

The Anti-Allergic Effects of Lactic Acid Bacteria Are Strain Dependent and Mediated by Effects on both Th1/Th2 Cytokine Expression and Balance

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Key Words

Lactic acid bacteria · IgE · Th1 · Th2 · *Lactobacillus paracasei* strain KW3110

Abstract

Background: There is growing interest in the immune-stimulating effect and in particular, the anti-allergic effect, of lactic acid bacteria (LAB). However, no comprehensive studies have been done that compare the immune-stimulating potential of LAB strains. **Methods:** The in vitro immune-stimulating effects on Th1/Th2 balance of more than 100 LAB strains were compared in splenocytes from ovalbumin-sensitized Th2-polarized mice. The in vivo anti-allergic ability of strain KW3110 was studied in the Th2-polarized model by detecting serum IgE concentration, Th1/Th2 cytokine secretion from splenocytes, and the expression of co-stimulatory molecules on macrophages. **Results:** In vitro studies from Th2-polarized splenocytes, using IL-12 as a Th1 parameter and IL-4 secretion as a Th2 parameter revealed a wide variety of IL-12-inducing and IL-4-repressing activities, depending on the strain of LAB, not depending on the species. However, evaluation of individual strains in vivo revealed that after exposure to *Lactobacillus paracasei* KW3110 strain, the serum IgE elevation elicited by re-

peated OVA injection of mice was strongly inhibited. Cytokine secretion from splenocytes 20 weeks after KW3110 administration showed increased IL-12 and decreased IL-4 expression. Both CD40 and B7-1 expression on macrophages was upregulated by administration of KW3110. **Conclusions:** Improving the consequences of the Th1/Th2 imbalance by administration of LAB was dependent upon the LAB strain rather than the LAB species. Oral KW3110 administration in the mouse allergy model directed the Th1/Th2 balance toward Th1 through the maturation of APCs and inhibition of serum IgE elevation.

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Introduction

Type I allergies, including hay fever, atopic dermatitis, allergic rhinitis and asthma, are characterized by an elevated production of IgE and mast cell degranulation that result in release of histamine as well as other chemical mediators of allergy [1]. Type 2 helper CD4+ T cells (Th2) are characterized by cytokines, such as IL-4 and IL-5, that promote the production of IgE. In contrast, type 1 helper CD4+ T cells (Th1) inhibit IgE production by secreting IFN- γ [2]. IL-12 produced by antigen-presenting cells

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(APCs), such as dendritic cells (DCs) and macrophages, is also known to stimulate both NK and Th1 cells to produce IFN- γ . Anti-allergic drugs, such as anti-histamines and anti-leukotrienes, relieve the discomfort of allergic diseases because they inhibit the release of chemical mediators in the effector phase of the allergic reaction. However, the induction phase of disease that involves sensitization by antigen and the differentiation to Th2 cells is not affected by such drugs.

A decade ago, proof of an inverse association between infections and allergy led to the 'hygiene hypothesis' [4]. There is now renewed interest in this hypothesis that focuses on the idea that exposure to certain types of infectious agents and endotoxins is essential for the maturation of the immune system, and that less exposure leads to an imbalance of certain types of immune responses. LAB are nonpathogenic, gram-positive bacteria. LAB are major components of the commensal microbial flora of the small and large intestinal tract of both humans and animals [5]. It is now widely accepted that probiotics exert health benefits beyond inherent basic nutrition [6, 7]. One potentially important function of probiotics is their involvement in development and maintenance of homeostasis in the gut-associated immune system. For example, LAB impact on cellular immune functions as bacterial antigenic stimuli, activators of NK cells [8], and enhancers of macrophage phagocytic activities [9]. The major role of Th1 immune response is to regulate cellular immune function. LAB prime immune cells for Th1 differentiation by secreting IL-12, a strong Th1-skewing cytokine [10]. LAB might moderate the allergic conditions that result from Th2-skewing by amplification of the Th1 response, resulting in normalization of the Th1/Th2 ratio.

The idea that all LAB have anti-allergic effects is questioned by reports showing that differences were observed among 6 strains as concerns the induction of IL-12. One species, *Lactobacillus casei*, was the strongest inducer of the Th1 response among 6 strains [11]. Also, strain dependency of induction of Th1 and Th2 cytokines was also studied on 8 LAB strains [12] and the cytokine profiles of two LAB strains and *E. coli* were compared on human PBMC [13]. However, those studies were considered not to be sufficient to infer that Th1 and Th2 cytokines are dependent on LAB strain. In this study we have tested over 100 LAB strains, representing 18 different species, to determine whether it is the species or the strain that determines the direction of the Th response. *Lactobacillus paracasei* KW3110 was selected from an in vitro study as being the most potent inducer of Th1 cytokines and repressor of Th2 cytokines. We have also investigated the

effect of oral administration of KW3110 in a mouse model of allergic disease. The gram-positive bacterial component, lipoteichoic acid (LTA), was shown to stimulate the immune system by activating transcription factor NF- κ B through TLR2 [14, 15]. The effect of LAB LTA from KW3110 on activation of NF- κ B signal was investigated.

Materials and Methods

Mice

Six-week-old female BALB/c mice were purchased from Charles River Japan.

Bacterial Strains

Bacterial strains used in this study were from the collections held at the Tokyo University of Agriculture, Japan Collection of Microorganisms, Institute for Fermentation Osaka, American Type Culture Collection, Japan Dairy Technical Association, Arla Foods, Rhodia Food, Christian Hansen, Kirin Brewery and Koiwai Dairy Products. Some strains were isolated from commercial products. All strains used in this study are listed in table 1. Cultures of bacterial strains were routinely grown at 37 °C for 48 h in MRS broth (Difco, USA). Cultured bacteria were washed twice with sterile distilled water, heat-killed at 100 °C, lyophilized, and suspended in PBS.

Cell Culture

For in vitro studies, ovalbumin (OVA)-primed splenocytes were used. BALB/c mice were injected intraperitoneally with 1 mg OVA absorbed onto 2 mg of Al(OH)₃ (Wako, Japan). Injections were given twice on day 0 and on day 6 after the initial immunization. Mice were sacrificed on day 13 to obtain splenocytes. After lysis of RBC, splenocytes were suspended at the concentration of 2×10^6 cells/ml in RPMI medium (Sigma) supplemented with 10% FCS and penicillin/streptomycin. Cells were cultured with 1 mg/ml OVA, in both the presence or absence of 1 μ g/ml bacterial strains. The effect of oral administration of KW3110 on cytokine and IgE production was tested by culturing splenocytes in complete RPMI medium with 100 μ g/ml OVA. All splenocyte cultures were sampled at 7 days for cytokines and at 14 days for IgE.

Recruitment of Peritoneal Macrophages

Mice were injected intraperitoneally with 1 ml of 10% thioglycolate (Sigma). Three days later, they were sacrificed and macrophages were harvested by peritoneal lavage using HBSS. Adherent cells were isolated by incubation on plastic plates for 2 h at 37 °C in 5% CO₂. The adherent cells were cultured in RPMI complete medium either with or without 1 μ g/ml of each of the bacterial strains. The strong inducer of IL-12 [15], OK432 (Chugai Pharma), was used as positive control at the concentration of 0.01 KE/ml. After 3 days, supernatants were collected for IL-12 quantification.

Removal of Specific Cell Populations from Splenocytes

Macrophage-depleted splenocytes from OVA-sensitized BALB/c mice were obtained using the MACS system (Miltenyi biotec). For FcR blocking, splenocytes were treated with mouse CD16/CD32 Ab (eBiosciences), incubated with CD11b microbeads (Miltenyi biotec) for 15 min on ice, and washed with PBS supplemented with 0.1%

Table 1. List of various strains of LAB used in this work

Strain ID	Genera	Culture collection	Strain ID	Genera	Culture collection
F19	<i>L. casei</i>	Arla Foods	NRIC1916	<i>L. paracasei</i>	NRIC
LBC80	<i>L. casei</i>	Rhodia Food	NRIC1917	<i>L. paracasei</i>	NRIC
LBC81	<i>L. casei</i>	Rhodia Food	NRIC1936	<i>L. paracasei</i>	NRIC
JCM1133	<i>L. casei</i>	JCM	NRIC1937	<i>L. paracasei</i>	NRIC
JCM1134	<i>L. casei</i>	JCM	NRIC1941	<i>L. paracasei</i>	NRIC
JCM2770	<i>L. casei</i>	JCM	NRIC1942	<i>L. paracasei</i>	NRIC
JCM8129	<i>L. casei</i>	JCM	NRIC1944	<i>L. paracasei</i>	NRIC
LCS	<i>L. casei</i>	Yakult	NRIC1945	<i>L. paracasei</i>	NRIC
KW3119	<i>L. casei</i>	isolated from dietary foods	NRIC1946	<i>L. paracasei</i>	NRIC
LB81	<i>L. casei</i>	Kirin Brewery	NRIC1962	<i>L. paracasei</i>	NRIC
B	<i>L. delbrueckii</i> subsp. <i>bulgaricus</i>	Koiwai dairy products	NRIC1963	<i>L. paracasei</i>	NRIC
KW3311	<i>L. delbrueckii</i> subsp. <i>bulgaricus</i>	Koiwai dairy products	NRIC1981	<i>L. paracasei</i>	NRIC
KW3315	<i>L. delbrueckii</i> subsp. <i>bulgaricus</i>	Koiwai dairy products	KW3110	<i>L. paracasei</i>	Koiwai dairy products
B-5b	<i>L. delbrueckii</i> subsp. <i>bulgaricus</i>	Japan dairy technical association	KW3939	<i>L. paracasei</i>	isolated from dietary foods
JCM1012	<i>L. delbrueckii</i> subsp. <i>bulgaricus</i>	JCM	LB82	<i>L. paracasei</i>	Kirin Brewery
La-5	<i>L. acidophilus</i>	Chrstian Hansen	JCM1131	<i>L. gasseri</i>	JCM
A	<i>L. acidophilus</i>	Koiwai dairy products	JCM1149	<i>L. plantarum</i>	JCM
L54-1	<i>L. acidophilus</i>	Japan dairy technical association	JCM2012	<i>L. johnsonii</i>	JCM
JCM1132	<i>L. acidophilus</i>	JCM	La1	<i>L. johnsonii</i>	Nestle
L-92	<i>L. acidophilus</i>	Calpis	JCM6124	<i>L. mesenteroides</i>	JCM
B-1	<i>L. helveticus</i>	Japan dairy technical association	KW4510	<i>L. lindneri</i>	Kirin Brewery
JCM1120	<i>L. helveticus</i>	JCM	KW4511	<i>L. lindneri</i>	Kirin Brewery
KW3712	<i>L. helveticus</i>	isolated from dietary foods	LGG	<i>L. rhamnosus</i>	ATCC
IFO3345	<i>L. brevis</i>	IFO	LB80	<i>L. rhamnosus</i>	Kirin Brewery
JCM1059	<i>L. brevis</i>	JCM	K5	<i>L. kefir</i>	Kirin Brewery
JCM1170	<i>L. brevis</i>	JCM	K7	<i>L. kefir</i>	Kirin Brewery
L8	<i>L. brevis</i>	Kirin Brewery	K29	<i>L. kefir</i>	Kirin Brewery
L37	<i>L. brevis</i>	Kirin Brewery	K45	<i>L. kefir</i>	Kirin Brewery
L40	<i>L. brevis</i>	Kirin Brewery	K61	<i>L. kefir</i>	Kirin Brewery
L41	<i>L. brevis</i>	Kirin Brewery	KW3610	<i>L. lactis</i>	Koiwai dairy products
L42	<i>L. brevis</i>	Kirin Brewery	H-61	<i>L. lactis</i> subsp. <i>cremoris</i>	Japan dairy technical association
L43	<i>L. brevis</i>	Kirin Brewery	527	<i>L. lactis</i> subsp. <i>lactis</i>	Japan dairy technical association
L45	<i>L. brevis</i>	Kirin Brewery	N-7	<i>L. lactis</i> subsp. <i>diacetylactis</i>	Japan dairy technical association
L50	<i>L. brevis</i>	Kirin Brewery	T	<i>S. salivarius</i> subsp. <i>thermophilus</i>	Koiwai dairy products
L52	<i>L. brevis</i>	Kirin Brewery	G	<i>S. salivarius</i> subsp. <i>thermophilus</i>	Koiwai dairy products
L53	<i>L. brevis</i>	Kirin Brewery	KW3212	<i>S. salivarius</i> subsp. <i>thermophilus</i>	Koiwai dairy products
L57	<i>L. brevis</i>	Kirin Brewery	KW3213	<i>S. salivarius</i> subsp. <i>thermophilus</i>	Koiwai dairy products
L61	<i>L. brevis</i>	Kirin Brewery	KW3214	<i>S. salivarius</i> subsp. <i>thermophilus</i>	Koiwai dairy products
L62	<i>L. brevis</i>	Kirin Brewery	E	<i>B. lactis</i>	Koiwai dairy products
L63	<i>L. brevis</i>	Kirin Brewery	JCM1192	<i>B. breve</i>	JCM
L107	<i>L. brevis</i>	Kirin Brewery	JCM1222	<i>B. infantis</i>	JCM
TUML43	<i>L. brevis</i>	Kirin Brewery	Bb536	<i>B. longum</i>	Morinaga Milk Industry
IFO12004	<i>L. paracasei</i>	IFO	L	<i>B. longum</i>	Koiwai dairy products
JCM1053	<i>L. paracasei</i>	JCM	<i>L. casei</i> = <i>Lactobacillus casei</i> ; <i>L. delbrueckii</i> subsp. <i>bulgaricus</i> = <i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i> ; <i>L. acidophilus</i> = <i>Lactobacillus acidophilus</i> ; <i>L. helveticus</i> = <i>Lactobacillus helveticus</i> ; <i>L. brevis</i> = <i>Lactobacillus brevis</i> ; <i>L. paracasei</i> = <i>Lactobacillus paracasei</i> ; <i>L. gasseri</i> = <i>Lactobacillus gasseri</i> ; <i>L. plantarum</i> = <i>Lactobacillus plantarum</i> ; <i>L. johnsonii</i> = <i>Lactobacillus johnsonii</i> ; <i>L. mesenteroides</i> = <i>Lactobacillus mesenteroides</i> ; <i>L. lindneri</i> = <i>Lactobacillus lindneri</i> ; <i>L. rhamnosus</i> = <i>Lactobacillus rhamnosus</i> ; <i>L. kefir</i> = <i>Lactobacillus kefir</i> ; <i>L. lactis</i> = <i>Lactococcus lactis</i> ; <i>S. salivarius</i> subsp. <i>thermophilus</i> = <i>Streptococcus salivarius</i> subsp. <i>thermophilus</i> ; <i>B. lactis</i> = <i>Bifidobacterium lactis</i> ; <i>B. breve</i> = <i>Bifidobacterium breve</i> ; <i>B. infantis</i> = <i>Bifidobacterium infantis</i> ; <i>B. longum</i> = <i>Bifidobacterium longum</i> .		
JCM1109	<i>L. paracasei</i>	JCM			
JCM1111	<i>L. paracasei</i>	JCM			
JCM1161	<i>L. paracasei</i>	JCM			
JCM1163	<i>L. paracasei</i>	JCM			
JCM1171	<i>L. paracasei</i>	JCM			
JCM1172	<i>L. paracasei</i>	JCM			
JCM1181	<i>L. paracasei</i>	JCM			
JCM1556	<i>L. paracasei</i>	JCM			
JCM2123	<i>L. paracasei</i>	JCM			
JCM2769	<i>L. paracasei</i>	JCM			
JCM8130	<i>L. paracasei</i>	JCM			
JCM8131	<i>L. paracasei</i>	JCM			
JCM8132	<i>L. paracasei</i>	JCM			
JCM8133	<i>L. paracasei</i>	JCM			

BSA. The negatively selected fraction from MACS yielded the macrophage-depleted splenocytes. DC, B cell, T cell and NK cell-depleted splenocytes were also obtained by using CD11c, CD45R, CD90 and DX5 microbeads (Miltenyi biotec), respectively.

NF- κ B Activation

NF- κ B activation was monitored in THP-1 human monocytic cells using a luciferase reporter gene assay. THP-1 cells were cultured in RPMI complete medium at 37°C and with 5% CO₂. The cells were co-transfected with 0.8 μ g of the firefly luciferase reporter vector (pNF- κ B luc reporter plasmid, Stratagene) and 0.2 μ g of the *Renilla* luciferase-bearing reporter vector pRL-TK (Promega) using the DEAE dextran method [16]. The transfection solution containing THP-1 cells was incubated for 7 min in a 37°C water bath. The transfected cells were resuspended in RPMI-1640 medium containing 10% FBS and plated in 24-well plates at a density of 2×10^5 cells per well. After 24 h, LTA fractions of either *Staphylococcus aureus* (Sigma), *Bacillus subtilis* (Sigma), or KW3110 were added to transfected cells. The LTA fraction of KW3110 was prepared as described [17]. Endotoxin contents were determined using limulus color KY test kit (Wako, Japan). Cells were harvested 16 h after the addition of the bacteria for measurement of luciferase activity. Activities derived from both firefly (NF- κ B constructs) and *Renilla* (pRL-TK) proteins were measured using the dual luciferase reporter assay system (Promega) and recorded using a Monolight 2010c luminometer (Diaia-tron). *Renilla* luciferase activities were used to standardize for transfection efficiency.

In vivo Experiments

The mice (n = 7 per group) were injected intraperitoneally on weeks 0, 2, 6, 10, 13 with 100 μ g of OVA (Sigma, grade V) and 2 mg Al(OH)₃. They were fed a diet containing 0.03% KW3110 strain. The mean food intake was about 3 g/day/mouse, indicating that each mouse was fed approximately 1 mg/day of KW3110 strain. Sera were collected every 7 days for total IgE quantification. On day 13, mice were sacrificed and splenocytes were harvested for culture.

ELISA

IFN- γ , IL-4, IL-5, IL-10, IL-12 and total IgE were measured in cell culture supernatants using the murine OptEIA ELISA set (BD Pharmingen). OVA-specific IgE levels were determined by a sandwich ELISA as previously described [18]. For the assay of OVA-specific IgE, biotinylated OVA was used instead of biotinylated rat anti-mouse IgE as the second antibody. OVA-specific IgE titers were expressed as the relative value compared with hyperimmunized mouse serum. The hyperimmunized mouse serum was obtained from Tg mice sensitized by means of intraperitoneal administration of OVA adsorbed onto alum, and the OVA-specific IgE level of this control serum was arbitrarily taken to be 1,000 units/ml.

Flow Cytometry

CD40 and CD80 expression on macrophages from mice fed a diet either with or without KW3110 strain were evaluated by flow cytometric analysis. Cells were incubated on ice for 30 min with PE-labeled mouse CD11b antibody (Ab) and FITC-labeled mouse CD40 Ab or FITC-labeled mouse B7.1 Ab (eBiosciences). After washing, CD40 and CD80 expression on CD11b+ cells was detected on an FACSsort flow cytometer (BD Pharmingen) with Cell Quest software.

Results

Cytokine Secretion from Splenocytes Was Dependent on Strain of LAB

The ability of different LAB strains to induce Th1 cytokine IL-12 and Th2 cytokine IL-4 production was tested in supernatants of splenocytes that had been cultured for 1 week with LAB. Over 100 strains from 18 species were tested using Th2-skewed splenocytes derived from OVA-sensitized BALB/c mice. The results in table 2 are expressed as percentage of *L. paracasei* strain KW3110 for IL-12 and percentage of control with no LAB strain added for IL-4. Absolute numbers for controls were 11.3 pg/ml for IL-12 and 321.6 pg/ml for IL-4. The level of IL-12 secretion was dependent upon the LAB strain. No strong IL-12 inducers were detected for either *Bifidobacterium* or *Lactobacillus kefir* species. Strong inducers of IL-12 also were strong inducers of IFN- γ (data not shown). The level of IL-4 also differed according to the LAB strain. Some strains, such as *Lactococcus lactis* strain N-7 and *Lactobacillus johnsonii* strain JCM2012, increased the IL-4 secretion from cells. There was a tendency for strong IL-12 inducers to be strong inhibitors of IL-4, although there were exceptions. *Lactobacillus lindneri* strain KW4510 and *Lactobacillus brevis* strain JCM1059 induced high levels of IL-12 despite weak inhibition of IL-4. Similar results were observed for both IL-4 and IL-5 secretion, but not for IL-10. IL-10 expression was induced to similar levels by the different LAB strains (data not shown). Among all strains tested, *L. paracasei* KW3110 strain was shown to be the strongest inducer of IL-12 and inhibitor of IL-4. To test the hypothesis that a species, such as *L. paracasei*, could be defined as a strong modulator of the Th1/Th2 balance toward Th1, the levels of IL-12 and IL-4 secretion induced by *L. paracasei* strains were compared (table 3). IL-12 and IL-4 secretion level varied widely within the same *L. paracasei* species. Strong IL-12 inducers also tended to be strong IL-4 inhibitors; similarly, weak IL-12 inducers were usually weak IL-4 inhibitors.

Effect of LAB Strains on IL-12 Expression by Peritoneal Macrophage

Because LAB may target APC, such as macrophages and DCs [11, 19], the effect of some strains on IL-12 secretion by thioglycollate-activated peritoneal macrophages was tested. KW3110, *L. casei* Shirota (LCS) and *Bifidobacterium longum* BB536 were chosen as the strong, medium, and weak IL-12 inducing LAB strains (table 2). KW3110 induced the secretion of about 700 pg/

Table 2. Production of cytokines induced by various LAB

IL-12			IL-4			IL-12			IL-4		
strain ID	IL-12, % of KW3110	SD	strain ID	IL-4, % of control	SD	strain ID	IL-12, % of KW3110	SD	strain ID	IL-4, % of control	SD
KW3110	100	–	KW3110	26.4	19.3	KW3212	19	6.1	JCM1556	69.8	4.2
KW4510	95.8	17.0	KW3119	27.5	5.8	L37	18.7	6.4	KW3712	70.7	4.3
JCM1149	93.9	16.5	NRIC1941	29.8	11.0	L42	18.1	8.1	JCM1134	72.4	21.7
T	77.7	10.4	JCM1109	29.8	8.8	KW3712	18	9.6	JCM8129	73.4	26.1
NRIC1917	77.1	10.4	JCM8132	30.2	16.4	JCM1161	17.9	5.1	IFO3345	74.6	4.1
JCM1059	76.1	21.8	NRIC1916	35.7	13.4	L54-1	17.1	9.8	L-92	74.6	15.6
JCM8132	70.1	9.4	JCM8130	36.3	14.3	JCM1163	17.1	4.0	JCM1192	74.7	15.3
KW3213	65.3	8.5	F19	36.3	12.6	IFO3345	16.4	9.7	JCM1120	75.0	15.7
JCM6124	57.4	20.6	JCM1131	36.7	12.8	JCM1171	14.4	4.5	L53	76.7	19.8
KW3119	56.0	7.5	JCM1149	36.7	18.9	JCM1170	13.9	7.0	L54-1	78.0	15.6
JCM1053	53.7	8.0	JCM1012	42.6	13.0	L-92	11.7	6.1	JCM1059	78.5	16.1
JCM1131	53.2	24.2	JCM1181	43.0	14.6	B-5b	10.7	8.9	Bb536	79.3	5.0
LGG	52.6	4.6	JCM6124	43.4	12.2	N7	10.6	11.3	L	80.0	10.3
LCS	48.8	4.8	LB81	43.9	17.2	NRIC1936	10.5	4.4	L62	80.9	11.3
L61	46.7	3.7	LGG	45	12.8	JCM2770	10.3	2.4	L61	81.3	18.4
A	44.2	17.6	NRIC1917	45.2	13.0	NRIC1962	10.0	3.3	La1	82.8	36.5
LBC80	43.6	4.2	JCM1053	45.3	16.8	IFO12004	9.0	4.4	IFO12004	83.0	23.0
NRIC1945	43.2	9.8	LCS	45.7	23.4	JCM1172	8.1	3.5	L63	87.2	8.6
F19	42.1	9.1	JCM1133	46.2	13.2	NRIC1946	7.9	3.5	L57	87.9	20.6
L8	41.5	18.4	NRIC1963	47.7	12.4	JCM1192	7.5	10.6	NRIC1937	89.7	26.9
NRIC1916	39.5	5.4	JCM1172	48.7	12.1	KW3315	7.4	3.8	KW3315	90.4	35.1
JCM8133	39.2	6.3	LBC80	48.8	12.4	L107	7.4	5.6	NRIC1942	93.2	10.9
LB81	38.8	7.9	JCM1111	49.0	10.4	L40	5.9	8.8	K61	94.3	20.1
La-5	38.7	6.9	La-5	49.2	23.1	JCM2769	5.5	2.9	K29	96.5	22.2
JCM1109	38.5	11.2	KW3214	49.5	2.9	H-61	5.3	4.6	TUML43	98.4	5.0
NRIC1941	38.4	12.5	T	49.9	3.1	JCM8131	5.1	2.2	control	100.0	–
LBC81	38.3	7.2	JCM1163	50.1	6.9	B-1	5.1	5.0	L52	101.5	12.2
LB82	38.3	6.7	LB82	50.3	14.3	L43	4.7	2.9	L50	101.5	25.2
L41	37.7	9.1	JCM8131	54	13.3	JCM1134	4.5	3.4	K45	103.5	27.8
TUML43	37.2	12.9	LBC81	54.8	13.5	JCM8129	4.5	3.8	KW3610	104.0	24.0
KW3214	37.2	8.4	KW3213	54.8	21.8	NRIC1937	4.3	2.8	E	105.5	15.4
NRIC1963	37.2	5.9	NRIC1936	54.9	13.4	B	3.6	3.9	527	107.8	15.8
G	37.1	7.3	LB80	55.4	13.5	K5	3.6	1.0	K5	109.0	31.3
NRIC1981	35.1	5.6	G	56.8	14.1	K7	2.6	0.0	L8	110.0	26.9
L63	34.3	2.8	JCM1161	57.0	15.2	KW3939	2.3	2.4	B	112.0	14.9
L53	33.8	2.3	NRIC1981	57.0	13.8	K61	2.3	0.8	L107	114.1	31.5
L52	33.0	12.5	KW3939	59.1	3.6	K29	2.1	0.6	L42	114.2	9.6
L50	30.8	3.0	JCM2123	59.7	13.9	NRIC1942	2.0	2.3	L43	119.8	26.4
JCM1181	30.7	10.0	JCM8133	59.8	13.3	JCM2012	1.8	5.4	L41	120.4	13.3
L62	29.5	12.6	JCM1171	61.1	14.1	L45	1.8	2.7	H-61	127.8	10.6
L57	28.8	9.4	NRIC1962	61.6	13.9	JCM1120	1.5	1.3	KW4511	134.4	4.0
JCM8130	28.2	7.3	JCM1132	63.6	12.7	LB80	1.3	2.0	K7	138.2	14.5
La1	26.7	23.4	JCM2769	63.7	14.2	JCM1556	1.2	1.7	L45	140.7	24.6
JCM1133	26.2	8.9	NRIC1945	63.7	34.7	JCM1222	1.2	1.5	JCM1222	143.5	37.2
JCM1132	25.6	13	A	66.7	21.4	KW3610	1.0	0.5	L40	151.0	35.0
JCM1012	21.9	6.8	KW3311	66.9	4.4	E	0.5	1.0	B-1	151.1	12.6
KW4511	21.4	7.7	KW3212	67.1	23.1	control	0.2	0.5	B-5b	162.0	39.1
JCM2123	19.5	6.8	NRIC1944	67.4	24.2	L	0.0	0.0	L37	168.7	37.3
KW3311	19.4	5.5	JCM2770	68.3	21.0	Bb536	0.0	0.0	JCM1170	171.1	37.8
JCM1111	19.3	3.7	NRIC1946	68.7	27.8	527	0.0	0.0	JCM2012	187.2	15.3
NRIC1944	19.1	6.6	KW4510	69.0	28.9	K45	0.0	0.0	N-7	187.8	38.4

Table 3. Comparison of cytokine production induced by various *L. paracasei* strains

IL-12			IL-4		
strain ID	IL-12, % of SD KW3110		strain ID	IL-4, % of SD control	
KW3110	100	–	KW3110	26.4	19.3
NRIC1917	77.1	10.4	NRIC1941	29.8	11.0
JCM8132	70.1	9.4	JCM1109	29.8	8.8
JCM1053	53.7	8.0	JCM8132	30.2	16.4
NRIC1945	43.2	9.8	NRIC1916	35.7	13.4
NRIC1916	39.5	5.4	JCM8130	36.3	14.3
JCM8133	39.2	6.3	JCM1181	43.0	14.6
JCM1109	38.5	11.2	NRIC1917	45.2	13.0
NRIC1941	38.4	12.5	JCM1053	45.3	16.8
LB82	38.3	6.7	NRIC1963	47.7	12.4
NRIC1963	37.2	5.9	JCM1172	48.7	12.1
NRIC1981	35.1	5.6	JCM1111	49.0	10.4
JCM1181	30.7	10.0	JCM1163	50.1	6.9
JCM8130	28.2	7.3	LB82	50.3	14.3
JCM2123	19.5	6.8	JCM8131	54	13.3
JCM1111	19.3	3.7	NRIC1936	54.9	13.4
NRIC1944	19.1	6.6	JCM1161	57.0	15.2
JCM1161	17.9	5.1	NRIC1981	57.0	13.8
JCM1163	17.1	4.0	KW3939	59.1	3.6
JCM1171	14.4	4.5	JCM2123	59.7	13.9
NRIC1936	10.5	4.4	JCM8133	59.8	13.3
NRIC1962	10.0	3.3	JCM1171	61.1	14.1
IFO12004	9.0	4.4	NRIC1962	61.6	13.9
JCM1172	8.1	3.5	JCM2769	63.7	14.2
NRIC1946	7.9	3.5	NRIC1945	63.7	34.7
JCM2769	5.5	2.9	NRIC1944	67.4	24.2
JCM8131	5.1	2.2	NRIC1946	68.7	27.8
NRIC1937	4.3	2.8	JCM1556	69.8	4.2
KW3939	2.3	2.4	IFO12004	83.0	23.0
NRIC1942	2.0	2.3	NRIC1937	89.7	26.9
JCM1556	1.2	1.7	NRIC1942	93.2	10.9
Control	0.2	0.5	control	100.0	–

ml of IL-12, while levels were undetectable following addition of BB536. IL-12 secretion following addition of LCS was approximately 160 pg/ml (fig. 1a).

The secretion levels of IL-12 induced by KW3110 strain in OVA-sensitized and Th2-polarized splenocytes depleted of various cell populations were compared with those of complete splenocytes. The KW3110 strain induced high amounts of IL-12 from complete splenocytes, while basal level induction occurred from CD11b+ cell-depleted splenocytes (fig. 1b). Decreases in IL-12 production were also observed when depleting CD11c+, CD45R+, CD90+ and DX5+ cells; however, the effects of

those cell depletions were much lower than CD11b+ cell depletion. These data underscore the major role of macrophages in IL-12 secretion elicited by LAB. High secretion levels of IL-4 from complete splenocytes without prior exposure to KW3110 were inhibited by adding the KW3110 strain. However, only basal levels of IL-4 secretion were detected in macrophage-depleted splenocytes, irrespective of the presence of the KW3110 strain (data not shown).

Activation of NF-κB Cascades by KW3110

The NF-κB signaling pathway activation is required for the expression of IL-12 in response to microbial stimuli [20]. The LTA, which is a membrane component of gram-positive bacteria, is also an immunostimulator of the NF-κB pathway [21]. For gram-negative bacteria, the NF-κB immunostimulator is a lipopolysaccharide (LPS), a major component of the outer membrane. Toll-like receptor (TLR)-4 is a receptor that initiates the induction and release of proinflammatory cytokines by monocytes and macrophages upon LPS stimulation [22]. In contrast, LTA induces activation of transcription factor NF-κB via TLR-2, but TLR-4 does not appear to be involved [23]. The ability of the KW3110 strain LTA fraction to induce the NF-κB signaling pathway was investigated in THP-1 cells transfected by NF-κB reporter plasmid. Dual luciferase reporter assays were done using *S. aureus* and *B. subtilis* LTA as positive controls. *S. aureus* initiated expression of reporter gene at a concentration of 10 µg/ml, whereas *B. subtilis* LTA effects were optimum at 1 µg/ml (fig. 2). Although a concentration of 10 µg/ml of KW3110 LTA also increased reporter expression, similar expression was observed with 1 µg/ml of the intact KW3110 strain. In addition, the reporter activities were higher with the intact KW3110 bacterial strain than those induced by the LTA fraction. Endotoxin contents were 69.2, 142.8, 32.1 and 1.1 EU/mg for *S. aureus* LTA, *B. subtilis* LTA, KW3110 LTA and intact KW3110, respectively. The concentrations were rather low and there were no relationships between endotoxin contents and the reporter activities.

Oral Administration of KW3110 Suppresses the Increase in Serum IgE in vivo Induced by OVA

To evaluate the anti-allergic potential of oral administration of KW3110 strain, BALB/c mice were injected with OVA at 2- to 4-week intervals for 14 weeks. This schedule of sensitization resulted in a marked increase in serum total IgE levels (fig. 3a, control). During the experiment, the test group was fed 1 mg heat-killed KW3110

Fig. 1. Role of macrophage in cytokine expression elicited by LAB. **a** Secretion of IL-12 from peritoneal macrophages. Culture supernatants from the peritoneal macrophage culture incubated in the presence of 1 µg/ml of LAB strains were assayed for IL-12 with ELISA. OK432 was used as a positive control at 0.01 KE/ml. Each bar represents mean ± SD of 6 mice. n.d. = Not detected. **b** Secretion of IL-12 from splenocytes depleted from various cell populations. Macrophages, DCs, B cells, T cells and NK cells were removed from OVA-sensitized Th2-polarized splenocytes using CD11b, CD11c, CD45R, CD90 and DX5 microbeads. Non-depleted splenocytes and splenocytes depleted of various cell populations were incubated with 1 mg/ml OVA, in both the presence and absence of 1 µg/ml *L. paracasei* strain KW3110. After culture for 7 days, supernatants were measured for IL-12 concentrations.

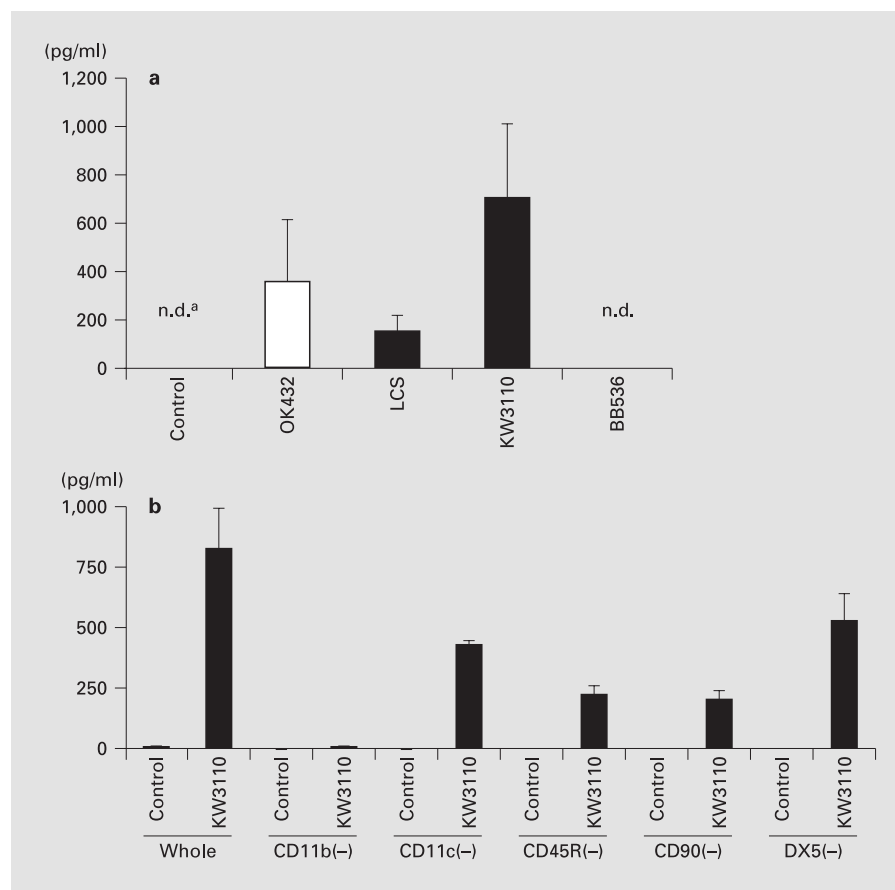
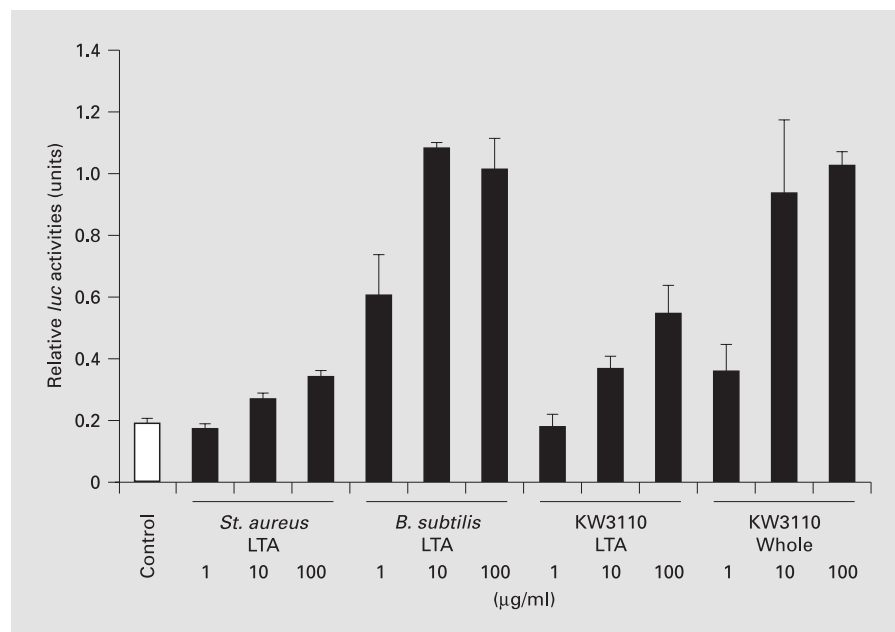


Fig. 2. Effect of KW3110 LTA fraction on NF-κB activation in human monocytes. THP-1 were transiently transfected with plasmids encoding for the firefly luciferase reporter gene joined to tandem repeats of NF-κB binding elements. In addition, plasmids encoding the *Renilla* luciferase reporter gene joined to HSV-TK were used. After stimulation with either bacterial LTA, fractions or whole KW3110 cells were lysed and chemiluminescence indicated by firefly luciferase and *Renilla* luciferase was quantified. Activation is indicated as relative luc activities (firefly luciferase/*Renilla* luciferase). Experiments were performed in triplicates and data from 3 representative experiments are shown. Each bar represents mean ± SD.



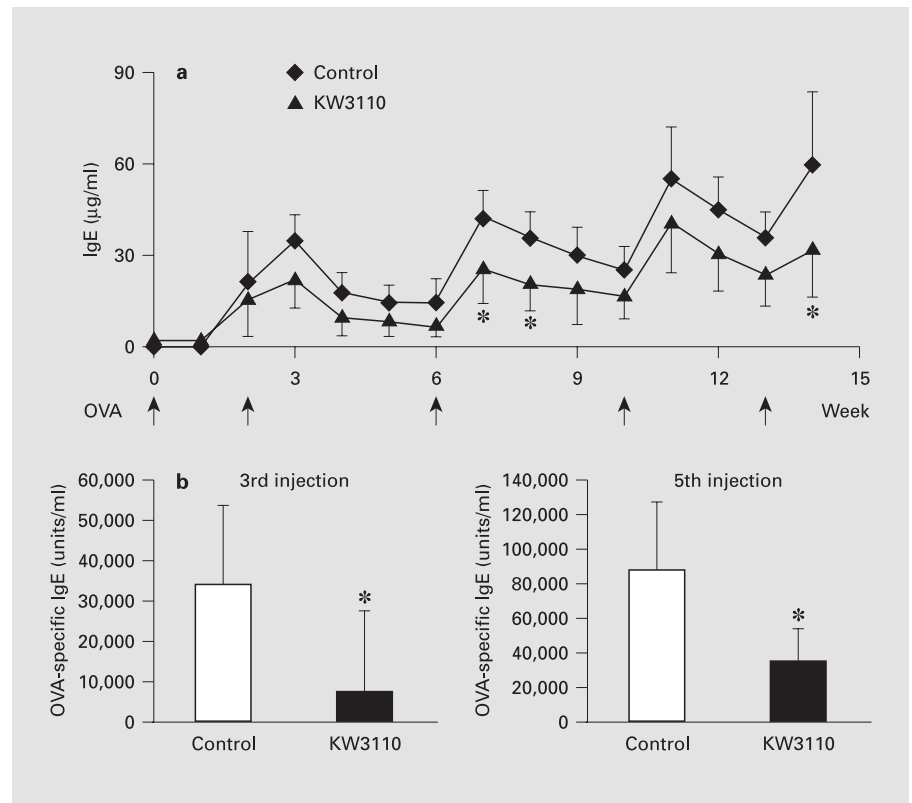


Fig. 3. Effect of oral feeding of KW3110 on IgE production in OVA-sensitized BALB/c mice. Mice were fed either with or without 1 mg of KW3110 strain daily for 14 weeks. OVA sensitization was performed on the week indicated by an arrow. **a** Sera were collected weekly and assayed for total IgE by ELISA. **b** OVA-specific IgE titers were determined on sera 1 week after the 3rd and 5th OVA injections. The values shown are the mean $\pm$ SD of 7 mice per group. p values <0.05 are represented by an * and were obtained by the Mann-Whitney U-test.

strain daily. The control group was not fed with LAB strains. Sera from both test and control groups were collected weekly for total IgE quantification. The group that was fed KW3110 showed significantly ($p < 0.05$) lower total IgE levels when compared with the control group after the 3rd and 5th OVA injection (fig. 3a). OVA-specific IgE titer was determined on 3rd and 5th OVA injection samples. The group fed KW3110 also showed significantly ($p < 0.05$) lower OVA-specific IgE levels compared to the control group. After 14 weeks, mice were sacrificed and splenocytes assayed for Th1/Th2 cytokine production. Splenocytes from the KW3110 group showed significantly ($p < 0.05$) higher IL-12 production in response to OVA stimulation compared to the control group (fig. 4). There were no differences between the 2 groups in IFN- γ production. In contrast, splenocytes from the KW3110 group produced less IL-4 than the control group. IgE synthesis in splenocytes from the mice that were fed KW3110 was significantly ($p < 0.05$) lower than that in the control group. These data indicate that oral administration of KW3110 strain regulates the Th1/Th2 immune response in vivo to induce suppression of serum IgE levels.

Expression of B7-1 and CD40 Costimulatory Molecule Expression Is Increased in Mice Fed KW3110

The effect of oral administration of KW3110 on APC maturation was tested by evaluating the expression of costimulatory molecules on macrophages. CD40L-CD40 costimulation and the B7-dependent costimulatory pathway both play major roles in regulating the Th1 response through direct induction of IL-12 from APCs [24, 25]. CpG oligodeoxynucleotides, that are strong inducers of IFN- γ and IL-12, upregulate both CD40 and B7 costimulatory molecules, with upregulation of B7.1 being greater than B7.2 upregulation [26]. When measured by flow cytometry, expression of both CD40 and B7-1 was upregulated in mice administered KW3110 when compared with control mice (fig. 5). The upregulation of costimulatory molecules on APCs and the promotion of maturation of APCs were associated with the administration of KW3110. The mature APCs might accelerate Th1 differentiation via enhanced IL-12 expression, thereby inhibiting both Th2 differentiation and IgE production.

Fig. 4. Profiles of Th1 (IFN- γ and IL-12), Th2 (IL-4) cytokines, and IgE secreted by splenocytes derived from BALB/c mice given oral KW3110. Controls received no KW3110 in their feed. The mice were sacrificed after the 5th OVA sensitization and splenocytes were cultured with OVA for 7 days for determination of cytokine production and for 14 days for IgE production. The values shown are the mean $\pm$ SD of 7 mice per group. p values <0.05 are shown by an * and were estimated using the Mann-Whitney U-test.

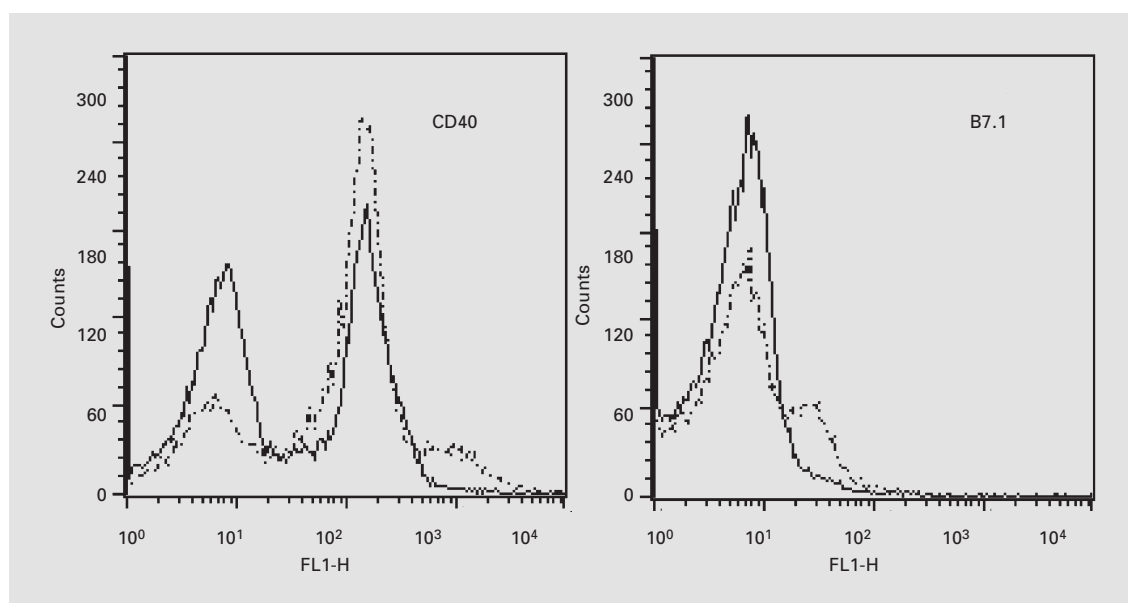
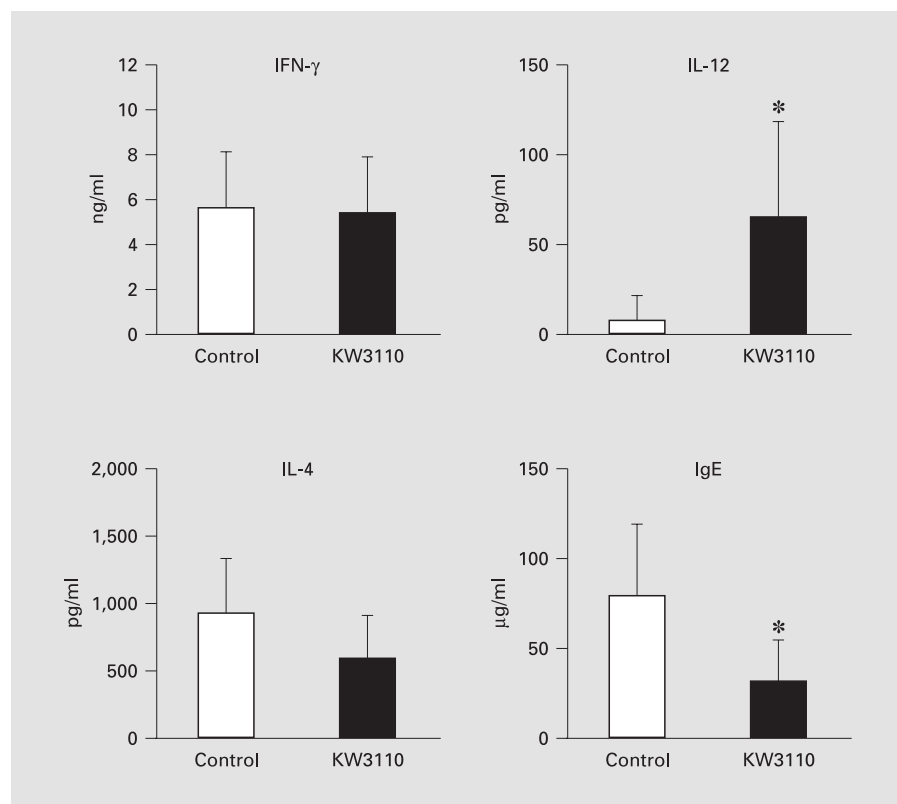


Fig. 5. B7.1 and CD40 expression is increased on macrophages prepared from mice given KW3110. Mice were sacrificed after the 5th OVA sensitization and splenocytes were analyzed by FACS. CD11b⁺ cells were gated to detect B7.1 and CD40 expression on macrophages. Cells were stained with the indicated FITC antibodies. The data from the KW3110 group are shown by a dotted line and the control by a full line. Each point represents data averaged from 5 mice.

Discussion

There is growing interest in the use of LAB as a means of protection from allergic diseases. It is well known that protective Th1 responses inhibit the Th2 activity that is required for IgE class switching [2]. In this study we have investigated whether all LABs possess anti-allergic effects. In addition, we asked whether differences in Th1-inducing and Th2-suppressing activities were at the strain or species level? Using OVA-sensitized BALB/c splenocytes, over 100 LAB strains were tested for IL-12 and IL-4 production. Each LAB strain, even those within the same species, had different activities. This result suggests APCs recognize highly specific features of a LAB strain. Since cell wall membrane compounds, such as LTA or peptidoglycan, and CpG DNA are immunologically active components of LAB, they would be logical candidates for the recognition by APCs [26–29]. When the effect of KW3110 LTA on NF- κ B signaling pathway was investigated, the LTA fraction effectively activated the NF- κ B cascade, however the same quantity of KW3110 in whole cell form also gave a strong activating effect. These data indicate that there are components on LAB, in addition to LTA, that have strong immunostimulating activity. Further investigation on the candidate components that govern the differences between strong and weak Th1-inducing Th2-repressing strains may give insight into the mechanism of the anti-allergic effects of LAB.

The oral administration of the strong IL-12-inducing strain repressed IgE synthesis elicited by repeated OVA injection in the OVA-sensitized mouse model of allergy. The splenocytes from mice given oral KW3110 for 100 days showed higher IL-12 secretion in response to OVA stimulus than the splenocytes from control mice. In spite of higher IL-12 secretion from KW3110 mice, IFN- γ production was similar in both groups. Although the macrophage target cell of LAB is a major producer of IL-12, it is also a major producer of the IL-10 repressor of IFN- γ producing cells [30]. If LAB induced both IL-12 and IL-10 production, the direction of Th1 differentiation might be determined by the reciprocal relationship between the 2 cytokines. There is concern that LAB could be associated with Th1-polarizing autoimmune diseases, such as multiple sclerosis, insulin-dependent diabetes mellitus and rheumatoid arthritis, since it is associated with induction of Th1 responses. However, if IL-10 and IL-12 were induced at the same time, concerns about the use of LAB as an immunomodulator might be unfounded. The splenocytes from the KW3110 group showed lower IL-4 and IgE production in response to OVA stimulus when

compared to control splenocytes. Therefore, repression of IL-4 production might be more important than induction of IL-12 in the mediation of an anti-allergic effect by LAB. The entry of bacteria into the intestinal mucosa occurs mainly through specialized epithelial cells, called M cells that are located in Peyer's patches. Recently, it was found that DCs express tight junction proteins, open the tight junctions between epithelial cells, and take up the microorganisms directly [31]. However how these mechanisms are working in vivo is still unclear. Our data showing that oral administration of KW3110 altered expression of splenocytes, cytokines, and cell surface costimulatory molecules suggest LAB has an impact on the peripheral immune system through intestinal mucosa pathogen entry pathways. Intestinal DCs might be accelerated to maturity by LAB stimulation and carried to the peripheral immune system to affect T cell differentiation.

In the allergic inflammatory process, not only IgE synthesis and Th1/Th2 balance, but also migration of lymphocytes has to be considered. Whether the chemokine expression that follows oral administration of LAB has effects on leukocyte adhesion and migration to the site of inflammation also requires further study.

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