



Lactobacillus plantarum CLP-0611 ameliorates colitis in mice by polarizing M1 to M2-like macrophages

Se-Eun Jang^{a,b}, Myung Joo Han^b, Se-Young Kim^c, Dong-Hyun Kim^{a,*}

^a Department of Life and Pharmaceutical Sciences, College of Pharmacy, Kyung Hee University, Seoul 130-701, South Korea

^b Department of Food and Nutrition, Kyung Hee University, Seoul 130-701, South Korea

^c R & D Center, CTOBIO Inc., Gyeonggi-do 445-913, South Korea

ARTICLE INFO

Article history:

Received 30 November 2013

Received in revised form 21 March 2014

Accepted 23 April 2014

Available online 9 May 2014

Keywords:

Lactobacillus plantarum CLP-0611

Colitis

TNF- α

Polarization

Macrophage

ABSTRACT

The TNF- α expression-inhibitory effect of lactic acid bacteria (LAB) isolated from kimchi were measured in lipopolysaccharide (LPS)-stimulated peritoneal macrophages. Among the LAB evaluated, *Lactobacillus plantarum* CLP-0611 inhibited the IL-1 β and IL-6 expression, as well as the NF- κ B and AP1 activation in LPS-stimulated peritoneal macrophages. Therefore, we investigated its inhibitory effect on 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis in mice. TNBS significantly induced colon shortening, as well as myeloperoxidase activity and macroscopic score. Oral administration of CLP-0611 significantly reduced TNBS-induced body weight loss, colon shortening, myeloperoxidase activity, IRAK-1 phosphorylation, NF- κ B and MAP kinase (p38, ERK, JNK) activation, and iNOS and COX-2 expression. CLP-0611 also inhibited TNBS-induced expression of TNF- α , IL-1 β , and IL-6. However, IL-10 expression was induced. CLP-0611 also induced the production of M2 macrophage markers (IL-10, arginase I and CD206). Based on these findings, CLP-0611 inhibits TLR-4-linked NF- κ B and MAPK signaling pathways and polarizes M1 to M2-like macrophages, thus ameliorating colitis.

© 2014 Elsevier B.V. All rights reserved.

1. Introduction

Inflammatory bowel disease (IBD), which includes ulcerative colitis and Crohn's disease, pertains to chronically relapsing disorders of the intestine [1,2]. Its pathogenic mechanism has been proposed to be the dysregulation of the intestinal immune response to gastrointestinal environmental antigens such as endotoxins of gut microbiota [2–4]. Therefore, IBD does not rapidly easy to significantly progress in germ-free animals [5]. Gut microflora, which consists of more than 1000 different bacterial species [5,6], might be strongly associated with the initiation and perpetuation of ulcerative colitis. Particularly, elevated levels of gram-negative bacteria such as Enterobacteriaceae are commonly observed in colitic patients, as well as in colitic mice treated with 2,4,6-trinitrobenzene sulfonic acid (TNBS) or dextran sulfate

sodium (DSS) [4]. Gram-negative bacteria produce bacterial endotoxins such as lipopolysaccharide (LPS). LPS activates the biosynthesis of a wide range of inflammatory cytokines including interleukin (IL)-1 β , IL-6, and tumor necrosis factor (TNF)- α via NF- κ B (NF- κ B) and mitogen-activated protein kinase (MAPK) pathways in macrophages [7]. Therefore, to improve IBD, dietary ingredients that inhibited LPS-stimulated NF- κ B and MAPK signaling pathways have been the focus of most recent studies [8,9].

Lactic acid bacteria (LAB) are safe and beneficial microorganisms that improve disturbed gut microbiota [10,11], possess anti-obesity effects [12], ameliorate infectious and inflammatory diseases [13], and impart anti-colitic effects [14,15]. For example, *Lactobacillus casei* inhibits expression of inflammatory cytokines in DSS-induced colitic mice [16]. *L. casei*, *Lactobacillus sordaryus*, *Lactobacillus brevis*, and *Bifidobacterium longum* also show anti-inflammatory activities in colitic animal models [17–19]. Among these, *L. brevis* G-101 induces IL-10 production, which imparts an anti-colitic effect by polarizing M1 macrophages to M2 macrophages. However, the macrophage-polarizing effect of TNF- α expression-inhibitory LAB has not been studied thoroughly.

Therefore, we isolated LAB from kimchi, measured their TNF- α expression-inhibiting effects in lipopolysaccharide (LPS)-stimulated peritoneal macrophages, isolated CLP-0611, which was identified to be *L. plantarum*, and then evaluated its anti-colitic and macrophage-polarizing effects in TNBS-induced colitic mice.

Abbreviations: LPS, lipopolysaccharide; LAB, lactic acid bacteria; TNBS, 2,4,6-trinitrobenzene sulfonic acid; IBD, inflammatory bowel disease; DSS, dextran sulfate sodium; IL, interleukin; TNF, tumor necrosis factor; NF- κ B, NF- κ B; MAPK, mitogen-activated protein kinase; DMEM, Dulbecco's modified Eagle's medium; HTAB, hexadecyl trimethyl ammonium bromide; RIPA, radioimmunoprecipitation assay; ELISA, enzyme-linked immunosorbent assay; MRS, de Man, Rogosa and Sharpe; CFU, colony forming unit.

* Corresponding author at: Department of Life and Pharmaceutical Sciences, College of Pharmacy, Kyung Hee University, 1, Hoegi, Dongdaemun-gu, Seoul 130-701, South Korea. Tel.: +82 2 961 0374; fax: +82 2 957 5030.

E-mail address: dhkim@khu.ac.kr (D.-H. Kim).

2. Materials and methods

2.1. Materials

TNBS, sodium thioglycollate, Dulbecco's modified Eagle's medium (DMEM), hexadecyl trimethyl ammonium bromide (HTAB), radio-immunoprecipitation assay (RIPA) lysis buffer, phosphatase inhibitor cocktail, protease inhibitor cocktail and tetramethyl benzidine were purchased from Sigma-Aldrich (St Louis, MO, U.S.A.). Enzyme-linked immunosorbent assay (ELISA) kits were purchased from Pierce Biotechnology (Rockford, IL, U.S.A.). Antibodies were purchased from Cell Signaling (Beverly, MA, U.S.A.). Enhanced chemiluminescence (ECL) immunoblot system was purchased from Pierce (Rockford, IL, U.S.A.). Gram stain kit was purchased from bioMérieux (Grenoble, France). Antibiotic-antimycotic was from Life Technologies (Grand Island, NY, U.S.A.). RNeasy Mini Kit and SYBR Green I were purchased from Qiagen (Hilden, Germany). Reverse-transcriptase and DNA polymerase were from Takara (Shiga, Japan). De Man, Rogosa and Sharpe (MRS) medium was purchased from BD (Sparks, MD, USA).

2.2. Bacterial strains and growth conditions

Seventy seven strains of LAB were isolated from Chinese cabbage kimchi using a MRS agar: 25 *Lactobacillus sakei* strains, 16 *Leuconostoc mesenteroides* strains, 21 *Lactobacillus plantarum* strains, 9 *Lactobacillus curvatus* strains, 5 *L. brevis* strains, and 1 *Lactobacillus pentosus* strains. Isolated LAB strains were identified using the Gram stain kit and 16S ribosomal DNA sequencing following the protocol previously reported by Jang et al. [19].

For anti-inflammatory activity assay of LAB in macrophage, LAB were anaerobically grown at 37 °C in MRS broth, collected by centrifuging at 10,000 ×g for 15 min and washed with saline twice. The pellet was suspended in 5 ml of phosphate-buffered saline (PBS), inactivated by heating in boiling water for 15 min, and used for experiments.

For in vivo study, *L. plantarum* CLP0611 was grown in MRS broth (3 to 4 OD at 600 nm), harvested by centrifuging at 10,000 ×g for 15 min, and washed with PBS twice. The resulting cells (1×10^8 and 1×10^9 CFU) were suspended in 50 mM sodium bicarbonate buffer with 1% glucose, and orally administered to mice [18].

2.3. Animals

All animal experiments were approved by the Committee for the Care and Usage of Laboratory Animals in the College of Pharmacy, Kyung Hee University, and performed according to the Kyung Hee University guideline for Laboratory Animals Care and Usage (KHP-2012-11-01-R1).

Male ICR mice (5 weeks old, 21–25 g) were supplied from the Central Lab. Animal Inc. (Seoul, Republic of Korea). Each group consisted of 6 mice, which were housed in a cage, fed on standard laboratory chow and water ad libitum, and maintained at $50 \pm 10\%$ humidity, 20–22 °C, and a 12-h diurnal light/dark cycle (light, 07:30–19:30 h).

2.4. Isolation and culture of peritoneal macrophages

The mice were intraperitoneally injected with 4 (w/v)% thioglycollate solution (2 mL) and sacrificed 4 days after the injection. The peritoneal cavity was collected with RPMI 1640 [19]. The peritoneal fluid was centrifuged at 300 ×g for 10 min. The cells (2×10^7 cells) were incubated in RPMI 1640 (5 mL) at 37 °C for 2 h and washed three times to remove nonadherent cells. The cells were then seeded and cultured in a 12-well plate at 37 °C in RPMI 1640 containing 10% FBS and 1% antibiotic-antimycotic. The attached cells were used for in vitro experiments. To evaluate anti-inflammatory effect of *L. plantarum* CLP-0611, peritoneal

macrophages (1×10^6 cells/well) were treated with LPS (100 ng/mL) in the absence or presence of *L. plantarum* CLP-0611 (1×10^3 CFU/well, 1×10^4 CFU/well and 1×10^5 CFU/well) for 24 h.

2.5. Preparation of colitic mice

The mice were divided into 7 groups randomly: normal control group treated with vehicle alone, control group treated with TNBS alone, two groups treated with *L. plantarum* CLP-0611 (1×10^8 CFU/mouse or 1×10^9 CFU/mouse) with TNBS, two groups treated with *L. plantarum* CLP-0611 (1×10^8 CFU/mouse or 1×10^9 CFU/mouse) without TNBS, and positive group treated with mesalazine (10 mg/kg) and TNBS. TNBS-induced colitis was induced by the intrarectal injection of 2.5% (w/v) TNBS solution (100 µL) dissolved in 50% ethanol into the colon of mice lightly anesthetized with ether [20]. The needle was inserted to 3.5–4 cm proximal to the anus. To distribute TNBS solution within the entire colon, the mice were vertically held for 30 s after TNBS injection. If TNBS solution was quickly excreted, the mouse was excluded. *L. plantarum* CLP-0611 (1×10^8 /mouse or 1×10^9 CFU/mouse) was orally given once a day for three days after treatment with TNBS. The mice were sacrificed 18 h after the 3rd administration of CLP-0611. The colon was quickly collected, opened longitudinally, and gently washed with PBS. Macroscopic colitic score was assessed: 0, no ulcer and no inflammation; 1, no ulceration and local hyperemia; 2, ulceration without hyperemia; 3, ulceration and inflammation at one site only; 4, two or more sites of ulceration and inflammation; 5, ulceration extending more than 2 cm [18]. The colon tissues were then stored at –80 °C until experiment.

2.6. Myeloperoxidase activity assay

Myeloperoxidase activity was assayed according to the method of Jang et al. [19]. The colon tissues were homogenized in 0.5% HTAB-contained 10 mM phosphate buffer (pH 7.0), and centrifuged at 20,000 ×g and 4 °C for 30 min. The supernatants were used as a crude myeloperoxidase solution. An aliquot (50 µL) of the crude enzyme solution was incubated in a reaction mixture containing 0.1 mM H₂O₂ and 1.6 mM tetramethyl benzidine at 37 °C and the absorbance at 650 nm was measured over time (Mullane et al., 1987). The protein was determined by the Bradford method [21].

2.7. Immunoblot analysis and ELISA

For the immunoblotting, peritoneal macrophages (5×10^5 cells) were incubated with LPS (100 µg/mL) for 120 min or 20 h in the absence or presence of CLP-0611 (1×10^3 CFU/well, 1×10^4 CFU/well and 1×10^5 CFU/well), lysed in RIPA lysis buffer (4 °C) containing 1% phosphatase inhibitor cocktail and 1% protease inhibitor cocktail, and centrifuged at 2000 ×g for 10 min [20]. The colons were also homogenized and lysed with the same lysis buffer and centrifuged at 15,000 ×g and 4 °C for 15 min. The lysate of macrophages and colons was separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 0.05% PBST containing 5% non-fat dried-milk proteins, and then probed with iNOS, COX-2, p65, p-p65, IRAK1, p-IRAK1, ERK, p-ERK, JNK, p-JNK, p38, p-p38, or β-actin antibody. The protein was detected with horseradish peroxidase-conjugated each secondary antibody for 50 min and its band was visualized with enhanced chemiluminescence reagent.

For the assay of cytokines, the supernatants of peritoneal macrophages and colon lysates were transferred to 96-well ELISA plates. The cytokine levels were measured by ELISA kits.

2.8. Real time-polymerase chain reaction (RT-PCR)

RNAs were extracted from the mouse colon tissues with the RNeasy Mini kit and first-strand cDNA for CD206, arginase (ARG) I, ARG II, IL-1 β , IL-10, TNF- α , and β -actin was synthesized using reverse-transcriptase. Real time-PCRs were carried out on the Rotor-Gene Q[®] using DNA polymerase and SYBR Green I (a reaction volume, 20 μ L). Primers for real-time PCR are described in Table 1. The normalized expression of each target gene, as for β -actin, was calculated for all samples using Microsoft Excel [22].

2.9. Immunohistochemistry

Macrophages were immunostained using anti-macrophage antigen (CD68, CD86, and CD206) antibodies and detected using 3,3'-diaminobenzidine (DAB) substrate kit from Thermo scientific (Rockford, IL). The serial sections were subjected to this procedure. Horseradish peroxidase activity was visualized with 3-amino-9-ethylcarbazole.

2.10. Statistical analysis

All experimental data are indicated as the mean \pm standard deviation (S.D.). The statistical significance was analyzed using one-way analysis of variance ($P < 0.05$).

3. Results

3.1. *L. plantarum* CLP-0611 inhibits the expression of proinflammatory cytokines in LPS-stimulated peritoneal macrophages

We isolated 77 LAB strains from kimchi and measured their TNF- α expression-inhibitory activities in LPS-stimulated peritoneal macrophages. Among the tested LAB (heat-treated), CLP-0611 potently inhibited TNF- α expression. CLP-0611 also inhibited LPS-induced IL-1 β and IL-6 expression (Fig. 1). The treatment with CLP-0611 (1×10^5 CFU/mL) significantly inhibited LPS-stimulated TNF- α , IL-1 β and IL-6 expression by 83.7%, 74.7% and 80.2%, respectively, compared to that treated with LPS alone. Furthermore, CLP-0611 inhibited LPS-induced activation of NF- κ B and AP1. Biochemical analysis and 16S ribosomal DNA sequencing revealed that CLP-0611 was *L. plantarum*.

3.2. Inhibitory effect of *L. plantarum* CLP-0611 on TNBS-induced colitis in mice

We examined the inhibitory effect of CLP-0611 on TNBS-induced colitis in mice. Intrarectal administration of TNBS into mice induced body weight loss, colon shortening and severe inflammation (Fig. 2). In the histological exam, the colon of TNBS-treated mice showed

bowel edema, and epithelial cell disruption. Treatment with CLP-0611 inhibited body weight losing, colon shortening, and colonic inflammation and thickening. CLP-0611 also inhibited TNBS-induced myeloperoxidase activity, which is a representative inflammatory marker. However, CLP-0611 did not influence body weight loss, colon length and myeloperoxidase activity in normal control mice (data not shown). The efficacy of CLP-0611 was potent compared to that of mesalazine.

TNBS also increased the activation of NF- κ B and MAPKs, as well as the expression of COX-2 and iNOS (Fig. 3). CLP-0611 blocked the induction of IRAK1, p65, ERK, JNK and p38 phosphorylation by TNBS. CLP-0611 also inhibited TNBS-induced expression of iNOS and COX-2. However, CLP-0611 did not influence NF- κ B activation and iNOS and COX-2 expression in normal control mice. The inhibitory effect of CLP-0611 (1×10^9 CFU) was potent compared to that of mesalazine (10 mg/kg). We also examined the levels of the proinflammatory cytokines, namely IL-1 β , IL-6, IL-10, and TNF- α by ELISA (Fig. 4). TNBS brought about an inverse in the protein expression of TNF- α , IL-1 β , and IL-6; however, it reduced the expression of IL-10. CLP-0611 treatment the reduced expression of IL-1 β , IL-6, and TNF- α ; however, CLP-0611 potently the increased expression of IL-10. Treatment with CLP-0611 (1×10^9 CFU/mice) inhibited expression of these cytokines by 57.7%, 77.6% and 57.1%, respectively, whereas it but restored IL10 expression to 75.3% relative to that of the normal control group.

3.3. Effect of *L. plantarum* CLP-0611 on expression of M1/M2 macrophage makers in TNBS-induced colitic mice

We measured the effect of CLP-0611 on the expression of IL-1 β , IL-10, TNF- α , CD206, ARG I, ARG II, and β -actin, which are markers of M1/M2 macrophages, in TNBS-induced colitic mice using real time-PCR (Fig. 5). Treatment with TNBS increased the expression of M1 macrophage markers IL-1 β , TNF- α , and ARG II, whereas it reduced expression of M2 macrophage markers IL-10 ARG I and CD206. CLP-0611 treatment blocked IL-1 β , TNF- α and ARG I expression, but increased IL-10, CD206, and ARG II expression. We also analyzed the effect of CLP-0611 on M1/M2 macrophage marker expression in TNBS-induced colitic mice using an immunohistological exam. TNBS significantly induced the expression of a M1 marker, CD86, but decreased the expression of CD206, which is a M2 marker. However, CLP0611 treatment significantly inhibited TNBS-induced CD86 expression, but increased CD206 expression.

4. Discussion

Acute and chronic inflammations are highly regulated by the release of cytokines and the transmigration of lymphocytes, neutrophils, and monocytes from the blood to the injured tissue [7,23]. Particularly, persistent and excessive chronic inflammation causes progressive damages in the body, resulting in a variety of diseases, such as colitis. Pro-inflammatory cytokines IL-1 β and TNF- α are activated via NF- κ B, and these cytokines also activate NF- κ B [24]. These inflammatory responses mainly proceed through signaling pathways [25]. Therefore, regulating the expression of these inflammatory cytokines should be beneficial in controlling chronic inflammatory diseases such as colitis [19,26]. To regulate the inflammatory reaction, prebiotics and probiotics have been developed. Among the probiotics, LAB suppress pathogen growth, restore gut microbiota disturbance [11], and ameliorate IBD [14,16,18,27]. However, the anti-colitic and macrophage-polarizing effects of LAB, except for *L. casei* G-101, have not been extensively investigated. Therefore, in the present study, we screened TNF- α expression-inhibiting LAB. The isolated LAB CLP-0611 did not influence TNF- α expression in resting macrophages, but potently inhibited TNF- α expression in LPS-stimulated macrophages. CLP-0611 also inhibited IL-1 β and IL-6 expression, whereas it activated NF- κ B and AP-1. CLP-0611 ameliorated TNBS-induced colon shortening, MPO activity

Table 1
Real time-polymerase chain reaction primers of polarization markers.

Molecule	Forward primer sequence	Reverse primer sequence
IL-1 β	5'-AACCTGCTGGTGTGTGACGT TC-3' (22mer)	5'-CAGCACGAGGCTTTTGTGTGT-3' (22mer)
IL-10	5'-ATGCTGCTGCTCTTACTGA CTG-3' (23mer)	5'-CCCAAGTAACCTTAAAGTCCTGC-3' (24mer)
TNF- α	5'-TCTTCTCATTCTGCTTG TGG-3' (21mer)	5'-GGTCTGGGGCATAGAACTGA-3' (20mer)
CD206	5'-CAGCGGTGGCAGTGGA-3' (17mer)	5'-CAGCTGATGGACTTCCTGGTAAG-3' (23mer)
Arginase I	5'-CAGAAGAATGGAAGAGTC AG-3' (20mer)	5'-CAGATATGCAGGAGTACC-3' (20mer)
Arginase II	5'-TGATTGGCAAAAGGCAGA GG-3' (20mer)	5'-CTAGGAGTAGGAAGGTGGTC-3' (20mer)
β -actin	5'-GTGCTATGTTGCTCTAGACT- 3' (20mer)	5'-CACAGGATTCATACCAAG-3' (20mer)

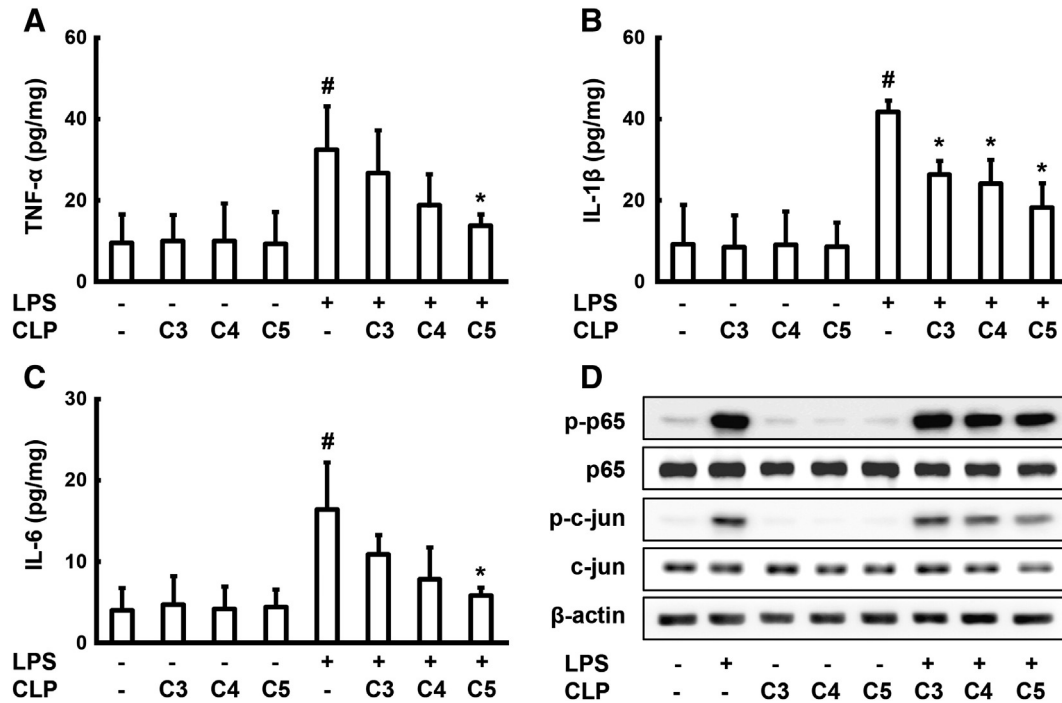


Fig. 1. Effect of *L. plantarum* CLP-0611 on the expression of TNF-α (A), IL-1β (B) and IL-6 (C) and the activation of NF-κB and AP1 (D) in LPS-stimulated peritoneal macrophages. The peritoneal macrophages (1×10^6 /well) were treated with 50 ng/mL LPS in the absence or presence of *L. plantarum* CLP-0611 (1×10^3 , 1×10^4 , 1×10^5 CFU/well) for 20 h. Levels of TNF-α, IL-1β, and IL-6 in culture supernatants were measured by ELISA. NF-κB (p65 and p-p65), AP1 (c-jun and p-c-jun) and β-actin were analyzed by immunoblotting. CON, normal control; LPS, treated with LPS alone; C3, treated with 1×10^3 CFU of *L. plantarum* CLP-0611 and LPS; C4, treated with 1×10^4 CFU of CLP-0611 and LPS; C5, treated with 1×10^5 CFU of CLP-0611 and LPS. CLP-0611 heated in boiling water bath for 10 min was used. Enzyme activity values are expressed as the mean \pm S.D. ($n = 3$). [#]Significantly different compared to the normal control group ($P < 0.05$). ^{*}Significantly different compared to the group treated with LPS alone ($P < 0.05$).

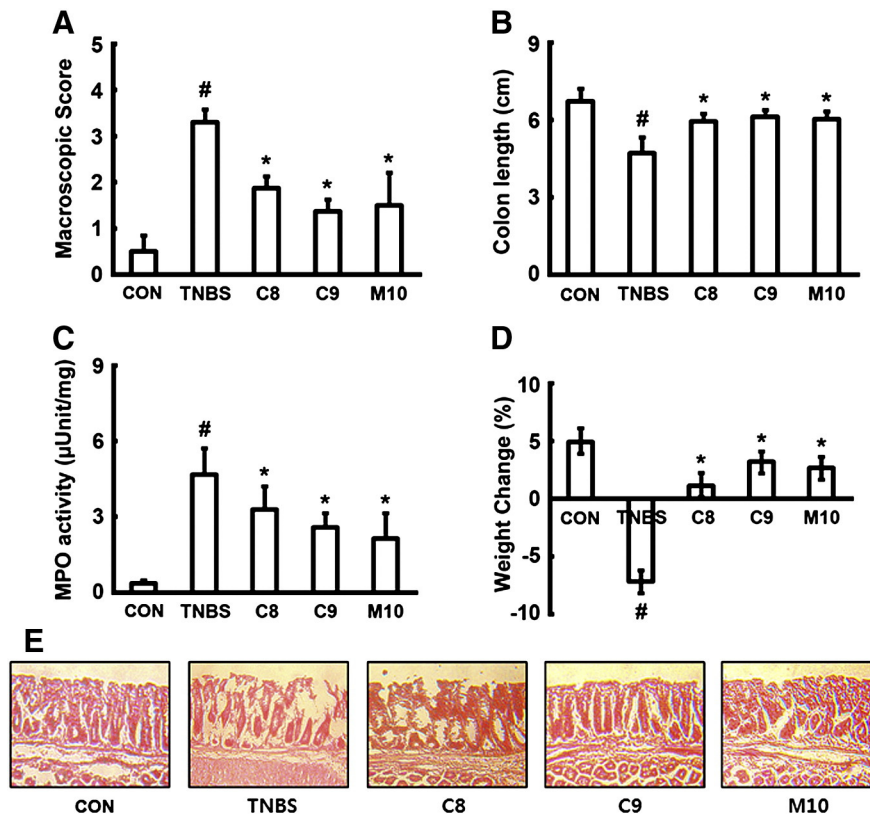


Fig. 2. Effect of *L. plantarum* CLP-0611 on macroscopic disease (A), colon length (B), colonic myeloperoxidase activity (C), body weight (D) and colonic histology (E) in TNBS-induced colitic mice. TNBS, except in the normal control group (CON, treated with vehicle alone), was intrarectally administered in the TNBS, C8, C9 and M10 groups. The test agents (TNBS, saline alone; C8, 1×10^8 CFU/mouse of *L. plantarum* CLP-0611 with TNBS; C9, 1×10^9 CFU/mouse of CLP-0611 with TNBS; M10, 10 mg/kg mesalazine with TNBS) were orally administered for 3 days after TNBS treatment. The mice were anesthetized and sacrificed 20 h after the final administration of LAB. All values are expressed as the mean \pm S.D. ($n = 6$). [#]Significantly different compared to normal control group ($P < 0.05$). ^{*}Significantly different compared to the group treated with TNBS alone ($P < 0.05$).

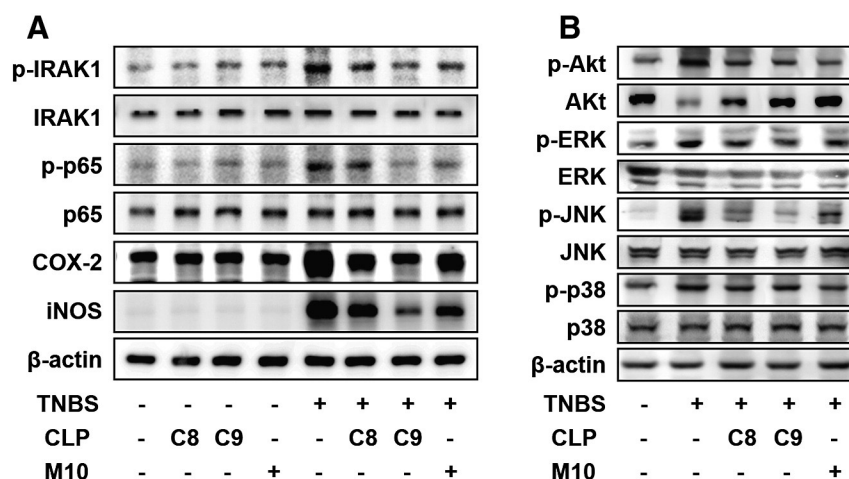


Fig. 3. Effect of *L. plantarum* CLP-0611 on NF-κB (A) and MAPK activation (B) in TNBS-induced colitic mice. TNBS, except in the normal control group (CON, treated with saline), was intrarectally administered in the TNBS, C8, C9 and M10 groups. The test agents (TNBS, saline alone; C8, 1×10^8 CFU/mouse of *L. plantarum* CLP-0611 with TNBS; C9, 1×10^9 CFU/mouse of CLP-0611 with TNBS; M10, 10 mg/kg mesalazine with TNBS) were orally administered for 3 days after TNBS treatment. The mice were anesthetized and sacrificed 20 h after the final administration of CLP-0611. The protein levels were measured by immunoblotting.

and proinflammatory cytokine expression, and NF-κB activation in mice. CLP-0611 also inhibited the activation of MAPKs, which are phosphorylated by TAK1 in the TLR4/NF-κB pathway [28], in TNBS-induced colitic mice. In addition, *L. plantarum* K8 lipoteichoic acid also inhibited TNF-α-stimulated and *Shigella flexneri* peptidoglycan-induced inflammation via the NF-κB signal pathway in HT-29, a human colon carcinoma cell line [29,30]. These results suggest that CLP-0611

inhibits inflammation by regulating the canonical TLR/NF-κB signaling pathway. Additionally, CLP-0611 inhibited the expression of IL-1β, TNF-α, and ARG II, which are M1 macrophage markers [29], but induced the expression of IL-10 ARG I and CD206, which are M2 macrophage markers. Thus, these results suggest that CLP-0611 induces the polarization of M1 to M2-like macrophages in TNBS-induced colitic mice. Furthermore, CLP-0611 might be capable of restoring a series of immune

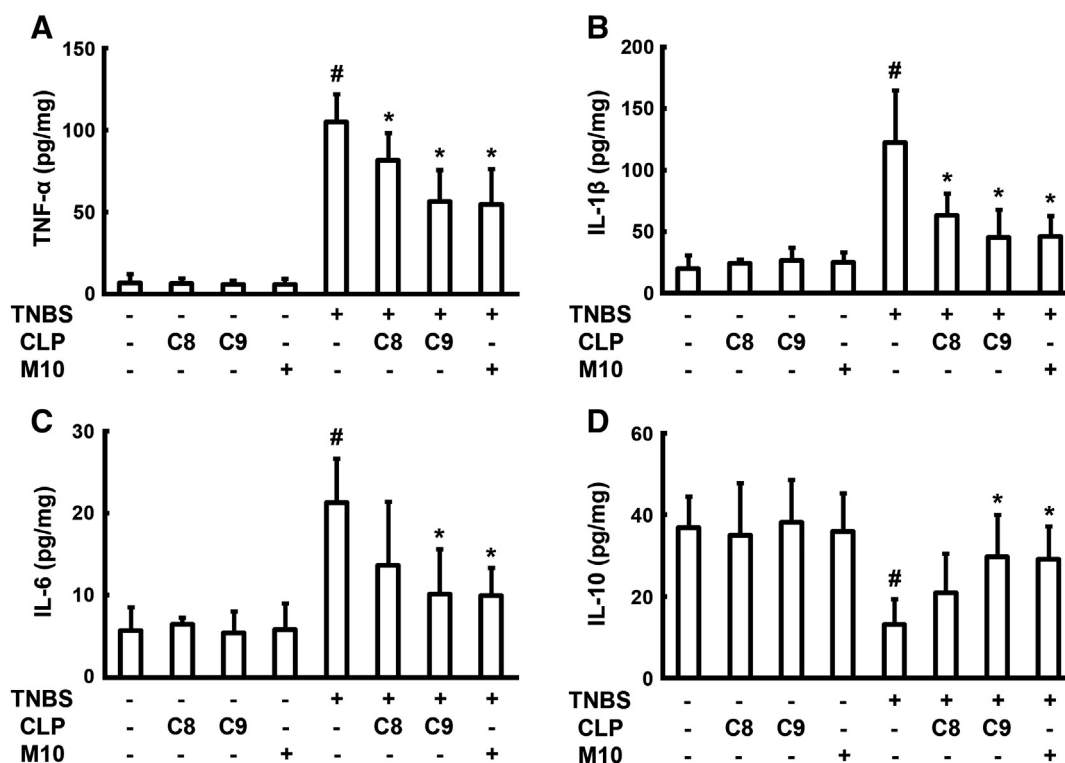


Fig. 4. The effect of *L. plantarum* CLP-0611 on the expression of proinflammatory cytokines TNF-α (A), IL-1β (B), and IL-6 (C) and anti-inflammatory cytokine IL-10 (D) in TNBS-induced colitic mice. TNBS, except in the normal control group (CON, treated with saline), was intrarectally administered in the TNBS, C8, C9 and M10 groups. The test agents [TNBS, vehicle (saline) alone; C8, 1×10^8 CFU/mouse of *L. plantarum* CLP-0611 with TNBS; C9, 1×10^9 CFU/mouse of CLP-0611 with TNBS; M10, 10 mg/kg mesalazine with TNBS] were orally administered for 3 days after TNBS treatment. The mice were anesthetized and sacrificed 20 h after the final administration of CLP-0611. Colonic cytokine levels were measured using ELISA. All values are expressed as the mean \pm S.D. ($n = 6$). #Significantly different compared to the normal control group ($P < 0.05$). *Significantly different compared to the group treated with TNBS alone ($P < 0.05$).

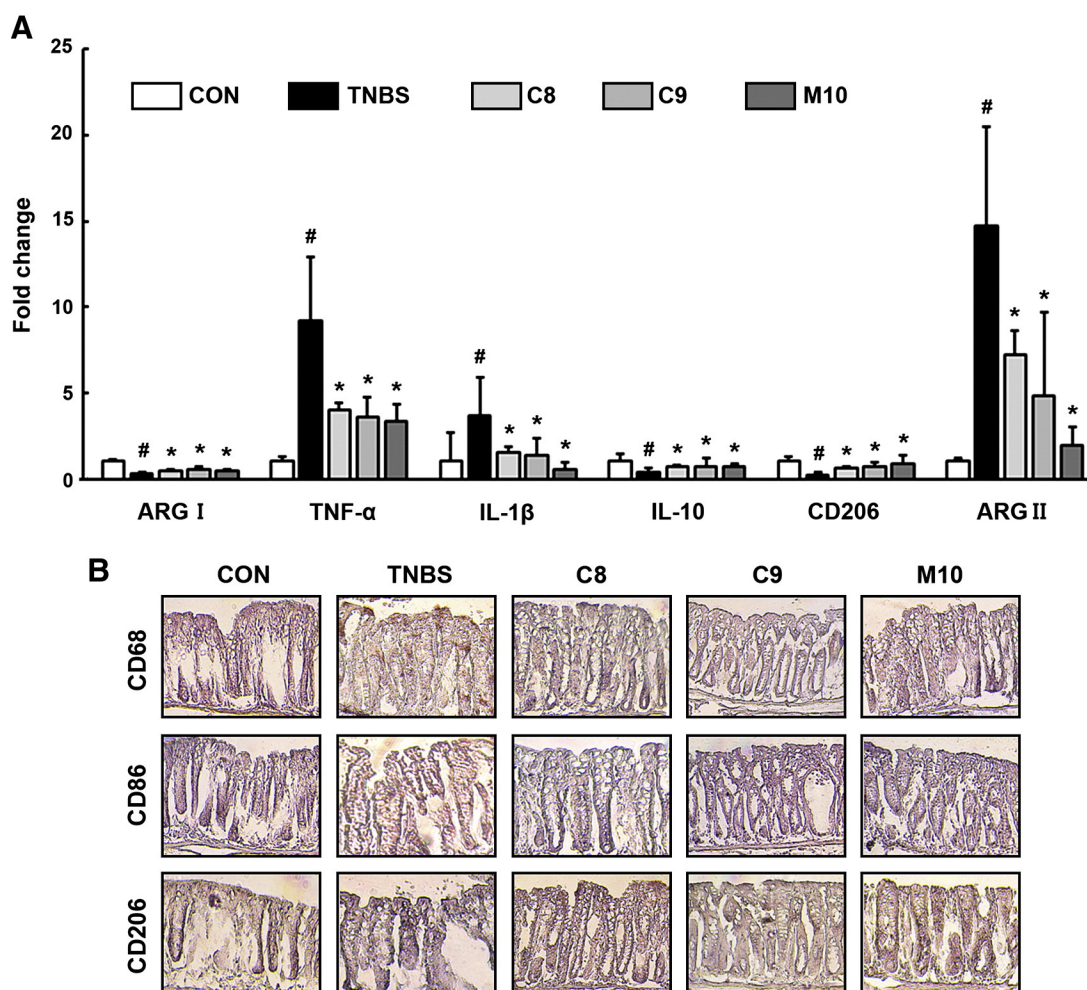


Fig. 5. Effect of *L. plantarum* CLP-0611 on expression of M1/M2 macrophage polarization markers in TNBS-induced colitic mice. The expression of macrophage polarization markers were measured by real-time PCR (A) and immunostaining (B). TNBS, except in the normal control group (CON, treated with saline alone), was intrarectally administered in the TNBS, C8, C9 and Me groups. The test agents [TNBS, vehicle (saline) alone; C8, 1×10^8 CFU/mouse of *L. plantarum* CLP-0611 with TNBS; C9, 1×10^9 CFU/mouse of CLP-0611 with TNBS; M10, 10 mg/kg mesalazine with TNBS] were orally administered for 3 days after TNBS treatment. The mice were anesthetized and sacrificed 20 h after the final administration of CLP-0611. Levels of colonic TNF- α , IL-1 β , IL-10, ARG I, ARG2 and CD206 were measured by real time-PCR. Colonic CD68, CD86, and CD206 were detected by immunostaining. All values are expressed as the mean \pm S.D. ($n = 6$). #Significantly different compared to the normal control group ($P < 0.05$). *Significantly different compared to the group treated with TNBS alone ($P < 0.05$).

responses caused by the inflammation process by polarizing M1 to M2-like macrophages. Its macrophage-polarizing effect was comparable to that of the previously reported G101 [19].

Based on these findings, CLP-0611 inhibits TLR-linked NF- κ B and MAPK signaling pathways and polarize M1 to M2-like macrophages, thus ameliorating colitis.

Acknowledgement

This study was supported by grants from the Bio & Medical Technology Development Program (2013M3A9B6076413) of the National Research Foundation (NRF) funded by the Korean government (MSIP).

References

- [1] Neuman MG, Nanau RM. Inflammatory bowel disease: role of diet, microbiota, life style. *Transl Res* 2012;160:29–44.
- [2] Shanahan F. Gut flora in gastrointestinal disease. *Eur J Surg Suppl* 2002;587:47–52.
- [3] Rafii F, Ruseler-Van Embden JG, van Lieshout LM. Changes in bacterial enzymes and PCR profiles of fecal bacteria from a patient with ulcerative colitis before and after antimicrobial treatments. *Dig Dis Sci* 1999;44:637–42.
- [4] Lee IA, Bae EA, Hyun YJ, Kim DH. Dextran sulfate sodium and 2,4,6-trinitrobenzene sulfonic acid induce lipid peroxidation by the proliferation of intestinal gram-negative bacteria in mice. *J Inflamm (Lond)* 2010;7(7).
- [5] Chandran P, Satthaporn S, Robins A, Eremin O. Inflammatory bowel disease: dysfunction of GALT and gut bacterial flora (I). *Surgeon* 2003;1:63–75.
- [6] Benno P, Leijonmarck CE, Monsén U, Uribe A, Midtvedt T. Functional alterations of the microflora in patients with ulcerative colitis. *Scand J Gastroenterol* 1993;28:839–44.
- [7] Aderem A, Ulevitch RJ. Toll-like receptors in the induction of the innate immune response. *Nature* 2000;406:782–7.
- [8] Lee IA, Park YJ, Joh EH, Kim DH. Soyasaponin Ab ameliorates colitis by inhibiting the binding of lipopolysaccharide (LPS) to Toll-like receptor (TLR)4 on macrophages. *J Agric Food Chem* 2011;59:13165–72.
- [9] Sartor RB. Therapeutic manipulation of the enteric microflora in inflammatory bowel diseases: antibiotics, probiotics and prebiotics. *Gastroenterology* 2004;126:1620–33.
- [10] Sanz Y, Nadal I, Sánchez E. Probiotics as drugs against human gastrointestinal infections. *Recent Pat Antiinfect Drug Discov* 2007;2:148–56.
- [11] Collins MP, Gibson GR. Probiotics, prebiotics, and synbiotics: approaches for modulating the microbial ecology of the gut. *Am J Clin Nutr* 1999;69:s1052–7.
- [12] Aggarwal J, Swami G, Kumar M. Probiotics and their effects on metabolic diseases: an update. *J Clin Diagn Res* 2013;7:173–7.
- [13] Romeo J, Nova E, Wärmberg J, Gómez-Martínez S, Díaz Ligia LE, Marcos A. Immunomodulatory effect of fibres, probiotics and synbiotics in different life-stages. *Nutr Hosp* 2010;25:341–9.
- [14] Peran L, Sierra S, Comalada M, Lara-Villoslada F, Bailon E, Nieto A, et al. A comparative study of the preventative effects exerted by two probiotics, *Lactobacillus reuteri* and *Lactobacillus fermentum*, in the trinitrobenzenesulfonic acid model of rat colitis. *Br J Nutr* 2007;97:96–103.
- [15] Daniel C, Poirat S, Goudercourt D, Dennin V, Leyer G, Pot B. Selecting lactic acid bacteria for their safety and functionality by use of a mouse colitis model. *Appl Environ Microbiol* 2006;72:5799–805.

- [16] Chung YW, Choi JH, Oh TY, Eun CS, Han DS. *Lactobacillus casei* prevents the development of dextran sulfate sodium-induced colitis in Toll-like receptor 4 mutant mice. *Clin Exp Immunol* 2007;151:182–9.
- [17] Peran L, Camuesco D, Comalada M, Bailon E, Henriksson A, Xaus J, et al. A comparative study of the preventative effects exerted by three probiotics, *Bifidobacterium lactis*, *Lactobacillus casei* and *Lactobacillus acidophilus*, in the TNBS model of rat colitis. *J Appl Microbiol* 2007;103:836–44.
- [18] Lee IA, Bae EA, Lee JH, Lee H, Ahn YT, Huh CS, et al. *Bifidobacterium longum* HY8004 attenuates TNBS-induced colitis by inhibiting lipid peroxidation in mice. *Inflamm Res* 2010;59:359–68.
- [19] Jang SE, Hyam SR, Han MJ, Kim SY, Lee BG, Kim DH. *Lactobacillus brevis* G-101 ameliorates colitis in mice by inhibiting NF- κ B, MAPK and AKT pathways and by polarizing M1 macrophages to M2-like macrophages. *J Appl Microbiol* 2013;115: 888–96.
- [20] Joh EH, Lee IA, Jung IH, Kim DH. Ginsenoside Rb1 and its metabolite compound K inhibit IRAK-1 activation — the key step of inflammation. *Biochem Pharmacol* 2011;82:278–86.
- [21] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:248–54.
- [22] Kim KA, Gu W, Lee IA, Joh EH, Kim DH. High fat diet-induced gut microbiota exacerbates inflammation and obesity in mice via the TLR4 signaling pathway. *PLoS One* 2012;7:e47713.
- [23] Johnson LN, Koval M. Cross-talk between pulmonary injury, oxidant stress, and gap junctional communication. *Antioxid Redox Signal* 2009;11:355–67.
- [24] Collins T, Read MA, Neish AS, Whitley MZ, Thanos D, Maniatis T. Transcriptional regulation of endothelial cell adhesion molecules: NF- κ B and cytokine inducible enhancers. *FASEB J* 1995;9:899–909.
- [25] David H, Masayuki F, Yasmin GH, John PS, Tyrlee G, Junsuke M, et al. Toll-like receptor 4 differentially regulates epidermal growth factor-related growth factors in response to intestinal mucosal injury. *Lab Invest* 2010;90:1295–305.
- [26] Kawaguchi K, Matsumoto T, Kumazawa Y. Effects of antioxidant polyphenols on TNF-alpha-related diseases. *Curr Top Med Chem* 2011;11:1767–79.
- [27] Lee JH, Lee B, Lee HS, Bae EA, Lee H, Ahn YT, et al. *Lactobacillus suntoryeus* inhibits pro-inflammatory cytokine expression and TLR-4-linked NF-kappaB activation in experimental colitis. *Int J Colorectal Dis* 2009;24:231–7.
- [28] Wei J, Feng J. Signaling pathways associated with inflammatory bowel disease. *Recent Pat Inflamm Allergy Drug Discov* 2010;4:105–17.
- [29] Ambarus CA, Krausz S, van Eijk M, Hamann J, Radstake TR, Reedquist KA, et al. Systematic validation of specific phenotypic markers for in vitro polarized human macrophages. *J Immunol Methods* 2012;375:196–206.
- [30] Kim H, Jung BJ, Jung JH, Kim JY, Chung SK, Chung DK. *Lactobacillus plantarum* lipoteichoic acid alleviates TNF- α -induced inflammation in the HT-29 intestinal epithelial cell line. *Mol Cells* 2012;33:479–86.