



Orally Administered *Lactobacillus paracasei* KW3110 Induces *in Vivo* IL-12 Production

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Lactic acid bacteria (LAB) are popularly used as probiotics, and some strains of LAB have anti-allergic functions *in vivo*. Although *in vitro* studies show that LAB modulate the T helper type (Th) 1/Th2 balance and inhibit IgE secretion by inducing IL-12, it is not known how LAB regulates allergies *in vivo*. In this study, we evaluated *in vivo* IL-12 production after oral administration of *Lactobacillus paracasei* KW3110, a strain reported to improve allergies, to mice. Orally administered KW3110 interacted with CD11b positive cells and induced IL-12 mRNA expression at Peyer's patch. In addition, blood IL-12 levels increased transiently 10 h after administration of KW3110. Based on these results, we found that oral administration of KW3110 induces IL-12 *in vivo*. Our findings should contribute to understanding of the *in vivo* function of LAB.

Key words: lactic acid bacteria; IL-12; oral administration; Peyer's patch

Lactic acid bacteria (LAB) are part of the commensal microbial flora of the intestinal tract and are considered to play important roles in maintaining the health of the host.¹⁾ Recently, they are widely used as probiotics, defined as living microorganism that have health benefits.²⁾ Among their several beneficial functions, a number of studies indicate that a specific lactic acid bacteria (LAB) strain has an anti-allergic function, especially against type1 allergies, characterized by increased serum IgE levels.^{3,4)} We have reported that *Lactobacillus paracasei* strain KW3110 reduces IgE production *in vivo* and is considered to have an anti-allergic function.⁵⁾ Although there are accumulating reports on LAB function, how these LAB regulate allergies *in vivo* is still elusive.

T helper type (Th) 1 cells promote the differentiation of cytotoxic T lymphocytes, and Th1 differentiation is caused by IL-12. Th2 cells promote IgE production by secreting IL-4 and -13, which induce a class switch to IgE of B cells. The ratio of Th1/Th2 cells is important. If the balance is skewed towards Th2, there may be an increase in IgE.⁶⁾ Because specific LAB induce a large increase in IL-12 levels *in vitro*,⁷⁾ the anti-allergic function can be explained by an increase in Th1 in a Th2-skewed immune response.⁸⁾ Indeed, some reports

show that introduction of IL-12 *in vivo* reduced serum IgE levels and improved allergic responses.^{9,10)}

The importance of IL-12 induced by LAB is not restricted to the regulation of allergies. Some LAB strains exhibit anti-tumor or anti-infection functions by activating innate immunity, *e.g.*, NK cells. Recently, it was found that an important mediator in the enhancement of NK cell activity by LAB stimulation is IL-12.^{11,12)} Although there are many reports indicating the importance of IL-12 for immunomodulation by LAB, there is no report that directly shows *in vivo* induction of IL-12 by LAB. In this study, we evaluated *in vivo* IL-12 induction after oral administration of *Lactobacillus paracasei* KW3110, a strain that induces high levels of IL-12 secretion *in vitro*.¹³⁾ Our results indicate that KW3110 is taken up by Peyer's patch (PP) cells and induces IL-12 production *in vivo*.

Materials and Methods

Materials. BALB/c mice were purchased from Charles River Laboratories Japan (Kanagawa, Japan). DO11.10 TCR-transgenic mice (DO11.10) were purchased from The Jackson Laboratory (Bar Harbor, ME). Experiments were performed in accordance with the guidelines for care and use of laboratory animals of Kirin Holdings Co., Ltd. (Tokyo). Fluorescein isothiocyanate (FITC) and collagenase were purchased from Sigma (St. Louis, MO). Anti-mouse CD11c, CD11b, B220, CD90, CD4, and anti-FITC microbeads were purchased from Miltenyi Biotec (Gladbach, Germany). Biotinylated anti-mouse CD11b (clone M1/70), biotinylated anti-mouse IL-12p40 (clone C17.8), biotinylated mouse IgG2a (clone R35-95), anti-DO11.10 clonotypic TCR-PE (clone KJ1.26), and anti-mouse IL-4-allophycocyanin (APC) (clone 11B11) were purchased from BD Pharmingen (San Diego, CA). Anti-mouse CD16/32 (clone 93) and anti-mouse IFN- γ -FITC (clone XMG1.2) were from e-bioscience (San Diego, CA). Streptavidin conjugated Alexa568 was from Invitrogen (Carlsbad, CA). Synthetic peptide, OVA323-339, was from Abgent (San Diego, CA). Heat-killed *Lactobacillus paracasei* KW3110 was prepared as described in a previous study.⁵⁾

Cytokine assay. The concentrations of IL-12p40 and IL-12p70 in the cell culture supernatants and the plasma IL-12p40 concentration were measured using a commercially available enzyme-linked immunosorbent assay kit (BD Pharmingen). The amount of plasma IL-12p70 (detection limit, >0.65 pg/ml) was determined by the Bip-Plex system (Bio-Rad Laboratories, Hercules, CA).

Cell preparation and culture of OVA specific T cells. PP cells were treated twice with 1 mg/ml of collagenase diluted in RPMI at 37 °C for 20 min, and a single cell suspension was prepared. APC was prepared

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Abbreviations: APC, antigen-presenting cell; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; LAB, lactic acid bacteria; MLN, mesenteric lymph node; PE, phycoerythrin; PP, Peyer's patch; SED, subepithelial dome; Th, T helper type

from PP and from spleen cells by negative selection using CD90 microbeads and autoMACS (Miltenyi Biotec). OVA specific CD4⁺ T cells from DO11.10 mice were positively isolated from splenocytes using CD4 microbeads and autoMACS. Cells were cultured in RPMI1640 (Sigma) supplemented with 10% fetal calf serum, 100 U/ml of penicillin, and 100 µg/ml of streptomycin (RPMI). T cells from DO11.10 mice (1×10^6) and APC from BALB/c PP cells (3×10^6 cells/ml) were cultured with and without KW3110 (1 µg/ml) and/or anti-IL-12 in 5 µM OVA peptide supplemented RPMI for 1 week. Cells were collected and used for re-stimulation and cytofluorescence.

Cytofluorescence. Cells were cultured with PMA (50 ng/ml; Sigma), ionomycin (2 µg/ml; Sigma) and BD GolgiStop (2 µg/3 µl; BD Pharmingen) for 6 h. After culturing, the cells were washed and incubated with anti-CD16/32 followed by KJ1.26-PE. They were then fixed and permeabilized with a Cytofix/Cytoperm kit (BD Pharmingen) and stained for intracellular IFN- γ and IL-4, according to the manufacturer's instructions. They were washed and analyzed on a FACSCant2 flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

PP cell culture. PP cells were treated with and without anti-CD11b, anti-CD11c, anti-FITC, or anti-B220 microbeads. Then 7.5×10^6 of PP cells separated by autoMACS (Miltenyi Biotec) and the negative cell fraction was collected. Negatively collected cells were suspended in RPMI (400 µl) and cultured with 1 µg/ml of KW3110 for 24 h. The cell culture supernatant was collected and IL-12p70 was measured.

Oral administration and sample collection. Mice were fasted for 12 h, and 50 mg of KW3110 suspended in 500 µl saline was orally administered. The mice were sacrificed 0, 2, 6, 10, and 24 h after administration, and plasma, mesenteric lymph nodes (MLN), and PP were removed; plasma was always collected before the removal of MLN and PP. The tissues were soaked in RNA later (Qiagen, Venlo, Netherlands) and kept at -80°C until RNA extraction. To measure mRNA from CD11b-positive cells, PP cells were removed 6 h after KW3110 administration. CD11b and CD11c positive cells were separated using anti-CD11b or anti-CD11c microbeads and autoMACS, as described above. The separated cells were dissolved in RLT buffer (Qiagen) and kept at -80°C until RNA extraction.

Quantitative RT-PCR. Total RNA were extracted from PP, MLN, and sorted cells, and cDNAs were synthesized using an RNeasy Mini Kit (Qiagen) and the ThermoScript RT-PCR System (Invitrogen), according to the manufacturers' instructions. cDNA reaction mixtures were diluted 1/20, and 5 µl of the diluted cDNA reaction mixture was added to 20 µl of SYBR Premix Ex Taq (Takara Bio, Otsu, Japan), containing IL-12p35 (gacctgtgcttgccttagcatc and tgccttccacaggagggttc), IL-12p40 (atgccctggagaaacagtga and ttgtggagcagcagatgtgagt), or GAPDH (gcctggagaaacctgccaagta and tgaagtcgaggagacaacctg) primer pairs at 1 µM. Reactions were performed using a Light Cycler 480 (Roche, Basel, Switzerland). Samples were quantified by comparison with a standard curve generated by cDNA templates using the respective primers. The value for the target gene was divided by the value for the GAPDH gene, and relative gene expression units were normalized by the mean gene value for the control mice.

Tissue immunohistochemistry. PP was dissected from the mouse ileum 8 h after oral administration of FITC-conjugated KW3110 (50 mg/head). PP cells were frozen in O.C.T. compound (Sakura Finetek, Torrance, CA) and sliced into 6-µm sections. The sections were fixed in cold acetone and stained for CD11b or IL-12p40 using the TSA Plus System (PerkinElmer, Waltham, MA), according to the manufacturer's instructions. In brief, endogenous peroxidase activity was quenched with 3% H₂O₂ PBS, and endogenous biotin was blocked with an Endogenous Biotin Blocking Kit (Invivogen). Sections were then blocked with TNB buffer (PerkinElmer) and anti-CD16/32 antibody, and biotinylated anti-CD11b, biotinylated anti-IL-12p40, or biotinylated rat IgG2a antibody was applied for 1 h at room temperature. The slides were washed and incubated with horseradish peroxidase (HRP)-conjugated streptavidin for 30 min. After washing, biotinylated Tyramide (PerkinElmer) followed by streptavidin-Alexa 568 was applied. The slides were mounted with Vectashield (Vector Laboratories, Burlingame, CA) and analyzed by confocal microscopy with TCS NT (Leica Microsystems, Wetzlar, Germany).

Results

KW3110 stimulated PP cells induced a Th1 skewed immune response in an IL-12 dependent manner

Orally administered KW3110 passes through the gastrointestinal tract and might interact with intestinal immune tissue, including Peyer's patch (PP). To determine whether PP cells secrete IL-12 after KW3110 stimulation and which cell type is the target of KW3110, we depleted B220, CD11b, and CD11c positive cells from whole PP cells using magnetic beads and cultured whole and depleted cells with KW3110. Whole PP cells secreted IL-12p70 (Fig. 1A). Depletion of CD11c positive cells did not reduce IL-12 production, while CD11b depleted cells did not produce IL-12. Depletion of B220 positive cells, the majority of PP, caused IL-12p70 production to be reduced to almost half the normal level.

To determine whether KW3110 induces a Th1 skewed immune response to PP cells IL-12 dependently, antigen presenting cells (APC) from BALB/c mice PP and naïve CD4⁺ T cells from DO11.10 mice splenocytes were cultured with OVA peptide in the presence and the absence of KW3110 and/or anti-IL-12 antibody. After culturing, the cells were stimulated using PMA/ionomycin and intracellular IL-4 and IFN- γ were measured. The addition of KW3110 induced an increase in IFN- γ positive cells and a decrease in IL-4 negative cells (Fig. 1B). Blocking IL-12 cancelled these changes. These results indicate that KW3110 induces a Th1-skewed immune response through IL-12 induction of PP cells.

Oral administration of KW3110 induced IL-12 gene expression in PP and MLN

Active IL-12 (IL-12p70) is a heterodimer of IL-12p35 and IL-12p40 subunit.¹⁴ To examine the *in vivo* IL-12 induction of KW3110, we measured IL-12p35 and p40 gene expression in intestinal immune tissues, PP and MLN, at different time points after oral administration of KW3110. The IL-12p40 mRNA levels in PP significantly increased 2 to 10 h after administration (Fig. 2A). The expression levels decreased almost to the base level 24 h after administration. In MLN, IL-12p40 mRNA levels also increased significantly 10 h after KW3110 administration (Fig. 2B). However, IL-12p35 mRNA did not change at any of the time points, in either PP or MLN (Fig. 2A, B). According to previous studies, the IL-12p35 gene is ubiquitously expressed, while IL-12p40 gene expression is restricted to APCs that produce IL-12p70.^{15,16} To evaluate IL-12p35 gene expression, CD11b and CD11c positive PP cells from KW3110-administered mice were concentrated by MACS and IL-12 mRNA expression was measured. Both IL-12p40 and p35 increased in the CD11b and CD11c positive cells, although IL-12p40 expression was higher in CD11b positive cells. In view of these results, it was suggested that orally administered KW3110 induces IL-12 in PP and MLN.

In situ analysis of PP after oral administration of FITC-labeled KW3110

To observe KW3110 in the PP, FITC-labeled KW3110 was orally administered to mice. Frozen

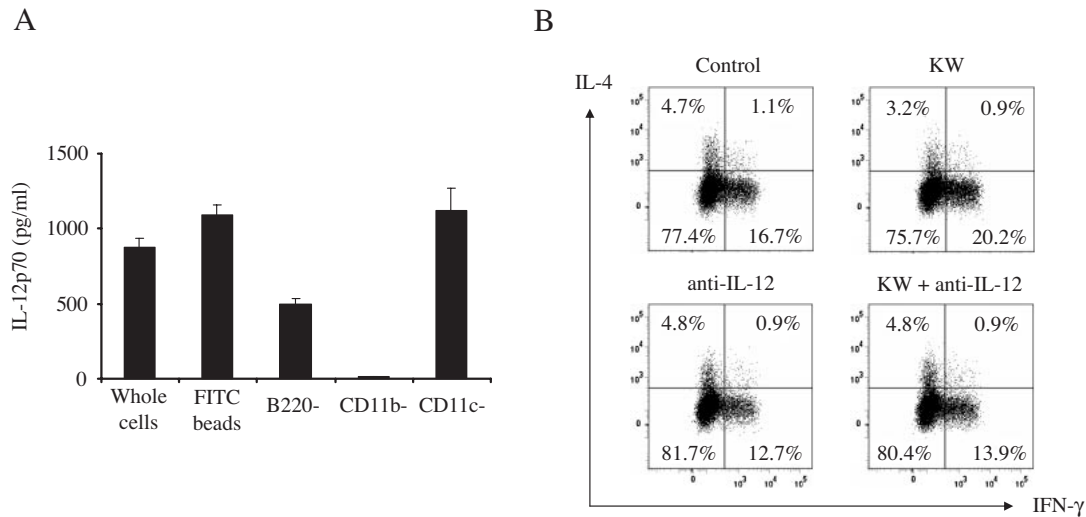


Fig. 1. KW3110 Induced IL-12 Is Crucial for a Th1 Skewed Immune Response.

A, PP cells were stained with anti-B220, anti-CD11b, anti-CD11c, or anti-FITC (control) microbeads, and magnetically separated. Negatively selected cells were cultured with 1 μ g/ml of KW3110 for 24 h, and IL-12p70 was measured. Data are representative of two experiments producing similar results, and each data value is the mean $\pm$ SD of triplicate cultures. B, T cells from DO11.10 mice (1×10^6) and APC from BALB/c PP cells (3×10^6 cells/ml) with and without KW3110 (1 μ g/ml) and/or anti-IL-12 were cultured with 5 μ M OVA peptide for 1 week. Cultured cells were stimulated with PMA/ionomycin and intracellular IFN- γ , and IL-4 of KJ1.26 positive cells were analyzed by FACS.

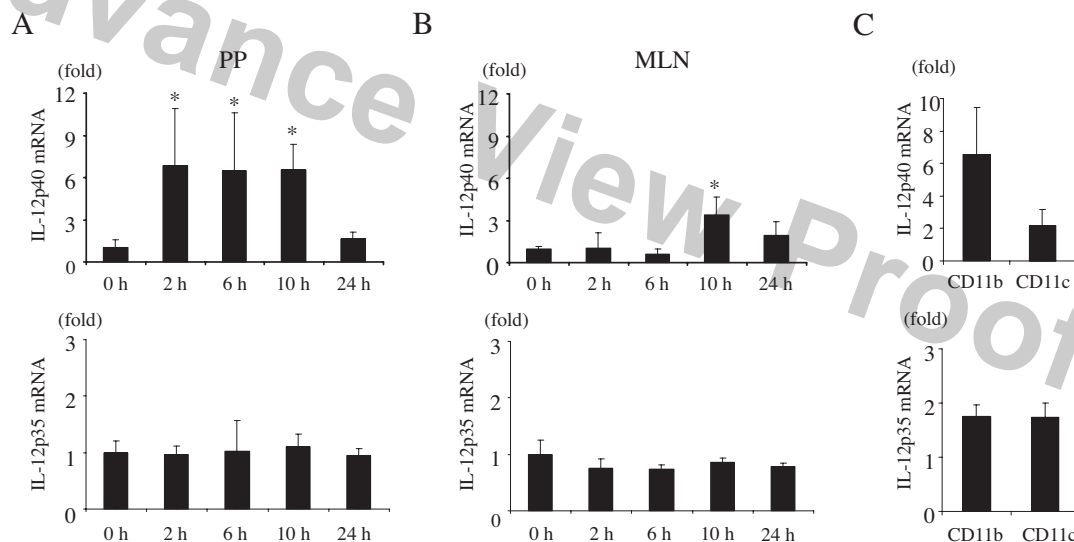


Fig. 2. Oral Administration of KW3110 Induced IL-12 Gene Expression in PP and MLN.

Total mRNA was extracted from (A) PP and (B) MLN 0, 2, 6, 10, and 24 h after oral administration of 50 mg of KW3110 ($n = 8$). The IL-12p40 and IL-12p35 gene expression values were estimated by quantitative RT-PCR and normalized using the GAPDH gene value. Bars show relative gene expression change in KW3110-administered mice as compared with the mean value in saline-administered mice. Data are representative of two experiments producing similar results. C, KW3110 or saline was administered to BALB/c mice ($n = 3$), and PP were removed 6 h later. The cells were pooled, and CD11b and CD11c positive cells were separated by MACS. Total mRNA was extracted, and the IL-12p35 and p40 gene expression values were estimated as in (A). Data are mean $\pm$ SE of four (for CD11b) or three (for CD11c) experiments. * $p < 0.01$ vs. 0 h (Dunnett's test).

sections of PP were stained by CD11b antibody and analyzed by confocal microscopy. Both FITC-labeled KW3110 and CD11b positive cells were detected in the subepithelial dome (SED) area (Fig. 3A, B). These data indicate that orally provided KW3110 interacts with CD11b positive cells in the PP. To confirm that KW3110 induces IL-12 protein in the PP, sections were stained with IL-12p40 antibody. Strong signals observed in SED area (Fig. 3C–E) indicated that IL-12 protein is secreted in PP after oral administration of KW3110.

Oral administration of KW3110 transiently enhanced blood IL-12 levels

To determine the effect of KW3110 administration on systemic IL-12 levels, we measured plasma IL-12. Plasma IL-12p40 significantly increased 6 to 10 h after oral administration of KW3110 (Fig. 4A). Twenty-four h after feeding, the levels decreased almost to the baseline levels. A significant increase in IL-12p70 levels was also observed 10 h after oral administration (Fig. 4B). These data indicated that oral administration of KW3110 affects IL-12 levels in the blood, and that this effect is transient.

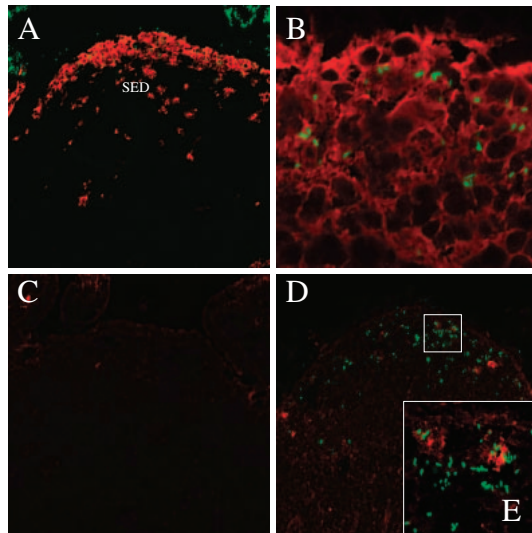


Fig. 3. Confocal Microscopic Analysis of KW3110 and IL-12p40 in PP.

PP was removed 8 h after oral administration of FITC-KW3110 (A, B, D, and E) or saline (C). A, PP sections were labeled with antibodies against CD11b (red). B, CD11b positive cells in the SED area are shown. C, D, PP sections were labeled with anti-IL-12p40 antibody (red). E, Part of Fig. 3D (white box) is expanded. FITC-KW3110 is shown in green. The original magnification was $\times 100$ (A, C, D) or $\times 630$ (B, E). Data are representative of two experiments yielding similar results.

Discussion

Our study indicates that *Lactobacillus paracasei* KW3110 induces IL-12 from PP CD11b⁺ cells but not from CD11c⁺ cells *in vitro* (Fig. 1A), and that induced IL-12 enhances the differentiation of IFN- γ and inhibits IL-4 producing T cells (Fig. 1B). These results correspond with a previous study using other LAB strains and splenocytes.¹⁷⁾ However, the *in vivo* mechanism of the anti-allergic function by LAB strains, including KW3110, is not understood. In this study, we examined IL-12 induction after oral administration of KW3110 *in vivo*.

Orally administered KW3110 is frequently found in the PP SED area, indicating that the PP is one of the main places for interaction between LAB and immune cells (Fig. 3A). Confirming this, IL-12p40 mRNA expression increased in PP after KW3110 administration (Fig. 2A). Dendritic cells that internalized commensal or orally administered bacteria migrated to the MLN from the PP.¹⁸⁾ An increase in IL-12p40 mRNA in MLN indicates cell migration that interacted with KW3110 (Fig. 2B). We observed an increase in IL-12p40 and IL-12p35 mRNA expression in PP CD11b and CD11c positive cells and IL-12p40 production in PP (Figs. 2C and 3D). These data strongly suggest that orally administered KW3110 induces IL-12p70 production in the PP. Differently from our *in vitro* study, it appeared that dendritic cells (CD11c⁺) also produced IL-12 after oral administration, although the IL-12p40 level was lower than that of the CD11b cells. Further studies on CD11c/CD11b positive dendritic cells and CD11b positive macrophages are necessary to understand their contribution to *in vivo* IL-12 induction by KW3110.

The increase in IL-12 was not restricted to the intestinal immune system, for blood IL-12 levels also

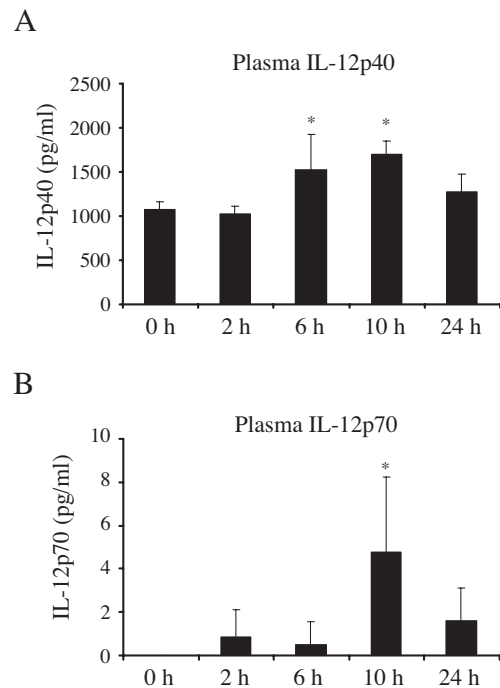


Fig. 4. Oral Administration of KW3110 Induces IL-12 Induction *in Vivo*.

A, KW3110 (50 mg/head) was administered to BALB/c mice ($n = 8$), and plasma was collected at 0, 2, 6, 10, and 24 h. The plasma IL-12p40 levels were measured using ELISA (A). The plasma IL-12p70 concentration was measured using the Bio-Plex system (B). Data are representative of two experiments yielding similar results, and each data value is the mean $\pm$ SD for eight mice. * $p < 0.01$ vs. 0 h (Dunnett's test).

increased after KW3110 administration (Fig. 4). Although we fed excess amounts of KW3110 to make detection of IL-12 production *in vivo* easier, we observed dose-dependent induction of plasma IL-12p40 (data not shown). This suggests that intake of LAB can induce IL-12 in the body.

Injection of IL-12 into mice inhibited Th2 cytokine expression and suppressed allergic responses.¹⁹⁾ Oral administration of a specific LAB strain modulated the Th1/Th2 balance, also suppressing allergic responses, but the *in vivo* involvement of IL-12 is not known.^{5,20,21)} We have indicated *in vivo* IL-12 induction by LAB, and it is suggested that LAB improves allergy by IL-12 induction. Since KW3110 have been indicated to induce IL-12 *in vivo*, IL-12 mediated modification of the Th1/Th2 balance contributes to the anti-allergic function of LAB, but it is not certain that the regulation of allergy is explained only by improvement of the Th1/Th2 balance. A recent study revealed that IL-17 plays an important role in some allergic models.²²⁾ Because IL-12 acts as antagonist of IL-23, an important mediator of Th17 development and inflammation,²³⁾ *in vivo* induced IL-12 perhaps demonstrates an anti-allergic function by inhibiting Th17 development. Indeed, we have reported that oral administration of KW3110 attenuates the development of atopic-dermatitis-like skin lesions in NC/Nga mice.²⁴⁾ There are reports indicating IL-12 independent anti-allergic mechanisms of LAB. Kanzato *et al.* suggested the induction of antigen specific apoptosis by oral administration of LAB.²⁵⁾ Although it was an *in vitro* study, Smits *et al.* indicated that

selective LAB induce regulatory T cells by modulating dendritic cells.²⁶⁾ It is important to study and evaluate the *in vivo* contribution of these potential mechanisms in order to understand the anti-allergic function of specific LAB.

Although there are reports indicating *in vivo* IL-12 induction using other microbes, they evaluated only mRNA expression after oral administration.^{15,27,28)} We found here for the first time that oral administration of LAB induces IL-12 production *in vivo* using KW3110. Our findings explain part of the mechanism, but further studies are necessary for our understanding of the anti-allergic response and other immune modulatory functions of LAB.

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