Enhanced natural killer cell activation by exopolysaccharides derived from yogurt fermented with *Lactobacillus delbrueckii* ssp. *bulgaricus* OLL1073R-1

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ABSTRACT

Yogurt is generally recognized as a beneficial food for our health, but research into its physiological effects has focused mainly on intestinal dysfunctions such as constipation and diarrhea. We previously found yogurt fermented with *Lactobacillus delbrueckii* ssp. *bulgaricus* OLL1073R-1 (hereafter OLL1073R-1) could reduce risks of catching the common cold and flu in human trials. It was assumed that immunostimulatory exopolysaccharide (EPS) produced from OLL1073R-1 play an important role in this context. However, few studies have examined the immunostimulatory effects of traditional Bulgarian yogurts fermented with different strains of lactobacilli and their metabolites. Therefore, we screened 139 *L. delbrueckii* ssp. *bulgaricus* strains and identified OLL1073R-1 as the most robust producer of EPS. This strain was also the only strain that induced the production of IFN- γ in vitro. Oral administration of the EPS or yogurt fermented with OLL1073R-1 and *Streptococcus thermophilus* OLS3059 (OLL1073R-1 yogurt) augmented natural killer (NK) cell activity and induced IFN- γ production in spleen cells in mice, whereas 2 other yogurts fermented with other strains had no effect on NK cell activity. Cellular preparations of the OLL1073R-1 strain also slightly augmented NK cell activity, but were less effective than EPS itself. The EPS-dependent stimulation of NK cell activity was abrogated in IFN- γ knockout mice and in myeloid differentiation factor 88 knockout mice. Furthermore, IFN- γ production from spleen cells stimulated with EPS was completely blocked with both anti-IL-12 and anti-IL-18 antibodies in vitro. These findings suggest that NK cell activation by OLL1073R-1 yogurt is EPS-dependent, occurs via IL-12- and IL-18-mediated IFN- γ production, and requires myeloid differentiation factor 88. We

showed that traditional Bulgarian yogurt could exert immunostimulatory effects by selecting starter strains and part of the mechanisms depend on IFN- γ inducible EPS produced from *L. delbrueckii* ssp. *bulgaricus*. Further investigations on processes of fermentation to increase of the EPS may lead to the development of new functional foods that keep our immune functions stable.

Key words: yogurt, exopolysaccharide, interferon- γ , natural killer

INTRODUCTION

Lactic acid bacteria have been widely used in fermented foods for thousands of years. Yogurt is possibly the most familiar fermented food worldwide, due in part to the belief that the regular consumption of yogurt was responsible for the unusually long lifespans of Bulgarian peasants (Metchnikoff, 1907). Traditional Bulgarian yogurts are made from milk by fermenting with *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*, and yogurt is currently defined in the Codex Alimentarius as a milk product obtained by fermentation of milk by the action of these 2 species (Codex Alimentarius, 2011). Yogurt promotes health by improving intestinal dysfunctions, such as the symptoms of lactose intolerance and diarrhea (Guarner et al., 2005); however, additional health benefits and their associated molecular mechanisms are relatively unexplored compared with probiotics.

Probiotics are defined as “live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host” (Reid et al., 2003). Most probiotics are derived from commensal bacteria that can be found in the human large intestine, including bifidobacteria and lactic acid bacteria. These bacteria act not only on the intestine, but also on the entire body, including the immune system (Xiao et al., 2006; Takeda and Okumura, 2007), metabolism (Kadooka et al., 2013), and mental state (Tillisch et al., 2013). Some

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were reported to reduce respiratory infections duration in the elderly (Guillemard et al., 2010) or frequency in infants and children (Hatakka et al., 2001; Leyer et al., 2009; Merenstein et al., 2010).

Ingestion of yogurt fermented with *L. delbrueckii* ssp. *bulgaricus* OLL1073R-1 (**OLL1073R-1**) can reduce the risk of catching the common cold and flu and can augment natural killer (NK) cell activity in subjects with low NK cell activity (Makino et al., 2010). Strain OLL1073R-1, which was originally isolated from traditional Bulgarian yogurt, produces high amounts of immunostimulatory exopolysaccharide (EPS; Kitazawa et al., 1998; Nishimura-Uemura et al., 2003); we have previously shown that EPS has IFN- γ inducing activity (Makino et al., 2006). Oral administration of the EPS derived from OLL1073R-1 and yogurt fermented with this strain enhances NK cell activity in mice (Makino et al., 2006) and exerts anti-influenza virus activity (Nagai et al., 2011). However, it remains unclear whether EPS has additional roles in the immunostimulatory effects of yogurt fermented with OLL1073R-1, and whether yogurts fermented with other strains have similar properties. Furthermore, the precise molecular mechanisms involved in the immunostimulatory effect of EPS in vivo remain unclear.

In the current study, we compared the amounts of EPS produced from 139 strains of *L. delbrueckii* ssp. *bulgaricus* and evaluated the ability of purified EPS to induce IFN- γ . We also studied the contribution of cytokines, such as IFN- γ , IL-12, IL-18, and the signal transduction molecule myeloid differentiation factor 88 (MyD88), on EPS-dependent activation of NK cells. We found differences between these strains in terms of EPS production, IFN- γ -inducing abilities, and NK cell activation. Molecular profiling revealed that NK cell activation required IL-12- and IL-18-dependent IFN- γ induction, which in turn operated via MyD88-driven signaling.

MATERIALS AND METHODS

Mice

C3H/HeJ mice were purchased from Japan SLC Inc. (Hamamatsu, Japan). BALB/c mice were purchased from CLEA Japan Inc. (Tokyo, Japan). Interferon- γ knockout (KO) mice on a BALB/c background were supplied from Juntendo University School of Medicine. Myeloid differentiation factor 88 KO mice on a BALB/c background were obtained from Oriental Bioservice (Kyoto, Japan). All mice were obtained at 7 wk of age and acclimatized for 1 wk. Mice were housed at 23 to 25°C with a 12-h light/dark cycle and supplied with a commercial diet (Oriental Yeast, Tokyo, Japan)

and tap water ad libitum. All animal experiments were performed according to the guidelines of the Ethical Committee for animal experiments of Meiji Co., Ltd.

Microorganisms and Culture

Lactobacillus delbrueckii ssp. *bulgaricus* strains (n = 139) originating from traditional Bulgarian yogurts and native plants in Bulgaria (Michaylova et al., 2007) were each subcultured twice at 37°C for 18 h in 10% (wt/wt) skim milk (SM). The preculture was inoculated (1%, wt/wt) into fresh 10% (wt/wt) SM and incubated at 37°C for 18 h to purify EPS and measure their amounts.

Strain OLL1073R-1 was cultured in de Man, Rogosa, Sharpe broth (Becton Dickinson, Sparks, MD) at 37°C for 18 h once a week to obtain live and heat-killed bacterial cells for oral administration to mice. The cultures were centrifuged at $10,000 \times g$ at 4°C for 15 min and the collected live bacterial cells were suspended in saline at 2.5×10^9 cfu/mL after washing twice. Inactivation of bacterial cells was performed by heating live cell suspensions at 75°C for 1 h. Live and heat-inactivated cells were preserved at 4°C until oral administration to mice.

Yogurts were prepared using a laboratory-scale manufacturing process. Starter cultures contained *L. delbrueckii* ssp. *bulgaricus* OLL1073R-1 and *S. thermophilus* OLS3059 (**OLL1073R-1** yogurt), *L. delbrueckii* ssp. *bulgaricus* OLL1245 and *S. thermophilus* OLS3059 (yogurt A), and *L. delbrueckii* ssp. *bulgaricus* OLL1256 and *S. thermophilus* OLS3295 (yogurt B). After pasteurization of 10% (wt/wt) SM by heating to 90°C and immediate cooling to 45°C, the SM was inoculated with 1% (wt/wt) of each yogurt starter culture. The cultures were allowed to ferment at 43°C for 4 h, with fermentation being halted by cooling. The acidity of each culture ranged from 0.84 to 0.88%.

Purification of EPS

The cultures of *L. bulgaricus* strains or yogurts were mixed with 100% (wt/wt) trichloroacetic acid (Wako, Tokyo, Japan) to a concentration of 10% (wt/wt) and centrifuged at $12,000 \times g$ at 4°C for 20 min. The culture supernatants were mixed with 2 volumes of ethanol (Wako) and incubated overnight at 4°C. The solutions were centrifuged at $12,000 \times g$ at 4°C for 20 min and the pellets were dissolved in MilliQ (Millipore, Billerica, MA) water (crude EPS solution). After dialyzing against MilliQ water, crude EPS solutions were incubated at 37°C with DNase (EC 3.1.21.1; Sigma Aldrich, St. Louis, MO), RNase (type I-AS, EC 3.1.27.5; Sigma Aldrich), and proteinase K (EC 3.4.21.64; Sigma Aldrich), as described previously (Makino et al., 2006).

The enzymes were subsequently inactivated by heating at 90°C for 10 min. Each solution was mixed with 2 volumes of ethanol, incubated overnight at 4°C, and centrifuged, as described previously. The EPS was dissolved in and dialyzed against MilliQ water using a dialysis membrane (molecular weight cutoff 6,000–8,000; Spectrum Laboratories Inc., Rancho Dominguez, CA) and lyophilized.

Exopolysaccharide purified from OLL1073R-1 cultures was dissolved in 0.02 M Tris-HCl buffer (pH 8.6) and fractionated into neutral EPS (NPS) and acidic EPS (APS) by DEAE Sepharose Fast Flow (GE Healthcare, Little Chalfont, UK). The NPS were obtained as the flow-through fraction, and APS were obtained as the fraction that was eluted with 0.5 M NaCl. Each fraction was then lyophilized after ethanol precipitation and dialysis, as described previously. To estimate the amounts of EPS in the crude EPS solutions, the latter were dialyzed against MilliQ water using a 96-well dialyzer (molecular weight cutoff 5,000; Harvard Apparatus, Holliston, MA), and the EPS concentrations were measured using a phenol-sulfuric acid method (Dubois et al., 1956).

Cell Culture

Mice spleen cells were cultured at 5×10^5 cells/well in 96-well plates (Corning, Corning, NY) at 37°C in a 5% CO₂ atmosphere in RPMI 1640 medium, which contained 25 mM HEPES, 300 mg/L of L-glutamine, 10% fetal bovine serum, 1% penicillin-streptomycin, and 5.5 μ M 2-mercaptoethanol (Becton Dickinson).

Spleen cells from male C3H/HeJ mice were suspended in RPMI 1640 medium and incubated for 72 h in the presence of EPS, NPS, or APS (100 μ g/mL final concentration). For the neutralizing antibody assay, spleen cells and APS were incubated with or without 10 μ g/mL of anti-mouse IL-12 (p40/p70; Becton Dickinson), anti-mouse IL-18 (Becton Dickinson), or both. Spleen cells from mice administered yogurts, EPS, or bacterial cells were suspended in RPMI 1640 medium and incubated with 1 μ g/mL of anti-mouse CD3e (eBioscience, San Diego, CA) for 48 h.

Oral Administration

Fifty female BALB/c mice were divided into 5 groups and administered 0.4 mL of distilled water (DW), 10% SM, OLL1073R-1 yogurt, yogurt A, or yogurt B once daily for 3 wk via oral gavage. Forty female BALB/c mice were divided into 4 groups and administered 0.4 mL of DW, 1×10^9 cfu of live or heat-killed OLL1073R-1 bacterial cells, or 100 μ g of EPS purified from a culture of OLL1073R-1 once daily for 3 wk via oral gavage.

Twenty female wild-type (WT) BALB/c mice or female IFN- γ KO mice were divided into 2 groups, with one being administered 0.4 mL of DW and the other being administered OLL1073R-1 yogurt or 100 μ g of EPS purified from a culture of OLL1073R-1 once daily for 3 wk via oral gavage. Twenty male WT BALB/c mice or male MyD88 KO mice were divided into 2 groups, with one being administered 0.4 mL of DW and the other being administered 100 μ g of EPS once daily for 3 wk via oral gavage.

NK Cell Activity

The NK cell activity of spleen cells was determined using standard 4-h ⁵¹Cr release assays (Takeda et al., 1996). Briefly, spleen cells were incubated at a 200:1 ratio with ⁵¹Cr-labeled YAC-1 cells in 96-well round-bottom microtiter plates for 4 h. The supernatants were harvested and their radioactivity counted using a gamma counter. Cytotoxicity was calculated as the percentage of released counts after subtracting counts released in the absence of spleen cells (spontaneous release).

Cytokine Assays

The concentrations of IFN- γ , IL-4, IL-6, and IL-10 in the culture supernatants were analyzed using OptEIA ELISA kits (Becton Dickinson).

Statistical Analysis

Experimental data were expressed as means and their standard deviations evaluated using Student's *t* tests and one-way ANOVA, followed by Dunnett's or the Tukey-Kramer post hoc test. All analyses were performed using StatView 5.0 software (Abacus Concept Inc., Berkeley, CA), with a *P*-value <0.05 being considered significant.

RESULTS

Selection of Strains Producing EPS and Evaluation of IFN- γ Induction

We first screened 139 strains of *L. bulgaricus* for EPS production, which we found varied considerably (Figure 1A). From cultures of the top 10 strains, we purified EPS and determined the amount following lyophilization. This revealed that only 3 strains (OLL1073R-1, *L. bulgaricus* OLL1251, and *L. bulgaricus* OLL1247) produced more than 100 mg/kg of EPS, with OLL1073R-1 being the highest EPS producer (154.6 mg/kg; Figure 1B). Among them, only EPS derived from OLL1073R-1

Figure 1. Estimation of the amounts of exopolysaccharide (EPS) produced by the 139 *Lactobacillus delbrueckii* ssp. *bulgaricus* strains using the phenol-sulfuric acid method (A) and the amounts of EPS purified from the cultures of the top 10 strains (B). Murine splenic cell IFN- γ production stimulated by EPS from strains producing over 100 mg/kg (C). An asterisk (*) represents significant difference ($P < 0.05$) compared with medium alone using Dunnett's multiple comparison test. Error bars represent the SD for 6 measurements.

was capable of eliciting IFN- γ production from mice spleen cells in vitro (Figure 1C).

Production of Cytokines upon Stimulation with EPS

We used an in vitro cytokine-production assay to compare the immunostimulatory properties of EPS purified from 3 different yogurts (OLL1073R-1 yogurt, yogurt A, and yogurt B). A significant increase of IFN- γ production was observed following stimulation with EPS purified from the OLL1073R-1 yogurt, but not with EPS purified from either of the other yogurts (Figure 2A). Although variable, an increased production of IL-6 and IL-10 was detected following treatment with all EPS samples (Figure 2B, C).

Increased NK Cell Activity and IFN- γ Production

We examined NK cell activity and cytokine production in murine spleen cells after oral administration of the OLL1073R-1 yogurt, yogurt A, or yogurt B. A sig-

nificant increase of NK cell activity was detected only in mice administered the OLL1073R-1 yogurt (Figure 3). In mice administered the OLL1073R-1 yogurt, splenic cell IFN- γ production was significantly greater compared with mice administered DW but not SM (Figure 4A). By contrast, no significant difference was noted between the DW group and yogurt A- or yogurt B-treated groups. No significant differences in IL-4, IL-6, and IL-10 production were observed between groups (Figure 4B-D).

Increased NK Cell Activity and IFN- γ Production after Treatment

The OLL1073R-1 yogurt contained EPS and the bacterial cells of OLL1073R-1 at concentrations of 41.5 $\mu\text{g/g}$ and 3.5×10^8 cfu/g, respectively. Therefore, we administered EPS or bacterial cells to mice at doses of 100 $\mu\text{g}/\text{mouse}$ and 10^9 cfu/mouse, respectively; doses equivalent to ~ 2.4 to 2.9 g of yogurt. The NK cell activity in the EPS-treated group markedly increased in

Figure 2. Interferon- γ (A), IL-6 (B), and IL-10 (C) production from mouse spleen cells stimulated by exopolysaccharide purified from yogurts fermented with different strains of *Lactobacillus delbrueckii* ssp. *bulgaricus*. OLL1073R-1 yogurt was fermented with *L. bulgaricus* OLL1073R-1 and *Streptococcus thermophilus* OLS3059. Yogurt A was fermented with *L. bulgaricus* OLL1245 and *S. thermophilus* OLS3059. Yogurt B was fermented with *L. bulgaricus* OLL1256 and *S. thermophilus* OLS3295. A single (*) or double asterisk (**) represents significant difference ($P < 0.05$ and $P < 0.01$, respectively) compared with medium alone, using Dunnett's multiple comparison test. Error bars represent the SD for 6 measurements.

comparison with the control group. The bacterial cells of OLL1073R-1, regardless of whether they were live or heat-killed, also augmented NK cell activity compared with saline, but the effects were significantly lower than

those observed with EPS (Figure 5A). Increased splenic cell IFN- γ production was detected in mice administered EPS, but not in those treated with bacterial cells (Figure 5B).

Enhanced NK Cell Activity Requires IFN- γ and MyD88

To confirm the involvement of IFN- γ in the activation of NK cells by OLL1073R-1 yogurt and EPS in vivo, we investigated effect of OLL1073R-1 yogurt and EPS on NK cell activity in IFN- γ KO mice. In WT mice, NK cell activity was increased significantly by oral administration of both OLL1073R-1 yogurt and EPS, whereas no increases were observed in IFN- γ KO mice (Figure 6A, B). We also investigated the involvement of the signal transduction molecule, MyD88. As observed in IFN- γ KO mice, the EPS-dependent increase in NK cell activity was attenuated in MyD88 KO mice (Figure 6C).

IL-12 and IL-18 Are Required for IFN- γ Induction

We previously showed that OLL1073R-1 EPS could be divided into NPS and APS subtypes, and that APS exerted IFN- γ -inducing activity (Makino et al., 2006). We confirmed this in the current study, as only APS stimulated the production of IFN- γ by murine splenic cells in vitro (Figure 7A). Addition of anti-IL-12 monoclonal antibodies (**mAb**) reduced, but did not completely block, APS-dependent IFN- γ production (Fig-

Figure 3. Natural killer cell activity of murine splenic cells treated with skim milk and 3 yogurts fermented with different strains of *Lactobacillus delbrueckii* ssp. *bulgaricus*. OLL1073R-1 yogurt was fermented with *L. bulgaricus* OLL1073R-1 and *Streptococcus thermophilus* OLS3059. Yogurt A was fermented with *L. bulgaricus* OLL1245 and *S. thermophilus* OLS3059. Yogurt B was fermented with *L. bulgaricus* OLL1256 and *S. thermophilus* OLS3295. The different letters (a,b) denote significant differences between groups by Tukey's multiple comparison test ($P < 0.05$). Error bars represent SD for 10 individual mice.

ure 7B). Although anti-IL-18 mAb also reduced IFN- γ production, the effect was lower than that observed with anti-IL-12 mAb (Figure 7B). Complete inhibition of APS-dependent IFN- γ induction was observed when splenic cells were incubated with both anti-IL-12 mAb and anti-IL-18 mAb (Figure 7B).

DISCUSSION

We demonstrated that EPS production varies among strains of *L. bulgaricus*, and that OLL1073R-1 produces the highest amounts of EPS among the tested strains. Furthermore, EPS derived from OLL1073R-1 specifically induces IFN- γ production by splenocytes, at least in the tested *L. bulgaricus* strains. This specific EPS, and yogurt fermented with OLL1073R-1 (but not other

2 yogurts which contained nonimmunostimulatory EPS), increased NK cell activity and IFN- γ production in mice. We inferred that OLL1073R-1, rather than *S. thermophilus* OLS3059, was the source of immunostimulatory effects. This is because yogurt A, which also contains *S. thermophilus* OLS3059, exerted no immunostimulatory activity. These results indicate that immunostimulatory properties of yogurts, at least augmentation of NK cell activity and of IFN- γ production, are dependent on the specific starter bacterial strains or the metabolites they produce. In OLL1073R-1 yogurt, EPS-dependent production of IFN- γ likely contributed to increased NK cell activity, because the effects of OLL1073R-1 cells alone were weaker than those observed with the EPS. A central role of IFN- γ in EPS-dependent NK cell activation is supported by

Figure 4. Interferon- γ (A), IL-4 (B), IL-6 (C), and IL-10 (D) production from murine splenic cells treated with skim milk and 3 yogurts fermented with different strains of *Lactobacillus delbrueckii* ssp. *bulgaricus*. OLL1073R-1 yogurt was fermented with *L. bulgaricus* OLL1073R-1 and *Streptococcus thermophilus* OLS3059. Yogurt A was fermented with *L. bulgaricus* OLL1245 and *S. thermophilus* OLS3059. Yogurt B was fermented with *L. bulgaricus* OLL1256 and *S. thermophilus* OLS3295. Splenic cells were incubated for 48 h with the anti-CD3 monoclonal antibody. A double asterisk (**) represents significant difference ($P < 0.01$) by Tukey's multiple comparison test. Error bars represent SD for 10 individual mice.

our observation that increases of NK cell activity by OLL1073R-1 yogurt and EPS were not observed in IFN- γ KO mice.

To investigate the mechanisms by which EPS is recognized by immune cells, we studied the immunostimulatory effect of EPS in MyD88 KO mice. As observed for the IFN- γ KO animals, EPS-dependent NK cell activation was not observed in MyD88 KO mice. Myeloid differentiation factor 88 is an important adapter molecule in toll-like receptor (TLR) signal transduction pathways; thus, it plays an essential role in the detection and elimination of invading microbes. MyD88 KO mice are unresponsive to ligands for TLR-2, TLR-4, TLR-5, TLR-7, and TLR-9 (O'Neill and Bowie, 2007). Additionally, phosphopolysaccharide from *Lactococcus*

Figure 5. Natural killer cell activity of murine splenic cells after administration of *Lactobacillus delbrueckii* ssp. *bulgaricus* OLL1073R-1 cells or exopolysaccharide (EPS) produced by this strain (A). Interferon- γ production from murine splenic cells after 48 h of incubation with the anti-CD3 monoclonal antibody (B). Different letters (a–c) denote significant differences between groups as determined by Tukey's multiple comparison test ($P < 0.05$). Error bars represent SD for 10 individual mice.

Figure 6. Natural killer cell activity of spleen cells of IFN- γ knock-out (KO) mice and myeloid differentiation factor 88 (MyD88) KO mice administered OLL1073R-1 yogurt or exopolysaccharide (EPS) produced by strain *Lactobacillus delbrueckii* ssp. *bulgaricus* OLL1073R-1. OLL1073R-1 yogurt was fermented with *Lactobacillus delbrueckii* ssp. *bulgaricus* OLL1073R-1 and *Streptococcus thermophilus* OLS3059. Different letters (a–c) denote significant differences between groups as determined by Tukey's multiple comparison test ($P < 0.05$). Error bars represent SD for 10 individual mice.

Figure 7. Interferon- γ production from murine splenic cells stimulated by neutral exopolysaccharides (NPS) and acidic exopolysaccharide (APS) produced by *Lactobacillus delbrueckii* ssp. *bulgaricus* OLL1073R-1 (A) and the effects of anti-IL-12 monoclonal antibodies (mAb) and anti-IL-18 mAb on IFN- γ production (B). A double asterisk (**) represents significant difference ($P < 0.01$) from 0 $\mu\text{g/mL}$ using Dunnett's multiple comparison test. Different letters (a-c) denote significant differences between groups as determined by Tukey's multiple comparison test ($P < 0.05$). Error bars represent the SD for 6 measurements.

lactis ssp. *cremoris* is recognized by the TLR homolog, RP105 (Tohno et al., 2007). Exopolysaccharide from OLL1073R-1 might therefore be recognized by pattern recognition receptors such as TLR or their homologs. However, further investigations are needed to clarify the mechanisms by which EPS is recognized by immune cells.

Pattern recognition receptors, such as TLR, are expressed mainly in innate immune cells such as dendritic cells (DC) and macrophages (Takeda et al., 2003). Direct interactions between intestinal immune cells and bacterial cells have been shown in several studies (Coombes and Powrie, 2008; Hiramatsu et al., 2011). Therefore, we inferred that orally administered OLL1073R-1 EPS was detected by DC and macrophages located beneath intestinal epithelial cells. In support of this hypothesis, we observed that OLL1073R-1 APS stimulated the secretion of IL-12 from murine bone marrow-derived

DC (data not shown). Alternatively, it is possible that immune cells in the lamina propria were indirectly affected by intestinal epithelial cells that had been stimulated by EPS. This would be consistent with results from Kishimoto et al. (2015), who showed that certain EPS molecules purified from cultures of *L. delbrueckii* ssp. *bulgaricus* strains stimulate murine macrophage RAW264.1 cells both directly and indirectly.

Interleukin-12 plays critical roles in the differentiation of CD4 T cells into IFN- γ -producing Th1 cells, as well as in the activation of NK cells to produce IFN- γ , which enhances their cytotoxic activity (Trinchieri, 2003; Watford et al., 2003). We also observed that anti-IL-12 mAb dramatically decreased IFN- γ production in splenic cells stimulated by APS. Furthermore, responses were completely inhibited when anti-IL-12 mAb and anti-IL-18 mAb were used in combination. Interleukin-12- and IL-18-dependent IFN- γ production from spleen cells stimulated by extracts of the mushroom, *Agaricus blazei* Murill, actually increased NK cell activity through IL-12-mediated IFN- γ production (Yuminamochi et al., 2007). Interleukin-18 can synergize with IL-12 to induce the development of Th1 cells and activate NK cells independently of IL-12 (Okamura et al., 1998). In a manner analogous to that elicited by glucans produced by *A. blazei*, it is likely that EPS derived from OLL1073R-1 activates NK cells through both IL-12 and IL-18 signaling pathways. However, it remains unclear how EPS-dependent activation of immune cells in the intestinal tissue leads to systemic changes to the immune system (such as an increase of NK cell activity of spleen cells, for example). Future studies should examine the changes in IL-12 or IFN- γ production from immune cells, as well as subpopulations of cells in the lymphoid tissue of intestine and spleen after oral administration of EPS.

In conclusion, we found that EPS plays important roles with regard to not only the quality and taste of yogurt, but also with respect to organismal health. These findings provide novel insight into the health-promoting effects of yogurt fermented with *L. bulgaricus* OLL1073R-1. Investigations of the fermentation process focused on the substantial increase of immunostimulatory EPS production in yogurt may lead to the future development of yogurt with enhanced immunostimulatory properties.

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