

• BASIC RESEARCH •

Distinct immune response induced by peptidoglycan derived from *Lactobacillus* sp

Jin Sun, Yong-Hui Shi, Guo-Wei Le, Xi-Yi Ma

Yong-Hui Shi, Guo-Wei Le, the Key Laboratory of Food Science and Security, Ministry of Education, Southern Yangtze University, Wuxi 214036, Jiangsu Province, China

Jin Sun, Yong-Hui Shi, Guo-Wei Le, Xi-Yi Ma, Institute of Food Nutrition and Safety, School of Food Science and Technology, Southern Yangtze University, Wuxi 214036, Jiangsu Province, China
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Correspondence to: Professor Yong-Hui Shi, Institute of Food Nutrition and Safety, School of Food Science and Technology, Southern Yangtze University, 170 Huihe Road, Wuxi 214036, Jiangsu Province, China. yhshi@sytu.edu.cn

Telephone: +86-510-5869236 Fax: +86-510-5869236

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INTRODUCTION

Lactobacilli have been shown to affect selected aspects of immune function that may involve one or several components of the humoral, cellular or nonspecific immunity^[1]. These functions suggest that the potential use of *Lactobacilli* in foodstuffs or medicines can prevent infection of pathogenic bacteria^[2] or induction of cancer^[3-5]. It is not known entirely, however, how *Lactobacilli* affect the immune system and exert these immunostimulative effects. It has been suggested that *Lactobacilli* activate innate immunity response and mediate a protective inflammatory response in the gut. The first defense mechanism in inflammation is innate immunity and involves epithelial barrier, phagocytes and release of cell-derived inflammatory mediators (i.e. cytokines). Innate immunity activates rapidly after exposure of the host to inflammatory stimuli. It has been found that pathogenic microbes express highly conserved molecular structures called pathogen-associated molecular patterns (PAMPs)^[6]. PAMPs include microbial cell wall components such as lipopolysaccharide (LPS), lipoteichoic acid and peptidoglycan (PG)^[7]. Active innate immune cells express pattern-recognition receptors (PRRs) which are capable of recognizing common features of PAMPs. Toll-like receptors (TLRs) are important PRRs involved in the activation of pro-inflammatory reactions^[6]. TLR2 is an important TLR member that recognizes PG. Binding of bacteria or bacterial products (i.e. PG) to TLRs leads to activation of intracellular signaling cascades, resulting in the activation of transcription factors, including nuclear factor κ B (NF- κ B), which regulates a wide variety of innate immunity responses. Activation of NF- κ B has been found to induce the expression of several inflammatory mediators such as pro-inflammatory cytokines and cell adhesion proteins^[8]. These factors acting alone, sequentially, or in combination, stimulate and amplify the inflammatory response. Recent studies indicate that, in addition to pathogenic microbes and their products, immune responses can be primed or activated by non-pathogenic commensal bacteria, including the probiotics such as *Lactobacillus* and *Bifidobacteria* species^[1]. Probiotics share distinct commensal-associated molecular patterns (CAMPs)^[9], which enhance epithelial barrier function in the gut, activate NF- κ B and cytokine production in monocytes/macrophages and natural killer (NK) cells and induce phagocytic activity in neutrophils^[10].

Abstract

AIM: To analyze the distinct immune responses induced by *Lactobacillus* peptidoglycan (PG).

METHODS: BALB/c mice were intraperitoneally injected with PG once a day for three consecutive days. Peritoneal macrophage and splenocyte mRNA was extracted and the gene expression profile was studied using high-density oligonucleotide microarrays. Inhibitory effects of *Lactobacillus* PG on colon tumor tissue were studied *in vitro* and *in vivo*.

RESULTS: The gene expression profiles revealed that the TLR-NF- κ B and Jak-STAT signaling pathways were highly activated. An inflammatory phenotype was induced when peritoneal macrophages were initially exposed to *Lactobacillus* PG and switched to a more complex phenotype when BALB/c mice were treated with three doses of *Lactobacillus* PG. A protective physiological inflammatory response was induced after three consecutive days of PG treatment. It was tending toward Th1 dominant immune response. *Lactobacillus* PG also appeared to induce a significant *in vivo* anti-colon tumor effect.

CONCLUSION: *Lactobacillus* PG is responsible for certain immune responses induced by *Lactobacilli*. Anti-tumor effects of *Lactobacilli* are likely to attribute to the activation of macrophages by PG expressed on the bacterial cell surface.

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Rakoff-Nahoum *et al.*^[11], demonstrated that the beneficial effects of commensal bacteria or probiotics are due to recognition of their surface molecules by TLRs. This recognition triggers TLRs to regulate genes that foster homeostasis of the intestinal epithelium. It also confers a protective effect on these cells and induces tissue repair. Elucidating the interaction between host and probiotic cell surface molecules is the key to this protective effect. However, there are few reports on systematic investigation of host cell responses to CAMPs from various probiotic strains. In an earlier study^[12], we found that *Lactobacillus* PG, a type of CAMP, enhances immune surveillance in mice. To delineate this response in a more comprehensive way, we undertook a genome-wide analysis of the immune response to *Lactobacillus* PG. The results reveal that the transcription of most genes participating in the immune response is modulated after PG treatment. Many of these genes are involved in the activation of TLR-NF- κ B and Jak-STAT signaling pathways. Activation of these signaling pathways is likely to invoke pro-helper T type-1 immune/cell-mediated immune response (Th1/CMI). Due to this induced response, anti-colon tumor effects of *Lactobacillus* PG were also studied and the results suggest that *Lactobacillus* PG can be used as a potential mediator in cancer immunotherapy.

MATERIALS AND METHODS

Preparation of peptidoglycan

A livestock feces derived *Lactobacillus* strain, *Lactobacillus* sp., was used. The strains were maintained at -80 °C as 15% glycerol stocks and subcultured in MRS broth or on MRS agar plates at 37 °C. *Lactobacillus* PG was prepared as previously described^[13] with minor modifications. Briefly, *Lactobacillus* sp. was grown in MRS liquid medium for 48 h at 37 °C without shaking. The culture was quickly chilled in an ice bath and the bacteria were harvested by centrifugation (2 000 r/min, 10 min, 4 °C) and washed several times with water. The cells were placed in a boiling water bath for 20 min before they were broken by sonication. The suspension was centrifuged at 1 000 r/min for 15 min to sediment unbroken bacteria and the supernatant containing the cell walls was decanted. After the centrifugation at 10 000×g for 10 min, the pelleted cell walls were incubated in 2% sodium dodecyl sulfate for 30 min at 60 °C. After being washed four times with water and twice with dehydrated alcohol, covalently bound proteins were digested with 0.02% trypsin in 0.1 mol/L Tris-HCl buffer (pH 7.5) for 20 h at 37 °C. The walls were washed several times with water and lyophilized, resuspended in 10% trichloroacetic acid and stirred for 20 min at 60 °C. PG was concentrated by centrifugation (10 000×g, 20 min) and washed extensively with water. All reagents used were sterile, and disposable plasticware or glassware baked at 180 °C was used to avoid contamination with endotoxin.

PG administration and preparation of macrophages and splenic lymphocytes

Three groups of BALB/c mice (three per group) were injected ip with either 0.5 mL of PBS (control) or PG

(25 mg/kg weight in 0.5 mL PBS) once a day for one day or three consecutive days. Mice were killed after administration of PG. Peritoneal macrophages and splenic lymphocytes were isolated and mixed together (1×10^7 cells each) for each treatment and stored at -70 °C in 2 mL TRIzol reagent for future use.

Analysis of gene expression by high-density oligonucleotide arrays

Gene expression analysis was performed using the Affymetrix MOE430A GeneChip system according to the manufacturer's instructions (Protocol for Eukaryotic Sample and Array Processing in <http://www.Affymetrix.com>). Briefly, total RNA was isolated using the RNeasy Mini Kit from QIAGEN. Double-stranded cDNA was synthesized using 0.2 µg poly (A)-RNA. Biotin-labeled cRNA was synthesized using the BioArray high-yield RNA transcript-labeling kit (Enzo Biochem) and equal amounts of fragmented RNA (30 µg) were hybridized to each array. The arrays were washed using the EukGW2 protocol on the GeneChip Fluidics Station 400 series and scanned using the GeneArray scanner.

Colon tumor inhibition by peptidoglycan

In order to determine *in vitro* inhibition of colon tumors by PG, CT26 colon tumor cells were collected in log phase, treated with 0.25% trypase, washed twice, and resuspended to 5×10^4 cells/mL in RPMI 1640 medium. The cell suspension (0.1 mL), and 0.1 mL of RPMI 1640 medium, with or without PG (100 µg/mL or 2 000 µg/mL), were added to each well of 40-well flat plates. After incubation for 48 h at 37 °C in a humidified atmosphere containing 50 mL/L CO₂ and 95% air, the cell supernatant was discarded and 100 µg methyl thiazolyl tetrazolium was added to each well. After another 4-6 h incubation, the suspension was centrifuged at 162 r/min at 4 °C for 5 min, the supernatant was discarded, and 100 µL dimethyl sulfoxide was added before A_{192} was determined.

To determine inhibition of colon tumors by PG *in vivo*, 30 BALB/c mice were inoculated with 0.2 mL CT26 cells (5×10^4 cells/mL) into the wall of left posterior limb and randomly allocated to three groups averagely. Mice were then administered ip with 0.5 mL physiologic saline, 0.1 or 0.5 mg PG in 0.5 mL physiologic saline, once in 2 d for 16 d. On d 17, tumor tissue and spleen were removed and stored at -80 °C for measurement of tumor weight, spleen weight, and analysis of tumor DNA by agarose gel electrophoresis.

Statistical analysis

The gene expression data were normalized and further processed using GeneChip analysis Microarray Suite 5.0 (Affymetrix) and Data Mining Tool Version 2.0 (Affymetrix). The GeneChip software performed statistical analysis of array data and generated signal intensity values representing the relative expression levels of each gene on the array. A comparison analysis was performed that directly compared probe pairs from one GeneChip to another. The signal log ratio (SLR) was used to estimate the change in expression level for a transcript between baseline and an experiment

array. This change was expressed as the \log_2 ratio. The fold change was calculated from the log ratio formula as follows: fold change = $2^{\text{single log ratio}}$ (if single log ratio ≥ 0) or $(-1) \times 2^{-\text{single log ratio}}$ (if single log ratio < 0). Therefore, a \log_2 ratio of 1 has a fold change of 2.

For annotation of genes, known or putative gene functions were ascertained from gene ontology (GO) classifications (provided at <http://www.NetAffx.com>). Probe sets can be found at <http://www.NetAffx.com> that provides oligonucleotide sequences of the probes and links to UniGene and other descriptive information. Pathways in KEGG (<http://www.genome.ad.jp/kegg/>) were analyzed to provide speculation on the possible biological pathways activated by PG, as suggested by the GeneChip data.

Inhibitions of colon tumors were analyzed using the SAS program. The statistical significance of the differences between group means was calculated by Student's two-tailed *t*-test for two groups, by single factor ANOVA, or by Tukey's test for three or more groups.

RESULTS

Global view of differentially expressed genes

To study the interaction between host cells and *Lactobacilli*, BALB/c mice were injected ip with PG derived from *Lactobacillus sp.* once a day for three consecutive days, and peritoneal macrophage and splenic lymphocyte mRNA was harvested. These mRNA samples were hybridized to oligonucleotide arrays containing 22 690 oligonucleotide probe sets (MOE430A GeneChip), each representing an expressed gene. The general information of gene expression is presented in Figure 1. Changes in gene expression were generally more marked after mice were treated once (e.g., expression of 5 085 genes was changed) than after three administrations during which only 3 995 genes were differentially regulated. When mice were treated once, a rapid and widespread response was triggered resulting in substantial changes in 3 332 genes (SLR was more than 1) and the total number of upregulated genes (1 762) was less than the number of genes whose expression was suppressed (3 323). When treated thrice, however, a slow but more specific response was induced resulting in transcriptional changes of 857 genes (SLR was more than 1) (Figure 1A). The total number of upregulated genes (1 846) was similar

to that of genes whose expression was suppressed (2 149). Of the 3 332 or 857 genes (absolute values of SLR were more than 1) in two different stimulating procedures, at least 125 upregulated and 191 downregulated genes of the mouse genome were mobilized in response to one or three times of stimuli (Figure 1B). Of these PG-responsive genes, 86 upregulated genes and 128 downregulated genes encoded proteins with known biological functions. These genes were divided into 10 functional categories (Figure 2) as defined by the Gene Ontology Consortium (<http://www.geneontology.org/>). The three categories of response to stress, signal transduction, and metabolism, accounted for 59% of the PG-responsive genes. Genes playing a role in defense and immunity (response to stress) or signal transduction comprised 44% of the PG-induced genes but only 14% of the downregulated genes. In contrast, genes with functions in intermediary metabolism made up 42% of the genes whose expression was suppressed by PG, but only 9% of the induced genes.

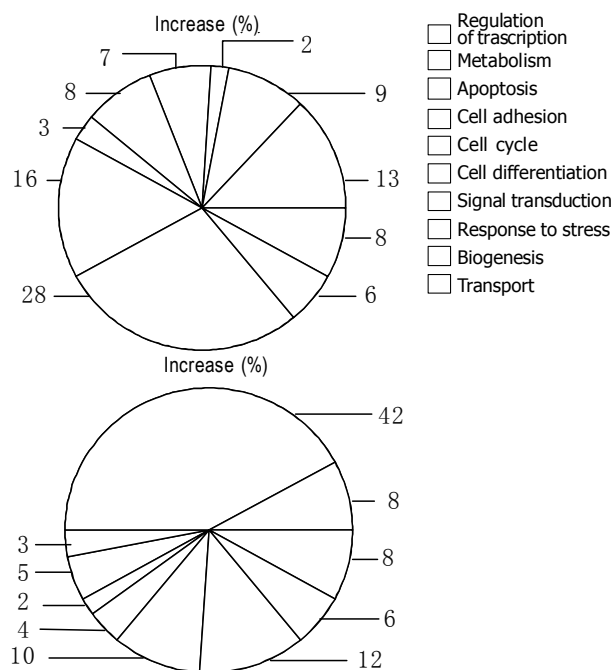
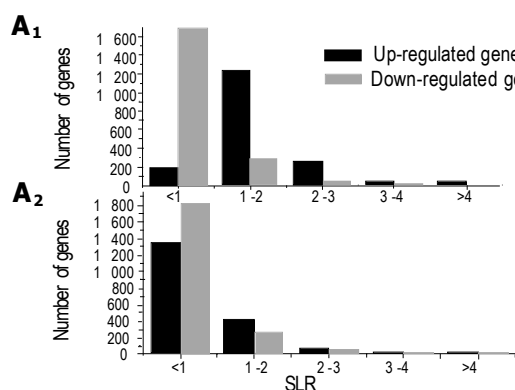


Figure 2 Functional profiles of expression changed by PG derived from *Lactobacillus sp.* in peritoneal macrophages and splenic lymphocytes.



B

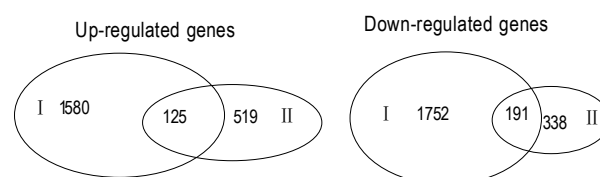


Figure 1 General information of gene expression change. **A:** Distribution of induced (black bars) and repressed genes (gray bars) based on their LSR; **B:** All induced and repressed genes with absolute value of LSR more than 1 after one and three doses of PG treatment and their intersection. I: One dose of PG treatment. II: Three doses of PG treatment.

Effects of peptidoglycan on genes involved in NF- κ B pathway

At least 361 and 57 stress response genes responded to PG when mice were treated once or thrice (absolute value of SLR was more than or equaled to 1). Expressions of 17 genes encoding important proteins that participate in TLR-NF- κ B pathway were regulated in different patterns after both treatments. According to the pathways defined at the KEGG database, a possibly regulated branch of TLR-NF- κ B signaling pathway is shown in Figure 3A. TLR2 could mediate response to PG and cell wall components of Gram-positive bacteria^[14]. Gene expression profile indicated that expression of TLR2 was only upregulated significantly after mice were treated with PG thrice. According to the findings of Anderson^[15], the strong expression of TLR2 would present in antigen presenting cells such as peritoneal macrophages. Different expressions of TLR2 in different treatments revealed that, in certain tissues, expression of certain TLR was different according to the time or frequency of cell interaction with particular probiotic CAMPs.

Several intracellular signal transduction pathways were activated when TLRs reacted with microbial products. The most prominent and best characterized of these is the NF- κ B signaling pathway^[16]. Expressions of downstream genes of TLR signal were analyzed. Results revealed that only certain branches of NF- κ B pathway were impacted by PG stimulation. Specifically, expression of toll-interleukin 1 receptor domain containing adaptor protein decreased after both PG treatments, suggesting that the cytoplasmic domain of TLRs was modified, affecting recruitment of myeloid differentiation primary response gene 88 (MyD88). Expression of thymoma viral proto-oncogene 1 (Akt1) was suppressed after a single PG dose. Expression of interleukin-1 receptor-associated kinase 1 (Trak1) was activated after a single PG stimulation. Expressions of Tnf receptor-associated factor 6 (Traf6) NF- κ B1 and NF- κ B2 were only induced after chronic stimulation. Furthermore, expression of mitogen-activated protein kinase 14 (P38) was upregulated after PG treatment.

It was demonstrated that once activated, Irak released from the receptor, and bound to and activated Traf6^[17]. In that study, increased expression of Irak was linked to the activation of Traf6. We postulate that the expression of Traf6 was induced during the later time of Irak stimulation. Akt1 is another important protein, which can directly phosphorylate inhibitor of kappaB kinase α (IKK α) and mitogen-activated protein kinase 6 (MKK6), leading to the activation of both Jun amino-terminal kinase (JNK)/p38 and NF- κ B signaling pathways^[18]. After single administration of PG, Akt1 expression decreased, indicating that in early period of stimulation Traf6 was a primary mediator of Ikk phosphorylation and led to nuclear transferring of NF- κ B. In three-dose stimulation, however, both TRAF6 and Akt1 took part in nuclear transport of NF- κ B. Perhaps the differential expression of Traf6 and Akt1 could lead to differential expression of products of NF- κ B signaling pathway. Many components of the NF- κ B pathway were stimulated only after the mice were treated thrice with PG. This finding could imply that constitutively expressed proteins comprising the NF- κ B pathway were

sufficient for early defense responses. Additional proteins might undergo degradation after initial PG stimulation. A series of genes encoding proteins of ubiquitin-proteasome proteolytic pathway were activated as shown in Table 2. Products of these genes could mediate destruction of NF- κ B pathway memberships. Tollip is a negative regulatory molecule in TLR signaling^[19]. In the present study, expression of Tollip was downregulated after acute injection of PG and the expression was resumed after mice received chronic PG treatment.

Some genes encoding products of NF- κ B pathway were upregulated (e.g., IL-1, IL-6, CD80, CD86) after PG treatment. IL-12 was only upregulated after acute PG treatment. It was reported that tumor necrosis factor (TNF) and IL-2 are molecules induced by some pathogens or probiotics exposed to macrophages^[16]. Our previous study suggested that PG could cause production of TNF in macrophages of cyclophosphamide-treated mice whose immune system was inhibited^[12]. In the present study, TNF gene expression was only upregulated when mice were treated chronically with PG. IL-2 expression, however, was not similarly induced. Therefore, expression of these molecules could be differentially regulated in healthy individuals compared to immune-compromised individuals. Because the expression of several cytokines such as interleukin-2 (IL-2), interferon- γ (IFN- γ), TNF- α and IL-3, as well as several growth factors, have been shown to be regulated by stabilization/destabilization of mRNA^[20], and IL-2 expression was not significantly affected by PG treatment in this study. IL-2 mRNA might go degradation, and was beneficial for the control of host immune response. After PG treatment, no change in iNOS (also named as NOS2 encoding a protein responsible for induced production of nitrogen monoxidum) mRNA levels was found. Although basic production of nitrogen monoxidum (NO) was beneficial for host to regulate defense response, too much NO induced a severe inflammatory response that might lead to tissue damage^[21]. *Lactobacillus* PG did not induce NO production, implying that inflammatory response induced by *Lactobacillus* was solely for enhancement of host immune surveillance.

Possible macrophage phenotype induced by PG

On the basis of macrophage gene expression patterns, Stoy^[22] has functionally classified macrophages into various phenotypes. External triggers, acting via cell membrane receptors (e.g., TLR2), and activation of the NF- κ B-dependent intracellular signaling pathway in macrophages, might be the most important determinants leading to the pro-inflammatory phenotype. NF- κ B could increase the release of cytokines (e.g., TNF- α , IL-1, IL-6, IL-12 and interferons), as well as amyloid A, adhesion molecules (e.g., Vcam-1 and Icam-1)^[23], chemokines, major histocompatibility complex (MHC) class I and II molecules^[24] from macrophages and a variety of other mammalian cell types. PG-induced proteins related to pro-inflammatory response and anti-inflammatory response are listed in Table 1. The innate response induced by *Lactobacillus* PG, reinforced over a limited period of time (one dose), sustained an elevation of IL-1 expression, increased IL-1 receptor type 2 mRNA

Table 1 Relationship between *Lactobacillus* PG-induced expression of proteins and pro-inflammatory and anti-inflammatory response

Probe set	Gene symbol	SLR		Probe set	Gene symbol	SLR	
		I	II			I	II
1450788_at	Amyloid A1	NC	1.1	1419282_at	Ccl12	-2.1	NC
1449326_x_at	Amyloid A2	-2.1	1.8	1420380_at	Ccl2	-4	NC
1450826_a_at	Amyloid A3	-2.4	NC	1417925_at	Ccl22	-1.2	0.5
1448162_at	Vcam1	2	1.6	1450488_at	Ccl24	-3.2	NC
1448862_at	Icam2	1.5	1.7	1421228_at	Ccl7	-4.3	0.6
1422930_at	Icam4	1.8	NC	1419684_at	Ccl8	-2.5	-1.3
1435290_x_at	H2-Aa	2	0.6	1417936_at	Ccl9	-1.6	NC
1451721_a_at	H2-Ab1	1.7	0.6	1419209_at	Cxcl1	NC	2.6
1422527_at	H2-DMa	1.4	0.6	1418930_at	Cxcl10	NC	3.6
1419744_at	H2-DMb1	1.6	0.6	1419697_at	Cxcl11	NC	4.5
1422891_at	H2-Ea	2.1	0.6	1417851_at	Cxcl13	NC	2.9
1417025_at	H2-Eb1	1.6	0.6	1449984_at	Cxcl2	NC	1.1
1419297_at	H2-Oa	2.4	NC	1418652_at	Cxcl9	NC	2.1
1418536_at	H2-Q7	1.3	0.7	1418480_at	Cxcl7	NC	-1.9
1449875_s_at	H2-T10	1.6	0.7	1418457_at	Cxcl14	-6.9	-1.3
1449556_at	H2-T23	1.2	0.7	1449195_s_at	Cxcl16	-1.1	NC
1422160_at	H2-T24	NC	0.6	1448995_at	Cxcl4	-1.5	NC
1452544_x_at	H2-K	NC	0.5	1419600_at	Defb4	-1.2	NC
1450534_x_at	H2-Q1	NC	0.5	1420653_at	Tgfb1	-2.2	0.6
1417314_at	H2-Bf	NC	1	1417455_at	Tgfb3	-2.5	NC
1426324_at	H2-D1	NC	1.1	1425947_at	Ifng	NC	0.9
1419413_at	Ccl17	1.5	1.7	1450330_at	Il10	NC	1.4
1419426_s_at	Ccl21a	3.8	NC	1418219_at	Il15	NC	3.5
1419561_at	Ccl3	2.6	1.9	1417268_at	Cd14	1.2	NC
1421578_at	Ccl4	2.5	2.4	1419532_at	Il1r2	1.2	NC

Probe set: probe used in Affymetrix GeneChip. NC: change in gene expression is not significant. Minus SLR: gene expression is downregulated. I: One dose of PG treatment. II: Three doses of PG treatment.

Table 2 Genes related to ubiquitin-dependent protein catabolism

Probe set	Gene symbol	SLR (I)	SLR (II)
1417172_at	Ubce8	2.7	NC
1422462_at	2700084L22Rik	1.7	NC
1452058_a_at	Rnf11	1.6	NC
1426404_a_at	Rnf11	1.1	NC
1454802_x_at	Arih2	1.1	1.1
1450696_at	Psmb9	1.3	NC
1422962_a_at	Psmb8	1	NC
1452402_at	Uchl3	1.5	NC
1449855_s_at	Uchl3	1.4	NC
1423107_at	Ube2b	1.1	NC
1454036_a_at	Usp15	1.2	NC
1450037_at	Usp9x	NC	1
1418191_at	Usp18	NC	2.1
1448420_a_at	Fbxl12	NC	1

Probe set: probe used in Affymetrix GeneChip. NC: change in gene expression is not significant. Minus SLR: gene expression is down-regulated. I: One dose of PG treatment. II: Three doses of PG treatment.

and induced (three doses) expression of PRRs, such as TLR2.

Serum amyloid (A1 and A2), intercellular adhesion molecules (Vcam1 and Icam2) increased after PG treatment. Icam4 was only induced after acute treatment. The majority of class II MHC genes were induced during the early period of stimulation with PG (SLR>1). Expressions of H2-T24, H2-K, H2-Q1 were induced during the later time period of stimulation with PG. H2-D1 and H2-b1, however, were induced after PG treatments. Eleven of C-C family chemotactic factors were inconsistently

induced or suppressed after either treatment. Expressions of seven C-X-C chemotactic factors were induced consistently after chronic PG treatment. These induced proteins, in addition to the pro-inflammatory cytokines (Figure 3A), were produced by activated macrophages and maintained the activated state. Upregulated expression of IL-12 and class II MHC genes mediated the initiation of the adaptive immune response of *Lactobacillus* PG by CD4⁺ T cells (Th cells). Studies have demonstrated adjuvant-like activity of *Lactobacillus*^[27], and factors induced by *Lactobacillus* PG in this study contributed to the enhancement of immune surveillance PG. Compared to one dose of PG treatment, a greater number of inflammatory genes were upregulated after three doses of PG treatment. Anti-inflammatory cytokines, such as TGF, were also upregulated during chronic PG treatment. This complex cytokine profile could lead to differential activation of helper T cells. Consequently, activated macrophages displayed an inflammatory phenotype, which switched to a complex phenotype induced by both inflammatory and anti-inflammatory factors. This differential response revealed the plasticity of macrophages when they were affected by *Lactobacillus* PG.

Immune processes underlying PG-mediated pro-Th1 signaling

One of the important signaling pathways induced by cytokines was involved in Janus tyrosine kinases (Jak kinases), signal transducers and activators of transcription (STAT proteins). At least 30 genes relating to T-cell proliferation and differentiation were observed to undergo significant alterations in expression after treatment. The Jak-STAT pathway was coordinately upregulated (Figure 3B), especially

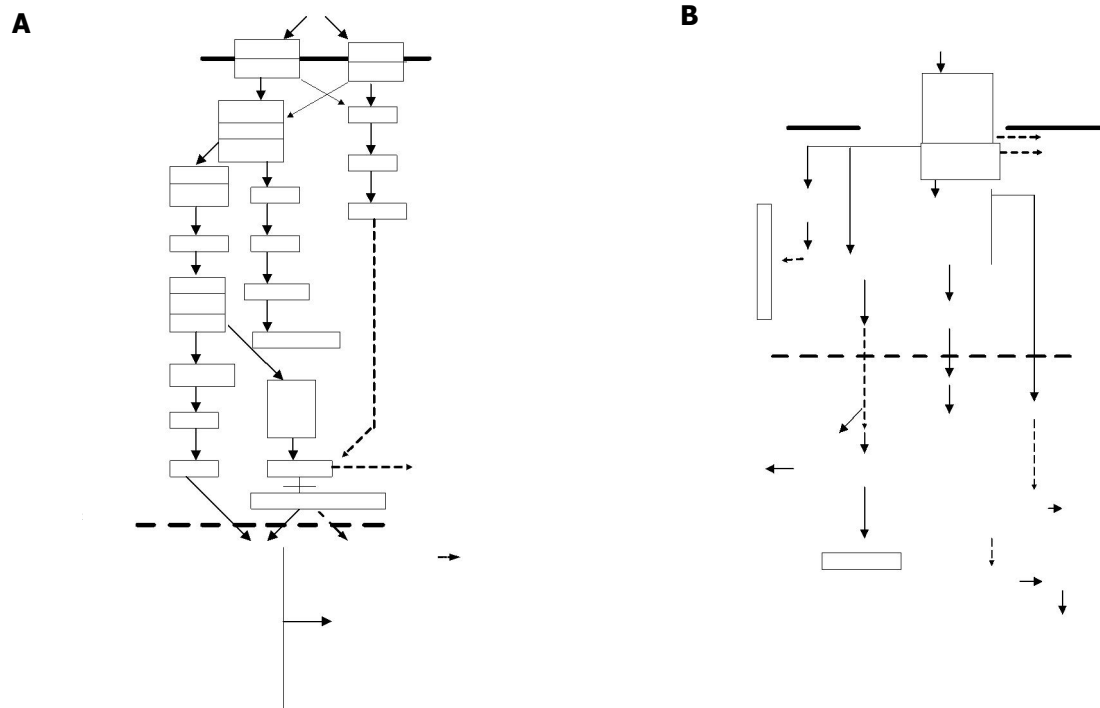


Figure 3 Functional mapping of genes to pathways for products of pro-

inflammatory cytokines (A) and IFN- γ and T-cell proliferation (B).

after consecutive PG treatment. After PG treatment, IL-2 receptor and proto-oncogene (Myb and Myc) were upregulated, essential for T-cell proliferation. This finding is coincident with those from our previous study^[12] in which proliferation of splenic lymphocytes was stimulated after single injection (ip) of *Lactobacillus* PG. Experimental evidence indicates that pro-Th1 immunoregulation is dependent on the ability of *Lactobacillus* to promote interferon production^[26], which in turn drives the production of pro-interferon monokines (principally IL-12 and IL-18). One dose PG treatment greatly induced expression of IL-12 and Stat4 (SLR was 1.9 and 2.6 respectively). Furthermore, expression of CD3 was also upregulated stimulating naive CD4⁺ T cell differentiated into type 1 helper T cells. It has been demonstrated that differentiation of T cells occurs 48-72 h after stimulation^[27]. In this experiment, upregulated expression of IFN- γ was observed after chronic PG treatment (about 72 h), suggesting the polarization of naive CD4⁺ T cells toward Th1 cells and the activation of the IL-12/IFN-axis. After either treatment, the gene encoding IL-18 receptor was also upregulated and served to maintain the development of CMI/Th1 response. Many downstream proteins involved in cytokine/cytokine receptor interaction within the Jak-STAT pathway were strongly activated after chronic PG treatment (Figure 3B). The major genes affected were Jak, Stat, cytokine inducible SH2-containing protein (cish), and pim-1 oncogene (Pim), suggesting that only certain branches of Jak-STAT pathway are activated. It should also be noted that continuous stimulation of mice with PG induced production of IL-10 and its receptor, likely to protect host from damage by CMI response. On the other hand, both Stat1 and Stat3 are activated by IL-6, IL-10^[28,29], which may lead to proliferation or differentiation of macrophages or lymphocytes via

activation of the Jak-STAT pathway.

Colon tumor suppression by *Lactobacillus* PG

Microarray analysis indicated that peritoneal macrophages were activated by *Lactobacillus* PG. We studied whether activation of macrophages could induce tumoricidal activity against CT26 colon cancer cells. Results indicated that there was a dose-dependent inhibition of CT26 colon cancer development in mice treated with PG ($P < 0.01$) (Figure 4B). The inhibition ratio of high PG dose was 54.2%. A DNA ladder appeared in the apoptosis analysis (Figure 5), indicating that cancer apoptosis was induced by PG. On the other hand, cancer cell line growth *in vitro* following PG treatment showed no direct inhibitory effect (Figure 4A), suggesting that PG-induced immune responses might be primarily responsible for these anti-tumor effects.

DISCUSSION

A recent study has shown that cytokine production by cells of the immune system can be altered by *Lactobacillus* strain^[1]. In the present study, immune response induced by *Lactobacillus* PG agreed with results induced by these *Lactobacillus* strains, indicating that *Lactobacillus* PG is responsible for certain immune responses induced by this type of important probiotic organism. *Lactobacillus* PG, as a distinct CAMP, could modulate the production of cytokines and other immunoregulatory factors of macrophages. Through these factors, macrophages play a pivotal immunoregulatory role in the Th1 and Th2 cell balance. In the present study, one dose of PG induced a temperate inflammatory reaction, and three doses of PG enhanced antigen presentation and T-cell differentiation. One dose of *Lactobacillus* PG induced the expression of IL-12 and

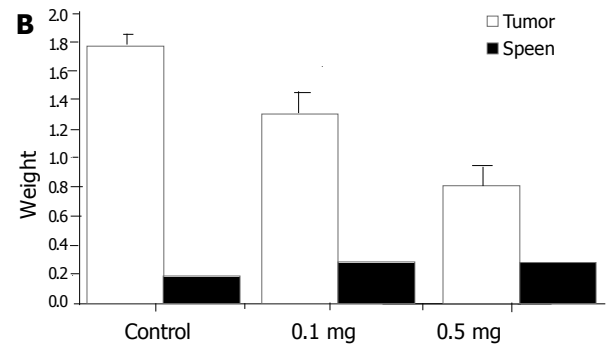
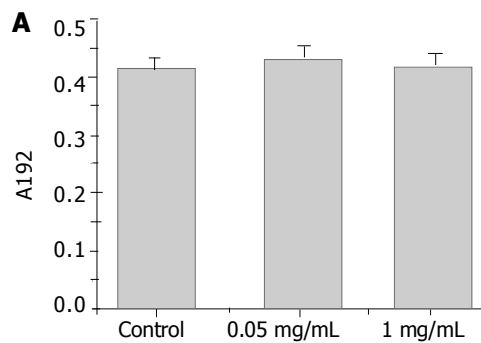


Figure 4 *In vitro* (A) and *in vivo* (B) inhibitory effects of PG on growth of CT26.

Values are mean±SD.

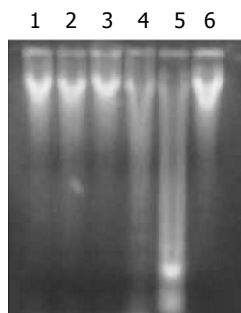


Figure 5 DNA of inoculated colon tumor in 2% agarose gel. Lane 1-3 and 6: control; lane 4: tumor-inoculated mice treated with low doses of PG; lane 5: tumor-inoculated mice treated with high doses of PG.

three doses of PG increased the expression of IFN- γ , suggesting that the induced immune response may develop toward Th1 immune response (CMI response). CMI response is important for tumor inhibition. The present study demonstrated that *Lactobacillus* PG could inhibit CT26 colon cancer development *in vivo*, which was mediated by increased CMI.

Enhanced phagocytic activity, NK cell activity, T- and B-cell function, and antigen-specific antibody responses are likely to result in an increased resistance to tumor incidence. All these types of tumoricidal activity require macrophage activation^[30]. Interaction between PAMPs and cell surface PRRs induce macrophage activation^[31]. Macrophages are important antigen-presenting cells that present throughout the gastrointestinal tract. Macrophages under lamina propria may be targets for modulation by gut microbes, including ingested probiotics. It has been found that foreign *Lactobacillus murinus* and cell fragments can penetrate the gut wall by translocation through the epithelial layer or through Peyer's patches^[31]. Indigenous intestinal bacteria including *Lactobacilli* are also able to cross the intestinal mucous layer and can survive in the spleen or in other organs for many days where they stimulate phagocytic activity. It is possible that physiologically translocated *Lactobacilli* carry PG or other CAMPs to macrophages in gut or other sites and can induce the production of modulatory molecules.

An important finding revealed by gene expression assay is that different doses of PG induce different cytokine profiles. In the present study, expression of Traf6 and Tollip was changed by different doses of *Lactobacillus* PG,

suggesting that expression of cytokine is regulated by activation or inhibition of Traf6 or Tollip expression. Zhang and Ghosh^[19] reported that overexpression of Tollip causes interaction with TLR2 and TLR4 and inhibits TLR signaling, indicating that Tollip plays a negative regulatory role in TLR signaling. These findings imply that *Lactobacillus* PG can modulate the activating state of TLR signaling partly, although it may change the expression of inhibitory molecules. On the other hand, in mammals, Traf proteins are key components in various NF- κ B activating pathways and overexpression of Traf2 or Traf6 activates NF- κ B^[32]. Traf6 knockout mice exhibit defects in IL-1 and LPS-induced NF- κ B activation^[33]. In the present study, three doses of *Lactobacillus* PG-induced high activation of TLR-NF κ B signaling might be a result of increased expression of TRAF6.

Although used widely, different strains of *Lactobacillus* can induce increased systemic or mucosal immune response at different levels of stimulation, and the stimulation is also dose-dependent^[27]. Maassen *et al.*^[34] have also found that the growth phase of orally administered individual *Lactobacillus* strains can differentially affect antigen-specific antibody subclasses IgG1 and IgG2a. This differential antibody response is likely due to growth phase-dependent differences in bacterial cell compositions^[31]. In the present study, we found that different doses of *Lactobacillus* PG led to different immune responses, suggesting that difference in CAMPs compositions and amount of CAMPs reaching to macrophages influences immune stimulatory effect of *Lactobacilli*.

In conclusion, the *Lactobacillus* PG-induced response is dominant in Th1, and a stimulatory effect is dose-independent. *Lactobacillus* PG influences the expression of signaling regulatory molecules modulating the cytokine profile and activation of lymphocytes. Pro-Th1 immune signals generated by probiotic *Lactobacilli* at the gut mucosal interface (and in some cases at other mucosal sites) can stimulate systemic CMI responses relating to cancer immunotherapy^[35].

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