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A component of polysaccharide peptidoglycan complex on *Lactobacillus* induced an improvement of murine model of inflammatory bowel disease and colitis-associated cancer

S. Matsumoto,[1] T. Hara,[1]
M. Nagaoka,[1] A. Mike,[1]
K. Mitsuyama,[2] T. Sako,[1]
M. Yamamoto,[1] S. Kado[1] and
T. Takada[1]

[1]*Yakult Central Institute for Microbiological Research, Tokyo, Japan, and* [2]*Department of Gastroenterology, Kurume University School of Medicine, Fukuoka, Japan*

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Correspondence: Dr S. Matsumoto, PhD, Yakult Central Institute for Microbiological Research, 1796 Yaho, Kunitachi, Tokyo 186-8650, Japan.
Email: satoshi-matsumoto@yakult.co.jp
Senior author: Satoshi Matsumoto

Summary

Interleukin-6 (IL-6)/signal transducer and activator of transcription 3 (STAT3) signals play key roles in the pathogenesis of inflammatory bowel disease (IBD). We previously described that both intact cells and a cell wall-derived polysaccharide–peptidoglycan complex (PSPG) in a strain of lactobacillus [*Lactobacillus casei* Shirota (LcS)] inhibited IL-6 production in lipopolysaccharide (LPS)-stimulated lamina propria mononuclear cells (LPMCs) isolated from murine IBD. Diets with LcS improve murine IBD by suppression of IL-6 synthesis in LPMCs. Moreover, LcS supplementation with fermented milk ameliorates disease activity in patients with active ulcerative colitis. Here, we focused on the specific roles of PSPG in LcS concerning their anti-inflammatory actions. PSPG derived from LcS, and no other strain of lactobacilli, inhibited IL-6 production in LPS-stimulated murine IBD LPMCs. Purified PSPG-I from LcS inhibited IL-6 synthesis in LPS-stimulated murine IBD LPMCs through the inhibition of nuclear factor-κB. The anti-IL-6 action of LcS PSPG was abrogated by masking with monoclonal anti-PSPG-I. Furthermore, PSPG-I-negative *L. casei* strains (PSPG-I-negative mutant LcS: $LC^{\Delta PSPG-I}$, *L. casei* ATCC 334) did not inhibit IL-6 production. Finally, we confirmed the effects of PSPG-I on LcS in the models of both IBD and colitis-associated cancer (CAC). In the IBD model, ingestion of LcS improved ileitis and inhibited activation of IL-6/STAT3 signaling, while ingestion of the $LC^{\Delta PSPG-I}$ strain did not. In the CAC model, treatment with LcS, but not the $LC^{\Delta PSPG-I}$ strain, showed tumour-suppressive effects with an inhibition of IL-6 production in the colonic mucosa. These results suggested that a specific polysaccharide component in an *L. casei* strain plays a crucial role in its anti-inflammatory actions in chronic intestinal inflammatory disorders.

Keywords: colitis-associated cancer; inflammatory bowel disease; interleukin-6; *Lactobacillus*; polysaccharide–peptidoglycan complex

Introduction

Inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), is a chronic inflammatory disorder of the intestine, and its pathogenesis is not well understood.[1,2] The disease is thought to be caused by the non-physiological response of the mucosal immune system towards commensal bacteria.[3,4] Various IBD models in rodents do not manifest intestinal inflammation under germ-free conditions.[5–7] Interleukin-6 (IL-6) is a multifunctional cytokine that modulates various immune responses.[8,9] It has been described that IL-6/signal transducer and activator of transcription 3 (STAT3) signaling is activated in the intestinal mucosa in murine models of IBD and colitis-associated cancer (CAC).[10,11] Moreover, negative regulation of this signaling pathway inhibits IBD in rodents.[10] Therefore, this signaling pathway may play a critical role in the pathogenesis of both IBD and CAC, and targeting this pathway may be useful for therapeutic management.

Several strains of lactobacilli and bifidobacteria have been used as probiotics, and numerous such probiotic strains have been tested for the prevention and treatment of intestinal inflammatory disorders, such as infectious

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colitis and IBD. For example, a strain of *Bifidobacterium breve* in a fermented milk product induced relief from disease symptoms in both a rodent model of IBD and in patients with UC.[12,13] In a separate study, the probiotic preparation VSL#3 (a mixture of eight different lactic acid bacteria) was shown to prevent pouchitis. The mechanisms underlying the beneficial effects of probiotics remain unknown. However, probiotics may, at least in part, act by modifying innate and adaptive immunity. Indeed, culture supernatants of the above probiotic bifidobacterium strain could repress interleukin-8 (IL-8) secretion in tumour necrosis factor- α (TNF-α)-stimulated HT-29 cells.[14]

Lactobacillus casei strain Shirota (LcS) is one such probiotic strain and is commercially available as a health food supplement in several countries. Treatment with LcS can induce improvement in murine models of inflammatory disorders such as arthritis, type I diabetes and systemic lupus.[15–17] Recently, we confirmed that treatment with heat-killed LcS induced improvement of both murine chronic colitis induced by dextran sodium sulphate (DSS) and ileitis in SAMP1/Yit mice accompanied by the down-regulation of IL-6 production in lamina propria mononuclear cells (LPMCs).[18] Moreover, both LcS supplementation as fermented milk suppressed disease activity in patients with active UC in a pilot study.[19] The mechanisms underlying the anti-inflammatory actions of LcS are still not understood. However, we have confirmed that both LcS and the cell wall-derived polysaccharide–peptidoglycan complex (PSPG) inhibited IL-6 production in lipopolysaccharide (LPS)-stimulated LPMCs isolated from a murine chronic IBD model.[18] In these experiments, down-regulation of the translocation of nuclear factor-κB (NF-κB) in the nucleus of target cells was caused by Toll-like receptor (TLR)2- and TLR4-independent pathways.[18] In a separate study, it was also reported that PSPG was an important component of LcS with regard to the appearance of the anti-infectious activity against *Listeria monocytogenes* and *Pseudomonas aeruginosa*.[20]

In the present study, we aimed to elucidate the specific mechanisms of inhibitory action of the PSPG components in LcS on the production of IL-6 in LPS-stimulated macrophages. Moreover, we confirmed the roles of these PSPG components in terms of their anti-inflammatory actions in both IBD and CAC in murine models *in vivo*.

Materials and methods

Animals

Female BALB/c mice (8 weeks of age) were purchased from SLC (Shizuoka, Japan). Mice were maintained under specific pathogen-free (SPF) conditions during the experiments. SAMP1/Yit mice (15 weeks of age) were bred under SPF conditions at our institute.[21] C3H severe combined immunodeficiency (SCID) mutant mice (C3H/SmnC Prkdc scid/J, 10 weeks of age) were purchased from Jackson Laboratory (Bar Harbor, ME) and were maintained in the barrier systems in the animal facility of our institute.

Lactobacilli

LcS (YIT 9029; Yakult Honsha, Co., Ltd, Tokyo, Japan), *Lactobacillus rhamnosus* ATCC 53103 and *L. casei* ATCC 334 were used in this study. PSPG-I-negative mutant LcS strains ($LC^{\Delta PSPG\text{-}I}$-A, -B, -C and -D), which lacked the PSPG-I component, were also used.[22,23]

Preparation of cell derivatives of LcS

Heat-killed strains of lactobacilli were prepared.[24] The isolation of PSPG has been described previously.[24] In brief, the heat-killed cells were exhaustively digested with *N*-acetylmuramidase SG (Seikagaku corporation, Tokyo, Japan), followed by treatment with DNase, RNase and trypsin. The digest was then dialyzed against water and lyophilized. PSPG-I and PSPG-II components in LcS were separated as described previously.[24] In brief, the PSPG fraction was subjected to gel filtration, and the hexose-containing fractions obtained were designated as type I PSPG (PSPG-I) and type II PSPG (PSPG-II). The polysaccharide moieties (PS-I and PS-II) were prepared from PSPG-I and PSPG-II by treatment with 47% hydrogenfluoric acid at 4° for 20 hr. Hydrogenfluoric acid treatment hydrolyzes the phosphodiester bonds between the polysaccharide and the peptidoglycan moiety.[24] The hydolyzate was then dialysed against water and lyophilized. The polysaccharide was obtained using this procedure.

Induction of chronic DSS colitis

Chronic DSS colitis was induced in BALB/c mice as described previously.[25] In brief, 10-week-old mice were subjected to 4% DSS (molecular weight = 40 000; MP Biomedicals, LLC, Solon, OH) dissolved in drinking water. Chronic colitis was induced by four administration cycles; each cycle comprised a regimen of 4% DSS for 7 days followed by drinking water without DSS for the next 7 days.

In vitro *IL-6 inhibition assay*

Colonic LPMCs were prepared from colitis-induced BALB/c mice by collagenase dissociation (Collagenase-Yakult S; Yakult Honsha) as previously described.[26] Colonic LPMCs, murine macrophage RAW264.7 cells and peripheral blood mononuclear cells (PBMCs) ($2{\cdot}0 \times 10^5$ cells/96-well plate) isolated from the patients with IBD were cultured in 10% fetal calf serum (FCS)/10 μM Hepes/penicillin–streptomycin/RPMI medium (complete

RPMI; Sigma, St Louis, MO) under 5% CO_2 at 37°. To assess the inhibitory action of heat-killed lactobacilli or PSPG on the production of IL-6 in LPS-stimulated colonic LPMCs, RAW264.7 cells and IBD PBMCs ($2{\cdot}0 \times 10^5$ cells/well), 100 ng/ml of LPS (Sigma, St Louis, MO) was added to the cultures with or without various doses of LcS, or mutant strains of LcS, PSPG, PSPG-I, PSPG-II, PS-I, PS-II or peptidoglycan (PG) components and the cells were cultured for 24 hr. After culture, the supernatants were collected and stored at −84° until required for enzyme-linked immunosorbent assay (ELISA) analysis to determine the IL-6 concentration. Data represent the percentage inhibition of IL-6 synthesis in the cultures stimulated with LPS plus various doses of LcS compared with those stimulated with only LPS. The inhibitory effects among other strains of lactobacillus on IL-6 production in LPS-stimulated colonic LPMCs were also determined using the same protocol.

Flow cytometric analysis

Heat-killed lactobacilli were dissolved in 1% FCS containing phosphate-buffered saline (PBS) (10 μg/ml). After centrifugation, the pellets were incubated with $F(ab')_2$-fragmented monoclonal antibody (mAb) raised against a specific mAb of PSPG-I of LcS for 30 min at 4°. The $F(ab')_2$ fragments were prepared by using an immunoglobulin M (IgM) fragmentation kit (Pierce Biotechnology, Inc., Rockford, IL). After the cells were washed with FCS/PBS, they were treated with fluorescein isothiocyanate (FITC)-conjugated mAb against the mouse Igκ chain for 30 min at 4°. The cells were washed with FCS/PBS and analyzed using an EPICS EL cell analyzer (Beckmann-Coulter, Inc., Fullerton, CA).

PSPG-I mAb inhibition assay

The PSPG complex isolated from several strains of lactobacilli dissolved in carbonate buffer (pH 9·5) and then coated on 96-well flat-bottom culture plates overnight at 4°. After several washes with complete RPMI, the wells were incubated with or without $F(ab')_2$ PSPG-I mAb (10 μg/ml) for 3 hr at 37°. The wells were then washed three times with complete RPMI. RAW264.7 cells ($2{\cdot}0 \times 10^5$ cells) were cultured in PSPG-coated wells with or without treatment with PSPG-I mAb. LPS (100 ng/ml) was subsequently added to the wells, and the culture supernatants were collected after 24 hr of culture. The amount of IL-6 in the culture supernatants was determined by ELISA as described in the following section.

ELISA

Murine monoclonal anti-IL-6 (clones MP5-20F3 and MP5-32C11) and human monoclonal anti-IL-6 (clones: 5IL-6 and 7IL-6) were purchased from BD PharMingen (Los Angeles, CA) and Pierce-Endogen (Woburn, MA). ELISA was performed as per the standard protocol recommended by the manufacturer.

Western blotting

Colonic LPMCs ($2{\cdot}0 \times 10^6$ cells) isolated from mice with induced chronic colitis were pretreated with PSPG isolated from LcS or *L. rhamnosus* ATCC 53103 and then subsequently stimulated with LPS (100 ng/ml). After 2, 4 or 8 hr of stimulation with LPS (100 ng/ml), the cells were washed with Hanks' balanced salt solution and treated with lysis buffer.[10] The supernatants of the cell lysate were stored at −80° prior to western blotting. In some experiments, RAW264.7 cells ($2{\cdot}0 \times 10^6$ cells) were treated with PSPG-I or PSPG-II; subsequently, these cells were stimulated with or without 100 ng/ml of LPS. After 3 and 6 hr, the cells were washed with Hanks' balanced salt solution and treated with lysis buffer using the methods described previously. In brief, 10 μg of protein was subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto a membrane (Immobilon-P; Millipore, Billerica, MA). Phospho-NF-κB or -IκB proteins were detected by immunoblotting using antibody to phospho-NF-κB or phospho-IκB (both polyclonal antibodies were purchased from Cell Signaling Technology, Beverly, MA) and enhanced chemiluminescence (ECL) plus a western blotting detection system (GE Health Sciences, Pittsburgh, PA). The signal was visualized using an LAS-3000 image analyzer (Fujifirm, Tokyo, Japan).

Nod2 mRNA analysis in RAW cells

RAW264.7 cells ($2{\cdot}0 \times 10^6$ cells) were stimulated with LPS (100 ng/ml), LcS, $LC^{\Delta PSPG\text{-}I}$, or *L. casei* ATCC 334. After 2, 4 and 6 hr of stimulation, the cells were harvested and total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA). Real-time quantitative reverse transcription–polymerase chain reaction (qRT-PCR) was performed using specific primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Nod2 (both from Qiagen GmbH, Hamburg, Germany) on an ABI-7500 (Applied Biosystems, Foster City, CA).

In vivo *effects of PSPG-I on chronic ileitis*

LcS or $LC^{\Delta PSPG\text{-}I}$ strains were cultured in MRS (de Man, Rogosa, Sharpe) broth for 12 hr. The cells were then washed twice with ice-cold PBS and resuspended in 1 ml of ice-cold PBS. Chronic ileitis was induced in C3H SCID mice by transferring $CD4^+$ αβ T cells from SAMP1/Yit mice, as previously described.[27] One week before the induction of ileitis, the SCID mice were

treated with 200 μl of the cell suspension of LcS or $LC^{\Delta PSPG-I}$ strains or with a gastric tube for 5 days once a week (approximately $5{\cdot}0 \times 10^8$ colony-forming units/mouse/day). Control mice were treated with PBS. Eight weeks after the induction of ileitis, the mice were killed and the severity of ileitis was compared among the groups ($n = 6$ in each group). Histological evaluation of ileitis in each group of mice was performed as previously described.[10]

In vivo *effects of PSPG-I on CAC*

CAC was induced in BALB/c mice as described previously with minor modifications.[28] In brief, chronic DSS colitis was induced in female BALB/c mice as described above.[25] Subsequently, the mice were treated with an additional five DSS cycles. LcS, *L. casei* ATCC 334 and $LC^{\Delta PSPG-I}$ strains were cultured, washed twice with PBS and resuspended in 1 ml of PBS. One week before the induction of CAC, the mice were treated with 200 μl of the cell suspension of LcS or $LC^{\Delta PSPG-I}$ strains via a gastric tube for 5 days once a week (approximately $5{\cdot}0 \times 10^8$ colony-forming units/mouse/day). Control mice were treated with PBS. After the final DSS treatment, the mice were killed, and the incidence and number of tumours were examined in each group ($n = 10$ in each group).

IL-6/STAT3 signaling in intestinal tissues in LcS-treated mice

Polyclonal antibodies against phospho-STAT3 or STAT1 were purchased from Cell Signaling Technology. Expression of phospho-STAT3 in ileal tissue extracts in each group of mice was examined by western blotting, as described above. The amounts of IL-6, suppressor of cytokine signaling 3 (SOCS3) and GAPDH mRNA in the ileal or colonic tissues were estimated by quantitative RT-PCR using specific primers on an ABI-7500 (Applied Biosystems). All primers were purchased from Qiagen (Qiagen GmbH, Hamburg, Germany). All data were calculated in relation to the housekeeping gene *GAPDH*. In some experiments, ileal cryosections were prepared. Immunofluorescence images of SOCS3 protein in the ileal tissues were obtained by confocal laser microscopy (LSM-500; Carl Zeiss GmbH, Oberkochen, Germany).

Statistics

All data were expressed as mean ± standard deviation (SD) and were evaluated by use of the Tukey or Tukey–Kramer test for multiple comparisons. The Student's *t*-test was used for the comparison of two independent groups. In all tests, P values of less than 0·05 were assumed to be statistically significant.

Results

PSPG derived from LcS, but not from *L. rhamnosus* ATCC 53103, inhibited IL-6 production in LPS-stimulated colitis-derived LPMCs

PSPG complex derived from *L. casei* ATCC 334 and *L. rhamnosus* ATCC 53103 did not inhibit the production of IL-6 in LPS-stimulated LPMCs isolated from chronic

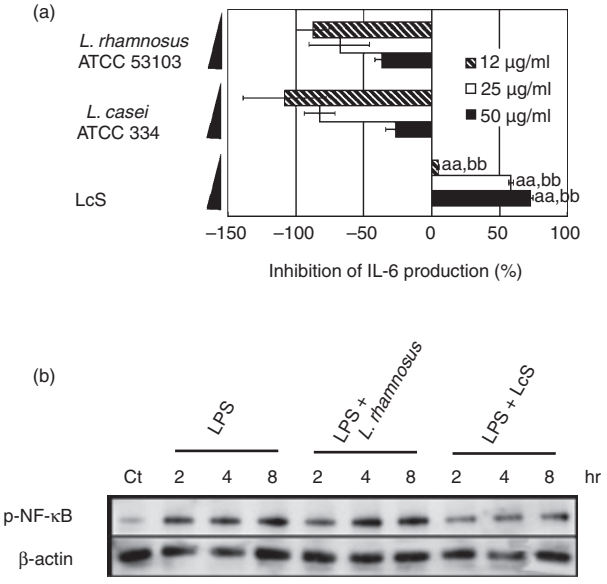

Figure 1. Inhibitory action of the polysaccharide–peptidoglycan complex (PSPG) isolated from *Lactobacillus casei* Shirota (LcS) on the production of interleukin (IL)-6 in lipopolysaccharide (LPS)-stimulated colonic lamina propria mononuclear cells (LPMCs) isolated from mice induced with chronic colitis (a). Colonic LPMCs were isolated from mice with induced chronic colitis and these cells ($2{\cdot}0 \times 10^5$ cells/well in 96-well culture plates) were stimulated with LPS (100 ng/ml) in the presence or absence of PSPG purified from lactobacillus strains. The interleukin-6 (IL-6) inhibition rate is shown, representing the percentage inhibition of IL-6 synthesis in the culture supernatants stimulated with LPS plus various doses of PSPG isolated from LcS, *L. casei* ATCC 334 or *Lactobacillus rhamnosus* ATCC 53103 compared to those stimulated with LPS only. Similar results were obtained from six independent experiments. Data represent the mean ± standard deviation (SD). Western blot analysis of phosphorylated nuclear factor-κB (p-NF-κB) expression in LPS-stimulated colonic LPMCs in the presence or absence of PSPG isolated from LcS or *L. rhamnosus* ATCC 53103 (b). Colonic LPMCs ($2{\cdot}0 \times 10^6$ cells/well) isolated from mice with induced chronic colitis were pretreated with PSPG isolated from LcS or *L. rhamnosus* ATCC 53103 and then subsequently stimulated with LPS (100 ng/ml). After 2, 4 or 8 hr of LPS stimulation, cell lysates were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and proteins were transferred onto Immobilon-P membrane. Western blotting was performed with antibody against phosphorylated NF-κB. Values (aa; $P < 0{\cdot}01$) differed significantly versus *L. casei* ATCC 334 treatment with the same doses. Values (bb; $P < 0{\cdot}01$) differed significantly versus *L. rhamnosus* ATCC 53103 treatments with the same doses. Ct, control.

colitis specimens induced in mice, whereas PSPG derived from LcS inhibited IL-6 production in LPS-stimulated colonic LPMCs in a dose-dependent manner (Fig. 1a). A similar result was obtained using an LPS-stimulated mouse macrophage cell line, RAW264.7 cells and PBMCs isolated from patients with UC (data not shown). Western blot analysis clearly showed that PSPG purified from LcS, but not from *L. rhamnosus* ATCC 53103, inhibited the phosphorylation of NF-κB in LPS-stimulated RAW cells (Fig. 1b).

Polysaccharide chain of PSPG-I played a critical role in inhibiting IL-6 production

In a previous report, Nagaoka *et al.* described that PSPG components of LcS are composed of two PSPG components, PSPG-I and PSPG-II.[24] Therefore, we purified PSPG-I, PSPG-II, PS-I and PS-II components, and then compared their inhibitory actions on IL-6 production in LPS-stimulated RAW cells. PSPG-I and PS-I both down-regulated IL-6 production in LPS-stimulated RAW cells. On the other hand, PSPG-II and PS-II did not inhibit IL-6 production (Fig. 2a). In western blotting analysis, the treatment of RAW cells with PSPG-I, but not with PSPG-II, inhibited the phosphorylation of both NF-κB and IκB after stimulation with LPS (Fig. 2b). Next, we examined the anti-IL-6 action of mutant LcS strains lacking the PSPG-I components in LPS-stimulated RAW cells. Flow cytometric analysis clearly showed that *L. casei* ATCC 334, *L. rhamnosus* ATCC 53103 and the mutant strains of LcS did not react with PSPG-I mAb (Fig. 2c). In accordance with the disappearance of the PSPG-I component from LcS, the anti-IL-6 action was completely abrogated in the mutant LcS strains (Fig. 2d).

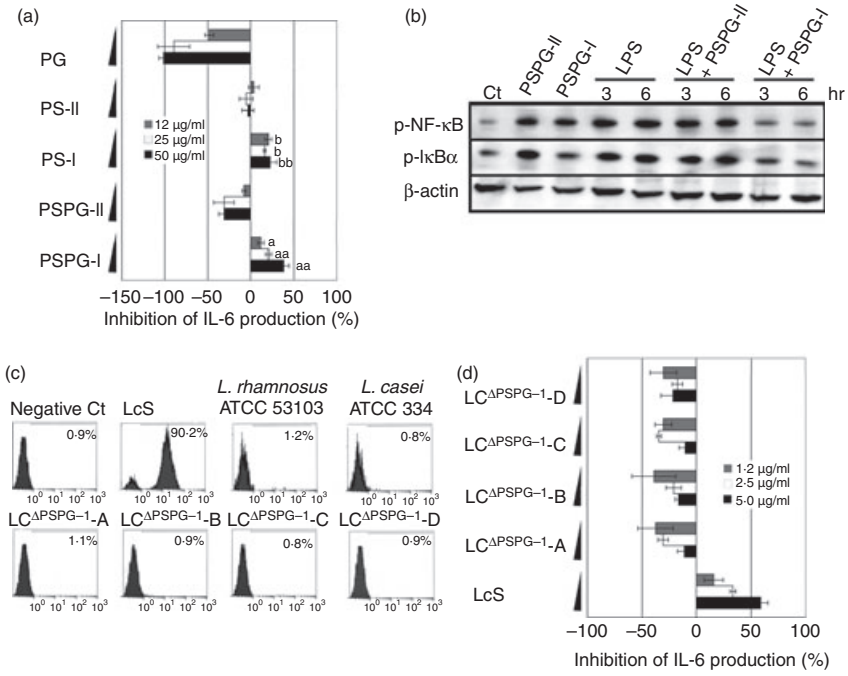

Figure 2. Inhibitory actions of polysaccharide–peptidoglycan complex (PSPG)-I and PSPG-II and of polysaccharide (PS)-I and PS-II isolated from *Lactobacillus casei* Shirota (LcS) on the production of interleukin-6 (IL-6) in lipopolysaccharide (LPS)-stimulated RAW cells. Data represent the mean ± standard deviation (SD) (a). To assess the inhibitory action of PSPG-I, PSPG-II, PS-I, PS-II or peptidoglycan (PG) components on the production of IL-6 in LPS-stimulated RAW cells, 100 ng/ml of LPS was added to the cultures ($2{\cdot}0 \times 10^5$ cells/well), with or without various doses of PSPG-I, PSPG-II, PS-I, PS-II or PG components, and the cells were cultured for 24 hr. After the culture, the supernatants were collected and the concentrations of IL-6 were determined using enzyme-linked immunosorbent assay (ELISA). (b) Western blot analysis of phosphorylated nuclear factor-κB (p-NF-κB) or phosphorylated IκBα (p-IκBα) expression in LPS-stimulated RAW cells in the presence or absence of PSPG-I or PSPG-II (b). Cell lysate was obtained from LPS-stimulated RAW cells ($2{\cdot}0 \times 10^6$ cells/well) in the presence or absence of PSPG-I or PSPG-II co-stimulation. Lysates were subjected to western blotting then probed using specific antibodies against p-NF-κB or p-IκBα. Flow cytometric analysis of the expression of PSPG-I on LcS and five PSPG-I-negative *L. casei* strains ($LC^{\Delta PSPG\text{-}I}$ A–D, *L. casei* ATCC 334 or *L. rhamnosus* ATCC 53130). control (Ct). (c) Inhibitory action of various doses of LcS or four PSPG-I-negative *L. casei* strains on the production of IL-6 in LPS-stimulated RAW cells. Data represent mean ± SD. (d) LPS (100 ng/ml) was added to the cultures ($2{\cdot}0 \times 10^5$ cells/well), with or without various doses of LcS or mutant LcS strains, and the cells were cultured for 24 hr. After the culture, the supernatants were collected and the concentrations of IL-6 were determined using ELISA. Similar results were obtained from two independent experiments. Values (aa; $P < 0{\cdot}01$, a; $P < 0{\cdot}05$) differ significantly versus PSPG-II treatment with the same doses. Values (bb; $P < 0{\cdot}01$, b; $P < 0{\cdot}05$) differ significantly versus PS-II treatment with the same doses.

PSPG-I mAb neutralized the inhibition of IL-6 production induced by PSPG

To confirm whether the anti-IL-6 action of PSPG is dependent on the internalization of the PSPG component by the activated macrophage, we examined the anti-IL-6 action of the immobilized PSPG component that was isolated from LcS. Immobilized PSPG components on the culture plate displayed an anti-IL-6 action similar to the soluble form of PSPG in LPS-stimulated RAW cells (Fig. 3). However, the immobilized PSPG on the microplate that was derived from *L. rhamnosus* ATCC 53103 or $LC^{\Delta PSPG-I}$ strains did not inhibit IL-6 production. Moreover, by masking PSPG-I in LcS PSPG coated with PSPG-I mAb, inhibition of the production of IL-6 in LPS-stimulated RAW cells was completely abrogated.

In vivo effect of PSPG-I in a chronic ileitis model

To examine the anti-inflammatory roles of PSPG-I *in vivo*, we administered cultured LcS or $LC^{\Delta PSPG-I}$ strains into models of mice with chronic ileitis. Histological scores of ileitis were repressed by treatment with LcS. However, the scores for the $LC^{\Delta PSPG-I}$ strains were comparable with those for the control (Fig. 4a). In accordance with the suppression of the histological scores, the amounts of IL-6 and SOCS3 mRNA in the ileal mucosa were lower in the mice treated with LcS than in the mice treated with $LC^{\Delta PSPG-I}$ strains or PBS (Fig. 4b). Both in control and $LC^{\Delta PSPG-I}$ strain-treated mice, ileal histology showed severe ileitis accompanied by infiltration of $CD4^+$ cells and class II major histocompatibility complex (MHC)-positive mononuclear cells in the lamina propria (Fig. 4c). Moreover, the expression of SOCS3 on ileal epithelial cells was pronounced in those groups. In contrast, ileitis in the mice treated with LcS was mild. Infiltration of the inflammatory cells and the induction of SOCS3 was repressed by treatment with LcS. Western blot analysis showed that the amounts of phospho-STAT3 proteins, but not of phospho-STAT1, in the ileal mucosa were lower in mice treated with LcS than in mice treated with $LC^{\Delta PSPG-I}$ strains or PBS (Fig. 4d).

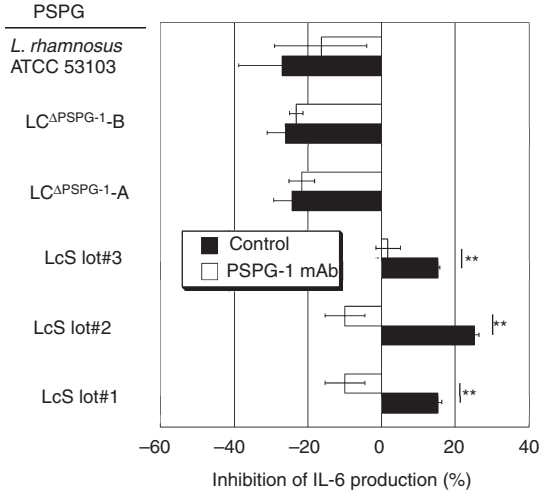

Figure 3. Masking of polysaccharide–peptidoglycan complex (PSPG)-I abrogated anti-interleukin-6 (IL-6) action of PSPG isolated from *Lactobacillus casei* Shirota (LcS) in lipopolysaccharide (LPS)-stimulated RAW264.7 cells. PSPG isolated from LcS, two PSPG-I-negative *L. casei* strains or *L. rhamnosus* ATCC 53103 were coated on the 96-well culture plate. After removing the unbound PSPG component, the plates were incubated with or without monoclonal antibody (mAb) raised against PSPG-I in LcS. After several washes with culture medium, RAW cells ($2{\cdot}0 \times 10^5$ cells/well) were stimulated with LPS, and the concentration of IL-6 in the culture supernatant was determined by enzyme-linked immunosorbent assay (ELISA). Values ($^{**}P < 0{\cdot}01$) differed significantly between each experimental group.

In vivo effect of PSPG-I in the CAC model

We also examined the anti-inflammatory effect of PSPG-I in CAC. During the induction of CAC, LcS or $LC^{\Delta PSPG-I}$ strains were ingested by mice with CAC, and their anti-CAC action was compared. As shown in Fig. 5(a), the incidence and number of tumours were repressed in mice treated with LcS, compared with those treated with $LC^{\Delta PSPG-I}$ strains and PBS. The tumour-suppressive effects of LcS were accompanied by a down-regulation of IL-6 and SOCS3 mRNA expression in the colonic tissues (Fig. 5b). There was no difference in TNF-α mRNA expression in the colonic tissues among the groups.

Nod2 mRNA induction by LcS

Recently, it had been reported that stimulation of Nod2 in macrophages with a muramyl dipeptide (MDP) component significantly decreased the production of pro-inflammatory cytokines.[29] Therefore, we examined the induction of Nod2 mRNA in RAW cells by stimulation with LcS or with PSPG-I-negative *L. casei* strains. As shown in Fig. 6, the concentration of Nod2 mRNA increased markedly after stimulation with LcS, compared with PSPG-I negative *L. casei* strains ($LC^{\Delta PSPG-I}$, *L. casei* ATCC 334).

Discussion

In previous studies, a probiotic *L. casei* strain, LcS, inhibited several murine experimental models of immune disorders, such as arthritis, type I diabetes, murine lupus and chronic IBD.[15–18] Furthermore, LcS-fermented milk ameliorated disease activity in UC patients in a pilot study.[19] In IBD models, we discovered that LcS and its intact cell wall component inhibited IL-6 production in LPS-stimulated LPMCs with chronic IBD. In addition, LcS inhibited the production of IL-6 in LPS-stimulated

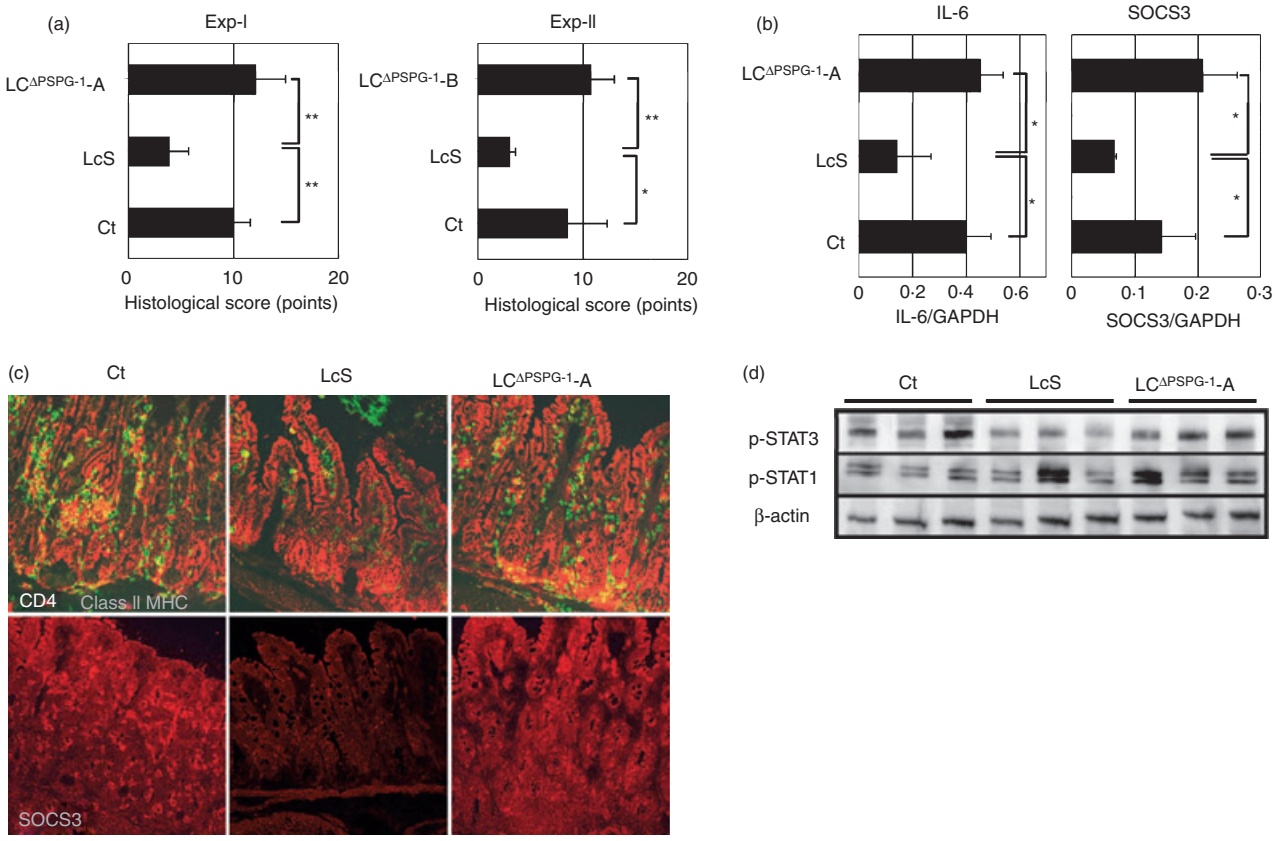

Figure 4. Anti-inflammatory actions of *Lactobacillus casei* Shirota (LcS) or polysaccharide–peptidoglycan complex (PSPG)-I-negative *L. casei* strains in an ileitis model. (a) Histological ileitis score and (b) quantitative reverse transcription–polymerase chain reaction (RT-PCR) analysis of interleukin-6 (IL-6) and suppressor of cytokine signaling 3 (SOCS3) mRNA expression in ileal tissues in severe combined immunodeficient (SCID) mutant mice with induced ileitis. The data were calculated in relation to the housekeeping gene *GAPDH*. (c) Ileitis was induced in C3H severe combined immunodeficiency (SCID) mice by injection with $CD4^+$ T cells isolated from SAMP1/Yit mice ($n = 6$ in each group). During the induction of ileitis in SCID mice, the mice were treated with LcS, PSPG-I-negative *L. casei* strains, or phosphate-buffered saline (PBS) for 5 days per week. After 8 weeks, the mice were killed and the severity of ileitis was compared. Confocal analysis of the infiltration of $CD4^+$ T cells (red), and the expression of class II major histocompatibility complex (MHC, green) and of SOCS3 proteins in ileitis-induced SCID in mice treated with LcS, PSPG-I negative *L. casei* strains, or PBS was analyzed. Original magnification: 100×. (d) Western blot analysis of phospho-STAT3 and phospho-STAT1 expression in ileal tissues in ileitis-induced SCID mice treated with LcS, PSPG-I-negative *L. casei* strains, or PBS. Similar results were obtained from two independent experiments. Values ($^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$) differed significantly among each experimental group.

PBMCs isolated from patients with IBD. Moreover, PSPG, the major soluble constituent of the intact cell wall, played a key role in the inhibitory effect on IL-6 production. In this study, we examined the mechanism of the anti-IL-6 action of LcS. We first compared the inhibitory effect of several PSPGs isolated from LcS or other strains of lactobacilli on the production of IL-6 in LPS-stimulated LPMCs isolated from mice with IBD. Then, we confirmed that PSPG derived from LcS, but not from the other strains of lactobacilli, inhibited the production of IL-6. It has already been described that PSPG in LcS comprises two components, namely PSPG-I and PSPG-II. Therefore, we confirmed the effect of these two components on IL-6 production in LPS-stimulated RAW cells. We obtained clear evidence that PSPG-I, but not PSPG-II, was critical for the anti-IL-6 action of LcS. Purified PSPG-I, but not PSPG-II, inhibited IL-6 production in LPS-stimulated RAW cells by down-regulation of the phosphorylation of NF-κB. Interestingly, we observed the action of anti-IL-6 in immobilized PSPG components in LPS-stimulated RAW cells and this action was completely abrogated by masking with the sugar chain-specific mAb raised against PSPG-I. Therefore, the anti-IL-6 effect of PSPG-I is not dependent on the internalization of the PSPG-I component by the activated macrophages. In addition, PSPG-I mutant LcS strains did not inhibit IL-6 production in LPS-stimulated RAW cells. Therefore, we concluded that the PSPG-I component in LcS plays a crucial role in the down-regulation of IL-6 production in LPS-stimulated LPMCs.

Recently, several authors have reported the anti-inflammatory property of probiotic strains. VSL#3-

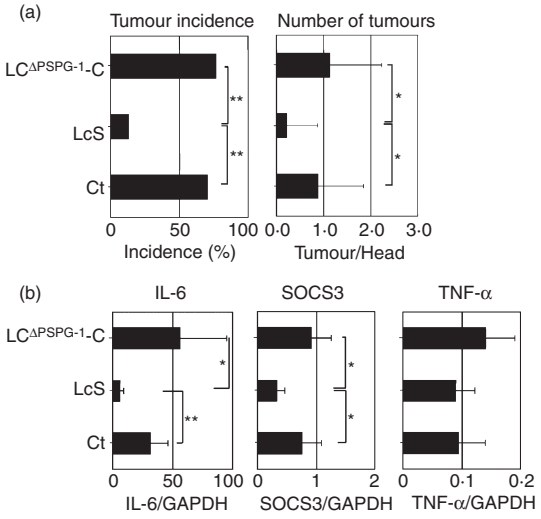

Figure 5. Anti-inflammation-based colon cancer action of *Lactobacillus casei* Shirota (LcS) was dependent on the presence of the polysaccharide–peptidoglycan complex (PSPG)-I component. (a) Colitis-associated cancer (CAC) was induced in BALB/c mice with repeated administration of dextran sodium sulphate (DSS) ($n = 10$ in each group). During CAC induction, the mice were treated with LcS or PSPG-I-negative LcS, orally, 5 days per week. After 20 weeks, the mice were killed, and the incidence and number of tumours were analyzed. (b) Quantitative reverse transcription–polymerase chain reaction (RT-PCR) analysis of interleukin-6 (IL-6), suppressor of cytokine signaling 3 (SOCS3) and tumour necrosis factor-α (TNF-α) mRNA expression in colonic tissues in CAC-induced BALB/c mice treated with LcS, PSPG-I-negative *L. casei* strains, or phosphate-buffered saline (PBS). The data were calculated in relation to the housekeeping gene *GAPDH*. Data represent the mean ± standard deviation (SD). Similar results were obtained from three independent experiments. Values ($^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$) differ significantly among each experimental group.

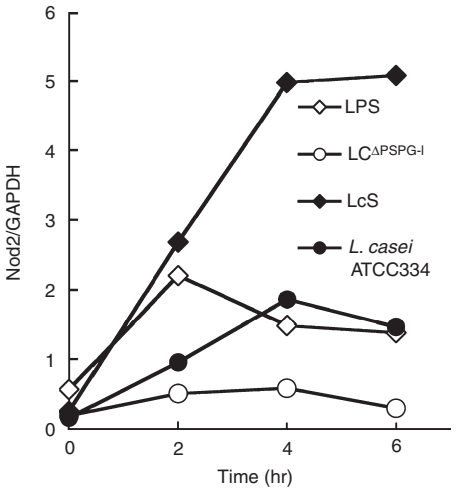

Figure 6. Quantitative reverse transcription–polymerase chain reaction (RT-PCR) analysis of Nod2 mRNA expression in RAW264.7 cells treated with *Lactobacillus casei* Shirota (LcS), polysaccharide–peptidoglycan complex (PSPG)-I negative *L. casei* strains, or *L. casei* ATCC 334. RAW cells ($1{\cdot}0 \times 10^6$ cells/well in 48-well culture plates) were incubated with lipopolysaccharide (LPS) (100 ng/ml) or heat-killed strains of lactobacilli (5 μg/ml). After 2, 4 or 8 hr of incubation, the cells were harvested and total RNA was purified. Quantitative RT-PCR of Nod2 was performed using an ABI-7500. All the data were calculated in relation to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*).

derived DNA was able to repress IL-8 production in intestinal epithelial cells in the presence of pro-inflammatory stimuli.[30] Among the probiotic strains, the anti-inflammatory actions of *L. casei* strains have been well documented by several investigators, including our reports. One strain of *L. casei*, *L. casei* DN-114 001, inhibited IL-6 and TNF-α synthesis in co-culture with ileal tissues isolated from CD patients.[31] Tien *et al.* suggested that this *L. casei* strain inhibited the synthesis of pro-inflammatory cytokines in invasive *Shigella flexneri*-stimulated cultured Caco2 cells.[32] In that report, he suggested that *L. casei* stabilized IκB by manipulation of the ubiquitin/proteasome pathway and inhibited NF-κB activation. These characteristics of *L. casei* DN-114 001 are very similar to those of LcS that we have observed previously, and in the present study.[18] We showed herein that phosphorylation of IκB kinase is inhibited by the presence of PSPG-I in LPS-stimulated RAW cells. The composition of the sugar of PSPG-I and PSPG-II in LcS were previously reported by Nagaoka *et al.*[24] According to that report, PSPG-I contains large amounts of glucose and rhamnose and small amounts of galactose and glucosamine. On the contrary, PSPG-II consists of large amounts of rhamnose, glucose, glucosamine and galactosamine. There may be other similarities in the form of the exo-polysaccharide chains of PSPG components or other characteristics between LcS and *L. casei* DN-114 001. Further studies should clarify this interesting issue.

It is well established that IL-6/STAT3 signaling is important for the pathogenesis of IBD as well as for CAC.[10,11] Moreover, recent reports state that the IL-6 signaling is important in breast and lung cancer development.[33,34] It is well established that various tumour tissues produce IL-6. Sansone *et al.* described that the conversion of mammary stem cells produced IL-6 and induced Notch3 ligand Jaggled-1 expression, which triggered the up-regulation of hypoxia-resistant protein, carbonic anhydrase IX, to promote the tumour malignancy.[33] We had discovered that inflammatory macrophages are a major cell source of IL-6 and modulated colon tumorigenesis in a mouse model of CAC. Indeed, treatment with sgp130–Fc fusion protein suppressed colon tumorigenesis[11] (S. Matsumoto, T. Hara, K. Mituyama, S. Rose-John, M. Yamamoto, unpublished data). Therefore, the IL-6 signal is one of the most appropriate targets for intestinal inflammation and cancer treatment. We showed here that PSPG-I down-regulated

IL-6 production in inflammatory macrophages *in vitro* and the PSPG-I-positive *L. casei* strain suppressed IBD as well as CAC development. However, we could not exclude PSPG-I interference with NF-κB activation in inflammatory macrophages. We had already described that TLR2 and TLR4 are not involved in the anti-IL-6 action of LcS.[18] Several negative regulatory signals for the TLR signaling pathway, such as A20, IκB NS, SOCS1, or SOCS3, have already been reported.[35–38] It is possible that PSPG-I up-regulates these negative regulators that interfere in the activation of the NF-κB signaling pathway. It was interesting that not only the soluble form of PSPG-I but also immobilized PSPG-I could interfere with LPS-induced IL-6 production in RAW cells. The binding of pathogenic bacteria to a human epithelial cell line induced the ubiquitinization of IκBα protein and inhibited the NF-κB signaling pathway.[39] It is well known that a myeloid inhibitory C-type lectin-like receptor (MICL) containing immunoreceptor tyrosine-based inhibitory motifs (ITIMs) expressed on the monocytes and its ligand inhibited zymosan-induced TNF-α synthesis.[40] Other ITIM-containing inhibitory receptors, such as Siglec, were reported by many investigators. Therefore, we speculated that a certain receptor for PSPG-I on inflammatory macrophages may contribute to the anti-IL-6 action of the LcS strain. Further analysis of the ligand for PSPG-I should clarify this important issue.

We investigated the induction of Nod2 in macrophages stimulated with LcS. This is important because studies have shown that stimulation of Nod2 with MDP significantly decreased pro-inflammatory production in activated macrophages.[29] In this study, we observed a marked induction of Nod2 mRNA in RAW cells by LcS but not by the control *L. casei* strain. Nod2 has been shown to recognize MDP, a component of peptidoglycan in the bacterial cell wall.[41] Polymorphisms in Nod2 are correlated with an increased incidence of the susceptibility to Crohn's disease.[42] It is well known that Nod2-deficient antigen-presenting cells manifest increased NF-κB activation.[43] Moreover, the stimulation of Nod2 by MDP significantly decreased pro-inflammatory cytokine production on TLR2 and TLR4 ligands.[29] In fact, Nod2-transgenic mice are resistant to colitis induced with 2,4,6-trinitrobenzene sulphonic acid (TNBS).[44] Therefore, Nod2 induction by LcS may be important for the anti-inflammatory and anti-cancer actions of LcS. The mechanisms underlying the activation of Nod2 by LcS remain unknown. Interestingly, Shida *et al.* reported that compared with other lactobacillus strains, LcS is resistant to intracellular digestion in macrophages.[45] This evidence may be a reason for the spontaneous activation of Nod2 in macrophages with LcS treatment *in vitro*.

In summary, we have shown that the anti-inflammatory and anti-tumour action of a probiotic *L. casei* strain is regulated by IL-6 production, which was dependent on the presence of PSPG-I on the probiotic *L. casei* strain. The safety of this probiotic strain is well documented, and the therapeutic efficiency of this strain in IBD had been confirmed in a pilot study.[19,46] Thus, this probiotic strain is useful in the treatment of intestinal inflammatory disorders.

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