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Full Paper

Mechanisms of interleukin-10 induction in murine spleen and RAW264 cells by *Latilactobacillus curvatus* K4G4 isolated from fermented *Brassica rapa* L.

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Lactic acid bacteria (LAB) are commonly used in fermented foods, and some LAB modulate the immune response. We aimed to investigate the mechanism by which LAB isolates from fermented *Brassica rapa* L. induce the production of anti-inflammatory interleukin (IL)-10 by the murine spleen and RAW264 cells. Spleen cells from BALB/c mice or the mouse macrophage cell line RAW264 were cultured with heat-killed LAB isolated from fermented *B. rapa* L., and the IL-10 level in the supernatant was measured. *Latilactobacillus curvatus* K4G4 provided the most potent IL-10 induction among 13 isolates. Cell wall components of K4G4 failed to induce IL-10, while treatment of the bacteria with RNase A under a high salt concentration altered K4G4 induction of IL-10 by spleen cells. In general, a low salt concentration diminished the IL-10 induction by all strains, including K4G4. In addition, chloroquine pretreatment and knock down of toll-like receptor 7 through small interfering RNA suppressed K4G4 induction of IL-10 production by RAW264 cells. Our results suggest that single-stranded RNA from K4G4 is involved, via endosomal toll-like receptor 7, in the induction of IL-10 production by macrophages. K4G4 is a promising candidate probiotic strain that modulates the immune response by inducing IL-10 from macrophages.

Key words: lactic acid bacteria, fermented *Brassica rapa* L., murine immune cells, interleukin-10, single-stranded RNA, toll-like receptor

INTRODUCTION

Lactic acid bacteria (LAB) are a group of gram-positive microorganisms that produce lactic acid as their main fermentation end product during carbohydrate metabolism [1]. They are widespread in nature and are commonly used in fermented food manufacturing. Thus, LAB are a highly represented group of bacteria in probiotics, which are defined as "living microorganisms that confer several health benefits when administered in adequate amounts to the host" by the World Health Organization [2]. LAB are involved in modulating the composition and function of the gut microbiome, and they help to improve intestinal barrier functions, immune modulation, competitive adhesion of pathogens to mucosal sites, attenuation of the virulent factors of pathogens, and the production of bioactive compounds with anti-infectious or other functional properties [1]. The immune-modulatory functions of LAB include the suppression and induction of inflammatory and anti-inflammatory cytokine production, respectively, as well as the regulation of the T helper (Th) 1/Th2 balance [3, 4].

Th1 cells are involved in cell-mediated immunity, while Th2 cells are involved in humoral immunity. Th2-skewed immune reactions are characterized by increased levels of Th2 cytokines, such as interleukin (IL)-4 and IL-5, and they contribute to the development of type I allergy [4]. IL-10 is one of the anti-inflammatory cytokines secreted mainly from macrophages, dendritic cells (DCs), monocytes, and regulatory T cells [5–7]. Some strains of LAB have been shown to induce IL-10, resulting in the suppression of allergic diseases [8]. IL-10 is also involved in the suppression of metabolic syndrome, inflammatory bowel diseases, and light-induced retinopathy [9–11].

Brassica rapa L., known as *Nozawana* in Japan, is often consumed as a lactic acid-fermented food called *Nozawana-zuke* in Nagano prefecture, Japan. Previous research identified *Latilactobacillus curvatus* as the most numerous LAB throughout the fermentation of *B. rapa* L., followed by *Lactiplantibacillus*

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plantarum and *Levilactobacillus brevis*, all of which induce IL-10 and interferon-γ production by mouse spleen cells [8]. Administration of a boiled-water-insoluble fraction of *B. rapa* L. has been reported to change the composition of the gut microbiota by increasing the number of butyrate-producing bacteria, and this fraction can also modulate the immune response, such as by increased IL-10 production by mouse spleen cells [12]. In addition, LAB isolated from fermented *B. rapa* L. can induce a Th1-type of immune modulation [13]. These results have raised interest in the functionality of LAB isolated from fermented *B. rapa* L.

Among the LAB isolated from fermented *B. rapa* L., the bacterial components that are involved in IL-10 induction, as well as the molecular mechanisms involved in such induction, are still unclear. Thus, our study focused on determining which LAB isolates from fermented *B. rapa* L. are able to induce anti-inflammatory cytokine IL-10 production and on investigating the causative mechanism of the IL-10 induction of LAB. Some bacterial components have been reported to induce cytokine production by immune cells. For example, the amount and structure of the teichoic acids (one of the bacterial cell wall components in gram-positive bacteria) of *L. plantarum* OLL2712 change according to the culture conditions, and thus culture conditions can affect the toll-like receptor (TLR) 2-dependent IL-10 production in bone marrow-derived dendritic cells (BMDCs) of BALB/c mice [3]. Furthermore, the amount of lipoteichoic acid (LTA) of *L. plantarum* L-137 is correlated with the expression levels of IL-12 p40 in DCs from the BALB/c mouse spleen [14]. However, the levels of IL-12 p70 production in the spleen and mesenteric lymph node cells of BALB/c mice are not correlated with the amount of LTA but rather are positively correlated with the levels of peptidoglycan (PGN) of LAB [15]. In the case of *Pediococcus acidilactici* K15, double-stranded (ds) RNA from this bacterium is involved in IL-6 or IL-12 induction, while single-stranded (ss) RNA is involved in IL-10 induction in DCs [16, 17]. In *Enterococcus faecalis* EC-12, ssRNA mainly contributes to the induction of IL-12 in human monocytes [18].

Here, we investigated the mechanism of IL-10 induction by LAB previously isolated from fermented *B. rapa* L. [8]. We focused on studying each isolate's cell wall fractions and RNA as the causative agents of IL-10 induction. We first treated spleen cells of BALB/c mice with different LAB strains, and then we compared the IL-10 induction activities among the different strains. We investigated RAW264 cells in a similar manner to clarify whether macrophages can be induced to produce IL-10 by LAB strains. In addition, both spleen and RAW264 cells were treated with cell wall fractions and ribonuclease (RNase)-treated LAB. Lastly, TLRs of RAW264 cells were knocked down with siRNAs to clarify the molecular mechanisms involved.

MATERIALS AND METHODS

Preparation of LAB

L. curvatus K4G4, K4G6, and K4G7 and *L. plantarum* K4G1, K4G2, K4G9, K4G13, K5G6, K5G8, K5G9, K5G18, K5G20, and K5G24 were isolated from fermented *B. rapa* L. [8]. LAB species were identified by 16S ribosomal RNA (rRNA) gene sequencing and species-specific PCR during previous research [19]. All LAB strains were cultured in de Man, Rogosa and Sharpe (MRS) broth (Becton Dickinson, Franklin Lakes, NJ, USA) at 37°C for 24 hr. Bacterial cells were centrifuged at 10,000 × g for 5 min and washed with distilled water. The cells were resuspended in 1 mL of distilled water, heat-killed by incubation at 65°C for 30 min, and lyophilized. The lyophilized cells were resuspended in phosphate-buffered saline (PBS) and stored at −80°C.

Preparation of cell wall components

Cell wall (CW), PGN, and defatted CW fractions of *L. curvatus* K4G4 were prepared as described in previous research [14, 20], with slight modifications. K4G4 was cultured in MRS broth (800 mL) at 37°C for 24 hr. The cells were collected by centrifugation at 10,000 × g at 4°C for 10 min, resuspended in 0.1 M phosphate buffer (pH 7.2), and then disrupted using an ultrasonic generator (VC-500, AS ONE, Osaka, Japan; set at an amplitude of 80 and disrupted for 3 min × 3) on ice. Unbroken cells were removed by centrifugation at 5,000 × g at 4°C for 30 min. The resulting supernatant was centrifuged at 19,000 × g at 4°C for 30 min, and the precipitate was resuspended in 4% SDS solution and boiled at 95°C for 40 min. After cooling to room temperature, the solution was pelleted by centrifugation at 19,000 × g at room temperature for 30 min, and the pellets were washed several times with sterilized water and resuspended in sterilized water. These suspensions were lyophilized and used as the CW fraction. To obtain the PGN fraction, the CW fraction was mixed with the same volume of 10% trichloroacetic acid, and the mixture was boiled for 20 min. After cooling to room temperature, the suspension was washed once with chloroform, twice with sterilized water, and then with ethanol. The solution was centrifuged at 20,600 × g at room temperature for 10 min, and the resulting pellets were lyophilized and used as the PGN fraction. The defatted CW fraction was prepared by mixing the CW fraction with the same amount of methanol and then centrifuging the mixture at 20,600 × g at room temperature for 10 min. The precipitate was delipidated through sequential suspension in methanol-chloroform-water (1:1:1, v/v), and methanol-water (1:1, v/v). The delipidated precipitate was completely air-dried and lyophilized, and the resulting product was used as the defatted CW fraction. All the different CW components were stored at −80°C prior to use.

Ribonuclease treatment

RNase A was used to digest dsRNA and/or ssRNA according to previous reports [16, 18]. Briefly, heat-killed LAB (1 mg) were suspended either in 120 µL of low-salt (10 mM Tris-HCl, pH 8.0) or high-salt (10 mM Tris-HCl, pH 8.0, 0.3 M NaCl) buffer, and RNase A (Macherey-Nagel, Düren, Germany) was added to a final concentration of 24 U/mL. To digest dsRNA, heat-killed LAB was digested with 100 U/mL of RNase III (New England Biolabs, Tokyo, Japan) in the high-salt buffer [18]. After 2 hr of incubation at 37°C, RNase-treated cells were washed twice with each respective buffer and resuspended in PBS. The cell suspensions were stored at −80°C prior to use.

Preparation and stimulation of splenocytes

Five-week-old female BALB/c mice (Charles River Laboratories Japan, Kanagawa, Japan) were housed at 23°C ± 2°C with a 12 hr light/dark cycle. The mice were rendered unconscious by CO_2 inhalation and euthanized by cervical dislocation. Their spleens were removed by laparotomy and then sandwiched between two nylon meshes. The spleens were then

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crushed using the plunger of a syringe. The crushed spleens were treated with hemolysis buffer (Tonbo Biosciences, San Diego, CA, USA) to deplete the tissue of red blood cells. After centrifugation, spleen cells were resuspended in RPMI-1640 medium (Nacalai Tesque, Kyoto, Japan) containing 10% (v/v) fetal calf serum (JRH Biosciences, Tokyo, Japan), 1% penicillin-streptomycin, and 50 μM 2-mercaptoethanol (Sigma-Aldrich, Tokyo, Japan). Spleen cells were cultured in 96-well flat-bottomed plates at a concentration of 5×10^5 cells/well in the presence of heat-killed LAB at 37°C for 48 hr in an atmosphere containing 5% CO_2. After incubation, the supernatants were collected and stored at −80°C until cytokine measurements.

All experimental procedures were conducted in accordance with the Regulations for Animal Experimentation of Obihiro University of Agriculture and Veterinary Medicine, Japan (approval no. 21-126 and 21-182).

Cultivation and stimulation of RAW264 cells

RAW264 cells derived from murine macrophages were purchased from the RIKEN BioResource Center (RCB0535; Tsukuba, Ibaraki, Japan). The cells were maintained in RPMI-1640 medium containing 10% (v/v) fetal calf serum and 1% penicillin-streptomycin at 37°C in an atmosphere containing 5% CO_2. RAW264 cells were seeded in 96-well plates at a concentration of 5×10^5 cells/well and then allowed to stabilize overnight. After seeding, the cells were pretreated with chloroquine (Nacalai Tesque, Kyoto, Japan) for 30 min [21] and then treated with heat-killed LAB (final concentration: 10 μg/mL) for 24 hr. After incubation, supernatants from LAB-treated cells were collected and stored at −80°C until subsequent measurements.

RNA interference

The RNA interference procedure is based on previous research [18]. TLR7 small interfering RNA (siRNA; Stealth siRNA, ID: 86644) and a control siRNA (Stealth siRNA negative control) were purchased from Thermo Fisher Scientific (Tokyo, Japan). RAW264 cells were seeded in 48-well plates at a concentration of 1.25×10^5 cells/well and allowed to stabilize overnight. Then, 48 μL of Lipofectamine RNAiMAX (Thermo Fisher Scientific) was diluted with 800 μL of Opti-MEM (Thermo Fisher Scientific). The siRNA stock solution was diluted with Opti-MEM to a concentration of 0.77 μM, and the siRNA solution was mixed with the same volume of mixture of the Opti-MEM/Lipofectamine solution (1:16.7). After a 5-min incubation, RAW264 cells were washed with Opti-MEM, and 50 μL of the Lipofectamine/siRNA mixture and 200 μL of Opti-MEM were added to each well. After 48 hr of further incubation, heat-killed K4G4 (final concentration: 10 μg/mL) was added. After 24 hr of incubation, the supernatants were collected and stored at −80°C until subsequent measurements.

Cytokine assay

The IL-10 concentrations of supernatants were determined with a commercial enzyme-linked immune sorbent assay (ELISA) kit (Invitrogen, Tokyo, Japan) according to the manufacturer's instructions.

Revers transcription polymerase chain reaction

Total RNA was isolated using an ISOGEN II kit (Nippon Gene, Tokyo, Japan) following the manufacturer's instructions. The first-strand complementary DNA (cDNA) was synthesized by reverse transcription using ReverTra Ace qPCR RT Master Mix with a gDNA Remover (Toyobo, Osaka, Japan) according to the manufacturer's protocols. Real-time polymerase chain reaction (PCR) was performed with a 7300 Fast Real-Time PCR System (Applied Biosystems), using a KAPA SYBR FAST Universal qPCR Kit (Kapa Biosystems, Boston, MA, USA) according to the manufacturer's instructions. The relative levels of gene expression were calculated using the delta-delta threshold cycle (Ct) method with normalization to glyceraldehyde-3-phospate dehydrogenase (*Gapdh*). All the primer sequences used in this study are listed in Supplementary Table 1.

Statistical analysis

All experiments were repeated at least twice. Data are presented as means ± standard deviation (SD). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by the Tukey–Kramer post hoc test using R version 4.0.5. Mean differences were considered statistically significant at $p<0.05$.

RESULTS

Screening of LAB strains from fermented B. rapa *L.*

Spleen cells from BALB/c mice were treated with 13 strains of heat-killed LAB (3 strains of *L. curvatus* and 10 strains of *L. plantarum*) for 48 hr. Analysis of the cell-free supernatants showed that *L. curvatus* K4G4 induced the highest level of IL-10 in the spleen cells (Fig. 1).

IL-10 induction activities of CW components of K4G4

To identify the bacterial components contributing to IL-10 production by mouse spleen cells, we prepared the CW, PGN, and defatted CW fractions from K4G4 cells and added each of them to cultured spleen cells. Although heat-killed K4G4 induced IL-10 production, none of the CW components induced significant IL-10 production by spleen cells of mice (Fig. 2).

Effects of RNase A treatment of LAB on IL-10 induction activity

To determine whether LAB RNA is involved in IL-10 induction activities, we selected 3 strains of *L. curvatus* and 4 strains of *L. plantarum* based on their IL-10 induction activities, and we treated these 7 LAB strains with RNase A under low (0 M) and high (0.3 M) NaCl concentrations prior to their cultivation with spleen cells. RNase A treatment under the low NaCl concentration clearly resulted in lower IL-10 induction activities in spleen cells, irrespective of the LAB strain evaluated (Fig. 3). In contrast, RNase A treatment under the high NaCl concentration induced different IL-10 activities among the LAB strains. In particular, the IL-10 induction activity of K4G4 and K5G18 after RNase A treatment under the high NaCl concentration was significantly lower than that of untreated strains, and the reduced IL-10 induction by RNase A treatment of K4G4 was more potent than that of K5G18. In contrast, the IL-10 induction activities of other LAB strains remained unchanged after RNase A treatment under the high NaCl concentration.

IL-10 induction activity of K4G4 in RAW264 cells and effects of RNase treatment

Among the immune cells of the spleen, we focused on macrophages as possible inducers of IL-10 in response to K4G4

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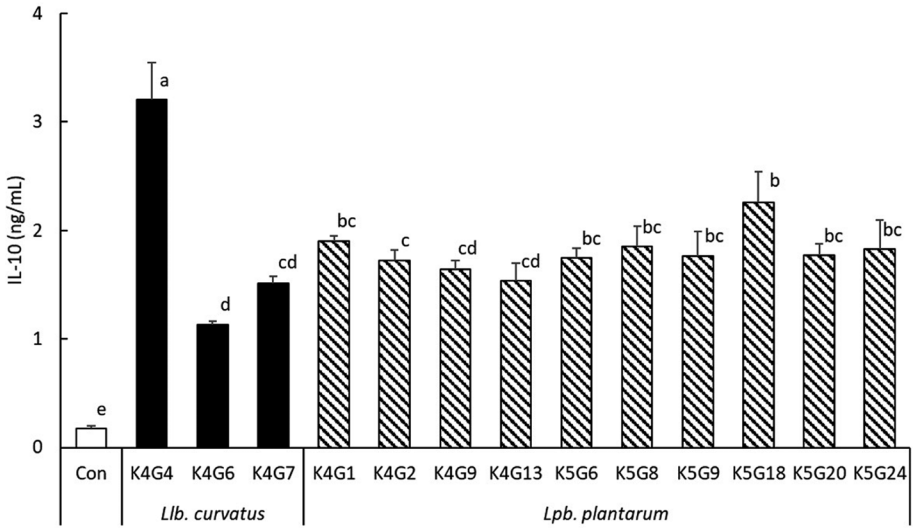

Fig. 1. IL-10 induction from spleen cells of BALB/c mice by 13 lactic acid bacteria (LAB) strains.

Spleen cells from BALB/c mice (5×10^5 cells/well) were treated without (Con) or with 10 μg/mL of heat-killed bacteria. After 48 hr of culture, cell-free supernatants were collected, and IL-10 levels were analyzed using enzyme-linked immune sorbent assay (ELISA) kits. Data are shown as the mean ± SD (n=3) and were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Values without common letters differ significantly ($p<0.05$). Con: control.

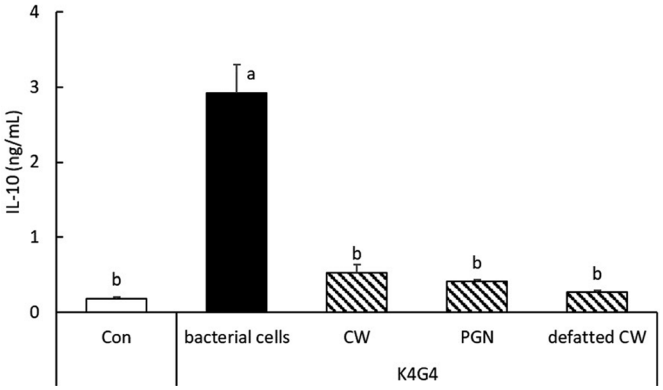

Fig. 2. IL-10 induction from spleen cells of BALB/c mice by cell wall components of K4G4.

Spleen cells from BALB/c mice (5×10^5 cells/well) were treated without (Con) or with 10 μg/mL of cell wall components of K4G4. After 48 hr of culture, cell-free supernatants were collected, and IL-10 levels were analyzed using enzyme-linked immune sorbent assay (ELISA) kits. Data are shown as the mean ± SD (n=3). Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Values without common letters differ significantly ($p<0.05$).

Con: control; CW: cell wall; PGN: peptidoglycan.

stimulation. We treated the murine macrophage cell line RAW264 with 13 strains of heat-killed LAB and then compared the IL-10 induction activities. IL-10 induction in RAW264 cells was observed only with K4G4 (Supplementary Fig. 1). The bacterial components of K4G4 (CW, PGN, defatted CW) failed to induce IL-10 in RAW264 cells (data not shown). RNase A treatment of K4G4 under both the high and low NaCl concentrations resulted in significantly lower levels of IL-10 induction, and RNase III treatment of K4G4 did not produce significant changes in IL-10 induction compared with untreated K4G4 (Fig. 4).

Identification of major receptors responsible for IL-10 induction by K4G4 in RAW264 cells

To clarify the mechanism of IL-10 induction by K4G4 RNA, we treated chloroquine-pretreated RAW264 cells with K4G4. Chloroquine pretreatment of RAW264 cells at concentrations of 10 μg/mL or higher resulted in significantly reduced IL-10 induction by heat-killed K4G4, and with chloroquine pretreatment at 20 μg/mL, K4G4 failed to induce IL-10 production (Fig. 5). Chloroquine was not cytotoxic at concentrations of 20 μg/mL or lower (data not shown).

We pretreated RAW264 cells with TLR7 siRNA. TLR7 siRNA pretreatment significantly reduced ($p<0.01$) *Tlr7* expression (Supplementary Fig. 2). TLR7 siRNA pretreatment of RAW264 cells resulted in suppressive IL-10 induction by heat-killed K4G4 (Fig. 6).

DISCUSSION

IL-10 is an anti-inflammatory cytokine that improves health problems [9–11]. Among 13 strains of LAB isolated from fermented *B. rapa* L., *L. curvatus* K4G4 induced the highest level of IL-10 production from murine immune cells, and we thus further investigated the mechanism of IL-10 induction in this strain.

The 13 LAB strains consisted of 3 strains of *L. curvatus* and 10 strains of *L. plantarum* isolated from fermented *B. rapa* L. [8]. The IL-10 induction activities were compared among the strains, because such induction activities are known to be strain specific [22]. IL-10 induction activities differed among the LAB strains, especially among those belonging to the species *L. curvatus* (Fig. 1). Furthermore, *L. curvatus* K4G4 induced the highest level

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of IL-10 production in spleen cells (Fig. 1). Thus, we selected K4G4 to investigate the mechanisms involved in IL-10 induction. The pro-inflammatory cytokine IFN-γ was induced by all strains, including K4G4, in spleen cells, and K4G4 had the lowest ability to induce IFN-γ production (data not shown). These observations are consistent with previous research [23].

CW components are known to be responsible for the immune modulation activities of LAB. For example, teichoic acids of *L. plantarum* OLL2712 induce IL-10 production in BMDCs of BALB/c mice [3], and LTA of *L. plantarum* L-137 induces the expression of IL-12 p40 in DCs of BALB/c mice [14]. Therefore, we prepared the CW, PGN, and defatted CW fractions of the K4G4 cell wall and compared their contributions to IL-10 induction. None of these CW components induced IL-10 production in murine spleen cells (Fig. 2).

Bacterial RNA is also known to contribute to cytokine induction by certain strains of LAB [16, 18]. By treating different LAB strains with RNase A under two different NaCl concentrations, we determined whether bacterial RNA contributed to IL-10 induction. RNase A digests only ssRNA at a high NaCl concentration (0.3

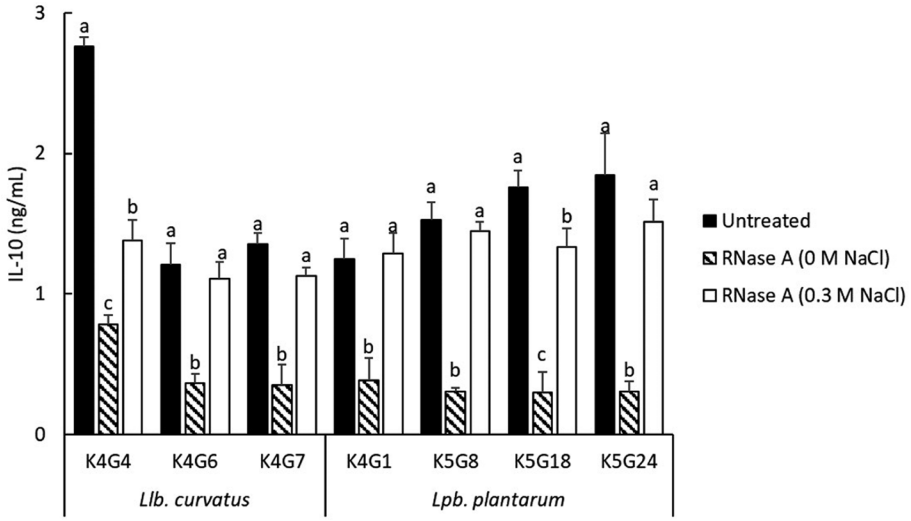

Fig. 3. Effects of ribonuclease (RNase) A treatment of lactic acid bacteria (LAB) strains on IL-10 production in mouse spleen cells.

LAB were treated with RNase A at a low (0 M) or high (0.3 M) NaCl concentration. Spleen cells from BALB/c mice (5×10^5 cells/well) were treated with 10 μg/mL of RNase-treated or untreated LAB. After 48 hr of culture, cell-free supernatants were collected, and IL-10 levels were analyzed using enzyme-linked immune sorbent assay (ELISA) kits. Data are shown as the mean ± SD (n=3). Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Within the same bacterial strain, treatment values without common letters differ significantly ($p<0.05$).

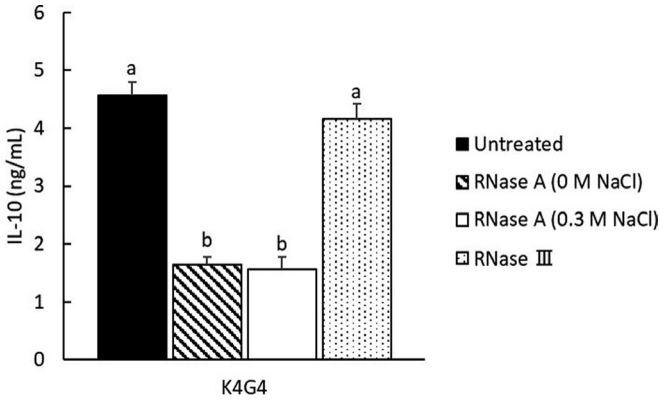

Fig. 4. Effects of ribonuclease (RNase) treatment of lactic acid bacteria (LAB) strains on IL-10 production in RAW264 cells.

LAB were treated with RNase A at a low (0 M) or high (0.3 M) NaCl concentration or with RNase III. RAW264 cells (5×10^5 cells/well) were treated with 10 μg/mL of RNase-treated or untreated LAB. After 24 hr of culture, cell-free supernatants were collected, and IL-10 levels were analyzed using enzyme-linked immune sorbent assay (ELISA) kits. Data are shown as the mean ± SD (n=4). Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Values without common letters differ significantly ($p<0.05$).

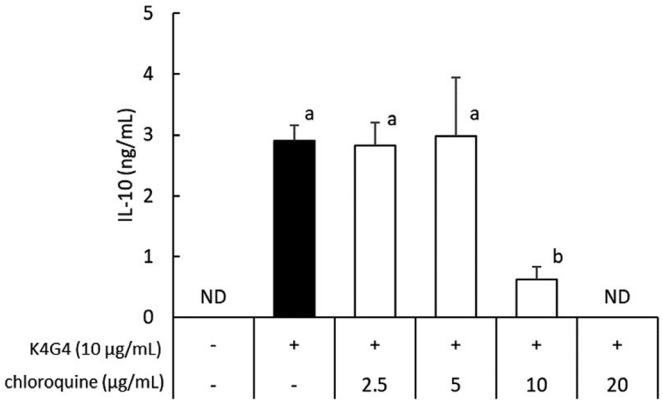

Fig. 5. Effects of chloroquine on K4G4-induced IL-10 production in RAW264 cells.

RAW264 cells (5×10^5 cells/well) were pretreated with 0, 2.5, 5, 10, and 20 μg/mL of chloroquine for 30 min, and then they were treated with 10 μg/mL of heat-killed K4G4. After 24 hr of culture, cell-free supernatants were collected, and IL-10 levels were analyzed using enzyme-linked immune sorbent assay (ELISA) kits. Data are shown as the mean ± SD (n=4) and were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Values without common letters differ significantly ($p<0.05$). ND: not detected.

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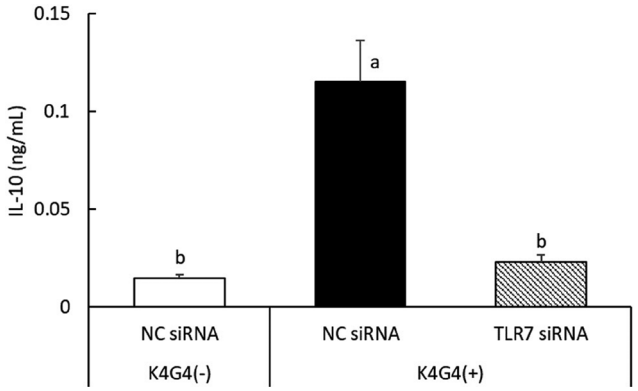

Fig. 6. Effects of toll-like receptor (TLR) knock down on K4G4 induction of IL-10 production in RAW264 cells.

RAW264 cells (1.25×10^5 cells/well) were pretreated with NC siRNA or TLR7 siRNA for 48 hr. After treatment, the cells were treated with 10 μg/mL of heat-killed K4G4. After 24 hr of culture, cell-free supernatants were collected, and IL-10 levels were analyzed using enzyme-linked immune sorbent assay (ELISA) kits. Data are shown as the mean ± SD (n=4) and were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Values without common letters differ significantly ($p<0.05$).

M), while it digests ssRNA, dsRNA, and RNA-DNA hybrids at a low NaCl concentration (0 M) [18]. Seven strains, including K4G4, were treated with RNase A under high and low NaCl concentrations, and IL-10 production in murine spleen cells was determined. IL-10 induction activity of K4G4 was remarkably lower after RNase treatment under the high NaCl concentration compared with that with untreated K4G4 (Fig. 3), suggesting that ssRNA, rather than dsRNA, of K4G4 was involved in the IL-10 induction activity. In contrast, the IL-10 induction activities of the other 6 LAB strains were not changed remarkably by RNase treatment at the high NaCl concentration (Fig. 3), suggesting that the ssRNAs of these 6 strains were not much involved in their IL-10 induction. In addition, the IL-10 induction activities of all the tested RNase A-treated LAB strains without NaCl were significantly decreased (Fig. 3), suggesting that the dsRNAs of all tested strains were involved in the IL-10 induction activity. Therefore, K4G4 induced high levels of IL-10 from spleen cells, possibly because both dsRNA and ssRNA were involved in this strain's IL-10-inducing activity.

IL-10 is secreted mainly from macrophages, DCs, monocytes, and regulatory T cells [5–7]. We investigated whether macrophages secrete IL-10 in response to K4G4 stimulation. When RAW264 cells were treated with 13 LAB isolates from fermented *B. rapa* L., only K4G4 induced IL-10 production (Supplementary Fig. 1). As described above, bacterial cell wall components (CW, PGN, and defatted CW) of K4G4 did not induce IL-10 production. In addition, RNase A treatment both in the absence and presence of NaCl significantly decreased IL-10 induction activities compared with untreated K4G4; however, RNase III treatment did not (Fig. 4). Overall, these results suggest that among the 13 strains isolated from fermented *B. rapa* L., only the ssRNA of K4G4 induced IL-10 production in RAW264 cells; moreover, in contrast to spleen cells, RAW264 cells were not stimulated by bacterial dsRNA. Macrophages and other immune cells are known to produce IL-10 in response to the recognition of bacterial ssRNA or both bacterial dsRNA and ssRNA. The reason why RAW264 cells did not react to bacterial dsRNA and induce IL-10 production is still unclear. In this study, we conducted RNase treatment of LAB to determine whether RNA contributed to the IL-10 induction. Thus, the influence of other bacterial components cannot be excluded. Therefore, it is necessary to determine the IL-10 induction by ssRNA extracted from K4G4. In addition, it is possible that K4G4 possesses specific ssRNA in terms of structure, amount, size, or sequences when compared with other LAB strains; however, the details of the mechanisms involved in the high levels of IL-10 induction by K4G4 need to be clarified.

At least 13 TLRs have been identified in mammals, and previous studies have localized TLR3, TLR7, TLR8, and TLR9 to the endosomes [24–26]. TLR3 specifically recognizes dsRNA, while TLR7 and TLR8 recognize ssRNA [24, 25]. TLR9 recognizes DNA, particularly hypomethylated CpG-containing DNA [24, 25]. Chloroquine is an anti-malaria drug that has also been used to treat immune-mediated inflammatory disorders such as rheumatoid arthritis and systemic lupus erythematosus [27]. It inhibits TLR3, TLR7, TLR8, and TLR9 signals (which are located in the endosome) by increasing the pH in the endosome and by binding to nucleic acids [21, 26, 28]. Here, we showed that pretreatment of chloroquine suppressed IL-10 induction by K4G4 in RAW264 cells in a dose-dependent manner (Fig. 5), suggesting that K4G4 stimulates endosomal TLRs to induce IL-10 production in RAW264 cells. Although TLR7 and TLR8 are close phylogenetic relatives, ssRNA stimulates human TLR7 and TLR8 and mouse TLR7 but does not stimulate mouse TLR8 [29]. As our results suggest that the ssRNA of K4G4 contributes to IL-10 induction in RAW264 cells, we knocked down TLR7 using its siRNA. Although the overall production of IL-10 was low due to fewer cells being seeded, the levels of IL-10 induction by heat-killed K4G4 were suppressed in TLR7-knocked down RAW264 cells (Fig. 6), suggesting that K4G4 is taken up by endosomes and that the ssRNA of K4G4 then stimulates TLR7 to induce IL-10 production in RAW264 cells. The TLR7 agonist imiquimod reportedly induced IL-10 production in macrophages with accompanying cellular cAMP elevation, whereas another agonist, loxoribine, failed to induce IL-10 because of the absence of cAMP elevation [30], suggesting the contribution of not only TLR7 signaling but also cAMP elevation to IL-10 induction in macrophages. Since the pathway from TLR7 to IL-10 induction is unknown, it will be necessary to evaluate the details of downstream signaling pathways in the future. In addition, it will also be necessary to evaluate the anti-inflammatory function of K4G4 via IL-10 induction using *in vivo* models.

Among LAB isolates from fermented *B. rapa* L., the bacterial components that are involved in IL-10 induction and the molecular mechanisms involved in such induction have not been well determined. We demonstrated here that *L. curvatus* K4G4 induced IL-10 production from both murine spleen cells and the RAW264 murine macrophage cell line. The mechanism probably involves the recognition of bacterial RNA, especially ssRNA of K4G4, by endosomal TLR7 of macrophages.

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CONFLICT OF INTEREST

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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