***Lactobacillus helveticus* SBT2171, a cheese starter, regulates proliferation and cytokine production of immune cells**

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ABSTRACT

Consumption of a *Lactobacillus helveticus* SBT2171 (LH2171)-containing cheese has been reported to exhibit immunoregulatory actions, including an increase in regulatory T cell population and reduction in proinflammatory cytokine production in mice. We examined the in vitro effects of LH2171 cells per se on immune cell function, specifically proliferation and cytokine production, which are primary reactions of the immune response. Immune cell fractions were prepared by mechanical disruption of mesenteric lymph nodes (MLN), Peyer's patches (PP), and spleens (SP) of mice. The cell fractions were dispensed into a culture plate and stimulated with anti-CD3/CD28 antibody beads in place of antigen-presenting cells or lipopolysaccharide (LPS) in the presence or absence of heat-treated LH2171 cells and other bacterial strains for comparison. After incubation, proliferation, cytokine production, and cell viability of the immune cells were determined. The LH2171 significantly inhibited the proliferation of MLN immune cells stimulated with anti-CD3/CD28 compared with other bacterial strains. The antiproliferative potency of LH2171 was effective not only on MLN but also on PP and SP stimulated with anti-CD3/CD28 or LPS. The LH2171 also decreased LPS-stimulated IL-6 production from MLN, PP, and SP, and IL-1 β production from SP, but LH2171 did not affect the viability of immune cells. The LH2171 inhibited immune cell proliferation and proinflammatory cytokine (IL-6 and IL-1 β) production. The inhibitory actions were not due to cytotoxicity to immune cells, suggesting that LH2171 is a dairy *Lactobacillus* strain with beneficial immunoregulatory properties.

Key words: *Lactobacillus helveticus*, immune cell, proliferation, cytokine production

INTRODUCTION

Lactobacillus helveticus SBT2171 (LH2171) has high protease activity (Tan et al., 1995) and has been used as a starter bacterial strain in the production of a Gouda-type cheese, and we recently reported that consumption of cheese containing LH2171 led to immunoregulatory actions, including induction of regulatory T cells, reduction in proinflammatory cytokine production, and alleviation of dextran sulfate sodium-induced colitis in mice (Hosoya et al., 2012). Other studies have also reported immunomodulatory effects due to cheese consumption in humans (Ibrahim et al., 2010) and rats (Lollo et al., 2012); however, the studies did not examine whether the starter bacterial cells used in the cheese production could induce immunomodulatory functions. Although it is not clear which components of cheese are responsible for the modulatory actions, we consider lactic acid bacteria (LAB) a possibility.

The immunomodulatory actions of LAB can be applied not only to activate immune responses to external invasion and tumors, but also to regulate excessive immune responses involved in certain abnormalities, including inflammatory bowel disease (IBD) and rheumatoid arthritis (RA), for example. An approach to ameliorate such excess responses involves using the immunoregulatory functions of LAB. Such regulatory functions include actions of LAB on immune cells, such as inducing apoptosis of antigen-stimulated immune cells (Kanzato et al., 2008) and suppressing immune cell proliferation (Peluso et al., 2007; Yoshida et al., 2011). Lactic acid bacteria have also been shown to prevent proinflammatory cytokine production (Matsumoto et al., 2005; Lee et al., 2008; Philippe et al., 2011), an excess production of which could cause inflammatory and autoimmune diseases (Atreya et al., 2000; Yamamoto et al., 2000).

The present study focused on the immunoregulatory actions of LH2171 on the basis of our previous study, which showed the immunoregulatory actions of cheese containing LH2171 and suggested a possible contribution of LH2171 itself to the regulatory function. Thus, we examined the effects of LH2171 bacterial cells on mouse immune cells treated in vitro with immune cell

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stimulants, an anti-CD3/CD28 antibody, and LPS, by measuring proliferation and inflammatory cytokine production.

MATERIALS AND METHODS

Mice

Male C57BL/6J mice (5 to 6 wk old) were purchased from Charles River Japan Inc. (Yokohama, Japan) and acclimatized for more than 1 wk with free access to pellet food (CE-2; Clea Japan Inc., Tokyo, Japan) and water. Mice were maintained under controlled conditions of temperature, humidity, and light ($23 \pm 2^\circ\text{C}$ temperature, $50 \pm 10\%$ humidity, and 12-h light-dark cycle). Experiments were carried out in accordance with NRC (1996) guidelines.

Preparation of Immune Cells

Mice were killed and dissected under anesthesia at 7 to 10 wk of age. Mesenteric lymph nodes (MLN), Peyer's patches (PP), and spleens (SP) of mice were mechanically disrupted in a cell culture medium (RPMI 1640 medium) containing 10% heat-inactivated fetal bovine serum, 10 mM HEPES buffer, 2 mM L-glutamine, 100 U of penicillin/mL, 100 μg of streptomycin/mL, and 0.05 mM 2-mercaptoethanol. Cell suspensions thus prepared were filtered through 70- μm cell strainers (BD Biosciences, San Jose, CA). Erythrocytes remaining in the spleen cell preparation were eliminated by density-gradient centrifugation with Lympholyte-M medium (Cedarlane Laboratories Ltd., Burlington, Ontario, Canada) according to the manufacturer's protocol. Cells were washed twice, resuspended in the cell culture medium, and stored on ice until the start of the cell culture experiments.

Preparation of Bacterial Cells

A total of 41 LAB strains used in this study, which originated from dairy products and the human intestine, are shown in Table 1. *Streptococcus thermophilus* and *Lactobacillus* strains were grown at 37°C , and *Lactobacillus kefiranoferiens*, *Lactobacillus plantarum*, and *Lactococcus lactis* were grown at 30°C for 16 h in de Man, Rogosa, and Sharpe (MRS) medium (Difco Laboratories Inc., Detroit, MI). *Bifidobacterium* strains were grown at 37°C and *Leuconostoc* strains were grown at 30°C for 16 h in GAM medium (Nissui Seiyaku Co., Tokyo, Japan) supplemented with 1% glucose. The incubation was carried out in stationary culture. Cells of LAB harvested by centrifugation at $8,500 \times g$ for

10 min at 4°C were washed twice with distilled water, and then lyophilized. Lyophilized bacterial cells were resuspended in distilled water at 10 mg/mL and heat treated at 80°C for 30 min.

Cell Proliferation Assay

Immune cells isolated from mice (5×10^4 cells/well) were stimulated with anti-CD3/CD28 antibody beads (Life Technologies Inc., Gaithersburg, MD; 5×10^4 beads/well) or LPS from *Escherichia coli* (Sigma-Aldrich Japan, Tokyo, Japan; 10 $\mu\text{g}/\text{mL}$) in the presence or absence of heat-treated LAB (10 $\mu\text{g}/\text{mL}$) in 96-well round-bottomed plates (Asahi Glass Co. Ltd., Yokohama, Japan) at 37°C for 3 d in 5% CO_2 in air. After stimulation, PrestoBlue Cell Viability Reagent (Life Technologies Inc.), which becomes highly fluorescent depending on the proliferation of cells, was added to each well according to the manufacturer's protocol, and incubated for about 7 h. After incubation, the fluorescence intensity (excitation: 530 nm; emission: 580 nm) of each well was measured.

Measurement of Cell Viability

Immune cells isolated from mice (5×10^4 cells/well) not stimulated with proliferative activators were cultured in the presence or absence of heat-treated LAB (10 $\mu\text{g}/\text{mL}$) in 96-well round-bottomed plates at 37°C for 3 d in 5% CO_2 in air. After incubation, CyQUANT Direct Cell Proliferation Assay Kit (Life Technologies Inc.), which is an assay measuring the number of viable cells by the fluorescence intensity based on DNA content and cell membrane permeability, whose increase reflects early apoptosis and cytotoxicity, was added to each well according to the manufacturer's protocol and incubated for 1 h. After incubation, the fluorescence intensity (excitation: 485 nm; emission: 530 nm) of each well was measured and the number of viable cells was calculated according to the manufacturer's instructions.

Cytokine Analysis by ELISA

Immune cells isolated from mice (2×10^5 cells/well) were stimulated with LPS (10 $\mu\text{g}/\text{mL}$) in the presence or absence of heat-treated LAB (10 $\mu\text{g}/\text{mL}$) in 96-well round-bottomed plates at 37°C for 2 d in 5% CO_2 in air. After stimulation, culture supernatants were collected and stored at -30°C until their use in cytokine assays. Levels of IL-6, IL-1 β , and tumor necrosis factor α (TNF- α) in the supernatant were measured by ELISA according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Table 1. Lactic acid bacteria (LAB) used in the present study

No.	LAB	Strain ¹
1	<i>Lactobacillus helveticus</i>	SBT0064
2	<i>L. helveticus</i>	SBT0621
3	<i>L. helveticus</i>	JCM1120 ^T
4	<i>L. helveticus</i>	SBT2161
5	<i>L. helveticus</i>	SBT2171
6	<i>Lactobacillus kefiranofaciens</i> ssp. <i>kefiranofaciens</i>	JCM6985 ^T
7	<i>L. kefiranofaciens</i> ssp. <i>kefirgranum</i>	JCM8572 ^T
8	<i>L. kefiranofaciens</i> ssp. <i>kefirgranum</i>	SBT11088
9	<i>L. kefiranofaciens</i>	SBT11223
10	<i>L. kefiranofaciens</i>	SBT11295
11	<i>Lactobacillus acidophilus</i>	SBT1372
12	<i>L. acidophilus</i>	SBT1921
13	<i>L. acidophilus</i>	SBT2062
14	<i>L. acidophilus</i>	SBT2068
15	<i>Lactobacillus. gasseri</i>	JCM1131 ^T
16	<i>L. gasseri</i>	JCM1025
17	<i>L. gasseri</i>	JCM5813
18	<i>L. gasseri</i>	JCM8788
19	<i>Lactobacillus buchneri</i>	JCM1115 ^T
20	<i>Lactobacillus. paracasei</i>	NCFB151 ^T
21	<i>Lactobacillus plantarum</i>	NCFB1752 ^T
22	<i>Lactobacillus delbrueckii</i> ssp. <i>bulgaricus</i>	JCM1002 ^T
23	<i>L. delbrueckii</i> ssp. <i>lactis</i>	JCM1248 ^T
24	<i>Lactobacillus rhamnosus</i>	JCM1136 ^T
25	<i>Lactobacillus zeae</i>	JCM11302 ^T
26	<i>Lactobacillus crispatus</i>	JCM1185 ^T
27	<i>Lactobacillus amylovorus</i>	JCM1126 ^T
28	<i>Lactobacillus johnsonii</i>	JCM2010 ^T
29	<i>Lactobacillus reuteri</i>	JCM1112 ^T
30	<i>Lactobacillus fermentum</i>	JCM1173 ^T
31	<i>Lactococcus lactis</i> ssp. <i>cremoris</i>	JCM16167 ^T
32	<i>L. lactis</i> ssp. <i>lactis</i>	JCM5805 ^T
33	<i>Bifidobacterium longum</i>	SBT2497
34	<i>B. longum</i>	SBT10841
35	<i>Bifidobacterium infantis</i>	SBT10258
36	<i>Bifidobacterium breve</i>	SBT10522
37	<i>Bifidobacterium animalis</i>	ATCC25527 ^T
38	<i>Bifidobacterium adolescentis</i>	SBT10830
39	<i>Leuconostoc mesenteroides</i> ssp. <i>mesenteroides</i>	JCM6124 ^T
40	<i>Leuconostoc pseudomesenteroides</i>	JCM9696 ^T
41	<i>Streptococcus thermophilus</i>	ATCC19258 ^T

¹T = type strain; SBT = Snow Brand Type Culture Collection (Saitama, Japan); JCM = Japan Collection of Microorganisms (Saitama, Japan); NCFB = National Collection for Food Bacteria (Reading, UK); ATCC = American Type Culture Collection (Manassas, VA).

Statistical Analysis

The 1-sample *t*-test was used to evaluate the difference between proliferation indices each LAB strain exhibited and the control value of 100 that was set as a proliferation index in the absence of LAB. The Tukey-Kramer test was used for multiple comparisons between the groups. A *P*-value less than 0.05 was considered statistically significant. Data were analyzed using StatView software (version 5.0; SAS Institute Inc., Cary, NC).

RESULTS

LH2171 Suppressed the Proliferation of Immune Cells

We first examined the proliferative response of immune cells using MLN cells stimulated with anti-

CD3/CD28 antibody beads in the presence or absence of heat-treated LH2171 bacterial cells and 40 other LAB strains (Table 1). Figure 1 shows that 16 out of 41 strains significantly suppressed the proliferation of MLN cells compared with the control containing no LAB cells. The 16 strains demonstrating suppressive activity were *Lactobacillus helveticus* (5 strains, including LH2171; from raw milk, fermented milk, and cheese), *Lactobacillus acidophilus* (4 strains, from the human intestine and fermented milk), *Lactobacillus kefiranofaciens* (4 strains, from fermented milk and raw milk), and 3 other strains from different species (*Lactobacillus gasseri*, *Lactobacillus delbrueckii*, and *Lactobacillus paracasei* from the human intestine, fermented milk, and a commercial milk product, respectively).

Figure 1. Effect of heat-treated lactic acid bacteria (LAB) on the proliferation of mesenteric lymph node cells stimulated with anti-CD3/CD28 antibody beads. Proliferation was represented as fluorescence intensity (%), in which the control value obtained by the anti-CD3/CD28 stimulation alone was set to 100%. Data are shown as means $\pm$ SD ($n = 3$). Asterisks indicate a significant difference ($P < 0.05$; 1-sample t -test) from the control. The top 3 LAB species in terms of the number of strains that showed a significant suppression are marked as follows: *Lactobacillus helveticus* (solid black bars), *Lactobacillus acidophilus* (striped bars), and *Lactobacillus kefirifaciens* (dotted bars).

We further compared the proliferative responses among MLN, PP, and SP cells stimulated with anti-CD3/CD28 or LPS in the presence or absence of LH2171 as well as 3 other LAB strains for comparison: *L. gasseri* JCM1131^T (LG1131^T), a strain with no suppression of immune cell proliferation, according to Figure 1, and close taxonomical proximity to *L. helveticus*, according to the analysis of their 16s rRNA; *Lactobacillus crispatus* JCM1185^T (LC1185^T), a strain with the greatest enhancement of proliferation among lactobacilli examined; and *L. helveticus* JCM1120^T (LH1120^T), a strain that suppressed proliferation to an extent comparable with LH2171. Figure 2 shows that LH2171 and LH1120^T inhibited the proliferation of MLN, PP, and SP cells when stimulated with anti-CD3/CD28, whereas LG1131^T and LC1185^T did not inhibit the proliferation of any of the immune cells. When

stimulated with LPS, LH2171 and LH1120^T inhibited the proliferation of the immune cells more significantly than LG1131^T and LC1185^T.

LH2171 Did Not Affect the Viability of Immune Cells

We measured the number of viable immune cells when they were incubated with LH2171 to examine whether its antiproliferative potency could be attributed to a reduction in cell viability. Figure 3 shows that LH2171 did not significantly reduce the viability of MLN, PP, and SP cells compared with the control containing no LAB. The LG1131^T strain, which was used as a comparison and had no or less suppressive action on the anti-CD3/CD28- or LPS-induced immune cell proliferation compared with LH2171, also caused no significant changes in the viability of MLN and PP

Figure 2. Effect of heat-treated lactic acid bacteria (LAB) on the proliferation of mesenteric lymph node (MLN), Peyer’s patch (PP), and spleen (SP) cells stimulated with anti-CD3/CD28 antibody beads or LPS. Proliferation was represented as fluorescence intensity (%), in which the control value obtained by the anti-CD3/CD28 or LPS stimulation alone was set to 100%. Data are shown as means ± SD (n = 3). Statistical differences between the groups were analyzed using the Tukey-Kramer test and significant differences ($P < 0.05$) are represented by different letters (a–d). LG1131^T = *Lactobacillus gasseri* JCM1131^T; LC1185^T = *Lactobacillus crispatus* JCM1185^T; LH1120^T = *Lactobacillus helveticus* JCM1120^T; LH2171 = *Lactobacillus helveticus* SBT2171.

cells, except in SP cells in which LG1131^T significantly enhanced proliferation compared with the control.

LH2171 Suppressed LPS-Stimulated IL-6 and IL-1β Production

We examined whether LH2171 suppressed the production of proinflammatory cytokines (IL-6, IL-1β, and TNF-α) in the culture supernatants of LPS-stimulated immune cells. Figure 4a shows that LH2171 suppressed IL-6 production of MLN, PP, and SP; by contrast, LG1131^T, whose inhibitory action on immune cell proliferation was small compared with LH2171, had no suppressive effect on the production of IL-6. Figure 4b

shows that LPS stimulation also induced IL-1β production of SP, and this induction was significantly inhibited by LH2171 and LG1131^T, in which LH2171 showed a more significant inhibition compared with LG1131^T. In PP and MLN, in contrast to SP, IL-1β was not induced by LPS stimulation (data not shown). *Lactobacillus helveticus* SBT2171 did not consistently regulate TNF-α production, and the levels also fluctuated between the 3 independent experiments (data not shown).

DISCUSSION

Several immunoregulatory actions of *L. helveticus* on mammalian cells have been reported, although the num-

Figure 3. Effect of heat-treated lactic acid bacteria (LAB) on the viability of mesenteric lymph node (MLN), Peyer’s patch (PP), and spleen (SP) cells. Viability was determined as the number of viable cells. Data are shown as means ± SD (n = 3). Statistical differences between the groups were analyzed using the Tukey-Kramer test and significant differences ($P < 0.05$) are represented by different letters (a and b). LG1131^T = *Lactobacillus gasseri* JCM1131^T; LH2171 = *Lactobacillus helveticus* SBT2171.

Figure 4. Effect of heat-treated lactic acid bacteria (LAB) on the proinflammatory cytokines IL-6 (a) and IL-1 β (b) produced by LPS-stimulated immune cells of mesenteric lymph nodes (MLN), Peyer's patches (PP), and spleens (SP). Data are shown as means $\pm$ SD ($n = 3$). Statistical differences between the groups were analyzed using the Tukey-Kramer test and significant differences ($P < 0.05$) are represented by different letters (a-c). LG1131^T = *Lactobacillus gasseri* JCM1131^T; LH2171 = *Lactobacillus helveticus* SBT2171.

ber of publications is limited. Oral administration of *L. helveticus* cells inhibited the expression of proinflammatory cytokines and the activation of nuclear factor κ B in LPS-stimulated mouse peritoneal macrophages (Joo et al., 2012), and the production of IL-8, a chemokine involved in acute-phase inflammation, was reduced in human intestinal epithelial cells treated with fermented preparations containing *L. helveticus* (Wagar et al., 2009). The present study further examined the immunoregulatory actions of *L. helveticus* using heat-treated LH2171 bacterial cells and immune cells, including PP, MLN, and SP cells, and demonstrated significant suppressive activity on immune cell proliferation among 41 LAB strains of various species and on the production of several inflammatory cytokines.

A well-known mechanism of the suppressive action is cell death, including apoptosis: *L. acidophilus* strain L-92 inhibited antigen-stimulated CD4⁺ T-cell proliferation by inducing apoptosis (Kanzato et al., 2008). By contrast, LH2171 did not affect the viability of immune cells, indicating that it did not induce apoptosis but inhibited immune cell proliferation by a mechanism different from that of the L-92 strain.

Metabolites of LAB have been shown to suppress immune cell proliferation. Peluso et al. (2007) showed that live *L. paracasei* ssp. *paracasei* B21060 directly inhibited human CD4⁺ T-cell proliferation and those authors showed that lactic acid produced from live bacteria suppressed CD4⁺ T-cell proliferation. However, we used heat-killed bacteria, which did not produce lactic acid in the assay system, so LH2171 could not have inhibited proliferation by its metabolites.

The inhibitory effect of heat-killed bacteria on lymphocyte proliferation has been reported with *L. gasseri* OLL2809 by Yoshida et al. (2011), who showed that the inhibitory effect required RNA from strain OLL2809, suggesting that the suppressive effect occurred through the toll-like receptor 7, a receptor for bacterial single-stranded RNA in mammalian cells. They also showed that bacterial cells and their RNA did not induce apoptosis, IL-10 secretion, or changes in the ratio of regulatory Foxp3⁺ cells in CD4⁺ T cells. Similarly, LH2171 did not induce IL-10 secretion or Foxp3⁺ cells (data not shown), both of which are essential factors in suppressing immune cell proliferation, suggesting that the mechanism by which LH2171 sup-

pressed cell proliferation might be similar to that of strain OLL2809.

We then examined whether LH2171 suppressed IL-6, IL-1 β , and TNF- α , which are proinflammatory cytokines whose production from immune cells is known to be strongly stimulated by LPS. Lipopolysaccharide is a critical factor in inflammatory disorders: intestinal overgrowth of *Bacteroides*, a group of gram-negative bacteria possessing LPS in its cellular components, induced severe intestinal inflammation in rats (Rath et al., 1999); colitis was less severe in mice lacking the gene for LPS recognition (*Lps* on mouse chromosome 4), which regulates the responsiveness to lipid A, compared with normal mice (Lange et al., 1996). The crucial role of these inflammatory cytokines is also well known; IL-6, IL-1 β , and TNF- α were more strongly induced in mice with colitis than in control mice (Philippe et al., 2011). The present study showed that LH2171 significantly suppressed IL-6 production in LPS-stimulated MLN, PP, and SP. *Lactobacillus helveticus* SBT2171 also significantly suppressed IL-1 β production of LPS-stimulated SP. However, such suppression was not observed with LG1131^T, which was used for comparison due to its taxonomic proximity to *L. helveticus* in the *Lactobacillus* genus, according to the analysis of their 16s rRNA (Felis and Dellaglio, 2007), suggesting the importance of strain LH2171 among lactobacilli.

We consider the suppression of IL-6 to be the most important issue, because IL-6 is secreted from lymphocytes in inflammatory diseases, such as IBD and RA; patients with IBD and RA produced more IL-6 than control patients (Reimund et al., 1996; Hosokawa et al., 1999; Hashizume and Mihara, 2011; Oranskiy et al., 2012) and *IL6*-gene-disrupted mice were resistant to dextran sulfate sodium-induced colitis (Suzuki et al., 2001) and RA (Alonzi et al., 1998). Some LAB and probiotic bacteria have also been reported to downregulate IL-6 production and alleviate inflammatory diseases (Matsumoto et al., 2005; Fitzpatrick et al., 2008). *Lactobacillus casei* strain Shirota downregulated IL-6 in the lamina propria mononuclear cells in the large intestine and improved murine chronic colitis (Matsumoto et al., 2005). In the present study, LH2171 exhibited significant suppression of IL-6 from MLN, PP, and SP in vitro, which warrants further examination of the effect in vivo in the future.

Lactobacillus helveticus SBT2171 significantly reduced cell proliferation and IL-6 and IL-1 β production. The results were consistent between the independent experiments. In contrast, TNF- α was not consistently regulated between the experiments (data not shown). Thus, the reduction in immune cell proliferation was correlated only with the reduction in IL-6 and IL-1 β production, but not with TNF- α . This also suggests

that reduced cell proliferation per se might not be a direct cause of decreased cytokine production. Pathways through which LH2171 inhibits cell proliferation and cytokine production remain to be investigated.

CONCLUSIONS

Lactobacillus helveticus SBT2171 significantly suppressed the proliferation of immune cells of MLN, PP, and SP stimulated with anti-CD3/CD28 antibodies and LPS. *Lactobacillus helveticus* SBT2171 also reduced the production of LPS-stimulated proinflammatory cytokines (IL-6 from MLN, PP, and SP; IL-1 β from SP). These regulatory effects on immune cells were not due to cytotoxicity, suggesting that LH2171 is a dairy *Lactobacillus* strain with beneficial immunoregulatory properties.

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