Title: Inhibitory effect of heat-killed *Lactobacillus* strain on immunoglobulin E-mediated degranulation and late-phase immune reactions of mouse bone marrow-derived mast cells

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Running Head: EFFECT OF *LACTOBACILLUS* ON MAST CELLS

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ABSTRACT

This study investigated the *in vitro* effect of *Lactobacillus* strains, a major group of probiotic lactic acid bacteria, on immunoglobulin (Ig)E- and antigen-induced mast cell degranulation and subsequent gene expression. Bone marrow-derived mast cells (BMMCs) from DBA/2 mice were cultured with heat-killed *Lactobacillus* strains for 24 h. Some strains significantly inhibited IgE- and antigen-induced β-hexosaminidase release from BMMCs. Furthermore, *Lactobacillus reuteri* NBRC 15892, which exhibited the strongest inhibitory activity, significantly reduced the elevated interleukin (IL)-4, IL-13, tumor necrosis factor-α, and cyclooxygenase-2 expression levels that was induced by 1–2 h of stimulation with IgE and antigens. The suppressive effect of NBRC 15892 strain on BMMC degranulation was significantly reduced in the presence of a toll-like receptor (TLR)2-neutralizing antibody. In addition, downregulation of cell surface FcεRIα expression was observed after 6 h of NBRC 15892 treatment. These results suggest that some *Lactobacillus* strains inhibited IgE-mediated mast cell degranulation and subsequent late-phase reactions involving mast cells via a TLR2-dependent mechanism with FcεRIα downregulation.

Key words: *degranulation, Lactobacillus, mast cell, toll-like receptor 2, type I allergy*

INTRODUCTION

Genus *Lactobacillus* is a group of lactic acid bacteria that were widely used in a variety of lactic acid-fermented products. Some *Lactobacillus* strains are probiotic owing to their beneficial effects on human or animal health. The anti-allergic effect of probiotic *Lactobacillus* strains has been extensively researched (Segawa *et al.* 2008; Tobita *et al.* 2009). The effect by probiotic *Lactobacillus* strains has been attributed to reduction of immunoglobulin (Ig)E production (Matsuzaki *et al.* 1998), remediation of the T helper cell (Th) 1/Th 2 balance (Pochard *et al.* 2002; Fujiwara *et al.* 2004), and promotion of the production of immunosuppressive cytokines by regulatory T cells (Pessi *et al.* 2000).

Some of the immunomodulatory activities of probiotic *Lactobacillus* are considered to be exerted via toll-like receptors (TLRs), which are highly conserved receptors of pathogen-associated molecular patterns (PAMPs). TLRs play a fundamental role in innate immunity by recognizing multiple microorganism-derived ligands as well as PAMPs (Medzhitov 2001; Takeda *et al.* 2003). Probiotic *Lactobacillus* -derived components including peptidoglycan, lipoteichoic acid (LTA), and particular oligonucleotides, have also been reported to act as ligands of TLRs (Matsuguchi *et al.* 2003; Shimosato *et al.* 2005; Asong *et al.* 2009). Most of the documented TLR-mediated effects of probiotic *Lactobacillus* strains on immune responses have been described for antigen-presenting cells including macrophages and dendritic cells (Mohamadzadeh *et al.* 2005, Miettinen *et al.* 2008). This is because TLRs are predominantly located on these cells (Anderson 2000; Kaisho & Akira 2000). On the other hand, recent studies have revealed that mast cells also have some TLRs and produce proinflammatory cytokines such as interleukin (IL)-6 and tumor necrosis factor (TNF)-α in response to TLR-mediated stimulation (McCurdy *et al.* 2001; Supajatura *et al.* 2001; Kulka *et al.* 2004). Furthermore, Yoshioka *et al.* (2007) reported that treatment with *Staphylococcus aureus*-derived LTA, a TLR2 ligand, suppressed a high-affinity receptor for IgE (FcεRI)-mediated degranulation of human mast cells. This finding suggests that TLR2-ligand-containing *Lactobacillus* strains suppress mast cell degranulation. However, whether or not the entire *Lactobacillus* structure has this suppressive effect remains unclear.

Therefore, in the present study, the effect of heat-killed *Lactobacillus* strains on IgE- and antigen-induced degranulation was investigated using mouse bone marrow-derived mast cells (BMMCs) —a commonly used model of mucosal mast cells. This study shows that the effect of the degranulation-suppressive strain on IgE- and antigen-induced gene expressions in mast cells, which are related to late-phase reactions of immediate-type allergy.

MATERIALS AND METHODS

Bacteria

The *Lactobacillus* strains used in this study (Table 1) were purchased from the Japan Collection of Microorganisms (JCM; Saitama, Japan) and the National Institute of Technology and Evaluation (NITE) Biological Resource Center (NBRC;

Table 1

Chiba, Japan). All bacteria were propagated in glucose, yeast extract, and peptone (GYP) medium and stored as frozen cultures in the medium at –80°C.
The bacteria were cultured in the GYP medium at 30°C for 24 h and harvested by the centrifugation at 700 × *g* for 10 min. The resultant precipitate was washed 3 times with distilled water and then freeze-dried. Prior to use, the bacteria were killed by heating at 70°C for 30 min in a thermal cycler (PTC-200 DNA Engine; MJ Research, Waltham, MA, USA).

Mice
Specific pathogen-free male DBA/2 Cr mice aged 6–8 weeks were purchased from Japan SLC (Shizuoka, Japan) and housed at 23°C ± 3°C under a 12-h light/dark cycle. All the animal protocols used in this study were approved by the Committee for Animal Experiments of Shinshu University.

Preparation of BMMCs
BMMCs were prepared from 6–8-week-old mice according to a previously described method (Lee *et al.* 2005). In brief, mice were killed by cervical dislocation and their intact femurs were aseptically harvested. Bone marrow cells were obtained by repeatedly flushing the femurs with RPMI1640 medium (Nissui Pharmaceutical, Tokyo, Japan) containing 100 IU/mL penicillin and 100 µg/mL streptomycin. The cells thus obtained were washed twice with the same medium by centrifugation at 700 × *g* for 10 min. The centrifuged cells were suspended in complete RPMI1640 medium supplemented with 10% fetal bovine serum (FBS; Equitech-Bio, Kerrville, TX, USA), 0.1 mM non-essential amino acids (Invitrogen Life Technologies, Carlsbad, CA, USA), 5 ng/mL recombinant murine IL-3 (Peprotech, Rocky Hill, NJ, USA), 50 µM 2-mercaptoethanol, 100 IU/mL penicillin, and 100 µg/mL streptomycin. They were then cultured at a density of 1×10^5 cells/mL in a humidified atmosphere of 5% CO_2/95% air at 37°C. After 4–5 weeks, the cells were subjected to flow cytometric analysis for the evaluation of cell surface FcεRI and c-kit expression and to a β-hexosaminidase release assay, as described below.

Cultivation of BMMCs
BMMCs were cultured at a density of 1×10^6 cells/mL in 48-well tissue culture plates (Greiner Bio-One, Frickenhausen, Germany). Cells were cultured in the presence or absence of 100 µg/mL heat-killed *Lactobacillus* strain for 24 h.

In order to investigate the involvement of TLR2 in the *Lactobacillus*-mediated suppression of mast cell degranulation, BMMCs were treated or not with 2.5 or 5.0 µg/mL of neutralizing anti-mouse TLR2 antibody (clone T2.5; BioLegend, San Diego, CA, USA) 1 h prior to the addition of 50 µg/mL of the strain sample. Untreated BMMCs were used as controls.

β-Hexosaminidase release assay
To evaluate IgE-mediated mast cell degranulation, a previously reported β-hexosaminidase release assay was used (Razin *et al.* 1983). BMMCs cultivated with or without *Lactobacillus* strain were washed with HEPES-Tyrode buffer (137

mM NaCl, 5.6 mM glucose, 2.7 mM KCl, 0.5 mM NaH_2PO_4, 1.0 mM $CaCl_2$, and 10 mM HEPES at pH 7.3) containing 0.1% bovine serum albumin and then suspended in the same buffer. After washing, 5×10^5 BMMCs were stimulated with 1 μg/mL anti-dinitrophenyl (DNP) mouse IgE (clone SPE-7; Sigma, St. Louis, MO, USA) for 2 h and then exposed to 10 ng/mL DNP-conjugated human serum albumin (HSA; Sigma) in a 96-well tissue-culture plate for 30 min at 37°C. After stimulation, 40 μL of the supernatant was transferred into a 96-well microtiter plate (Nunc; Thermo Fisher Scientific, Roskilde, Denmark) and mixed with 40 μL of 1 mM *p*-nitrophenyl-*N*-acetyl-D-glucosaminide (Sigma) in a 0.1 M citrate buffer (pH 4.5) and incubated for 1 h. The reaction was stopped by adding 160 μL of a 0.1 M carbonate buffer (pH 10.0). The absorbance of the mixture was measured at 405 nm with a microplate reader (model 550; Bio-Rad Laboratories, Hercules, CA, USA). The relative ß-hexosaminidase activity was calculated using the following formula: Relative release (%) = $\{(T - N)/(P - N)\} \times 100$, where T, P, and N represent the absorbance of equal portions of supernatants derived from degranulated *Lactobacillus*-treated cells, degranulated control cells (positive controls), and non-degranulated control cells (treated with antigen alone; negative controls), respectively. In addition, the growth and viability of the cells in the presence or absence of *Lactobacillus* strain were evaluated by counting the cells with a hematocytometer after staining them with trypan blue.

Induction of late-phase immune reactions

BMMCs were cultivated with or without 100 μg/mL *Lactobacillus* strains in a 48-well tissue-culture plate (Greiner Bio-One) for 24 h. The cells were harvested and washed twice with the HEPES-Tyrode buffer. The washed cells were suspended in the same buffer in a sterilized centrifuge tube (BM Equipment, Tokyo, Japan) at a concentration of 1×10^7 cells/mL. Some of the cells were stimulated with 1 μg/mL of anti-DNP mouse antibody and 10 ng/mL DNP-labeled HSA for the indicated time at 37°C. After stimulation, the cells were collected by centrifugation. The resultant pellet was washed twice with a phosphate-buffered saline (PBS; pH7.2) and stored –80°C until use.

Reverse transcription-polymerase chain reaction

BMMCs (2×10^6 cells) were degranulated for the indicated time, and total RNA was extracted from them by using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. The extracted RNA (1 μg) was reverse transcribed in the PTC-200 with 1 mM of each dNTP, 2.5 U/μL of M-MLV reverse transcriptase (Invitrogen), and 10 pmol/μL of oligo$(dT)_{18}$ primer at 42°C for 50 min. The resulting cDNA was subjected to polymerase chain reaction (PCR) with SYBR Premix Ex Taq (Takara Bio, Shiga, Japan) and 10 pmol/μL of the primers listed in Table 2. The PCR consisted of 30 cycles of denaturation (94°C, 1 min), primer annealing (55°C, 1 min), and extension (72°C, 1 min) and was performed in a Thermal Cycler Dice Real Time System TP800 (Takara). The PCR products were analyzed by the ΔΔCt method using Multiplate RQ software (Takara). The amount of the PCR products was normalized against the expression level of the *GAPDH* gene.

Table 2

Flow cytometric analysis

The surface expression of FcεRI on BMMCs after treatment with or without 100 μg/ml *Lactobacillus* strain was determined using flow cytometric analysis. *S. aureus*-derived LTA (Sigma) was used as positive control after heated (70°C for 30 min) or not. For FcεRI labeling, 1×10^6 BMMCs were washed twice with PBS (pH7.2) and treated with 0.125 μg/mL fluorescein isothiocyanate (FITC)-conjugated Armenian hamster anti-mouse FcεRIα IgG (clone MAR-1; eBioscience, San Diego, CA, USA) and phycoerythrin (PE)-conjugated rat anti-mouse c-kit IgG (clone 2B8; eBioscience) at room temperature for 30 min. FITC-conjugated Armenian hamster IgG isotype control (clone eBio299Arm; eBioscience) and PE-conjugated rat IgG isotype control (clone eB149/10H5; eBioscience) were used as a negative control. After the cells were stained, they were washed with PBS and analyzed using FACSCalibur (BD Biosciences, San Jose, CA). All data were analyzed using the CellQuest Pro software (BD Biosciences).

Statistical analysis

The data were statistically analyzed using a two-sided Student *t*-test or Dunnett multiple comparison test. A *P*-value of less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Effect of heat-killed *Lactobacillus* strain on β-hexosaminidase release from BMMCs

Degranulation of mast cells or basophils induces immediate hypersensitivity reactions, which are classified as type I allergic reactions (Coombs & Gell 1968). In this study, the effects of *Lactobacillus* strains on BMMC degranulation were initially evaluated. Heat-killed *Lactobacillus* strain decreased β-hexosaminidase release from the BMMCs by 14–72%; further, this inhibitory effect was strain dependent, with *Lactobacillus reuteri* NBRC 15892 exhibiting the strongest inhibitory activity (Fig. 1). Treatment with the *Lactobacillus* strain alone had no obvious effect on the growth rate and viability of BMMCs and on spontaneous β-hexosaminidase release from BMMCs (data not shown). β-Hexosaminidase release has been widely accepted as an indicator of the degree of degranulation (Schwartz *et al.* 1979). Therefore, these results suggest that some of heat-killed *Lactobacillus* strains inhibited degranulation of BMMCs without affecting the viability of them.

Figure 1

Effects of the NBRC 15892 strain on gene expression in BMMCs

Type I allergic responses are followed by late-phase reactions that occur hours after the exposure to the allergen (Metcalfe *et al.* 1997). These late-phase reactions are mainly characterized by the release of newly synthesized inflammatory cytokines and arachidonic acid-derived lipid mediators. Thus, the effect of *Lactobacillus* strain on the expression of genes related to late-phase reaction

mediated by BMMCs was further investigated using the most potent inhibitory strain—NBRC 15892. IL-4, IL-13, and TNF-α were transiently expressed in BMMCs after the cells were stimulated with IgE and antigens. The marked elevation in the expression of these cytokines was consistent with previously reported findings (Plaut *et al.* 1989; Reddy *et al.* 2000). The expressions of all the above cytokines peaked at 1—2 h after exposure to IgE and the antigens and then decreased within 4 h of the exposure. In the presence of heat-killed NBRC 15892, the expression of these cytokines tended to decrease throughout the treatment period (Fig. 2). The IL-4 levels after 1 and 2 h, the IL-13 level after 1 h, and the TNF-α level after 2 h of the IgE-induced stimulation were statistically significant compared with that under NBRC 15892-untreated condition. In late-phase reactions, IL-4 and IL-13 play essential roles in the enhancement of local IgE production and subsequent IgE-related inflammation (Pawankar *et al.* 2000). TNF-α up-regulates endothelial and epithelial adhesion molecules and promotes the secretion of chemokines (Tonnel *et al.* 1996). This suggests that, by inhibiting the expression of these 3 cytokines at the transcriptional level, NBRC 15892 weakens the inflammatory responses. In addition, heat-killed NBRC 15892 significantly decreased the expression of cyclooxygenase (COX)-2—an inducible form of the enzyme responsible for prostaglandin (PG) production (Mitchell *et al.* 1995)—in BMMCs that had been stimulated with IgE for1 h. BMMCs are reported to produce PGD_2 via the induction of COX-2 expression peaked 1—2 h after stimulation with IgE and antigens (Ashraf *et al.* 1996). PGD_2 that is synthesized de novo after mast cell degranulation is involved in allergic inflammatory symptoms (Matsuoka *et al.* 2000). Reduced COX-2 expression suggests that NBRC 15892 suppresses inflammatory PG production.

Involvement of TLR2 in NBRC 15892-mediated suppression of BMMC degranulation

Recent studies have shown that BMMCs express TLRs, including TLR2 (Supajatura *et al.* 2001; Matsushima *et al.* 2004). In order to determine the involvement of TLR2 in the NBRC 15892-mediated suppression of BMMC degranulation, the effects of this strain were evaluated in the presence of a neutralizing anti-mouse TLR2 antibody. The inhibition of BMMC degranulation was significantly reduced in the presence of the neutralizing antibody (Fig. 3), indicating that TLR2-mediated signal transduction is required for the inhibitory activity of NBRC 15892. Furthermore, to elucidate the mechanism underlying the NBRC 15892-induced inhibition of BMMC degranulation, the level of FcεRI expression was measured using flow cytometric analysis. The expression of the FcεRI α chain, an extracellular subunit of FcεRI (Blank *et al.* 1991), of BMMCs was measured after treatment with NBRC 15892 for 6 and 24 h. The FcεRIα expression was reduced after treatment with NBRC 15892 and LTA for 6 h (Fig. 4A); however, the effect almost disappeared after 24-h treatment (Fig. 4B). In the study by Yoshioka *et al.* (2007), the suppressive effect of the same LTA on human mast cell degranulation was observed after over 24 h of treatment. The distinct difference in the time dependence for FcεRI downregulation between human mast cells and BMMCs is still obscure. Furthermore, the suppressive effects of

Figure 3

Figure 4

heat-treated LTA and untreated LTA on FcεRIα expression are comparable (Fig. 4A). Therefore, the distinct effect in BMMCs is not attributed to the heat treatment. Mast cell type or species specific factors may influence the duration of TLR2-medidated responses.

Time dependence of the suppressive effect of NBRC 15892 on β-hexosaminidase release from BMMCs

The effect of time length of NBRC 15892 treatment on the β-hexosaminidase release from BMMCs was further evaluated. As shown in Fig. 5, the suppressive effect was strongest at 6 h of treatment and then gradually weakened as the duration of treatment increased. This result is correlates with the downregulation of FcεRIα. However, it should be noted that considerable suppression was observed despite the recovery of FcεRIα expression. This result suggests that other mechanisms are involved in the suppression of BMMC degranulation.

Figure 5

The role of TLR2-mediated signals in mast cell degranulation is not yet fully understood. Supajatura *et al.* (2002) reported that a TLR2 ligand, a peptidoglycan derived from *S. aureus*, induced BMMC degranulation. Qiao *et al.* (2006) reported that TLR2-mediated signals synergistically enhance IgE-induced cytokine production from BMMCs. These finding imply that TLR2 ligand-mediated signals in mast cells are very complex, and have the potential to enhance, suppress, or trigger the degranulation and subsequent processes. Further studies are required to clarify the *Lactobacillus*-induced suppression of mast cell degranulation.

In conclusion, this study showed that some heat-killed *Lactobacillus* strains inhibit IgE- and antigen-induced degranulation of BMMCs. Furthermore, NBRC 15892, the most potent inhibitory strain, suppresses the expression of IL-4, IL-13, TNF-α, and the COX-2 genes, which are related to the late-phase immune reactions of mast cells. Degranulation and subsequent late-phase reactions of mast cells play essential roles in the progression and chronicity of type-I allergic symptoms. The results of this study suggest that some *Lactobacillus* strains have probiotic effects and may suppress type-I allergic reactions by inhibiting degranulation and subsequent late-phase reactions of mast cells.

ACKNOWLEDGMENTS

This work was supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science.

I would like to express my sincere gratitude to my supervisor, Professor Hajime Otani of the Laboratory of Food Bioscience, Graduate School of Agriculture, Shinshu University, for providing me the encouragement and the opportunity to study in his laboratory. This work was also technically supported by the Collaborated Research Center for Food Functions, Faculty of Agriculture, Shinshu University (CREFAS).

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FIGURE LEGENDS

Figure 1. β-Hexosaminidase activity in culture supernatant of IgE- and antigen-exposed BMMCs after treated with *Lactobacillus* strains. Data are represented as a relative percentage against the results obtained for the positive control (without *Lactobacillus* strain treatment). Data are represented as the mean ± standard deviation (SD) (n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. the positive control (Student *t*-test).

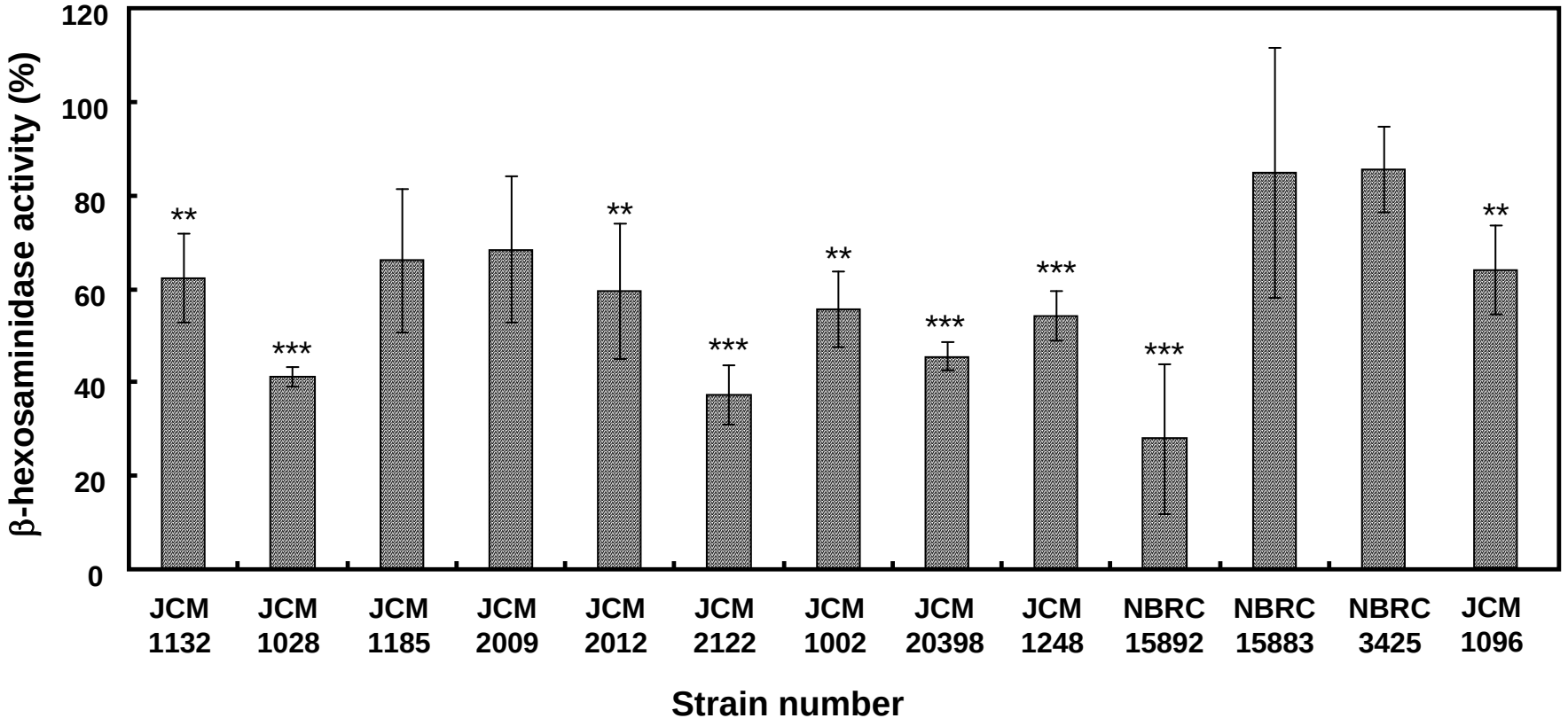
Figure 2. Temporal gene expression in BMMCs. Gray, black, and white circles represent gene expression in IgE- and antigen-exposed BMMCs after NBRC15892 treatment, IgE- and antigen-exposed BMMCs that were not treated with NBRC 15892, and normal BMMCs that were not treated with NBRC 15892, respectively. The black arrow indicates the time at which the cells were exposed to IgE and antigens. Data are represented as a relative value against the values obtained for normal BMMCs at 0 h. Data are represented as the mean ± SD (n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. the positive control (Student *t*-test).

Figure 3. β-Hexosaminidase activity in culture supernatant of IgE- and antigen-exposed BMMCs after cultivation with the NBRC 15892 strain in the presence of a TLR2-neutralizing antibody. Data are represented as the relative percentage against the results obtained with the positive control (without NBRC 15892 treatment). Data are represented as the mean ± SD (n = 3). The different letters indicate significant difference at $^{*}P < 0.05$ in Dunnett multiple comparison test.

Figure 4. Expression of FcεRIα and c-kit on the surface of BMMCs. Cells were analyzed after treatment with NBRC 15892 strain for 6 h (A) and 24 h (B). The numbers in the plots indicate the percentage of sorted cell population in each quadrant.

Figure 5. Time dependence of the suppressive effect of NBRC 15892 on β-hexosaminidase release from BMMCs. Data are represented as a relative value against the value obtained for the positive control (without NBRC 15892 treatment). Data are represented as the mean ± SD (n = 3). $^{***}P < 0.001$ vs. the positive control (Student *t*-test).

Figure 1


β-hexosaminidase activity (%)
120
100
80
60
40
20
0
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JCM 1132
JCM 1028
JCM 1185
JCM 2009
JCM 2012
JCM 2122
JCM 1002
JCM 20398
JCM 1248
NBRC 15892
NBRC 15883
NBRC 3425
JCM 1096
Strain number

Figure 2

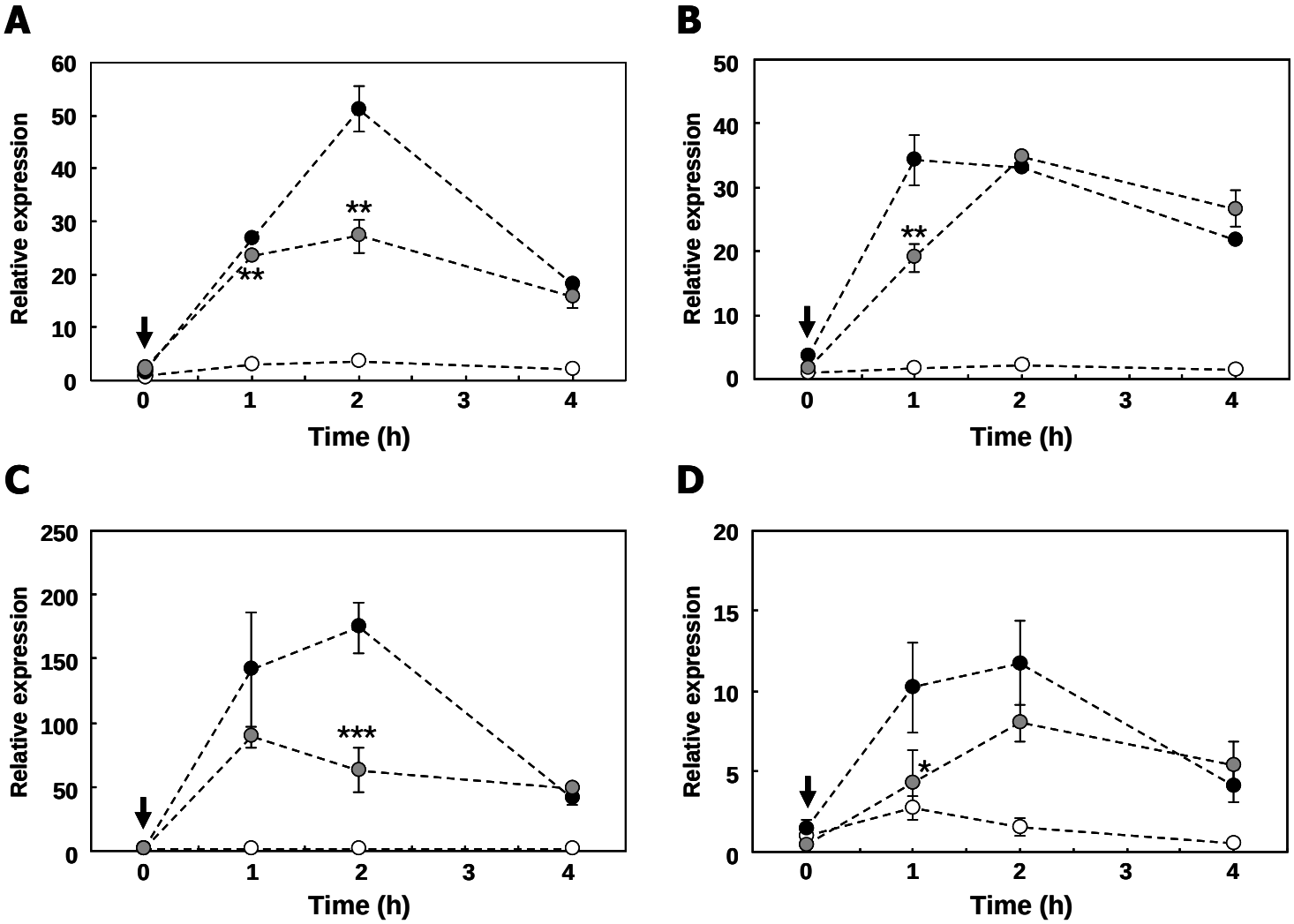

Figure 3

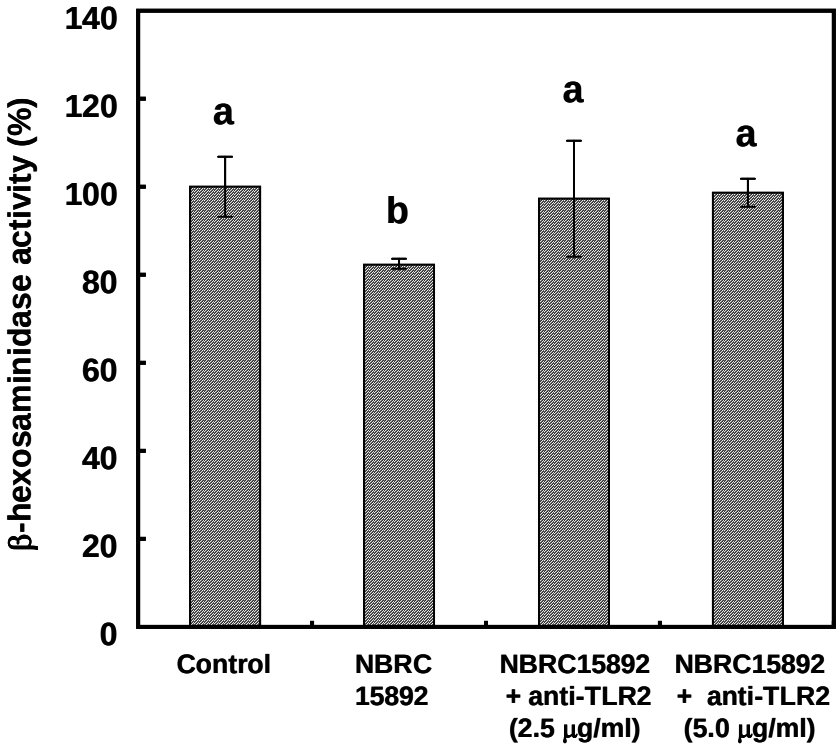
β-hexosaminidase activity (%)
140
120
100
80
60
40
20
0
a
b
a
a
Control
NBRC 15892
NBRC15892 + anti-TLR2 (2.5 μg/ml)
NBRC15892 + anti-TLR2 (5.0 μg/ml)

Figure 4

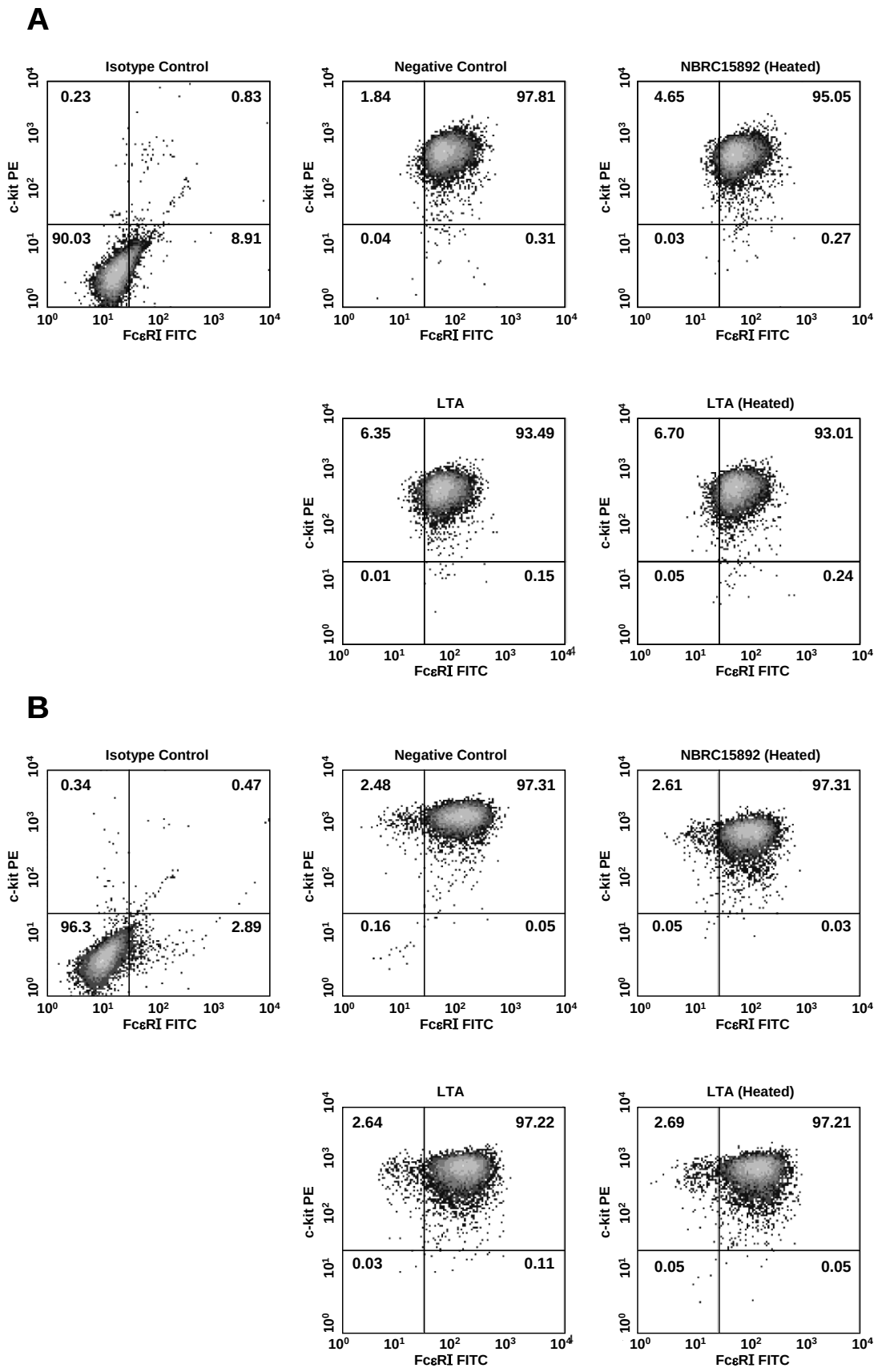
A
Isotype Control
0.23
0.83
90.03
8.91
Negative Control
1.84
97.81
0.04
0.31
NBRC15892 (Heated)
4.65
95.05
0.03
0.27
LTA
6.35
93.49
0.01
0.15
LTA (Heated)
6.70
93.01
0.05
0.24
B
Isotype Control
0.34
0.47
96.3
2.89
Negative Control
2.48
97.31
0.16
0.05
NBRC15892 (Heated)
2.61
97.31
0.05
0.03
LTA
2.64
97.22
0.03
0.11
LTA (Heated)
2.69
97.21
0.05
0.05
c-kit PE
FcεRI FITC

Figure 5

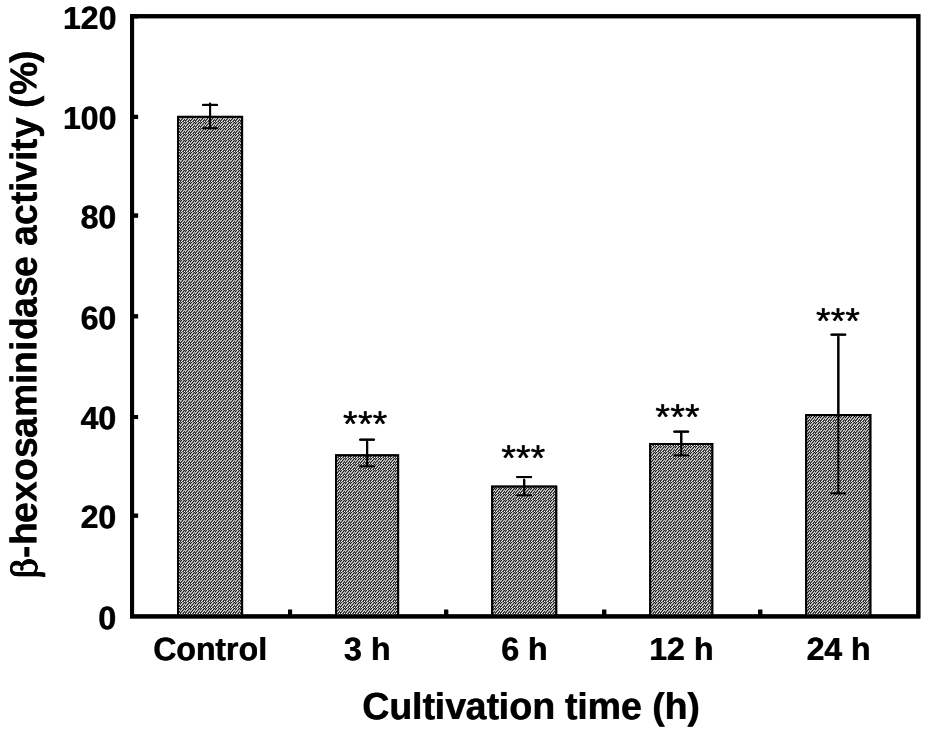
β-hexosaminidase activity (%)
120
100
80
60
40
20
0
***
***
***
***
Control
3 h
6 h
12 h
24 h
Cultivation time (h)

Table 1 LAB strains used in this study

Species	Strain	Source
Lactobacillus acidophilus	JCM1132	Human feces
Lactobacillus acidophilus	JCM1028	Human intestine
Lactobacillus crispatus	JCM1185	Human eye
Lactobacillus crispatus	JCM2009	Urine
Lactobacillus johnsonii	JCM2012	Human blood
Lactobacillus johnsonii	JCM2122	Sour milk
Lactobacillus delbrueckii subsp. *bulgaricus*	JCM1002	Bulgarian yogurt
Lactobacillus delbrueckii subsp. *bulgaricus*	JCM20398	Sour milk
Lactobacillus delbrueckii subsp. *lactis*	JCM1248	Emmental cheese
Lactobacillus reuteri	NBRC15892	Adult intestine
Lactobacillus casei	NBRC15883	Cheese
Lactobacillus rhamnosus	NBRC3425	(Unknown)
Lactobacillus curvatus	JCM1096	Milk

Table 2 Primer sequences used in this study

Target	Forward primer	Reverse primer	Product size	Accession Number
IL-4	TCAACCCCCAGCTAGTTGTC	TGTTCTTCGTTGCTGTGAGG	177	M25892
IL-13	CAGCATGGTATGGAGTGTGG	AGGCCATGCAATATCCTCTG	117	NM_008355
TNF-α	TAGCCAGGAGGGAGAACAGA	TTTTCTGGAGGGAGATGTGG	127	M13049
COX-2	TTGGGGAGACCATGGTAGAG	CATTGATGGTGGCTGTTTTG	231	M94967
GAPDH	CACTGAGCATCTCCCTCACA	GTGGGTGCAGCGAACTTTAT	111	BC096042