Heat-Killed Cells of Lactobacilli Skew the Immune Response Toward T Helper 1 Polarization in Mouse Splenocytes and Dendritic Cell-Treated T Cells

LISA CHUANG,† KEH-GONG WU,‡ CINDY PAI,† PEI-SHAN HSIEH,§ JAW-JI TSAI,∥ JYH-HERNG YEN,⊥ AND MEEI-YN LIN*,†

Department of Food Science and Biotechnology, National Chung Hsing University, Taichung, Taiwan, Department of Pediatrics, Taipei Veterans General Hospital and National Yang-Ming University, Taipei, Taiwan, ProMD Biotech. Co. Ltd., Southern Taiwan Science Park, Tainan, Taiwan, Department of Medical Education and Research, Taichung Veterans General Hospital, Taichung, Taiwan, and Agricultural Extension Center, National Chung Hsing University, Taichung, Taiwan

It is believed that probiotics play an important role for the health of the host, including modulation of immune responses. Most studies have focused on the immunomodulatory effects of viable cells of lactic acid bacteria; however, we investigated those of heat-killed cells of lactic acid bacteria in this study. We first observed the effects on immune functions via stimulating splenocytes with three heat-killed Lactobacillus strains. Furthermore, we also investigated the effect of mouse dendritic cells (DCs) treated with these heat-killed Lactobacillus strains on T cell responses. The results showed that these Lactobacillus strains were able to stimulate cell proliferation and interleukin (IL)-10, IL-12 p70, and interferon (IFN)-γ production but not transforming growth factor (TGF)-β in splenocytes. In addition, these heat-killed Lactobacillus strains also stimulated high-level secretion of IL-12 p70 in DCs and switched T cells to T helper (Th) 1 immune responses, as evidenced by the elevated secretion of IFN-γ but not IL-5, IL-13, and TGF-β. These results showed that lactobacilli play a potentially important role in modulating immune responses and allergic reactions.

KEYWORDS: Lactobacillus; splenocytes; dendritic cells; T helper 1; cytokines

INTRODUCTION

Probiotics are defined as “living microorganisms, which on ingestion in certain numbers, exert health benefits beyond inherent basic nutrition” (1). Lactic acid bacteria including lactobacilli are members of the commensal microorganisms of the gastrointestinal tract of humans and mammals and are generally recognized as probiotics (2, 3). It is widely believed that the intestinal lactic acid bacteria play an important role for the health of the host, including modulation of immune responses (1, 4–6). One potential function of lactic acid bacteria is their involvement in the development and maintenance of homeostasis in the intestine-associated immune system (5, 7). It has been shown that peptidoglycan and other cell-wall components of lactic acid bacteria may play a significant role in stimulating immunocompetent cells in the intestinal tract (4, 5, 8, 9). Intestinal lactic acid bacteria including various species of Lactobacillus interact regularly with intestine cells, which include antigen-presenting cells (APCs) and intestinal epithelial cells (10–12). It has been reported recently that lactobacilli may moderate the allergic reaction by maintaining the balance between Th1 and Th2 responses (5, 13–15). This balance is thought to be maintained by specialized subsets of T-regulatory (Treg) cells that produce suppressive cytokines, such as interleukin (IL)-10 and transforming growth factor (TGF)-β (16, 17). Most studies have focused on the immunomodulatory effects of viable lactic acid bacteria; however, we investigated those of heat-killed lactic acid bacteria in this study. Heat-killed lactic acid bacteria have the advantages of allowing a longer product shelf-life, easier storage, and transportation.

Dendritic cells (DCs) are bone-marrow-derived professional APCs that can induce adaptive immune responses against invading pathogens (18). Immature DCs migrate through the bloodstream into nonlymphoid tissues, such as skin and mucosa, where they can capture and process antigens. DCs then migrate to the T cell areas of lymphoid...
organs, such as the lymph node, spleen, and mucosal-associated lymphoid tissues (MALT), where they lose the antigen-processing activity and mature to become potent immunostimulatory cells (18, 19). DCs in the intestine mucosa may be modulated by lactic acid bacteria, including the intestinal inhabitants and those administered orally (20). They interact indirectly with these bacteria that have gained access via M cells in Peyer’s patches (21) and directly with luminal bacteria, bypassing their dendrites between epithelial tight junctions into the intestinal lumen (11, 22). These microbial stimuli induce the maturation process of DCs, including the upregulation of costimulatory molecules and production of cytokines and chemokines, and contribute to the type of T cell responses. For example, DCs upregulate the costimulatory molecules, CD80 (B7-1) and CD86 (B7-2), and produce IL-12, which contribute to Th1 responses (23). They also produce IL-4 and IL-10, which promote Th2 or Treg responses (24–26).

Studies have demonstrated that lactic acid bacteria can effectively stimulate the production of IL-12 and interferon (IFN)-γ and modulate immune responses in mice and human (13, 14, 27). It has been shown recently that Lactobacillus strains can regulate DC surface molecule expressions and cytokine productions (28–30). Lactic acid bacteria, including the intestinal inhabitants and those administered orally, are in close proximity to DCs in the gut mucosa. It appears reasonable to expect that lactic acid bacteria, including lactobacilli, may have immunoregulatory effects through DCs in the gut modulating Th1, Th2, or Treg responses. In the present study, the effects of three heat-

Figure 1. Effects of heat-killed Lactobacillus strains on splenocyte proliferation. Splenocytes were treated with heat-killed Lactobacillus strains, phytohemagglutinin (PHA), or no stimulants. Data are presented as mean ± SD (n = 3). Bars with different letters are significantly different (p < 0.05).

Figure 2. Production of IL-10 (A), IL-12 p70 (B), IFN-γ (C), and TGF-β (D) in splenocytes treated with heat-killed Lactobacillus strains. Splenocytes were treated with heat-killed Lactobacillus strains, phytohemagglutinin (PHA), or no stimulants. Data are presented as mean ± SD (n = 3). Bars with different letters A–D are significantly different among different treatments (p < 0.05). Bars with different letters x–z are significantly different among different time points (p < 0.05).
killed Lactobacillus strains on mouse splenocyte proliferation and cytokine productions were examined. Furthermore, we also investigated the effect of these heat-killed Lactobacillus strains on the activation of mouse DCs and examined T cell responses to DCs that have been treated with these heat-killed lactobacilli.

Figure 3. Production of IL-4 (A), IL-10 (B), and IL-12 p70 (C) in DCs treated with heat-killed Lactobacillus strains. DCs were treated with heat-killed Lactobacillus strains, lipopolysaccharide (LPS), or no stimulants. ND = not detectable. Data are presented as mean ± SD (n = 3). Bars with different letters A—D are significantly different among different treatments (p < 0.05). Bars with different letters x and y are significantly different among different time points (p < 0.05).
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MATERIALS AND METHODS

Lactobacillus Strains. Three Lactobacillus strains used in this study were from the collections held at ProMD Biotech Co., Ltd., in Southern Taiwan Science Park, Tainan, Taiwan. Heat-killed cells of Lactobacillus acidophilus PM-A0002 (A2), Lactobacillus gasseri PM-A0005 (A5), and Lactobacillus salivarius PM-A0006 (A6) were used, respectively. L. acidophilus A2, L. gasseri A5, and L. salivarius A6 were cultured in de Man, Rogosa, and Sharpe (MRS) broth (Difco, Detroit, MI) at 37 °C for 24 h and collected by centrifugation at 2000g for 10 min. Cells were washed twice with sterile distilled water, suspended in phosphate-buffered saline (PBS; 0.85% NaCl, 2.68 mM KCl, 10 mM NaHPO₄, and 1.76 mM KH₂PO₄ at pH 7.7), and then heat-killed at 100 °C for 15 min.

Preparation of DCs. Mouse DCs were generated from the bone marrow of 6–10-week-old female BALB/c mice purchased from National Laboratory Animal Center (Taipei, Taiwan). Bone marrow was obtained from the thighbone of mice, washed twice with PBS, and then suspended in RPMI-1640 (GIBCO-BRL, Grand Island, NY) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS; GIBCO-BRL). The mouse bone marrow cells were cultured at 1 × 10⁶ cells/mL RPMI-1640 containing 10% heat-inactivated FBS in 24-well plates (Falcon, Oxnard, CA) with mouse granulocyte macrophage-colony-stimulating factor (mouse GM-CSF; 800 units/mL) and mouse IL-4 (500 units/mL). Fresh medium containing mouse GM-CSF and mouse IL-4 was added every 2–3 days. Mouse bone-marrow-derived DCs were used routinely at day 6 of cultures.

Preparation of Splenocytes and T Cells. Female BALB/c mice were sacrificed by cervical dislocation following deep anesthesia. Their spleens were aseptically removed. A single cell from the spleen was suspended in PBS. Splenocytes were obtained by centrifugation at 400 g for 30 min with Ficoll-Hypaque (Pharmacia, Uppsala, Sweden), and the light-density fraction from the 42.5–50% interface was recovered. CD4⁺ T cells were purified by negative selection using biotin-antibody cocktail and antibiotin microbeads in conjunction with the MiniMACS system following the instructions of the manufacturer (Miltenyi Biotec., Auburn, CA). The mouse CD4⁺ T cells were cultured at 2 × 10⁶ cells/mL of RPMI-1640 containing 10% heat-inactivated FBS in 96-well plates (Falcon).

Stimulation of Splenocytes with Lactobacillus Strains. Splenocytes (4 × 10⁶ cells/mL) were treated with heat-killed cells of Lactobacillus at a ratio of 1:3 and incubated at 37 °C for 24 h, 48 h, 72 h, and 5 days in a humidified atmosphere supplemented with 5% CO₂. Phytomagens-glutamin (PHA; GIBCO-BRL) at 1 μg/mL was used as a positive control. The supernatants from 24, 48, and 72 h cultures were collected and assayed for IL-10, IL-12 p70, INF-γ, and TGF-β by enzyme-linked immunosorbent assay (ELISA) kits following the instructions of the manufacturer (R&D system, Minneapolis, MN). The 5 day cultures were proceeded with the MTT assay using Sigma CGD-1 following the instructions of the manufacturer (Sigma-Aldrich, St. Louis, MO) at 0.1 μg/mL as a positive control. The supernatants from 24, 48, and 72 h cultures were collected and assayed for IL-4, IL-10, and IL-12 p70 by ELISA kits following the instructions of the manufacturer (R&D system and ebioscience). DCs treated with heat-killed cells of Lactobacillus for 24, 48, and 72 h were then arrested by mitomycin C (25 μg/mL) at 37 °C for 1 h.

Stimulation of T Cells with Lactobacillus-Treated DCs. Arrested DCs were incubated with T cells (1 × 10⁶ cells/mL) at a ratio of 1:10 at 37 °C for 2 and 5 days in a humidified atmosphere supplemented with 5% CO₂. The supernatants of the cocultures from 2 day cultures were collected and assayed for IL-5, IL-13, INF-γ, and TGF-β by ELISA kits following the instructions of the manufacturer (R&D system and ebioscience). The 5 day cultures were proceeded with the MTT assay using Sigma CGD-1 following the instructions of the manufacturer (Sigma-Aldrich) to determine T cell proliferation.

RESULTS

Lactobacillus Strains Induced Splenocyte Proliferation. Three heat-killed Lactobacillus strains, including L. acidophilus A2, L. gasseri A5, and L. salivarius A6, were tested for their capacity to promote activation of splenocytes (Figure 1). PHA (1 μg/mL) was used as a positive control in this experiment. L. salivarius significantly induced the proliferation of splenocyte as compared to the negative control (an increase of about 11-fold).

Lactobacillus Strains Induced Cytokine Production in Splenocyte Cultures. To determine whether these heat-killed Lactobacillus strains induce the cytokine secretion of splenocytes, the production of IL-10, IL-12 p70, INF-γ, and TGF-β in the supernatants of splenocytes treated with heat-killed Lactobacillus strains for 24, 48, and 72 h were examined (Figure 2). PHA (1 μg/mL) was used as a positive control in this experiment. We observed that all three Lactobacillus strains induced the secretion of IL-10 (Figure 2A) but much lower levels than those of IL-12 p70 (Figure 2B). In addition, all three strains of lactobacilli were potent stimulators of IL-12 p70 production in splenocyte cultures. As shown in Figure 2C, it appeared that L. salivarius was the strongest INF-γ inducer, corresponding to the results of splenocyte production. When splenocytes were stimulated with L. acidophilus for 24 h and L. gasseri or L. salivarius for 48 h, the highest INF-γ production was observed. However, no marked effect on TGF-β production in splenocyte cultures was observed as compared to the negative control (Figure 2D).

Lactobacillus Strains Induced Cytokine Production in DC Cultures. To determine whether these heat-killed Lactobacillus strains affect cytokine production in DCs, we examined the production of IL-4, IL-10, and IL-12 p70 in the supernatants of DCs treated with these heat-killed Lactobacillus strains for 24, 48, and 72 h (Figure 3). LPS (0.1 μg/mL) was used as a positive control in this experiment. As shown in Figure 3A, the production of IL-4 by three Lactobacillus strain-treated DCs were significantly lower than that of the negative control at 48 h.

Figure 2A

Figure 2B

Figure 2C

Figure 2D

Figure 4. Effects of DCs treated with heat-killed Lactobacillus strains on T cell proliferation. DCs were treated with heat-killed Lactobacillus strains, lipopolysaccharide (LPS), or no stimulants for 24, 48, and 72 h and then cocultured with T cells. Data are presented as mean ± SD (n = 3). Bars with different letters A–C are significantly different among different treatments (p < 0.05). Bars with different letters x and y are significantly different among different time points (p < 0.05).

Statistical Analysis. Data are presented as mean ± standard deviation (SD) (n = 3). Statistical comparisons were analyzed by analysis of variance (ANOVA), followed by Duncan’s multiple-range test. p values of less than 0.05 were considered to be statistically significant.
but not detectable at 24 and 72 h ($p < 0.05$). There was no significant change in the production of IL-10 by DCs treated with these Lactobacillus strains for 24, 48, and 72 h (Figure 3B). However, the IL-12 p70 productions of DCs were strongly induced by all three Lactobacillus strains (Figure 3C).

**DCs Treated with Lactobacillus Strains Induced T Cell Proliferation.** To test whether the maturation of DCs was sufficient to promote the activation of T cells, DCs were first treated with these heat-killed Lactobacillus strains and then cocultured with T cells for 5 days. The results presented in Figure 4 show that DCs treated with these Lactobacillus strains for 24 h indeed induced the proliferation of T cells (increased about 2–3-fold). Except L. gasseri-treated DCs, the proliferation induced by DCs treated with L. acidophilus and L. salivarius for 48 or 72 h were approximately 1.5-fold higher than that in the negative control ($p < 0.05$).

**DCs Treated with Lactobacillus Strains Induced Cytokine Production in T Cell Cultures.** To further determine the cytokine secretion pattern of T cells, we also examined the production of IL-5, IL-13, IFN-γ, and TGF-β in the supernatants of T cell cultures incubated with these heat-killed Lactobacillus strain-treated DCs for 2 days (Figure 5). There were no significant changes in the IL-5 levels (Figure 5A), and the IL-13 levels were lower than that in the negative control (Figure 5B). However, DCs treated with these Lactobacillus strains stimulated IFN-γ production in T cell cultures (Figure 5C). When T cells were stimulated with L. acidophilus- or L. salivarius-treated DCs for 24 h and L. gasseri-treated DCs for 48 h, the highest IFN-γ production was observed. However, TGF-β production had no significant changes, except in T cell cultures stimulated with L. gasseri-treated DCs for 24 h (Figure 5D).

**DISCUSSION**

Lactic acid bacteria including lactobacilli have been known to confer health benefits, including modulation of immune responses. Most studies have focused on the immunomodulatory effects of viable cells of lactic acid bacteria or their cell-wall extracts; however, we investigated those of heat-killed cells of lactic acid bacteria in this study.

The allergic reaction is characterized by a disruption of the Th1/Th2 balance toward Th2 immune reactions (31). Macrophage-derived IL-12 and T-cell-derived IFN-γ are Th1 cytokines, which enhance cell-mediated immunity. IL-12 stimulates IFN-γ production in T and NK cells and enhances the development of naive CD4+ T cells into Th1 cells (32, 33). IFN-γ also increases IL-12 production and reduces the proliferation and activation of Th2 cells, which enhance humoral immunity (34). IL-10 and TGF-β, the immunosuppressive cytokines, can inhibit the activation and functions of effector T cells, such as Th1 and Th2 cells. They also play a vital role in differentiation and function of a Treg cell subset (16, 17). Our results showed that L. salivarius induced the great proliferation of mouse spleno-
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cytes but that *L. acidophilus* and *L. gasseri* only had a little increase (not statistically significant), corresponding to the results of IFN-γ production in splenocytes. However, all three *Lactobacillus* strains induced high levels of IL-12 p70 but low levels of IL-10 and no TGF-β production by splenocytes, indicating that Th1-skewed cells were strongly stimulated. It has been demonstrated that lactobacilli can preferentially promote Th1-type cytokine production (35). Splenocytes stimulated with *L. casei* Shirota were also able to induce the production of IL-12 and IFN-γ (27). Regular ingestion of *Lactobacillus* strains has been reported to enhance the capacity of murine splenic leukocytes to produce IFN-γ following mitogenic stimulation, while IL-4 or IL-5 production is unaffected (36, 37).

It is believed that the intestinal lactic acid bacteria may have immunoregulatory effects through DCs in the gut. DCs produce IL-12, which contribute to Th1 responses (23), and also produce IL-4 and IL-10, which promote Th2 or Treg responses (24–26). IL-5 and IL-13 secretion by Th2 cells recruits granular effector cells, such as eosinophils, basophils, and mast cells to the site of allergic inflammation (38, 39). In this study, we observed that three heat-killed *Lactobacillus* strains stimulated IL-12 p70 secretion but not IL-4 and IL-10 by mouse DCs. DCs treated with these *Lactobacillus* strains stimulated T cell proliferation and produced high levels of IFN-γ, indicating that the maturation of DCs in response to these *Lactobacillus* strains was sufficient to promote the activation of T cells and switched T cells toward Th1 immune responses. Mohamadzadeh et al. (29) concluded that *L. johnsonii*, *L. reuteri*, and *L. gasseri* stimulated IL-12 production but not IL-10 by human monocyte-derived DCs that skewed T cells toward Th1 polarization. It has also been found that *L. casei* and *L. plantarum* Lb1 strongly induced IL-12 production by mouse bone-marrow-derived DCs but that *L. reuteri* DSM12246 induced IL-10 production (28). Recently, it has been reported that *L. acidophilus* and *L. paracasei* induced strong IL-12 production by human monocyte-derived DCs (30). The mechanisms that various *Lactobacillus* species or strains induced the differential DC responses are still unknown. The differences in immunomodulatory responses may result from several reasons, such as different lactic acid bacteria strains and human or mouse immune cells that are used. According to previous observations and our results, it appears to have a strain-dependent manner concerning lactobacilli-induced cytokine secretion.

We found that heat-killed *Lactobacillus* strains have immunomodulatory effects in vitro in this study. It has been reported that heat-killed *L. plantarum* L-137 induced IL-12 and IFN-γ production, inhibited IgE synthesis induced by IL-4, and suppressed Th2 immune responses in mice (40). Moreover, it has been demonstrated that stimulating splenocytes or oral feeding with the heat-killed *L. casei* Shirota was able to induce the production of IL-12 and IFN-γ, Th1 cytokines (27, 41). The results from this study suggest that heat-killed lactic acid bacteria are also effective in immunomodulation. Therefore, probiotic bacteria in the form of live cells may not be required for this purpose.

In summary, three heat-killed *Lactobacillus* strains used in this study, including *L. acidophilus* A2, *L. gasseri* A5, and *L. salivarius* A6, were able to activate mouse splenocytes and DCs to induce T cells toward Th1 immune responses. The results from this study suggest that the immunomodulatory effects of heat-killed lactic acid bacteria are strain-dependent and heat-killed cells of certain strains of lactic acid bacteria may play a potentially important role in modulating immune responses. Heat-killed lactic acid bacteria have the advantages of allowing a longer product shelf-life, easier storage, and transportation. Oral administration of heat-killed lactic acid bacteria in the form of dietary supplementations is convenient, especially for those people who travel frequently, and may meet the purpose of immunophrophaxis.

LITERATURE CITED


