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Sublingual administration of *Lactobacillus paracasei* KW3110 inhibits Th2-dependent allergic responses via upregulation of PD-L2 on dendritic cells

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KEYWORDS

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Abstract Lactic acid bacteria have potential in immunomodulation therapy, but their clinical efficacy and underlying mechanisms are unclear. We aimed to clarify the anti-allergic immune responses induced by intragastric and sublingual administration of heat-killed *Lactobacillus paracasei* KW3110 and *Lactobacillus acidophilus* L-92. The KW3110 strain (but not the L-92 strain) enhanced ovalbumin (OVA)-induced expression of CCR-7 and PD-L2 in murine dendritic cells (DCs), and strongly inhibited IL-5 and IL-13 production *in vitro* in co-cultures with Th2-skewed CD4⁺ T cells from DO11.10 transgenic mice. Sublingual administration of low-dose KW3110 (but not L-92) to OVA-sensitized mice selectively suppressed serum IgE production and Th2 cytokine expression in cervical lymph nodes, and significantly improved symptoms after OVA provocation *in vivo*. KW3110 probably accelerates DC migration into the regional lymph nodes and inhibits Th2 cytokine production through enhanced CCR-7 and PD-L2 expression. Thus, sublingual KW3110 administration may be effective in reducing allergic inflammation.

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Abbreviations: PBMC, Peripheral blood mononuclear cell; SLIT, Sublingual immunotherapy; DC, Dendritic cell; OVA, Ovalbumin; BM, Bone marrow; LPS, Lipopolysaccharide; CLN, Cervical lymph node; ILN, Inguinal lymph node; ELISA, Enzyme-linked immunosorbent assay; ELISPOT, Enzyme-linked immunospot; TLR, Toll-like receptor; LGG, *Lactobacillus rhamnosus* GG; FACS, Fluorescein-activated cell sorter; FITC, Fluorescein isothiocyanate; GM-CSF, Granulocyte macrophage colony-stimulating factor; TCR, T cell receptor; OD, Optical density

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1. Introduction

An increased prevalence of allergic rhinitis has been observed in many countries over the recent years [1]. A variety of medications have been used to relieve the symptoms of this condition; however, these drugs do not treat the underlying disease and have a high risk of adverse events, particularly when taken over a long period [2]. Antigen-specific immunotherapy can effectively change the natural course of allergic disease, prevent the development of other allergic diseases, and reduce new allergic sensitization [3]. However, conventional subcutaneous administration necessitates frequent visits to the physician and is associated with a risk, albeit low, of anaphylactic shock [4].

The efficacy of lactic acid bacteria in suppressing the development of allergic diseases has been demonstrated [5]. For example, when expectant mothers were treated with *Lactobacillus rhamnosus* GG (LGG), and their infants were also treated with LGG from birth to 6 months of age, there was inhibition of the development of atopic dermatitis at ages 2 and 4 years [6]. In a study of infants with milk allergy treated with LGG, placebo or other *Lactobacillus* strains, LGG significantly enhanced IFN- γ production from peripheral blood mononuclear cells (PBMCs) [6]. However, in another study, there was no evidence of significant clinical benefit or differences in cytokine production from PBMCs in infants treated with LGG compared to a placebo [7].

Xiao et al. [8] observed marked improvements in nasal symptoms and modulation of Th2-skewed immune responses after 13 weeks of administration of the probiotic strain *Bifidobacterium longum* for treatment of Japanese cedar pollinosis. There is no other evidence of benefit after 22 weeks of administration of LGG for treatment of birch pollinosis [7]. The use of different probiotics and the study methodologies, such as dose, period of administration, and study sample size may explain these contradictory results. Intestinal health and factors such as diet and antibiotic therapy can also have a significant influence, which makes it difficult to evaluate the roles of probiotics [8,9].

In this study, we examined 2 strains of heat-killed lactic acid bacteria, *Lactobacillus paracasei* KW3110 and L-92, which are widely used in Japan and are reported to produce unique and different immune responses *in vitro*. KW3110 induces higher levels of IL-12 *in vitro* than the LGG strain, making it a more effective strain [9]. KW3110 may inhibit Th2 cytokine-mediated allergic inflammation through mechanisms independent of toll-like receptors (TLRs) [10,11], since the induction of IL-12 is not abrogated in bone marrow (BM)-derived dendritic cells (DCs) in TLR2-, TLR4- and TLR9-deficient mice [12]. L-92 activates DCs in a TLR2-dependent manner [13] and may exhibit anti-allergic activity through induction of regulatory T cells *in vivo* [14]. We administered the *Lactobacillus* strains directly to the oral mucosa, rather than as probiotics, and examined the influence of the KW3110 and L-92 strains on allergic responses in antigen-sensitized mice *in vivo* and *in vitro*.

We have previously observed higher CCR-7 and PD-L2 expression in mature DCs upon stimulation with KW3110 but not with L-92, which accelerated DC migration to draining lymph nodes and increased inhibitory signals for Th2 cytokine production. Similar immunomodulation was observed in cervical lymph nodes (CLNs), which play an important role in allergic rhinitis as draining lymph nodes, after

sublingual administration of KW3110 *in vivo*. This therapeutic approach may therefore be effective for allergic rhinitis.

2. Material and methods

2.1. Mice

Seven-week-old female BALB/c mice were purchased from SLC Inc. (Hamamatsu, Japan) and were maintained under specific pathogen-free conditions. The use of these mice was approved by the Chiba University Institutional Animal Care and Use Committee and the experiments were conducted in conformity with the guidelines of the committee.

2.2. Reagents and medium

The heat-killed KW3110 [11] and L-92 [14] strains were obtained from the Central Laboratories for Frontier Technology, Kirin Holdings Co. (Kanagawa, Japan), and from the R&D Center, Calpis Co. (Yokohama, Japan) respectively. Ovalbumin (OVA) (grade 5) was purchased from Sigma-Aldrich (St. Louis, MO, USA). We used RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FCS, L-glutamine (2 μ M), penicillin (100 U.mL⁻¹), streptomycin (100 μ g.mL⁻¹), HEPES (10 mM), 2-mercaptoethanol (55 μ M), 1% non-essential amino acids, and 1 mM sodium pyruvate (all from Gibco BRL, Grand Island, NY, USA) in the cell culture experiments. OVA was dissolved in endotoxin-free D-PBS (Wako Pure Chemical Industries, Osaka, Japan), which had an endotoxin level below the detection limit (0.05 ELISA units per milligram protein). The reagents used for stimulation were also tested for endotoxin contamination; these also had levels below the detection limit.

2.3. Maturation of DCs

DCs from murine BM were cultured in a 100-mm bacteriological petri dish with 10 ng.mL⁻¹ granulocyte macrophage colony-stimulating factor (GM-CSF) (PeproTech, Rocky Hill, NJ) [15]. The medium was replaced twice. The immature DCs were stimulated with 1 mg.mL⁻¹ of OVA in endotoxin-free PBS with or without 1 μ g.mL⁻¹ KW3110 or L-92, and 10 μ g.mL⁻¹ LPS (Lipopolysaccharide) (O111:B4, Sigma-Aldrich), and cultured for 24 h in 24-well plates at 1×10^6 cells per well. The DCs were analyzed by fluorescein-activated cell sorter (FACS) analysis of surface markers (FACSCalibur; Becton Dickinson, Sunnyvale, CA).

2.4. Phagocytosis by DCs *in vitro*

KW3110 or L-92 (1 mg of each) was suspended in 1 mL of 100 mM carbonate buffer (pH 9.5), reacted with fluorescein 5(6)-isothiocyanate (100 μ g.mL⁻¹) (Sigma-Aldrich) at 37 °C for 60 min, and then washed with sterile PBS [16]. Fluorescein isothiocyanate (FITC)-labeled *Lactobacillus* (1 μ g.mL⁻¹) was cultured with immature DCs (1×10^6 cells.mL⁻¹) for 24 h. After washing with PBS, the DCs were fixed with a cold methanol:acetone (1:1) solution for 10 min and then stained with biotin-labeled CD11c (N418; BioLegends, San Diego, CA, USA), followed by Cy5-coupled streptavidin. For

FACS analysis, incubated DCs were stained with Cy5-labeled CD11c and MHC class II Alexa555, and then fixed with 4% paraformaldehyde for 10 min at room temperature, and permeabilized with 0.5% Triton X-100 (in 50 mM NaCl, 5 mM EDTA, and 0.02% NaN_3 ; pH 7.5) for 10 min on ice. After blocking with 3% BSA in PBS for 15 min, the cells were washed thoroughly with PBS supplemented with 1% FCS and 0.1% NaN_3 . Phagocytosis of DCs was examined by confocal microscopy (Olympus, Tokyo, Japan) and FACS analysis. We used the fluorescence intensity of immature DCs after incubation for 24 h with labeled *Lactobacillus* as an indicator of *Lactobacillus* uptake.

2.5. Cytokine production in the co-culture of DCs with Th2 cells

Th2-skewed CD4^+ T cells were prepared from OVA-specific T cell receptor (TCR) (DO11.10) transgenic mice in a BALB c background, expressing TCR α/β specific for OVA peptide, presented in the context of I-Ad [17]. Immature DCs (5×10^5) prepared from BALB c mice using the method described above, were pre-incubated with 0.4 μM OVA_{323–339} peptide (Loh15) with 1 $\mu\text{g mL}^{-1}$ of KW3110 or L-92 for 24 h, and were then co-cultured with Th2-skewed CD4^+ T cells (1×10^5 cells) for 48 h. After preincubation, some DCs were treated for 30 min with 10 $\mu\text{g mL}^{-1}$ of a neutralizing rat monoclonal antibody directed against PD-L2 (TY25 [18], rat IgG2a; BioLegends, San Diego, CA, USA) or a control isotype rat IgG2a monoclonal antibody. The cells were washed 3 times to remove excess antibody before co-culture. The culture supernatants were collected for ELISA analysis of cytokine production.

2.6. Migration of DCs after sublingual administration

We administered 0.5 mg of FITC-labeled KW3110 with 100 μg of OVA in 0.01 mL PBS sublingually to BALB c mice, using a micropipette tip placed under the tongue, while holding the back of the mouse for 20 s. The oral mucosa and CLNs were obtained, and freshly frozen 6- μm thick sections were fixed in a cold methanol:acetone (1:1) solution for 10 min. These were examined after staining with biotin-labeled CD11c followed by Cy5-coupled streptavidin.

2.7. In vivo immunization study protocol

BALB c mice were sensitized intraperitoneally with 100 μg of OVA and 4 mg of alum (Pierce, Rockford, IL, USA) once a week for 3 weeks. From 1 week after the last sensitization, 500 μg of OVA in 20 μL PBS was administered intranasally for 7 consecutive days [19]. Sensitized mice ($n=18$ in each group) received 5 mg or 0.5 mg of KW3110 or L-92 in 0.1 mL PBS, administered through a gavage tube for 7 consecutive days at the same time every day. A second group of sensitized mice ($n=16$ in each group) received 0.5 mg of KW3110 or L-92 in 0.01 mL PBS by sublingual administration for 7 consecutive days, as described above. After the last nasal administration of OVA, the behavior of the mice was recorded by video camera, and sneezing and nasal rubbing events were counted for 5 min [19]. The mice were then sacrificed and the serum, CLNs, spleens, inguinal lymph

nodes (ILNs), and BM were collected. Serum OVA-specific IgE Ab was analyzed by ELISA.

2.8. Treatment with a neutralizing anti-PD-L2 antibody

We investigated the preventive effect of treatment *in vivo* with a neutralizing anti-PD-L2 antibody. The OVA-sensitized mice were injected intravenously with 250 μg of anti-mouse PD-L2 mAb (TY25, rat IgG2a) or rat control IgG2 mAb 12 h after the last sensitization. We then administered 0.5 mg of KW3110 sublingually for 7 consecutive days.

2.9. Analysis of cytokine production from CD4^+ T cells in CLNs

Single-cell suspensions were prepared from CLNs and incubated with biotinylated anti- CD4 antibody (GK1.5; BioLegends, San Diego, CA, USA) at 4 °C for 30 min, followed by incubation with anti-biotin beads (Miltenyi Biotec, Bergisch Gladbach, Germany). CD4^+ cells were sorted using the MACS systems (Miltenyi Biotec) and suspended in complete RPMI 1640 medium. Cells were then cultured at a density of 1.5×10^5 cells per well in the presence of 1 mg mL^{-1} of OVA with CD4^+ T cell-depleted and irradiated splenic feeder cells (5×10^5 cells per well) in round-bottom 96-well microculture plates for 48 h. Cytokines in the supernatants were then analyzed by ELISA.

2.10. Detection of OVA-specific IgE-secreting cells

The number of OVA-specific IgE-secreting cells among CLN, ILN, spleen, and BM cells was evaluated in 96-well filtration plates (Multiscreen; Millipore Corp., Bedford, MA, USA) coated with 50 $\mu\text{g mL}^{-1}$ OVA or BSA in PBS at 4 °C overnight, and then blocked with 10% FCS in RPMI. CLN cells (3×10^5 cells per well) were incubated on the plates at 37 °C under 5% CO_2 . After incubation for 5 h, the plates were washed with PBS containing 0.1% Tween 20 (Sigma Chemical Co.) and then stained with alkaline phosphatase-conjugated anti-IgE antibodies (23 G3; Southern Biotechnology, Birmingham, AL, USA). Alkaline phosphatase activity was visualized using a BCIP/NBT phosphatase substrate (KPL, Gaithersburg, MD, USA). The plates were scanned and spots were counted automatically using an ELISPOT image analysis system (CTL Analyzers LLC, Cleveland, OH, USA).

2.11. ELISA analysis

Cytokine levels in the culture supernatant were measured by ELISA. Flat-bottom 96-well Nunc-Brand Immuno plates (Nalge Nunc International) were coated overnight at 4 °C with affinity-purified anti-IL-4 Ab (11B11), anti-IL-5 Ab (TRFK5) (all from Mabtech AB, Nacka, Sweden) or anti-IL-13 Ab (the ELISA Ready-SET-Go! Kit; eBioscience, San Diego, CA, USA). The wells were then washed and blocked with 1% BSA in PBS, and the culture supernatants were titrated onto a treated plate and incubated overnight at 4 °C. Bound cytokines were detected using biotin-conjugated anti-IL-4 Ab (BVD6-24G2), anti-IL-5 Ab (TRFK4) (all Mabtech AB), or anti-IL-13 Ab (eBioscience) followed by streptavidin-alkaline phosphatase

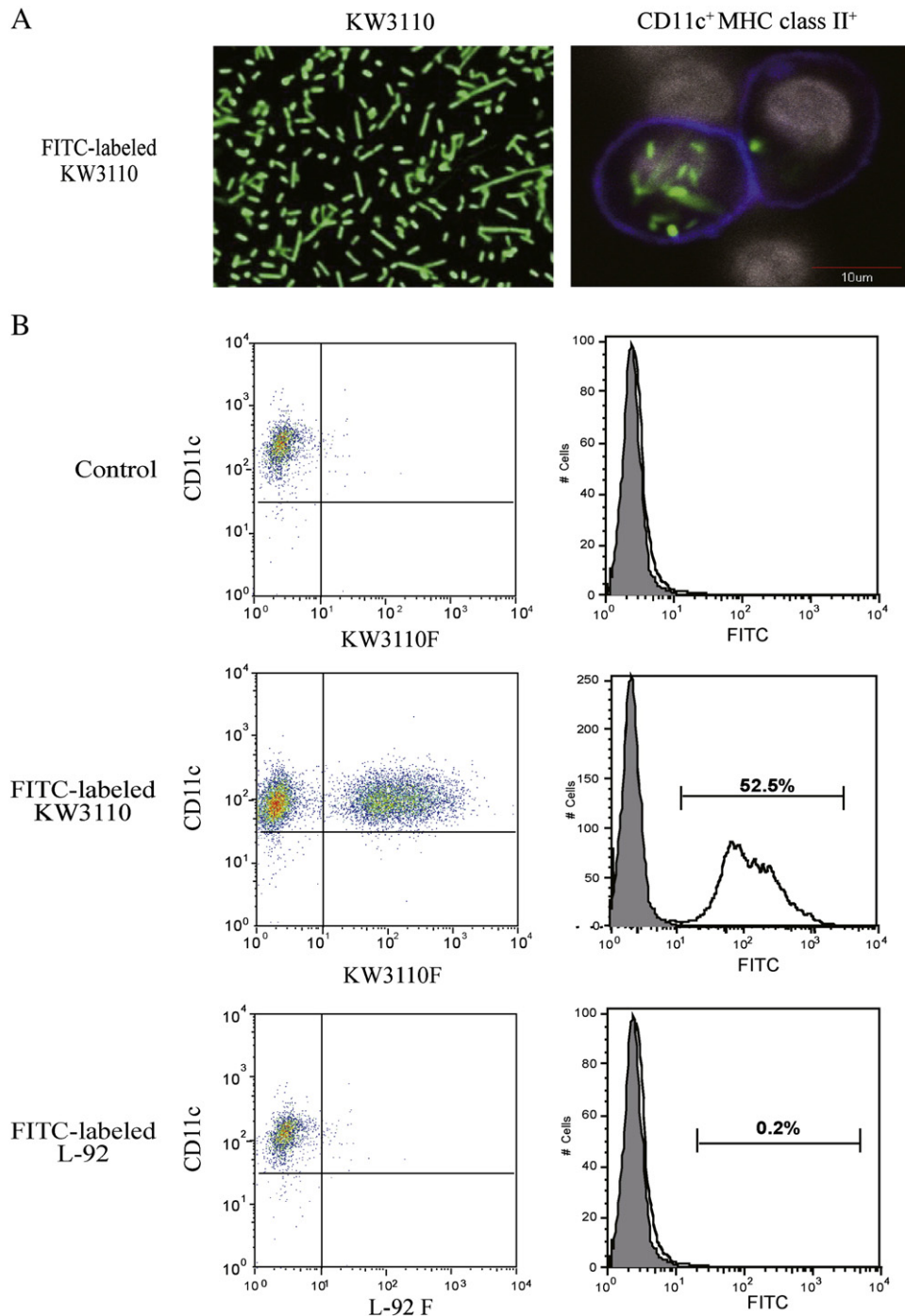


Figure 1 Phagocytosis of immature DCs analyzed by confocal microscopy (A) (green: KW3110, white: DAPI (nuclear), blue: CD11c-Cy5, red: MHC class II) and flow cytometry; (B) magnifications are 200 $\times$ and 400 $\times$ for the left and right images respectively. DCs phagocytosed many KW3110 bacteria in their intact form, and 52% of the cultured CD11c⁺ DCs were found to contain FITC-labeled KW3110 after 24 h, based on FACS analysis. Only a few L-92 bacteria were phagocytosed.

(AP). The plates were developed with *p*-nitrophenyl phosphate tablets (Pierce), with the reaction terminated by the addition of 2N NaOH. Specific absorbance was measured and optical density (OD) was quantified at 410 nM using a Multiskan JX plate reader (Thermo LabSystems, Beverly, MA, USA). OVA-specific IgE was measured with a mouse anti-OVA IgE antibody assay kit (Chondrex, Redmond, WA, USA).

2.12. Flow cytometric analysis

Four-color cytometry was performed as follows. Briefly, cells were harvested from culture plates, preincubated with 10 mg.mL⁻¹ of unlabeled anti-CD16/32 (24G2) (BD Biosciences), and then stained on ice with a combination of Abs. We used combinations of FITC-conjugated mouse CD11c (N418)

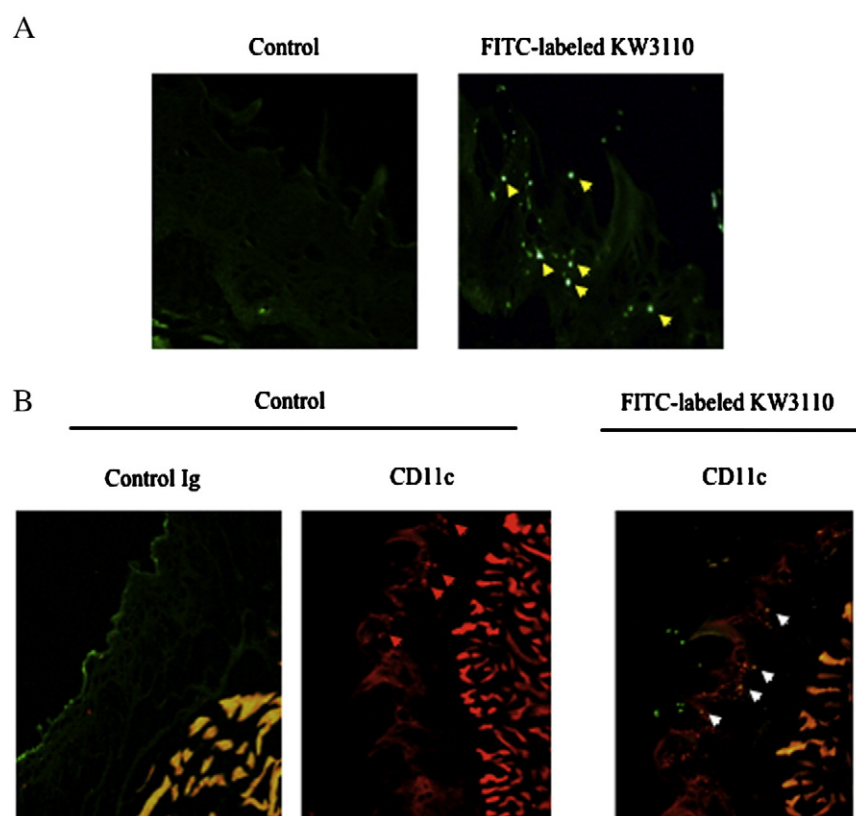


Figure 2 FITC-labeled KW3110 bacteria were detected in the oral mucosa 1 h after the sublingual administration, by confocal microscopy (A, yellow arrow). Many fragmented and some intact forms of KW3110 were observed to be phagocytosed by DCs in the double staining with anti-CD11c (B, white arrow). Magnification is 100 $\times$ for all images.

(eBioscience) with PE-conjugated Abs to MHC Class II IA+IE (M5/114.15), CD80 (16-10A-1), CD86 (GL-1), B7RP-1 (HK5.3), PD-L2 (122), OX40L (RM134L), 4-1BBL (TKS-1) (all eBioscience), PD-L1 (9G2), or APC-conjugated CCR-7 (4B12) (BioLegend). Data were analyzed using the Cellquest software (Becton Dickinson).

2.13. Statistical analysis

Statistical analysis was performed using the Mann–Whitney *U*-test. Data are shown as mean $\pm$ SEM or SD.

3. Results

3.1. KW3110 enhanced CCR-7 and PD-L2 expression

After incubation for 24 h with LPS, L-92, or KW3110, the expression of MHC class II^{high}, CD40, CD80, CD86, B7RP-1 and PD-L1 similarly increased; however, that of CCR-7 and PD-L2 was enhanced only by KW3110 in OVA-stimulated DCs, and not by LPS or L-92 (data not shown). Administration of *Lactobacillus* without OVA stimulation induced a low level of the surface markers, and the concentration of *Lactobacillus* and LPS required for expression of the highest levels of these markers with OVA stimulation was determined to be 10 $\mu\text{g}\cdot\text{mL}^{-1}$ in a preliminary analysis.

3.2. KW3110 was phagocytosed in the intact form by DCs

As shown in Figure 1A, the DCs phagocytosed KW3110 bacteria in the intact form, and 52% of cultured CD11c⁺ DCs showed uptake of FITC-labeled KW3110 over 24 h based on FACS analysis (Figure 1B). In contrast, only a few L-92 bacteria were phagocytosed, and even the uptake of FITC was very low.

3.3. KW 3110 decreased cytokine production from Th2 cells

In co-culture study of DCs and Th2-skewed CD4⁺ T cells from DO11.10 Tg mice, KW3110 decreased IL-5 and IL-13 production (from 0.210 $\pm$ 0.13 pg/mL to 0.041 $\pm$ 0.06 pg/mL for IL-5, and from 0.206 $\pm$ 0.35 pg/mL to 0.082 $\pm$ 0.19 pg/mL for IL-13, respectively); however, L-92 did not have this influence. IL-4 production was low, with no difference between various stimulations. Treatment of KW3110-treated DCs with anti-PD-L2 antibodies abrogated the inhibition of IL-5 and IL-13 production. The level of PD-L2 on DCs was the same with stimulation by OVA alone and with L-92 in OVA-stimulated DCs. In the response provoked by OVA alone, the PD-L2 antibody did not affect IL-5 or IL-13 production.

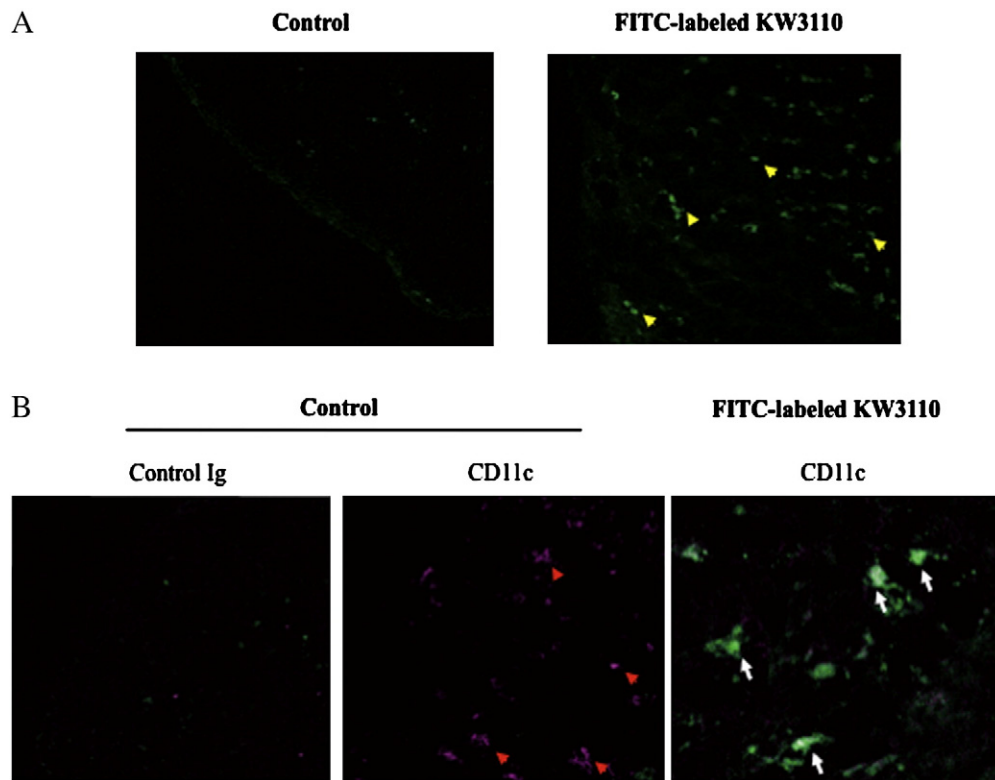


Figure 3 At 3 h after sublingual administration of FITC-labeled KW3110 stimulated with OVA, KW3110 bacteria were detected in CLNs by confocal microscopy (A, yellow arrow). CD11c-positive cells were identified by treatment with biotin-labeled anti-CD11c⁺ (B, red arrow). Many fragmented and some intact KW3110 (CD11c⁺ FITC⁺ cells) bacteria were phagocytosed by DCs, as identified by double staining (B, white arrow), but were not found in the CLNs of control mice that did not receive KW3110. Magnification is 400× for all images.

3.4. Sublingually administered KW3110 migrated to CLNs

FITC-labeled KW3110 was detected in the oral mucosa 1 h after the sublingual administration, by confocal microscopy (Figure 2A). The presence of CD11c-positive cells was shown by treatment with biotin-labeled anti-CD11c. Double staining with anti-CD11c showed that many fragmented and some intact KW3110 (CD11c⁺ FITC⁺ cells) organisms were phagocytosed by DCs (Figure 2B). Similarly fragmented and some intact forms of KW3110 were phagocytosed by DCs in CLNs 3 h after administration (Figure 3). Twelve hours later, these KW3110 were rarely detected in the oral mucosa and the CLNs.

3.5. KW3110 attenuated the nasal symptoms and serum IgE level in the OVA sensitized mice through PD-L2 expression

After OVA nasal provocation, the frequency of sneezing and nasal rubbing events decreased significantly in mice that received sublingual administration of KW3110 at 0.5 mg/day compared with those that received PBS (Table 1A). This response was dose-dependent and was stronger in mice receiving 0.5 mg.mL⁻¹ KW3110 compared to those receiving a 0.05 mg.mL⁻¹ dose. The response reached a plateau at a

dose of 0.5 mg.mL⁻¹ and there was no significant difference between mice receiving 0.5 and 5 mg.mL⁻¹ KW3110 (data not shown). The frequency of sneezing and nasal rubbing events was not decreased in OVA-sensitized mice receiving sublingual L-92 or 0.5 mg of intragastric KW3110. Nasal symptoms were decreased to some extent at a ten-fold higher intragastric dose (5 mg.day⁻¹) of KW3110, but the effect was less marked than for sublingual administration at 0.5 mg/day (data not shown). Serum OVA-specific IgE was significantly reduced in mice receiving sublingual KW3110 but not in others, including those receiving intragastric KW3110 at 5 mg.day⁻¹. Treatment of OVA-sensitized mice with anti-PD-L2 mAb before KW3110 sublingual administration abrogated the effects of KW3110 (Table 1B).

3.6. Sublingually administered KW3110 decreased Th2 cytokine production and the number of IgE-secreting cells in CLNs of sensitized mice

Cytokine production from CD4⁺ T cells in CLNs is shown in Table 2B. Of the Th2 cytokines examined, the IL-4, IL-5, and IL-13 levels were reduced in CD4⁺ T cells from mice that received KW3110 sublingually, but not in those from mice that received intragastric KW3110. Reduction of IFN-γ or enhancement of IL-10 was not observed in CD4⁺ T cells from any mice.

Table 1A Influence of intragastric administration via a gavage tube or sublingual administration of KW3110 or L-92 on nasal symptoms and serum OVA-specific IgE and on cytokine production in CD4⁺ T cells in CLNs.

		Sneezing		Nasal rubbing		Serum anti-OVA IgE	
		Counts		Counts		(ng /ml)	
<i>Nasal symptoms and serum IgE</i>							
Gavage tube (0.5 mg) (n=18 in each group)	PBS	61±15	} n.s. } n.s.	48±08	} n.s. } n.s.	425±015	} n.s. } n.s.
	KW3110	51±13		54±08		478±029	
	L92	68±23		44±06		468±087	
Sublingual. (0.5mg) (n=16 in each group)	PBS	55±06	} * } n.s.	48±10	} * } n.s.	568±054	} * } n.s.
	KW3110	23±05		20±03		278±054	
	L92	50±15		35±05		457±084	
Non immunized mice (control)		10±04	**	15±11	**	n.d.	

Values are presented as the mean±SD. *P<0.05; n.s., not significant; n.d., not detectable. **P<0.01, compared with data from other immunized group.

Table 1B Influence of intragastric administration via a gavage tube or sublingual administration of KW3110 or L-92 on nasal symptoms and serum OVA-specific IgE and on cytokine production in CD4⁺ T cells in CLNs.

		IL-4	IL-5	IL-13	IFN- γ	IL-10
		(ng /ml)	(ng /ml)	(ng /ml)	(ng /ml)	(ng /ml)
<i>Cytokine production in CD4⁺ T cells derived from cervical lymph nodes</i>						
Gavage tube (0.5 mg) (n = 18 in each group)	PBS	0.48±0.07	2.55±0.56	3.47±0.97	23.08±2.98	0.23±0.04
	KW3110	0.51±0.18	2.22±0.58	3.89±0.18	29.25±6.24	0.18±0.04
	L92	0.39±0.18	2.93±0.43	4.07±0.58	12.53±3.14	0.28±0.05
Sublingual. (0.5 mg) (n = 16 in each group)	PBS	0.52±0.08	2.47±0.48	4.62±0.08	24.62±4.08	0.12±0.06
	KW3110	0.22±0.13	1.09±0.23	1.57±0.13	22.16±0.13	0.15±0.03
	L92	0.36±0.03	2.38±0.17	3.17±0.71	28.23±3.46	0.14 ± 0.02
Non-immunized mice (control)		n.d.	n.d.	** n.d.	n.d.	n.d.

Values are presented as the mean±SD. *P<0.05; n.s., not significant; n.d., not detectable.

The number of IgE-secreting cells was higher in CLNs than in BM cells, ILNs, and the spleen from OVA-sensitized mice (Figure 4A). This number was significantly decreased in CLNs from mice that received submucosal administration of KW3110. Treatment with anti-PD-L2 antibody abrogated the reduction of Th2 cells (Tables 2A and 2B) and IgE-secreting cells (Figure 4B).

4. Discussion

DCs play a crucial role in the induction of primary T-cell-dependent immune responses [20]. Upon uptake of antigens on the mucosal surface, immature DCs in the oral mucosa are thought to migrate to the regional CLNs [21]. This suggests that the oral mucosa provides a potential route for vaccine administration and thus the interaction of oral DCs with T cells in regional lymph nodes is of interest [22,23]. In this study, we showed that *Lactobacillus* spp. administered sublingually might migrate to the CLNs and influence the maturation of DCs induced by uptake of antigen, but that the patterns differ significantly among strains.

KW3110 strongly enhanced the expression of CCR-7 and PD-L2 on DCs, compared to L-92. Sublingual administration of low doses of KW3110 in OVA-sensitized mice *in vivo* decreased IgE

production and nasal symptoms induced by nasal OVA provocation, whereas the same dose of the L-92 strain or intragastric administration of KW3110 had no effect. Th2 cytokine expression and IgE synthesis in CLNs (the major area of antigen presentation and induction of effector cells in the OVA-sensitized mice) were strongly suppressed by sublingual KW3110 but not by L-92 treatment. Pre-treatment with neutralizing antibodies to PD-L2 abrogated the anti-allergic effects of sublingual KW3110. Earlier studies have shown that CCR-7 expression accelerates migration of DCs to the regional lymph nodes [24]. It is also reported that PD-L2 expression on DCs is associated with inhibition of Th2 cytokine production [18,24,25]. Matsumoto et al. showed that PD-L2 was highly expressed on pulmonary DCs and macrophages of the allergic sensitized mice, and administration of anti-PD-L2 antibody enhanced airway hyperreactivity and production of Th2 cytokines [18]. These observations suggest that DCs with high CCR-7 expression that phagocytose KW3110 migrate to the CLNs and subsequently suppress production of Th2 cytokines and IgE, mainly through expression of PD-L2.

The anti-allergic actions of L-92 observed in previous studies were brought about by induction of Treg through TLR2 [13]. However, L-92 may not stimulate DCs directly to induce CCR-7 or PD-L2, and may not effectively activate DCs in the oral submucosa when administered sublingually, due to poor

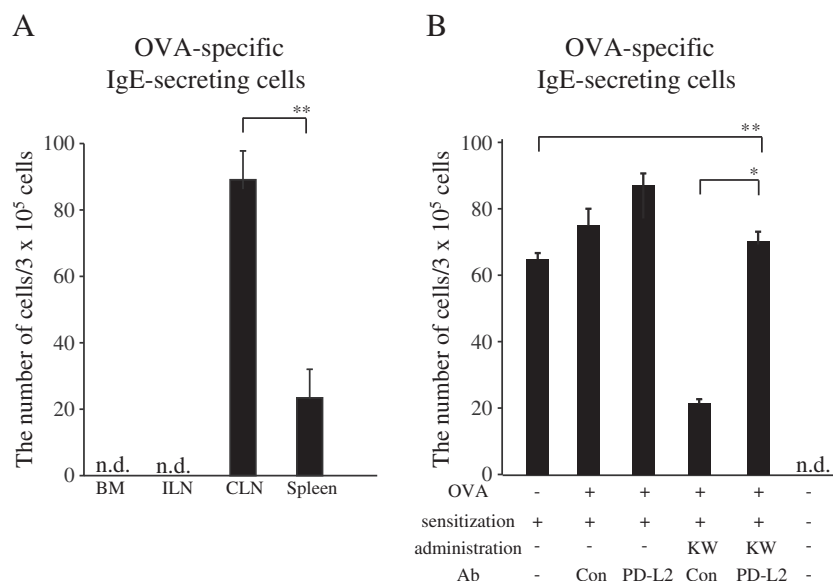


Figure 4 Inhibition of Ag-specific IgE production by KW3110. The number of OVA-specific IgE-secreting cells among cells from lymphoid organs and BM was analyzed by ELISPOT (A). Anti-PD-L2 treatment before oral submucosal administration of KW3110 blocked the reduction of IgE production in cells derived from CLNs (B).

phagocytosis. *In vitro*, DCs phagocytose a higher number of KW3110 organisms than L-92, as shown in this study. This may be because the L-92 strain is twice as large as the KW3110 strain. It is unclear if regulatory T cells were induced by sublingual administration of KW3110, because IL-10 induction or suppression of IFN- γ was not observed in the CLNs. Although the different responses among the strains of *Lactobacillus* may reflect different surface compositions and interactions with different TLRs, the roles of TLR2 or TLR9 in the immune responses induced by KW3110 are unclear [12].

KW3110 is used widely as an additive in yoghurt, beverages, and other foods, and its safety has been certified. However, in our previous study of patients with Japanese cedar pollinosis, 12 weeks intake of 50 mg day⁻¹ of heat-killed KW3110, which was commenced 4 weeks before pollen dispersal, had only a limited clinical effect [26]. The KW3110 in that study was administered in capsule form; this is digested and dissolved by gastric juice or digestive enzymes, but the metabolites are unknown. In the present study, there was no benefit from intragastric administration of KW3110, but direct

administration of the same dose of intact KW3110 to the oral mucosa did have an effect, with improvement in symptoms and reduced Th2 cytokine production.

5. Conclusions

Our results suggest that sublingual administration of KW3110 may reduce allergic inflammation, but the optimum dose and administration protocol require clarification. A recent review of randomized controlled studies of sublingual immunotherapy (SLIT) for allergic rhinitis suggests that this approach is safe and may be effective as an alternative route of administration [27]. Improved efficacy of SLIT could be achieved by adjuvant therapy with sublingual administration of KW3110, since the anti-allergic effects of sublingual administration were enhanced by simultaneous stimulation with an antigen in this study. A clinical study in patients with allergic rhinitis will be performed to examine this hypothesis, and may yield further information on the potential of *Lactobacillus* therapy.

Table 2A Effect of anti-PD-L2 antibody treatment after KW3110 sublingual administration.

Antigen	Administration	Neutralizing Ab	Sneezing Counts	Nasal rubbing Counts	Serum anti-OVA IgE (ng/ml)
<i>Nasal symptoms and serum IgE</i>					
-	-	-	61±9	68±11	651±051
OVA	-	Control Ig	52±12	67±07	695±037
OVA	-	PD-L2	64±13	70±19	677±017
OVA	KW3110	Control Ig	18±05	24±08	202±058
OVA	KW3110	PD-L2	49±07	54±17	623±015
Non-immunized mice (control)			01±02	05±03	n.d.

Values are presented as the mean ± SD. Number of mice in each group was 18. *P<0.05; n.s., not significant; n.d., not detectable. **P<0.01, compared with data from other immunized group.

Table 2B Effect of anti-PD-L2 antibody treatment after KW3110 sublingual administration.

Antigen	Stimulation	Neutralizing Ab	IL-4 (ng/ml)	IL-5 (ng/ml)	IL-13 (ng/ml)
<i>Cytokine production in CD4⁺ T cells derived from cervical lymph nodes</i>					
–	–	–	0.41±0.13	2.94±0.14	4.16±0.14
OVA	–	Control Ig	0.34±0.05	2.83±0.58	3.15±0.25
OVA	–	PD-L2	0.49±0.19	2.80±0.32	3.49±0.36
OVA	KW3110	Control Ig	0.14±0.04	0.78±0.07	1.08±0.09
OVA	KW3110	PD-L2	0.48±0.09	2.56±0.27	3.17±0.14
Non immunized mice (control)			n.d.	n.d.	n.d.

Values are presented as the mean ± SD. Number of mice in each group was 18. *P<0.05; n.s., not significant; n.d., not detectable.

6. Conflict of interest statement

The authors declare that there are no conflicts of interest.

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