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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-1β 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-1β) 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 ± 2°C temperature, 50 ± 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 m*M* HEPES buffer, 2 m*M* L-glutamine, 100 U of penicillin/mL, 100 μg of streptomycin/mL, and 0.05 m*M* 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 kefiranofaciens*, *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 × *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 μg/mL) in the presence or absence of heat-treated LAB (10 μg/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 μg/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 μg/mL) in the presence or absence of heat-treated LAB (10 μg/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-1β, 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).