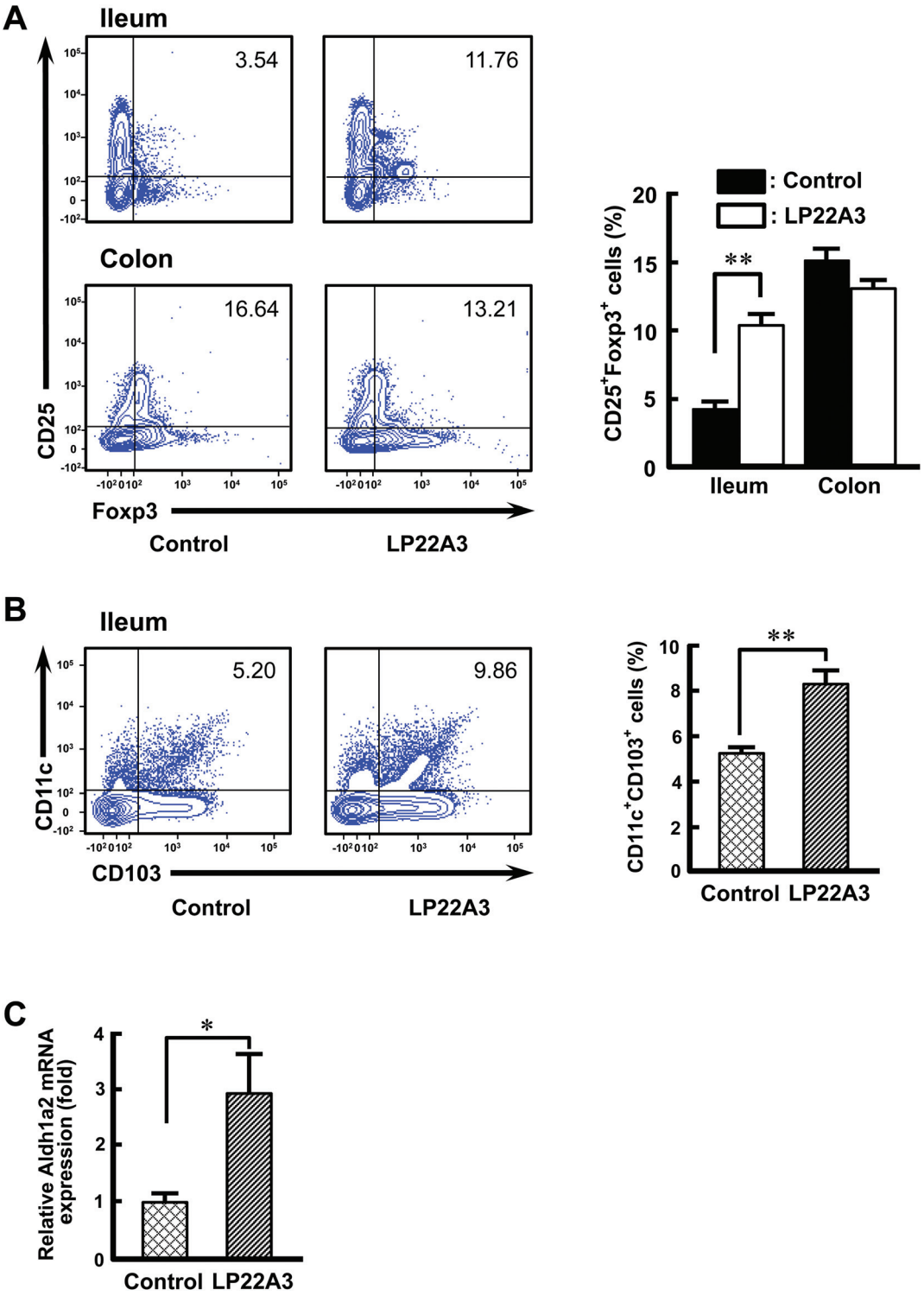

Fig. 8 LP22A3 promotes Foxp3$^+$ Treg and CD103$^+$ DC development. LP22A3 was orally administrated for one week and lamina propria lymphocytes were isolated for flow cytometry analysis. (A) Representative flow cytometric plots gated on CD4$^+$ population presenting CD25$^+$ and Foxp3$^+$ populations and gated on lamina propria lymphocytes presenting CD11c$^+$ and CD103$^+$ populations. (B) Frequency of CD25$^+$ Foxp3$^+$ cells and CD103$^+$ dendritic cells. (C) mRNA expression of *Aldh1a2* in lamina propria lymphocyte. Values represent the means ± SE (n = 3). *P < 0.05, **P < 0.01.

tissue. Tregs are also reported to migrate to the inflamed tissue responding to inflammatory signals such as chemokines.[29,30] It can be speculated that the Treg population promoted in the small intestine was possibly recruited to the colon when colitis and inflammation developed. This hypothesis is supported by the finding in which Foxp3 mRNA

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expression in the colon tissue was elevated only in DSS-treated mice that developed colitis and not in control mice that were only administrated with LP22A3 (Fig. 5). Moreover, recent studies have revealed that the homing of T-cells to the large intestinal mucosa is mediated by a G protein-linked chemoattractant receptor, GPR15, which is upregulated by TGF-β but not retinoic acid.[31] It is possible that LP22A3's ability to create TGF-β1-rich conditions, as indicated by the increase in TGF-β1 serum level, may further influence the homing of Tregs to the large intestine.

5. Conclusion

The data obtained in this study demonstrated the therapeutic properties of LP22A3 and its possible mechanism. LP22A3 induced TGF-β1 secretion from IECs of the small intestine through TLR2, and resulted in the promotion of $Foxp3^+$ Treg and $CD103^+$ DC populations. Both cells secreted high levels of anti-inflammatory cytokines, including TGF-β1 and IL-10, providing a protective condition in the intestine, which is less susceptible to inflammation. It was reported that TGF-β is induced through a cooperation between TLR2-AP-1 and TGF-β-Smad signaling pathways.[28] Therefore, it would be important to study Caco-2 cells which knocked down Smad2 and 3 genes. Also, at the time of inflammation, regulatory T-cell population promoted by LP22A3 may be recruited to the inflamed tissue and regulate the excessive inflammatory response. As there is much unknown regarding T-cell homing to the colon and its relevance to the small intestine, we would like to prove the mechanism in more detail by conducting experiments using KikGR mice which could confirm the migration of target cells.[32] It was demonstrated that LP22A3 administration is a promising alternative therapeutic strategy for IBD and possibly other inflammatory disorders.

Abbreviations

Aldh1a2	Aldehyde dehydrogenase-1A2
CD	Crohn's disease
DC	Dendritic cell
DMEM	Dulbecco's modified eagle's medium
DSS	Dextran sulfate sodium
ELISA	Enzyme linked immunosorbent assay
FBS	Fetal bovine serum
HBSS	Hank's balanced salt solution
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cell
IL-6	Interleukin-6
LP	Lamina propria
LP22A3	*Lactiplantibacillus plantarum* 22A-3
LPS	Lipopolysaccharide
MEM	Eagle's minimum essential medium
NEAA	Non-essential amino acids
PCA	Passive cutaneous anaphylaxis
PCR	Polymerase chain reaction
RA	Retinoic acid
TGF-β	Transforming growth factor-β
TLR2	Toll-like receptor 2
TNF-α	Tumor necrosis factor-α
UC	Ulcerative colitis.

Author contributions

Jarukan Lamubol: methodology, formal analysis, investigation, writing-original draft. Nobuaki Ohto: investigation, validation. Hiroshige Kuwahara: validation, resources. Masashi Mizuno: conceptualization, writing-review and editing, supervision, project administration.

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Conflicts of interest

The authors have declared no conflicts of interest.

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