International Journal of Food Sciences and Nutrition

ISSN: 0963-7486 (Print) 1465-3478 (Online) Journal homepage: http://www.tandfonline.com/loi/iijf20

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To cite this article: Ryoichi Shin, Yukie Itoh, Motoyuki Kataoka, Shiori Iino-Miura, Ryosuke Miura, Takeo Mizutani & Tomohiko Fujisawa (2016): Anti-tumor activity of heat-killed Lactobacillus plantarum BF-LP284 on Meth-A tumor cells in BALB/c mice, International Journal of Food Sciences and Nutrition

To link to this article: http://dx.doi.org/10.1080/09637486.2016.1185771

Published online: 19 May 2016.

INTERNATIONAL JOURNAL OF FOOD SCIENCES AND NUTRITION, 2016
http://dx.doi.org/10.1080/09637486.2016.1185771

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RESEARCH ARTICLE

Anti-tumor activity of heat-killed *Lactobacillus plantarum* BF-LP284 on Meth-A tumor cells in BALB/c mice

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ABSTRACT

Probiotics exert numerous effects on human well-being. Here, heat-killed *Lactobacillus plantarum* BF-LP284 (H-Lp) was isolated as a potent immuno-modulator among 15 strains of lactobacilli in terms of TNF-α induction ability in peritoneal macrophages. *In vitro* TNF-α and IFN-γ induction in Peyer's patch (PP) cells was higher when incubated with H-Lp than with live *L. plantarum* BF-LP284 (L-Lp). Suppression of syngeneic Meth-A tumors in a murine model by oral administration of H-Lp was also greater than that of L-Lp and of controls. H-Lp stimulated IFN-γ production in spleen cells, which displayed inhibited tumor growth in Winn assays when treated with H-Lp. Moreover, H-Lp increased the ratio of $CD3^+$ cells among peripheral blood mononuclear cells in Meth-A tumor-bearing mice, suggesting an H-Lp-mediated anti-tumor mechanism whereby immune cells that are activated by H-Lp in PP and acquire anti-tumor activity in the spleen migrate to tumor sites through lymphocyte homing to inhibit tumor growth.

ARTICLE HISTORY
Received 26 January 2016
Revised 21 April 2016
Accepted 1 May 2016
Published online 20 May 2016

KEYWORDS
Heat-killed; IFN-γ; Lactobacillus plantarum; probiotics; TNF-α; tumor

Introduction

Typically derived from the genera *Lactobacillus* and *Bifidobacteria*, probiotics are defined as nonpathogenic live microbial supplements that confer positive effects on human health through interaction with the gastrointestinal microflora and direct action on the immune system. It is widely believed that probiotics may relieve allergic symptoms, alleviate intestinal disorders, decrease serum cholesterol, protect the host from infection, and augment anti-tumor activity (Howarth and Wang 2013).

Probiotics have been shown to reduce the incidence of tumor formation and inhibit tumor growth by altering intestinal metabolism, degrading carcinogens, producing anti-mutagenic compounds, and augmenting host innate and adaptive immunity in animal studies and clinical trials (Aso et al. 1995; Kumar et al. 2010; Shida and Nomoto 2013). It has also been reported that differences in the immunomodulating ability of heat-killed and viable lactic acid bacteria might depend on the strain (Adams 2010). Ou et al. (2011) demonstrated that heat-killed *Enterococcus faecalis* YM-73 and *Lactobacillus salivarius* AP-32 possessed superior immunomodulatory ability among 11 strains of heat-killed and viable forms of lactic acid bacteria, while Asano et al. (1986) showed that both viable and nonviable *Lactobacillus casei* LC-9018 via various routes of administration inhibited the growth of MBT-2 tumors in mice. Heat-killed probiotics provide the advantages of longer product shelf life, easier storage, and more convenient transportation as compared with their living counterpart (Adams 2010). Moreover, immunodeficient individuals may be at increased risk of adverse effects from treatment with live probiotics, which are causative of several pathologies of their own, and therefore the use of heat-killed probiotics appears to be a generally safer alternative.

Although the anti-tumor mechanisms of orally administered probiotics are not well defined, it is believed that the means of host immunity augmentation by heat-killed probiotics is the same as that of viable probiotics (Adams 2010). Specifically, probiotics administered orally were incorporated into macrophages and dendritic cells (DCs) in Peyer's patch through M cells and digested to form active components (Kumar et al. 2010; Hiramatsu et al. 2011; Uccello et al. 2012). These cells subsequently gained the ability to produce TNF-α, IL-12, and IFN-γ, and other cytokines (Foligne et al. 2007; Kim et al. 2012) to induce anti-tumor action by modulating host immunity. Matsuzaki and Chin (2000) demonstrated

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that heat-killed *L. casei* LC9018 treatment prolonged the survival of tumor-bearing mice by the induction of TNF-α, which was the first cytokine secreted from activated macrophages and DCs. Soltan Dallal et al. (2012) reported that oral administration of *L. casei* spp *casei* decreased adenocarcinoma breast tumor growth and prolonged survival in a murine model via augmentation of natural killer (NK) cell activity by increasing the production of IL-12 and IFN-γ. These activated immune cells in PP trafficked to the spleen through the mesenteric lymph nodes (MLN) (Matsuno et al. 2010; Miura et al. 2012).

We recently reported that the product of soybean milk fermented with several strains of lactic acid bacteria, including *Lactobacillus plantarum* BF-LP284, reduced the incidence of colon cancer in mice by 1,2-dimethylhydrazine dihydrochloride induction and augmented the inhibition of transplantable syngeneic Meth-A tumor growth *in vivo* through improvement of the intestinal environment and activation of host immunity (Shin et al. 2014). The anti-tumor activity of the fermented product was considered to be at least partly related to the activation of host immunity by the lactic acid bacteria used in the fermentation.

In this study, the possible application of heat-killed *L. plantarum* LP284 (H-Lp) as a supplement to enhance host immunity was investigated following selection from among 16 lactobacillus strains according to TNF-α induction in peritoneal macrophages. Thereafter, the anti-tumor activity of orally administered H-Lp was determined in comparison with that of viable bacteria in transplantable syngeneic Meth-A tumor mice. The anti-tumor mechanisms of oral H-Lp were also investigated using a tumor-bearing mice model.

Materials and methods

Microorganisms

Lactobacillus plantarum BF-LP284, *L. acidophilus* ALAL005, *L. casei* ALAL003, *L. fermentum* ALAL013, *L. mali* ALAL014, *L. reuteri* ALAL001, and *L. rhamnosus* ALAL004 were isolated from fermented food products and stored at the Central Institute for Health Science of the A. L. A. Corporation. *Lactobacillus plantarum* JCM1055, JCM1057, JCM1149, JCM1551, JCM6651, JCM8341, JCM8342, and JCM8344 were obtained from the Japan Collection of Microorganisms (RIKEN BioResource Center, Tsukuba, Japan). The organisms were cultured for 24 h at 30 °C or 37 °C in de Man–Rogosa–Sharpe broth (Difo Laboratories, Detroit, MN). All strains were washed twice with distilled water and then heat-killed by autoclaving at 115 °C for 15 min. After heat-killing, each organism was lyophilized and stored at 4°C until use. Live *L. plantarum* BF-LP284 was also lyophilized and stored at 4°C until testing.

Animals

Test mice were housed in an animal room set at a temperature of 23 ± 1 °C, humidity of 50 ± 10%, and a 12 h fixed light/dark cycle from 8:00 am. The animals had access to standard laboratory chow (MF; Oriental Yeast Co., Ltd., Tokyo, Japan) or purified chow (AIN93M Oriental Yeast Co., Ltd., Tokyo, Japan) and tap-water *ad libitum*. Mice were acclimated for 1 or 2 weeks prior to the experiments. All experimental protocols were approved by the guidelines of the Animal Care and Use Committee of the Central Institute for Health Science, A. L. A. Corporation.

Screening of heat-killed Lactobacillus strains to produce TNF-α

Peritoneal exudate cells (PECs) were prepared according to the procedure of Ueda et al. (2004). Briefly, 7-week-old male ICR mice ($n = 10$, SLC Inc., Shizuoka, Japan) were sacrificed by CO_2 inhalation, and PECs were harvested using RPMI 1640 medium (Gibco BRL, Life Technologies, Gaithersburg, MD) supplemented with 1% (v/v) heat-inactivated fetal calf serum (FCS; Boehringer Mannheim GmbH, Germany). PECs were adjusted to 2×10^6 cells/ml by RPMI 1640 medium with 5% FCS. Macrophages were prepared as follows: PECs (200 µl) were cultured in a 96-well flat-bottom culture plate (Nunc®, Sigma-Aldrich Co., LLC) for 2 h at 37 °C in a 5% CO_2 incubator and non-plastic adherent cells were removed. Then, a heat-killed bacterial suspension (0.2 µg/200 µl of RPMI 1640 medium with 5% FCS) was added to each well and the culture supernatant was harvested after cultivation for 24 h. The amount of TNF-α in each culture supernatant was determined using a Duo Set™ Mouse TNF-α (R&D Systems, Minneapolis, MN) enzyme-linked immunosorbent assay (ELISA).

Cytokine production by Peyer's patch (PP) cells

PPC suspensions were prepared according to the method of Hill and Pohl (1990). PP samples were excised from 7-week-old female BALB/c mice, and PPCs were prepared by collagenase treatment (1mg/ml) at 37 °C for 1h. Thereafter, PPCs were adjusted to

5×10^6 cells/ml in RPMI 1640 medium with 5% FCS. PPC suspensions (5×10^5 cells/well) were cultured in well plates with H-Lp or L-Lp (0.1 μg/ml each) or medium only in RPMI 1640 medium with 5% FCS for 3 d in a 5% CO_2 incubator before harvesting culture supernatants. The levels of TNF-α and IFN-γ in the supernatants were detected using ELISA assay kits (R&D Systems, Minneapolis, MN).

Anti-tumor activity

Five-week-old female BALB/c mice (Charles River Japan, Kanagawa, Japan) were separated into three groups of equally distributed weight ($n = 6$/group). Live *L. plantarum* BF-LP284 (L-Lp) or H-Lp were administered orally (10 mg/day/head) every day for 3 weeks, and then every 2 d for 20 d after inoculation of Meth-A tumor cells. Physiological saline (Fuso Pharmaceutical Industries, Osaka) was administered orally to a control group according to the same protocol. Meth-A tumor cells (1×10^6 cells/0.2 ml physiological saline) were inoculated into the inguinal subcutaneous region of the animals in all three groups. Tumor size was calculated by the square root of the major axis × minor axis (mm) every 3–4 d throughout the experiment. Meth-A tumor cells were a kind donation from Tohoku University Cell Resource Center.

Production of TNF-α by PPCs of tumor-bearing mice

PPCs were aseptically removed from mice administered H-Lp or physiological saline on day 16 following Meth-A tumor cell inoculation. PPC suspensions (5×10^5 cells/well) were then cultured in well plates with H-Lp (2.5 μg/ml) or medium only in RPMI 1640 medium with 5% FCS for 7 d in a 5% CO_2 incubator prior to harvesting of culture supernatants. TNF-α in supernatants was detected using ELISA assay kits (R&D Systems, Minneapolis, MN).

Cytokine production by spleen cells of tumor-bearing mice

Single spleen cells were prepared according to the protocol of Saito et al. (1983). Briefly, spleens were aseptically removed from mice administered H-Lp or physiological saline on day 16 after Meth-A tumor cell inoculation and disrupted with a syringe plunger in RPMI 1640 medium with 1% FCS before filtration through a nylon wool 200 mesh. Spleen cells were suspended at 5×10^6/ml in RPMI 1640 medium with 5% FCS. Cell suspensions (5×10^5 cells/well) were cultured in well plates with H-Lp (0.02 μg/ml) or medium only in RPMI 1640 medium with 5% FCS for 3 d in a 5% CO_2 incubator, and culture supernatants were harvested. The levels of TNF-α, IL-12p40, and IFN-γ in the supernatants were detected using ELISA assay kits (R&D Systems, Minneapolis, MN).

Winn neutralization assay

Winn neutralization assays were performed as reported by Saito et al. (1984). Spleens were isolated from mice ($n = 6$/group) administered H-Lp or physiological saline on day 16 following Meth-A tumor cell inoculation. Spleen cells were prepared as described above and subsequently adopted as effector cells in Winn assays. Admixtures of effector cells (1×10^7) and Meth-A tumor cells (1×10^6) at a 10:1 ratio were injected subcutaneously into the inguinal regions of syngeneic intact BALB/c mice. Tumor growth inhibition was determined by measuring tumor size every 3–4 d.

Immunophenotyping of peripheral blood mononuclear cells (PBMCs) by flow cytometric analysis

PBMCs were analyzed according to the protocol of Lee et al. (2004) after preparation as described by McFarland et al (2006). Briefly, peripheral blood samples were collected from 6 mice per group on day 18 after Meth-A tumor cell inoculation through the inferior vena cava using a heparinized syringe and pooled for each group. Then, PBMCs (5×10^6 cells/ml) were suspended in RPMI1640 with 5% FCS after hemolysis with a 1% ammonium chloride solution. The PBMCs were incubated with phycoerythrin (PE)-Cy™5-conjugated anti-mouse CD3ε, fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD4, and PE-conjugated anti-mouse CD8a (Biolegend, San Diego, CA) for 60 min on ice. Flow cytometric analysis was carried out using a Coulter Epics XL device (Beckman Coulter Inc., Fullerton, CA).

Statistical analysis

Statistical analysis was performed with one-way analysis of variance using StatMateIV software (ATMS Co., Ltd., Tokyo, Japan). Significant differences between groups were determined by Tukey's or Dunnett's multiple comparison tests. A p value of less than 0.05 was considered to be statistically significant.

Results

Screening of heat-killed Lactobacillus strain to production of TNF-α

As shown in Table 1, the heat-killed *L. plantarum* BF-LP284 strain induced TNF-α (1.14 ± 0.11 ng/ml) to a significantly higher degree than did other *Lactobacillus* strains (Experiment 1). Moreover, *L. plantarum* BF-LP284 induced the highest level of TNF-α (1.04.± 0.04 ng/ml) among the 9 strains of *L. plantarum* tested (Experiment 2).

Cytokine production by PPCs

TNF-α induction in PPCs was observed by H-Lp stimulation only (15.7 ± 1.7 pg/ml), while the induction of IFN-γ in PPCs by H-Lp stimulation was 5-fold stronger than that by L-Lp (Figure 1).

Tumor growth inhibition

Tumor growth was significantly suppressed by oral administration of H-Lp from the 12th day (7.54 ± 2.89 mm) to the 20th day (12.52 ± 4.01 mm) after Meth-A tumor cell inoculation in comparison with the control group (11.82 ± 0.46 mm and 19.21 ± 1.18 mm, respectively) ($p < 0.01$) (Figure 2). L-Lp administration produced significant suppression on the 14th day only (11.16 ± 1.39 mm) as compared with controls (13.05 ± 1.05 mm) ($p < 0.05$).

Table 1. Screening of heat-killed *Lactobacillus* for production of TNF-α by peritoneal exudate cells (PECs) in ICR mice.

	Strain	TNF-α (ng/ml)
Experiment 1	*Lactobacillus plantarum* BF-LP284	1.14 ± 0.11^{a}
	Lactobacillus acidophillus ALAL005	0.80 ± 0.05^{b}
	Lactobacillus casei ALAL003	0.74 ± 0.06^{b}
	Lactobacillus fermentum ALAL013	0.56 ± 0.05^{c}
	Lactobacillus mali ALAL014	0.75 ± 0.08^{b}
	Lactobacillus reuteri ALAL001	0.53 ± 0.03^{c}
	Lactobacillus rhamnosus ALAL004	0.75 ± 0.09^{b}
Experiment 2	*Lactobacillus plantarum* BF-LP284	1.04 ± 0.04^{a}
	Lactobacillus plantarum JCM1055	0.34 ± 0.03^{d}
	Lactobacillus plantarum JCM1057	0.76 ± 0.03^{b}
	Lactobacillus plantarum JCM1149	0.77 ± 0.03^{b}
	Lactobacillus plantarum JCM1551	0.73 ± 0.05^{b}
	Lactobacillus plantarum JCM6651	0.68 ± 0.07^{b}
	Lactobacillus plantarum JCM8341	0.21 ± 0.05^{d}
	Lactobacillus plantarum JCM8342	0.68 ± 0.11^{b}
	Lactobacillus plantarum JCM8344	0.56 ± 0.06^{c}

PECs were cultured in well plates for 2 h at 37 °C and non-plastic adherent cells were removed. Heat-killed bacterial suspensions of *Lactobacillus* strains (Experiment 1) and 9 strains of *Lactobacillus plantarum* (Experiment 2) were added to each well (0.2 μg/200 μl), and then culture supernatants were harvested after cultivation for 24 h. The amount of TNF-α in the supernatants was determined by ELISA kits. Each value is expressed as mean ± SD ($n = 3$). Superscript letters indicate statistically significant differences among the strains ($p < 0.05$, Tukey's test). The results shown are representative of three independent experiments.

TNF-α induction in PPCs of tumor-bearing mice

As seen in Figure 3, TNF-α (24.14 ± 3.33pg/ml) was produced by the PPCs of mice administered H-Lp on the 16th day after Meth-A tumor cell inoculation. H-Lp stimulation was necessary to induce the production of TNF-α in PPCs from tumor-bearing mice that received H-Lp. TNF-α production in PPCs in tumor-bearing control mice was not detectable, even by H-Lp stimulation.

Cytokine production by splenocytes

Figure 4 discloses the induction of IFN-γ, TNF-α, and IL-12p40 in splenocytes of tumor-bearing mice administered H-Lp or physiological saline. IFN-γ was significantly more induced by stimulation with H-Lp (4.2 ± 1.5 pg/ml) than by no stimulation (2.1 ± 0.5 pg/ml) ($p < 0.05$). In control mice that had been administered physiological saline, no difference was seen between stimulation (2.12 ± 1.52pg/ml) and no stimulation (2.12 ± 0.40pg/ml) with H-Lp for IFN-γ. No apparent induction of TNF-α or IL-12p40 was noted in control splenocytes, regardless of the stimulant.

Winn neutralization assay

Tumor size in the H-Lp group (7.87 ± 2.04 mm) was significantly smaller than that in the control group (9.94 ± 0.91 mm) on the 10th day after Meth-A tumor cell inoculation ($p < 0.05$) (Figure 5). The number of

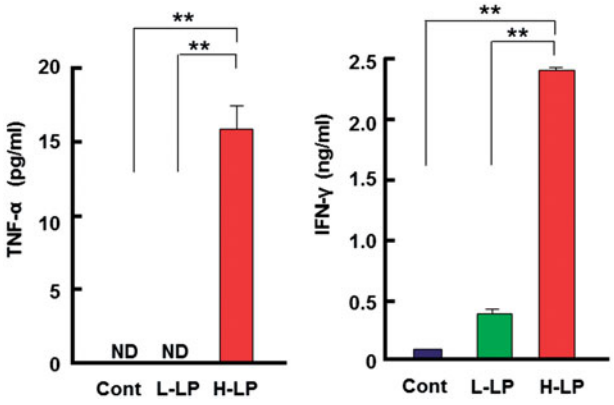

Figure 1. Cytokine production by Peyer's patch (PP) cells of BALB/c mice by stimulation with heat-killed cells of *Lactobacillus plantarum* BF-LP284 (H-Lp), live Lp (L-Lp), or medium alone (Cont). PPC suspensions (5×10^5 cells/well) were cultured in well plates with H-Lp or L-Lp (0.10μg/ml each) or medium only for 3 d. The amounts of TNF-α and IFN-γ in each culture supernatant were measured using ELISA kits. Values are expressed as mean ± SD ($n = 3$). ND: not detected. **$p < 0.01$ versus control or L-Lp values (Tukey's test). The results shown are representative of three independent experiments.

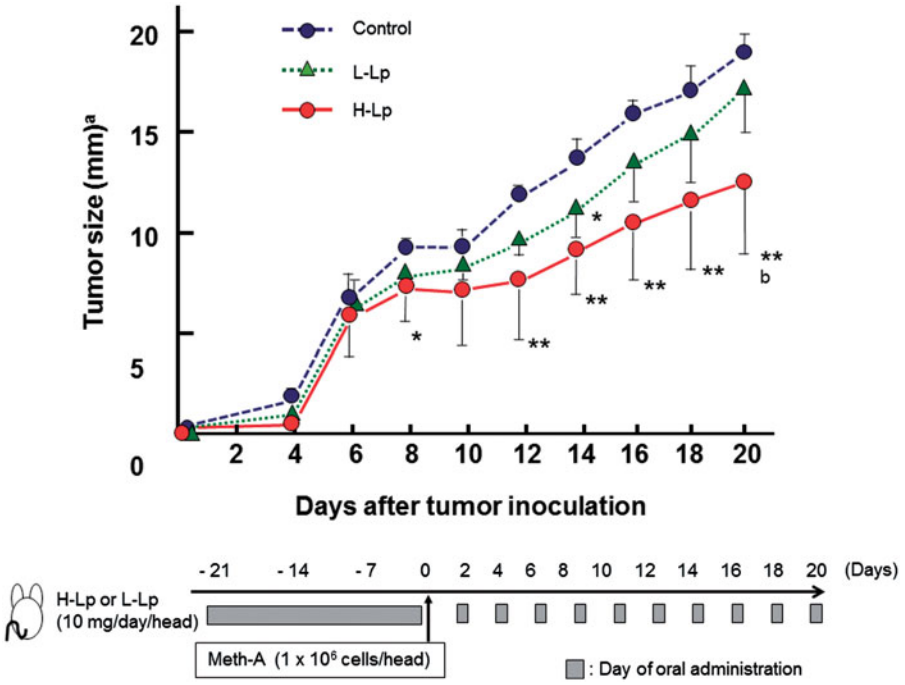

Figure 2. Growth inhibition of Meth-A tumors by oral administration of heat-killed *Lactobacillus plantarum* BF-LP284 (H-Lp) or live Lp (L-Lp) in BALB/c mice. H-Lp or L-Lp was administered orally (10 mg/day/head) every day and saline was used as a control. After 3 weeks of administration, Meth-A tumor cells (1×10^6 cells/0.2 ml/head) were implanted subcutaneously into the inguinal regions of the mice, and then animals were dosed every other day for 20 d. Values are expressed as mean ± SD ($n = 6$). [a]Tumor size was calculated by the square root of the value of the major axis × minor axis (mm). *$p < 0.05$ and **$p < 0.01$ versus control values (Tukey's test). [b]$p < 0.05$ versus values of L-Lp (Tukey's test). The results shown are representative of three independent experiments.

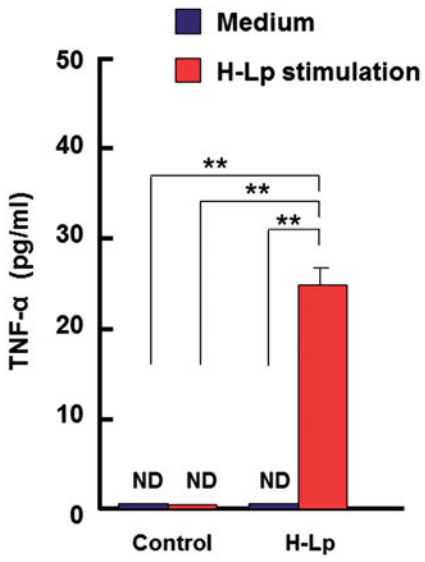

Figure 3. Production of TNF-α by Peyer's patch (PP) cells in tumor-bearing BALB/c mice administered heat-killed *Lactobacillus plantarum* BF-LP284 (H-Lp). PPC suspensions (5×10^5 cells/well) prepared from mice administered H-Lp or physiological saline (Control) were cultured in well plates with H-Lp (2.52μg/ml) or medium only for 7 d. The amounts of TNF-α in culture supernatants were measured using ELISA kits. Values are expressed as mean ± SD ($n = 3$). ND: not detected. **$p < 0.01$ versus control values (Tukey's test). The results shown are representative of three independent experiments.

spleen cells in the H-Lp and control groups was $6.37 \pm 1.84 \times 10^7$ and $6.30 \pm 2.03 \times 10^7$ cells/head, respectively, on day 16 after tumor cell inoculation. Spleen cell number in these groups were approximately 2.5-fold higher than that in intact mice (2.50×10^7 cells/head).

Analysis of PBMCs by flow cytometry

The ratio of $CD3^+$ T cells in the peripheral blood of mice administered H-Lp (22.6%) was 1.33 times greater than that of control group mice (17.0%) on the 18th day after Meth-A tumor cell inoculation (Figure 6). However, the ratios of $CD4^+$ cells (i.e. helper T cells) and $CD8^+$ cells (i.e. killer T cells) were comparable between the groups.

Discussion

The immunomodulation imparted by viable cells in probiotics can also be obtained from corresponding killed cells (Adams 2010). Several lines of evidence support that oral administration of live probiotics reduces tumor incidence and inhibits tumor growth in animals and humans by augmentation of host immune

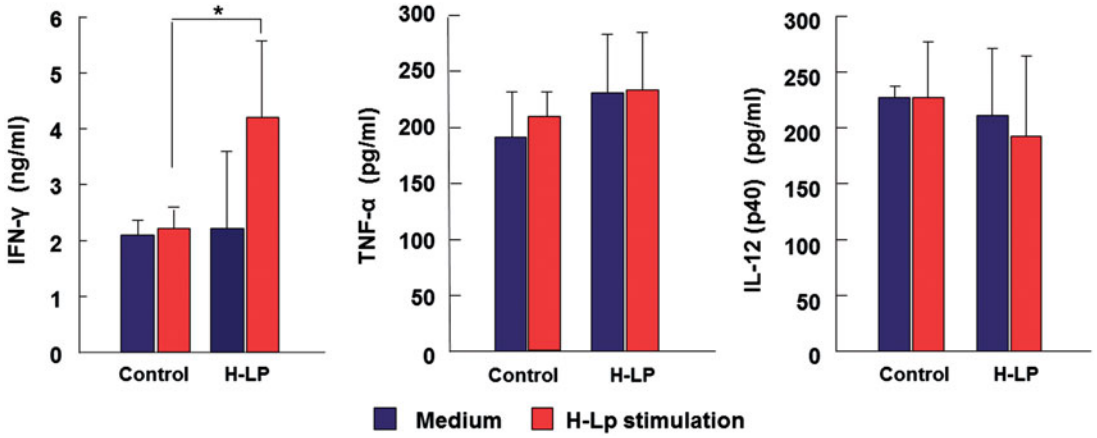

Figure 4. Cytokine production in spleen cells of Meth-A tumor-bearing BALB/c mice administered heat-killed *Lactobacillus plantarum* BF-LP284 (H-Lp). Cell suspensions (5×10^5 cells/well) prepared from mice administered H-Lp or physiological saline (Control) were cultured in well plates with H-Lp (0.02 μg/ml) or medium only for 3 d. Values are expressed as mean ± SD ($n = 4$). *$p < 0.05$ versus control values (Tukey's test). The results shown are representative of three independent experiments.

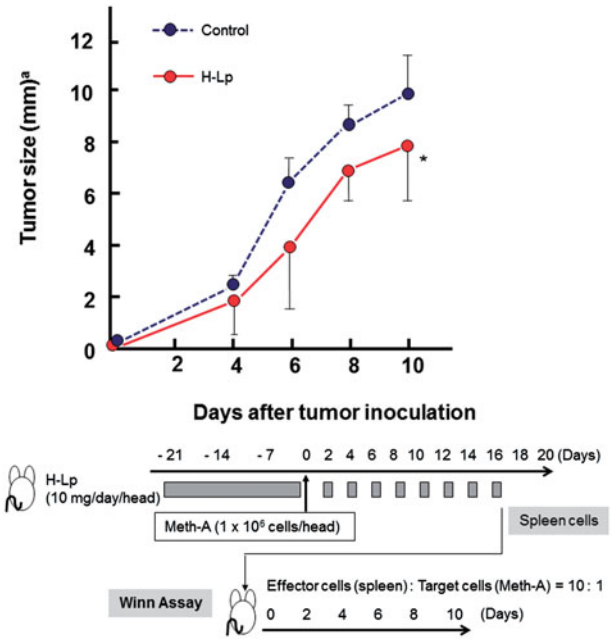

Figure 5. Winn neutralization assay of growth inhibition of Meth-A tumors by administration of heat-killed *Lactobacillus plantarum* BF-LP284 (H-Lp) in BALB/c mice. Spleens were obtained from mice ($n = 6$/group) administered H-Lp or physiological saline on day 16 after Meth-A tumor cell inoculation. Admixtures of spleen cells (1×10^7) and Meth-A tumor cells (1×10^6) at a 10:1 ratio were injected subcutaneously into the inguinal regions of syngeneic intact BALB/c mice. Values are expressed as mean ± SD. [a]Tumor size was calculated by the square root of the value of the major axis × minor axis (mm). *$p < 0.05$ versus control (Dunnett's test). The results shown are representative of three independent experiments.

systems (Kumar et al. 2010). However, the anti-tumor activity of live versus heat-killed probiotics is not well studied. Accordingly, we assessed for differences in anti-tumor activity between H-Lp and L-Lp using transplantable Meth-A tumors *in vivo*. Oral H-Lp clearly produced stronger inhibition of tumor growth than did control or L-Lp groups. These results might reflect differences in the stimulation ability of H-Lp and L-Lp specifically in PPCs.

As a penicillin and heat-killed *Streptococcus pyogenes* preparation and not a probiotic, OK-432 is a well-known potent biologic response modifier for cancer immunotherapy, even via oral administration (Nio et al. 1989). Orally administered OK-432 was incorporated into macrophages and DCs in PP to stimulate host immune systems against tumor cells, which established that the stimulatory activity of these cells in PP was indispensable in tumor-bearing mouse treatment with H-Lp. Here, high induction of TNF-α by H-Lp indicated that bacterial components were phagocytized into macrophages and DCs to induce cytokine production. Many reports have indicated that macrophages and DCs stimulated by antigens traffic to the MLN (Hiramatsu et al. 2011; Miura et al. 2012). Thus, probiotics can modulate immunity not only in the gut, but also in systemic immune organs, such as the MLN and spleen (Matsuno et al. 2010). During our preliminary examination, we witnessed that MLN cells from tumor-bearing animals induced higher levels of TNF-α in H-Lp-treated mice than in non-treated mice, which suggested that immune cells in PP activated by administration of H-Lp might have trafficked to the MLN.

We observed that IFN-γ-inducing activity in the spleen was enhanced by H-Lp in Meth-A tumor-bearing mice. Murosaki et al. (2000) and Fujiki et al. (2012) suggested that IFN-γ led to Th1-cell differentiation and formed a link between innate and adaptive immunity for tumor growth inhibition. Our results also implied that the immune cells stimulated by H-Lp produced high levels of IFN-γ, which contributed to

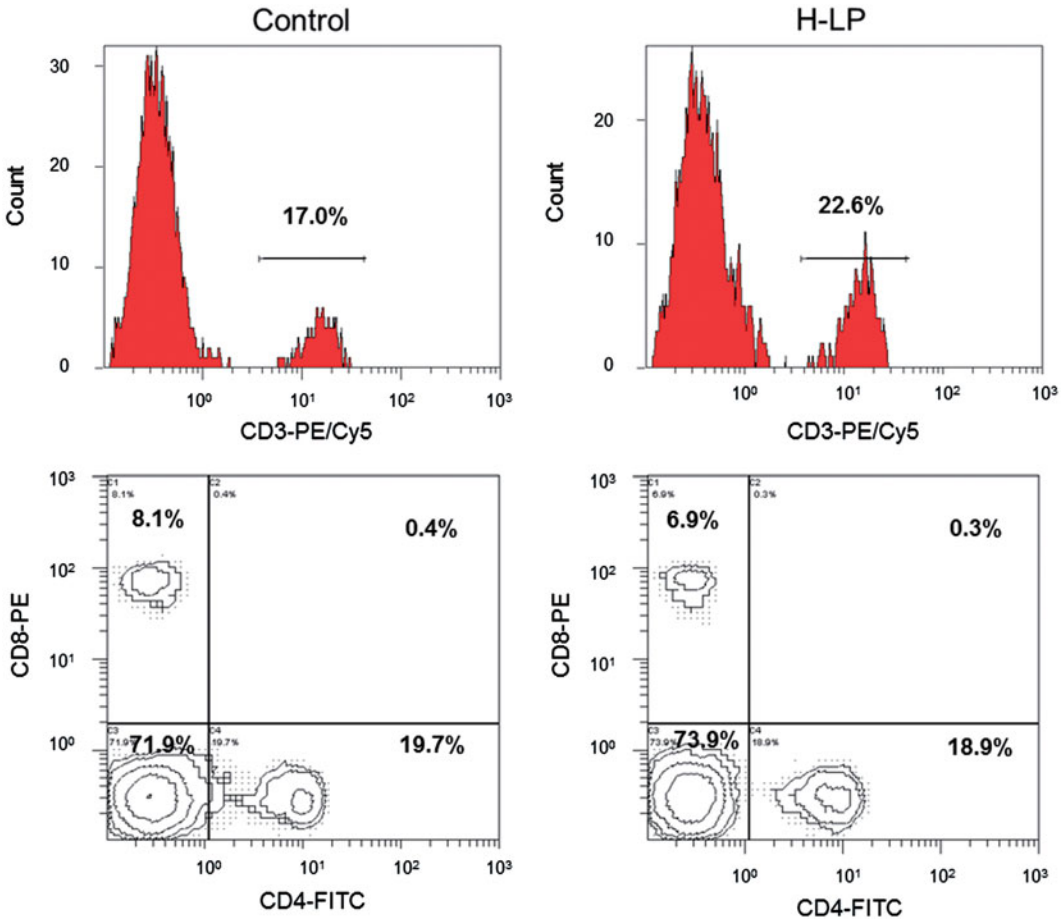

Figure 6. Flow cytometric analysis of peripheral blood mononuclear cells (PBMCs) in Meth-A tumor-bearing BALB/c mice. PBMCs were prepared from 6 mice per group on day 18 after Meth-A tumor cell inoculation and pooled for each group. PBMCs were stained with PE/Cy5-conjugated anti-mouse CD3ε, FITC-conjugated anti-mouse CD4, and PE-conjugated anti-mouse CD8a, and cell distribution ratio was evaluated using a Coulter Epics XL device. The results shown are representative of three independent experiments.

adaptive immunity for tumor growth inhibition. Takeda et al. (2013) demonstrated that gene expression of IFN-γ and IL-12p40 became elevated over that of non-treated controls by oral administration of *L. plantarum* 06CC2. However, IL-12p40 was not markedly affected by H-Lp in this study. To clarify this discrepancy, we examined the number of spleen cells on day 16 following tumor cell inoculation. The number of spleen cells was higher in tumor-bearing mice than in controls, which suggested that the increase in the number of spleen cells was due not to H-Lp, but to the presence of cancer. Rises in the number of splenocytes in tumor-bearing mice might depend on the migration and accumulation of immune cells induced by tumor antigens (Matsuno et al. 2010).

There was no difference in IL-12p40 induction between H-Lp and non-treated controls on the number of spleen cells in the present study. This could have been due to a smaller number of immune cells trafficking from PP by stimulation with H-Lp than by tumor antigens. Yazdi et al. (2010) and Maroof et al. (2012) stated that oral administration of *L. acidophilus* modulated immune responses by increasing IFN-γ and IL-12 production in splenocytes stimulated by tumor antigens and promoted Th1 responses to activate anti-tumor immune cells under oncogenic conditions. Furthermore, immune cells accumulated in the spleen from the tumor site to expand and differentiate into effector cells for tumor growth inhibition (Saiki et al. 2001; Ueta et al. 2008). Our findings and these reports demonstrate that the induction of various anti-tumor effector cells in the spleen, such as cytotoxic lymphocytes, NK cells, and cytotoxic macrophages, may be the key for tumor inhibition by H-Lp. The Winn assay also supported that the potent anti-tumor effector cells in the spleen might have been induced by H-Lp.

On the other hand, Lim et al. (2002) reported that levels of splenic $CD3^+$, $CD4^+$, and $CD8^+$ T lymphocytes factored prominently in suppressing bladder tumor formation in mice fed *L. rhamnosus* GG than in

control tumor-bearing mice, and Lee et al. (2004) demonstrated that the numbers of $CD3^+$ and $CD8^+$ cells in PBMCs were increased in tumor-bearing mice by oral administration of cytoplasmic fractions of *L. casei* and *Bifidobacterium longum*. These immune effector cells, which were found to be required for preventing and suppressing the development of cancer (Zamarron and Chen 2011), might have homed to tumor sites through the blood stream (Matsuno et al. 2010). Furthermore, Lim et al. (2002) reported that increased infiltration of immune cells at tumor sites inhibited tumor growth through a regime of *L. rhamnosus* GG. These immune cells ($CD3^+$, $CD4^+$, and $CD8^+$) may travel to tumor sites from the spleen in tumor-bearing mice. The higher levels of $CD3^+$ cells brought about by H-Lp in this study raised the possibility of increased trafficking of $CD3^+$ cells to tumor sites from the spleen through the blood stream. However, $CD4^+$ helper T-cell and $CD8^+$ killer T-cell populations were comparable between the H-Lp and control groups. Further studies are needed to clarify this discrepancy.

To summarize our results, H-Lp was shown as a potent immune modifier to inhibit tumor growth *in vivo* by the induction of TNF-α and IFN-γ in PPCs. Macrophages and DCs activated by H-Lp in PP and then trafficked to the spleen via the MLN, which was confirmed by the Winn assay. Lastly, that anti-tumor immune cells (i.e. $CD3^+$ cells) migrated to tumor sites through the bloodstream and inhibited tumor growth.

In conclusion, H-Lp may be a useful supplement to augment the host immune system. Additional study is required to clarify the mechanisms of anti-tumor immunity induction by oral administration of H-Lp.

Acknowledgements

We are grateful for the review of this article from the clinical standpoint by Dr. Morie Sekiguchi and thank Mrs. T. Ogata for her excellent technical support.

Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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