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ARTICLE

Heat-Treated *Lactobacillus crispatus* KT Strains Reduce Allergic Symptoms in Mice

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In the current study, we investigated the effects of heat-treated *Lactobacillus crispatus* KT strains on allergic response in mice. We found that the number of interferon (IFN)-γ^+CD4$^+$ cells was higher in C3H/HeN mouse spleen cultures incubated with *L. crispatus* KT strains than in those cultured with *Lactobacillus* JCM type cultures. The serum immunoglobulin E levels in NC/Nga mice that were administered KT strains were lower than those in the mice that were not given any bacterium. The ratio of spleen IFN-γ^+CD4$^+$/interleukin-4$^+$CD4$^+$ was highest in mice given *L. crispatus* KT-11. *L. crispatus* KT-11 also increased the expression of Toll-like receptor (TLR) 2, nucleotide-binding oligomerization domain (NOD) 1, and NOD2 in C3H/HeN mouse Peyer's patch cells. These results suggest that the *L. crispatus* KT-11 strain reduces allergic symptoms in NC/Nga mice via the adjustment of the type 1 helper T cell and type 2 helper T cell balance via TLR2, NOD1, and NOD2.

KEYWORDS: *Lactobacillus crispatus*; mite allergy; Th1/Th2 balance; Toll-like receptor; nucleotide-binding oligomerization domain

INTRODUCTION

Type I allergic symptoms are generally caused by degranulation that occurs following antigen cross-linking of immunoglobulin (Ig) E molecules present on mast cells (*1*), and thus, IgE is essential for type I allergic reactions. It is well established that interleukin (IL)-4, IL-5, and IL-13, which are produced by type 2 helper T (Th2) cells, stimulate IgE production (*1*). In particular, IL-4 is known to play a crucial role in IgE synthesis (*2*). In contrast, the type 1 helper T (Th1) cells mainly secrete interferon (IFN)-γ, which inhibits IL-4 production by the Th2 cells (*3*−*6*). As a result, a strict balance between Th1 and Th2 activity is essential for the control of IgE production.

Toll-like receptors (TLR) and nucleotide-binding oligomerization domains (NOD) comprise a family of pattern-recognition receptors that are known to be related to microbial specific molecular patterns (*7*−*9*). For example, the TLR2 recognizes lipoteichoic acid, peptidoglycan, and lipoprotein, while NOD1 and NOD2 detect distinct substructures of bacterial peptidoglycans (*10*). The stimulation of TLR results in the activation of various transcription factors including nuclear factor κB (NF-κB) and activator protein-1 (AP-1) (*11*, *12*), and induces the production of cytokines such as IL-1β, IL-6, and IL-12 (*13*). Similarly, NOD1 and NOD2 mediate the activation of mitogen-activated protein kinases and NF-κB via the caspase recruitment domain (CARD)-dependent recruitment of receptor-interacting protein 2 (RIP2) (*10*). As IL-12 is known to induce the release of IFN-γ from Th1 cells (*14*), it may therefore be possible that antiallergic activity depends on the quality and quantity of these receptors on host cells.

In recent years, numerous studies have reported on the beneficial effects of probiotic lactic acid bacteria. Various lactic acid bacterial strains exhibit the ability to prevent and alleviate type I allergic disease by modulating immune system responses. For example, *Lactobacillus acidophilus* inhibited nasal symptoms of allergic rhinitis, while *L. brevis* suppressed total and antigen-specific IgE production through the enhancement of Th1/Th2 balance in mice (*15*, *16*). We previously investigated the number of IFN-γ^+CD4$^+$ cells in C3H/HeN mouse spleen cells cultured in the presence of 29 lactic acid bacteria strains (KT-1 to KT-29) isolated from the feces of infant volunteers and found that *L. crispatus* KT-11, KT-23, and KT-25 strains significantly increased the total number of IFN-γ^+CD4$^+$ cells.

In the current study, we report the effects of heat-treated *L. crispatus* strains on allergic immune responses induced by the mite allergen *Dermatophagoides farinae* (Df) in NC/Nga mice and investigate the effects of KT-11 on TLR2, NOD1, and NOD2 expression in C3H/HeN mouse Peyer's patch cells in culture.

MATERIALS AND METHODS

Materials. Phycoerythrin (PE)-labeled antimouse IL-4 monoclonal antibody (mAb, clone 11B11), PE-labeled antimouse IFN-γ mAb (clone XMG1.2), biotin-labeled antimouse CD4 mAb (clone RM4−5), biotin-labeled antimouse CD11b mAb (clone M1/70), and phycoerythrin/cyanine 5 (PE/Cy5)-labeled streptavidin were purchased from BioLegend (San Diego, CA). Antimouse TLR2 polyclonal antibody (clone H-175) and PE-labeled antirabbit IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Brefeldin A (BFA), ionomycin, streptomycin, and phorbol 12-myristate 13-acetate (PMA) were purchased from Wako Pure Chemical Industries (Osaka, Japan). IntraPrep was obtained from

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Beckman Coulter (Marseille, France). Defined fetal bovine serum (FBS) was obtained from HyClone Laboratories (Road Logan, UT). Penicillin was purchased from MP Biomedicals (Costa Mesa, CA). RPMI-1640 was purchased from Nissui Pharmaceutical (Tokyo, Japan). TRIzol Reagent, dNTP, and M-MLV reverse transcriptase were obtained from Invitrogen Life Technologies (Carlsbad, CA). Two aliquots of SYBR premix Ex Taq mixture were purchased from Takara Bio (Shiga, Japan). Df-extract, a mite allergen, was obtained from LSL (Tokyo, Japan). Horseradish peroxidase (HRP)-labeled antimouse IgE and IgG were obtained from Bethyl Laboratories (Montgomery, TX). TMB was purchased from KPL (Gaithersburg, MD). All chemicals used in this study were of the highest analytical grade commercially available.

Lactic Acid Bacteria. *L. crispatus* KT-11, KT-23, and KT-25 were obtained as stock cultures from Kitii Co., Ltd. (Kanagawa, Japan). Additional lactic acid bacterial strains were obtained from the Japan Collection of Microorganisms (JCM, Saitama, Japan). The lactic acid bacteria were inoculated in MRS broth and cultivated for 72 h at 37 °C, collected by centrifugation, washed three times with sterile saline and lyophilized. Lactic acid bacteria prepared in this manner were then heated in 0.01 M phosphate-buffered saline (PBS, pH7.2) at 65 °C for 30 min prior to use in this study.

Spleen and Peyer's Patch Cell Suspensions and Cell Cultures. Six-week-old male C3H/HeN mice were obtained from Japan SLC (Shizuoka, Japan). Peyer's patches were collected from all mice entered into this study, while spleens were only collected from 2 mice chosen at random. The spleen tissue and Peyer's patches were then homogenized in RPMI-1640 medium containing 5% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. The resulting cell suspension was washed three times in this medium and adjusted to 1×10^6 viable cells/mL. One thousand microliters of the cell suspension was then plated into the wells of a 24 well flat bottom plate (Sarstedt, Inc., Newton, NC) and 100 μL of a lactic acid bacterium solution at a final whole cell concentration of 0 or 100 μg/mL added. The cells were cultured at 37 °C in a humidified 5% CO_2 incubator for 48 h (for cell functional analysis) or 3 h (for analysis of mRNA expressions). All animal experimentation undertaken during this study was conducted in accordance with the guidelines for the Regulation of Animal Experimentation at Shinshu University and according to Law No. 105 and Notification No. 6 of the Japanese government.

Cell Functional Analysis. Spleen cells were cultured at 37 °C in RPMI-1640 medium supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 20 μg/mL BFA, 2 μg/mL ionomycin, and 20 ng/mL PMA for 4 h. The cell surface markers for CD4 were then labeled using a specific biotin-conjugated antimouse mAb for 15 min at 4 °C, followed by incubation with PE/Cy5-labeled streptavidin for 15 min at 4 °C. Intracellular cytokines were measured following permeabilization and labeling with a PE-labeled antimouse cytokine mAb specific for IL-4 or IFN-γ. In order to achieve this, cells were fixed with IntraPrep reagent 1 for 15 min, washed, and permeabilized with IntraPrep reagent 2. The cells were then incubated with the appropriate antibodies and intracellular cytokine levels determined using a Guava personal cell functional analyzer (Guava PCA, Guava technologies, Hayward, CA). Peyer's patch cells were incubated with biotin-labeled antimouse antibody specific for CD11b for 15 min at 4 °C and then incubated with PE/Cy5-labeled streptavidin for 15 min at 4 °C. The cells were then incubated with antimouse antibody specific for TLR2 for 15 min at 4 °C and then incubated with PE-labeled antirabbit antibody for a further 15 min at 4 °C. The intracellular cytokine levels were then determined using a Guava PCA. This analysis was carried out at least in triplicate, and representative results are presented.

Preparation of Total RNA and Real-Time Reverse Transcription (RT)−Polymerase Chain Reaction (PCR). Analysis of mRNA expression was measured using real-time RT-PCR (Q-PCR). Total RNA from Peyer's Patch cells was extracted using TRIzol Reagent (Invitrogen) in accordance to the manufacturer's recommendations. The RT reaction was carried out by adding 1 mM of each dNTP, 2.5 units/μL M-MLV reverse transcriptase, and 10 pmol/μL oligo $d(T)_{18}$ primer to 0.5 μg of total RNA before incubating at 42 °C for 50 min. Q-PCR was conducted using a Thermal Cycler Dice Real Time TP800 system (Takara Bio) using 2 × SYBR premix Ex Taq mixture according to the manufacturer's instructions. The primer sequences for amplifying TLR2, NOD1, and NOD2 were as follows: TLR2 (sense), 5′-TGG TGT CTG GAG TCT GCT GTG-3′; TLR2 (antisense), 5′-CGC TCC GTA CGA AGT TCT CAG-3′; TLR4 (sense), 5′-CAG TGG TCA GTG TGA TTG TGG-3′; TLR4 (antisense), 5′-TTC CTG GAT GAT GTT GGC AGC-3′; NOD1 (sense), 5′-TCA CCC TCT GGT CCT GCT GGC TAA-3′; NOD1 (antisense), 5′-TCC TCC TGG CCA AAC ACA AAG A-3′; NOD2 (sense), 5′-GAG GAG CTT CCA GGA GTT TCT C-3′; NOD2 (antisense), 5′-AAG ACA GGG AGG TGG CAC AAA C-3′. The primer sequence for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been reported previously (*17*). The Q-PCR reaction involved 40 cycles of 95 °C for 5 s and 60 °C for 30 s. The relative amount of each mRNA was normalized using GAPDH expression as an internal control. An expression index was calculated from the normalized relative amount in the absence of the bacteria to the normalized relative amount in the presence of the bacteria. This analysis was carried out at least in triplicate, and representative results are presented.

Oral Administration. Four-week-old male NC/Nga mice were obtained from Charles River Japan (Kanagawa, Japan), and the mice were assigned to the test regimes for 2 weeks. After preliminary breeding, the mice were divided into 4 groups, in which they were administered saline (control), *L. crispatus* KT-11, KT-23, or KT-25 (control and KT-23, $n = 5$; KT-11 and KT-25, $n = 4$). For this experiment, mice were inoculated daily via oral administration of 0 or 1 mg of heat-treated lactic acid bacteria suspended in a sterile saline at a total volume of 500 μL from 6 to 21 weeks of age. A mite allergy was induced according to the description of Sasakawa et al. (*18*). The mice were intradermally injected with 5 μg of Df-extract dissolved in 10 μL of sterile saline into the ventral side of their right ear and their back weekly from 7 to 21 weeks of age. The thickness of the ear injected with Df-extract was measured weekly prior to the first injection and 24 h after each intradermal injection. Commercial mouse pellets (MF, Oriental Yeast Company, Tokyo, Japan) and water were supplied ad libitum throughout the course of the experiment. The mice were housed at 22 ± 2 °C under a standard 12-h light−dark cycle. The serum and spleen were collected immediately following a lethal dose of ether at 21 weeks of age and analyzed individually to investigate antibody levels and cell function.

Allergic Score. Allergic symptoms in the ear injected with Df-extract were scored in accordance to the description of Matsuda et al. (*19*). The degree of erythema, hemorrhaging, edema, scab/excoriation, and scaling/dryness was scored from 0 points (none) to 2 points (severe). The score was observed weekly and at random by 2 scorers. The sum of these scores was defined as the allergic score.

Analysis of Antibodies. Total-IgE and IgG levels in the serum were measured using a mouse enzyme-linked immunosorbent assay (ELISA) Quantitation Kit (Bethyl Laboratories) according to the manufacturer's protocol. Serum samples for the measurement of total-IgE or total-IgG were diluted 500-fold or 10000-fold in PBS containing 0.05% Tween 20 and 2% polyvinylpyrrolidone (PBS-T-PVP). Df-specific IgE and IgG levels were measured using ELISA. Briefly, the wells of microtiter plates (Nunc, Roskilde, Denmark) were coated with 100 μL of Df-extract (100 μg/mL) in 0.1 M carbonate buffer (pH 9.6) overnight at 4 °C and washed with PBS containing 0.05% Tween 20 (PBS-T). The plate was then postcoated with 300 μL of 0.1 M carbonate buffer (pH 9.6) containing 0.4% bovine serum albumin for 2 h at 25 °C and washed with PBS-T. The serum sample for Df-specific IgE or Df-specific IgG was diluted to 10-fold or 1000-fold in PBS-T-PVP and added to each well in a volume of 100 μL. The plate was then incubated for 2 h at 25 °C. After washing the plate as described above, 100 μL of optimally diluted HRP-labeled antimouse IgE or IgG in PBS-T-PVP was added to each well (IgE: 0.2 μg/mL, IgG; 0.1 μg/mL), the plate incubated for 1 h at 25 °C, and washed in PBS-T. One hundred microliters of 3,3′,5,5′-tetramethyl benzidine (TMB) was used to detect the peroxidase reaction. The plate was then incubated for 30 min at 25 °C and the reaction stopped by the addition of 100 μL of 4 N H_2SO_4. Total antigen levels were then read at 450 nm on a Bio-Rad model 550 microplate reader (Bio-Rad Laboratories, Hercules). The Df-specific antibody level was calculated using the following formula: antibody level = ELISA value (A_{450} nm) × dilution fold of the test sample.

Statistical Analysis. Data is presented as the mean ± standard deviation (SD). Statistical analyses were performed using the Dunnett's test for one-way analysis of variance. Differences were considered significant when P values were less than 0.05.

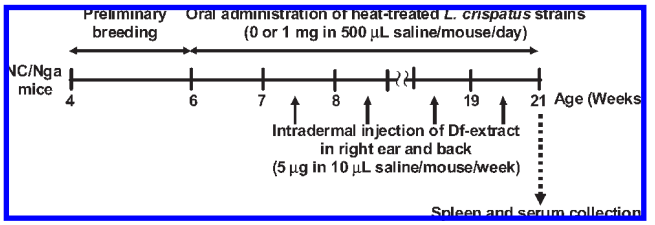

Figure 1. Schedules for the oral administration of heat-treated *L. crispatus* KT-11, KT-23, and KT-25, and for the intradermal injection of Df-extract. After preliminary breeding for 2 weeks, NC/Nga mice were inoculated daily with 0 or 1 mg of heat-treated bacteria suspended in a total volume of 500 μL of sterile saline between 6 and 21 weeks of age. Mice were intradermally injected with 5 μg of Df-extract dissolved in 10 μL of sterile saline into the ventral side of the right ear and their back weekly between 7 and 21 weeks of age.

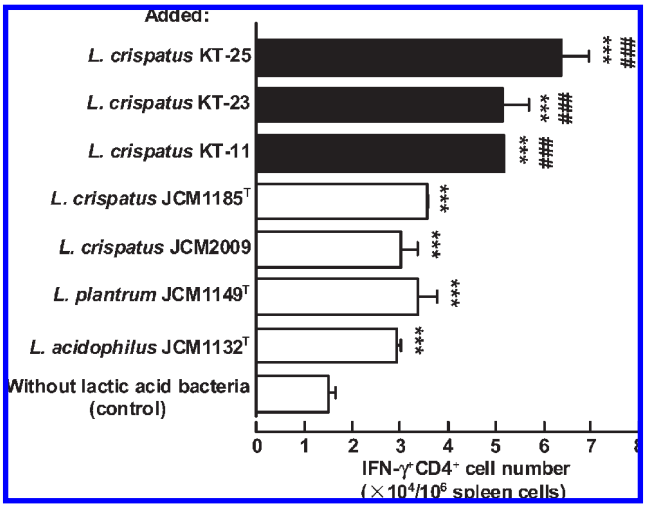

Figure 2. The total number of IFN-γ^+CD4$^+$ cells in C3H/HeN mouse spleen cultured in the presence of 3 *L. crispatus* KT strains and *Lactobacillus* type cultures. The spleen cells were cultured with heat-treated lactic acid bacteria for 48 h. The total number of IFN-γ^+CD4$^+$ cells was determined using Guava PCA. The data is presented as the mean ± SD (n = 3). ***P < 0.001 (compared to control). ###P < 0.001 (compared to *L. crispatus* JCM1185^T).

RESULTS AND DISCUSSION

Effects of Heat-Treated *L. crispatus* and Additional *Lactobacillus* Type Strains on the Number of IFN-γ^+CD4$^+$ Cells in Mouse Spleen Cell Cultures. Figure **2** shows the number of IFN-γ^+CD4$^+$ cells present in C3H/HeN mouse spleen cells cultured with heat-treated *Lactobacillus* KT and JCM strains. The total number of IFN-γ^+CD4$^+$ cells was more than 3 times higher in the cultures containing the lactic acid bacteria, in particular the KT strains, than in the cultures without any lactic acid bacterium (control). The total cell numbers were higher in the cultures treated with *L. crispatus* KT-11, KT-23, or KT-25 than in the cultures treated with *L. crispatus* JCM1185^T or JCM2009. Ishida et al. (*20*) reported that the immunostimulatory activities of lactic acid bacteria depend on the strain rather than the species. Our results appear to support this finding.

Effects of Oral Administration of Heat-Treated *L. crispatus* KT-11, KT-23, or KT-25 on Immune Response and Allergic Symptoms in Mice. In our study, NC/Nga mice were orally administered with heat-treated *L. crispatus* KT-11, KT-23, or KT-25 for 15 weeks (between 6 to 21 weeks of age). Under these conditions, we observed no significant differences in body weight between the mice treated with *L. crispatus* and the mice that were not given any bacterium (data not shown). **Figure 3** presents the ear thickness (part A) and allergic score (part B) of the NC/Nga mice. The ear thickness at 21 weeks of age was reduced in all mice administered with bacteria when compared to that of the mice that were not given any bacterium (control). The allergic score of the 21 week old mice was also lower in the mice given the bacterium than in the controls. **Figure 4** shows the serum levels for both total and Df-specific IgE and IgG. We found that the antibody levels were lower in the mice given the bacteria than the controls. In particular, the levels of total IgG and Df-specific IgE and IgG in the mice given *L. crispatus* KT-11, the levels of total and Df-specific IgE in mice given *L. crispatus* KT-23, and the levels of total IgE and Df-specific IgG in mice given *L. crispatus* KT-25 were significantly lower than those in the control treated mice. **Figure 5** shows the number of IFN-γ^+CD4$^+$ and IL-4$^+$CD4$^+$ cells, and the ratio of IFN-γ^+CD4$^+$/IL-4$^+$CD4$^+$ in the spleen of 21 week old mice. We observed a likely higher number of IFN-γ^+CD4$^+$ cells in the mice treated with bacteria when compared to the control treated mice, while the IL-4$^+$CD4$^+$ cells was significantly lower in mice given the bacteria. The ratio of IFN-γ^+CD4$^+$/IL-4$^+$CD4$^+$ was higher in mice given *L. crispatus* KT-11 when compared with the mice given *L. crispatus* KT-23 or KT-25. These results indicate that *L. crispatus* KT-11, KT-23, and KT-25 improve allergic symptoms by shifting the Th1/Th2 balance from a Th2-dominant state toward a Th1-dominant state. However, it is well established that *L. crispatus* belongs to the *L. acidophilus* group along with *L. gasseri*, *L. amylovorus*, *L. johnsonii*, and *L. gallinarum* (*21*). The *L. acidophilus* group is thought to play an important role in the human intestine (*22*, *23*). *L. acidophilus* and *L. johnsonii* strains were also reported to suppress antigen-specific IgE production and to reduce allergic reactions when administered orally

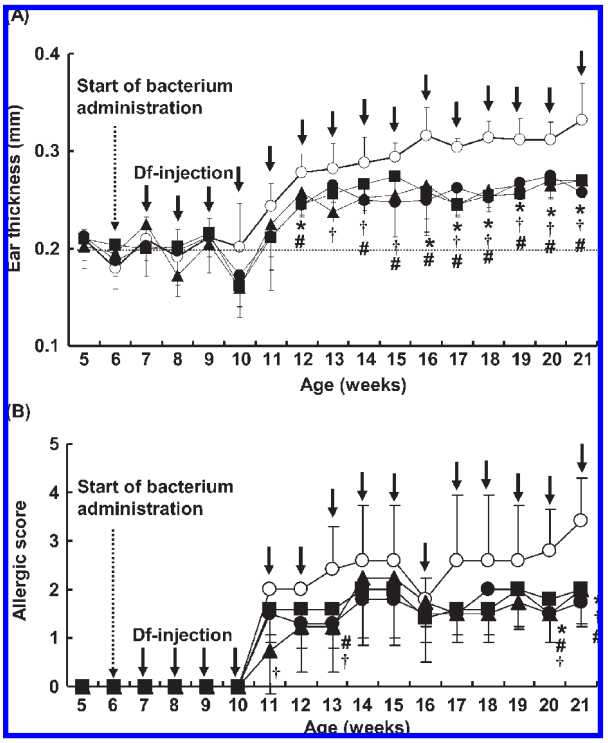

Figure 3. Total ear thickness (**A**) and allergic scores (**B**) for NC/Nga mice administered with *L. crispatus* KT-11, KT-23, or KT-25. The bacterium was given every day from 6 weeks of age. ○, control; ▲, KT-11; ■, KT-23; ●, KT-25. Each point represents the mean ± SD (n = 4−5). †, *, #P < 0.05 (†KT-11, *KT-23, or #KT-25 when compared with that of the control group). The arrows on the graph show the injection of Df-extract.

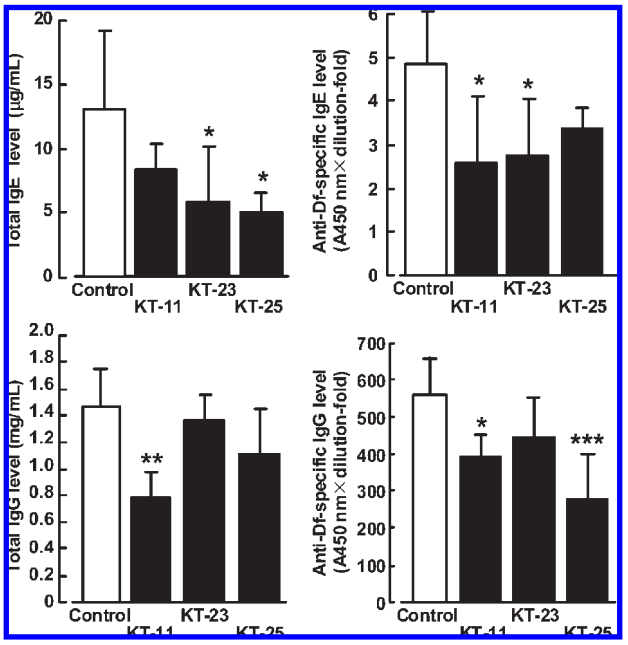

Figure 4. Total serum levels of Df-specific IgE and IgG in 21 week old NC/Nga mice given *L. crispatus* KT-11, KT-23, or KT-25. Total and Df-specific IgE and IgG levels were determined by ELISA. The data is presented as the mean ± SD ($n = 4-5$). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ (compared to those of the control groups).

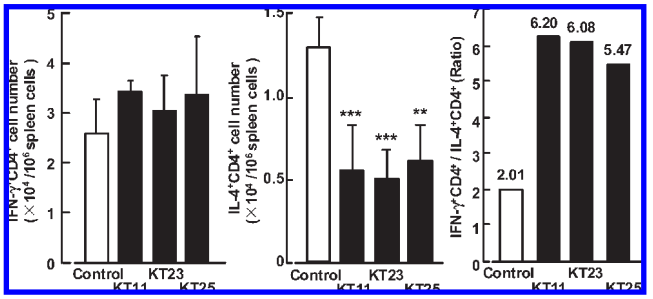

Figure 5. IFN-γ^+CD4$^+$ and IL-4$^+$CD4$^+$ cell numbers and their ratio in the spleen of 21 week old NC/Nga mice given *L. crispatus* KT-11, KT-23, or KT-25. The number of IFN-γ^+CD4$^+$ and IL-4$^+$CD4$^+$ cells was determined using Guava PCA. The data is presented as the mean ± SD ($n = 4-5$). **$P <$ 0.01 and ***$P < 0.001$ (compared to those of the control group). The IFN-γ^+CD4$^+$/IL-4$^+$CD4$^+$ ratio was calculated from the mean IFN-γ^+CD4$^+$ cell number against the mean IL-4$^+$CD4$^+$ cell number.

to allergic animal models (*24*, *25*). In the current study, we found that *L. crispatus* KT-11, KT-23, and KT-25 suppressed antigen-specific IgE production and reduced allergic symptoms in NC/Nga mice. Thus, we propose that *L. crispatus* exhibits an anti-allergic effect that is similar to other members of the *L. acidophilus* group.

Effects of Heat-Treated *L. crispatus* KT-11 and JCM1185^T on TLR2, NOD1, and NOD2 mRNA Expression on Mouse Peyer's Patch Cells. Figure 6A shows the expression of TLR2, NOD1, and NOD2 mRNA on C3H/HeN mouse Peyer's patch cells cultured in the presence of heat-treated *L. crispatus* KT-11 and JCM1185^T. The expression of TLR2, NOD1, and NOD2 mRNA was higher in cultures treated with *L.crispatus* KT-11 than in the cultures treated with *L. crispatus* JCM1185^T or those without any lactic acid bacterium treatment (control). No significant differences were observed in the expression of TLR4 mRNA in the presence of *L. crispatus* KT-11, *L. crispatus* JCM1185^T, or control treatment. **Figure 6B** shows the total number of

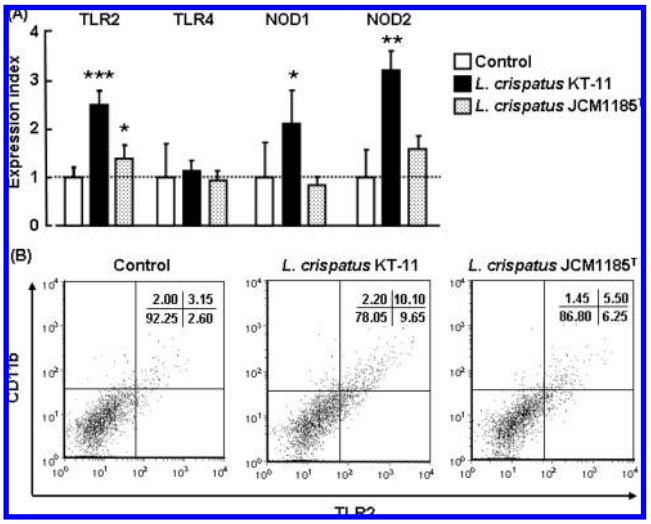

Figure 6. Expression index of TLR2, NOD1, and NOD2 mRNA (**A**), and the ratio of TLR2$^+$CD11b$^+$ cells (**B**) in C3H/HeN mouse Peyer's patch cells cultured with heat-treated *L. crispatus* KT-11 or JCM1185^T. Peyer's patch cells were cultured without the bacterium (control) or with 100 μg/mL bacteria for 3 h (**A**) or 48 h (**B**). The expression of TLR2, NOD1, and NOD2 mRNA was analyzed using Q-PCR. The data is presented as the mean ± SD ($n = 3$). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ (compared to those of the control). The number of TLR2$^+$CD11b$^+$ cells was determined using Guava PCA.

TLR2$^+$CD11b$^+$ cells in the Peyer's patch cells. The total cell number was higher in the cultures treated with *L.crispatus* KT-11 than in the cultures treated with *L. crispatus* JCM1185^T or the controls. TLR2, NOD1, and NOD2 are known to recognize bacterial lipoteichoic acid, lipoprotein, peptidoglycan, and their distinct substructures. These bacterial components are considered to suppress allergic responses via the production of Th1 cytokines (*26*−*28*). As described above, *L. crispatus* KT-11, KT-23, and KT-25 appeared to reduce allergic symptoms by shifting the Th1/Th2 balance from a Th2-dominant state toward a Th1-dominant state. The shifting of the Th1/Th2 balance by *L. crispatus* KT-11, KT-23, and KT-25 is thought to be associated with TLR2, NOD1, and/or NOD2. Recently, it was reported that specific DNA motifs of some lactic acid bacteria enhanced Th1 response via TLR9 (*29*, *30*). Therefore, with the exception of peptidoglycan, some cell components may also be related to the antiallergic effects of *L. crispatus* KT-11 via pattern recognition receptors.

In conclusion, we propose that *L. crispatus* KT strains, in particular KT-11, reduce mite-induced allergic symptoms in NC/Nga mice through the adjustment of Th1/Th2 balance via TLR2, NOD1, and/or NOD2. Thus, *L. crispatus* KT-11 may be expected to be utilized as a food material with potential antiallergic effects.

ABBREVIATIONS USED

AD, atopic dermatitis; AP-1, activator protein-1; BFA, brefeldin A; CARD, caspase recruitment domain; Df, *Dermatophagoides farinae*; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; Guava PCA, Guava personal cell functional analyzer; HRP, horseradish peroxidase; IFN, interferon; Ig, immunoglobulin; IL, interleukin; JCM, Japan Collection of Microorganisms; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; NOD, nucleotide-binding oligomerization domains; PBS, phosphate-buffered saline; PE, phycoerythrin; PE/Cy5, phycoerythrin/cyanine 5; PMA, phorbol 12-myristate 13-acetate; PVP, polyvinylpyrrolidone; RIP2, receptor-interacting protein 2; RT-PCR, reverse transcription−polymerase chain

reaction; Th1, type 1 helper T; Th2, type 2 helper T; TLR, Toll-like receptors; TMB, 3,3′,5,5′-tetramethyl benzidine.

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