

Induction of Th1 cytokines by *Leuconostoc mesenteroides* subsp. *mesenteroides* (KCTC 3100) under Th2-type conditions and the requirement of NF- κ B and p38/JNK

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ABSTRACT

Leuconostoc mesenteroides subsp. *mesenteroides* (LMM) KCTC 3100, is one of the prominent species in the fermentation of kimchi, a traditional Korean food. In the present study, we investigated the capacity of this microorganism in inducing Th1 cytokines in the presence of Th2 signals in vitro and in vivo and the requirement of NF- κ B and MAPK signaling. Stimulation with heat-killed LMM in mouse splenocytes induced the expression of IFN- γ , which was dependent on IL-12 production by LMM. Pre-treatment with LMM in vitro augmented the production of IFN- γ and IL-4 in response to anti-CD3 plus recombinant IL-4 (rIL-4). LMM administration to mice, beginning either before or after the development of OVA sensitization, increased OVA-restimulated IFN- γ production in the splenocytes and reduced serum total and OVA-specific IgE levels. However, only the pre-sensitization treatment induced a slight reduction in IL-4 from the same cells, but the post-sensitization treatment did not. Induction of IL-12 by LMM in peritoneal macrophages involved NF- κ B, p38 and JNK, but not ERK1/2. In conclusion, our data presented the upregulation of IFN- γ by LMM under the pro-Th2 conditions and the requirement of NF- κ B, p38 and JNK for IL-12 production. These observations suggest that this microorganism can be a useful Th1-inducing agent in modulating the Th1/Th2 imbalance.

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1. Introduction

IFN- γ , produced by Th1 cells, is implicated in cell mediated and inflammatory immune responses and is able to reciprocally inhibit the proliferation of IL-4 and IL-5 secreting Th2 cells [1,2]. Th2 cytokines are responsible for humoral responses including immunoglobulin E (IgE) isotype switching in B cells [3–5]. IL-12, produced mainly by antigen presenting cells (APC) such as macrophages and dendritic cells in response to microbial stimuli, plays an important role in the differentiation of Th1 cells [6–8]. IL-12, upon binding to the IL-12 receptor on T cells, has been reported to be able to stimulate the expression of IFN- γ in a cytokine dependent manner, which is different from the T cell receptor (TCR) dependent pathway [9–12].

As high hygienic standards and frequent antibiotic uses have decreased exposure to microbial infection, the increasing incidence of allergic responses to common environmental and food antigen has been attributed in part to a defect in the Th1 immune response

[13–18]. Allergy or hypersensitivity is mainly an IgE-mediated immune response, due to the overproduction of IL-4 and IL-5 from Th2 cells. In this regard, the use of immunostimulatory agents which are able to boost the Th1 response has been proposed as one effective approach toward a skewed Th2 response.

Several strains of lactic acid bacteria have caught much attention since a number of studies have shown that treatment with those lactic acid bacteria is beneficial in preventing or modulating allergic immune responses [19–25]. Lactic acid bacteria, like other pathogenic Gram-positive bacteria, can evoke innate immune responses by producing Th1 cytokines. *Lactobacillus rhamnosus* GG, for example, was reported to stimulate the production of IL-12 and IFN- γ through NF- κ B and STAT pathways in macrophages [26,27]. *Lactobacillus casei* was demonstrated to involve activation of NF- κ B and p38 mitogen-activated protein kinase (MAPK) in order to promote Th1 cytokines [28].

Kimchi is a traditional Korean fermented vegetable combination with cabbage being the main constituent. A variety of microbial populations such as subsets of *Leuconostoc*, *Lactobacillus*, and *Weissella* genera were isolated from fermented kimchi [29]. *Leuconostoc mesenteroides* subsp. *mesenteroides* (LMM) are used as start bacteria for

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taste and quality control in some of the commercially available kimchi products manufactured in Korea [30]. This microorganism is one of the major species in the fermentation of kimchi and predominates during the first part of fermentation. In our study, we investigated the potential use of LMM as probiotic strain in upregulating and maintaining the expression of IFN- γ despite the dominating Th2 environment *in vitro* and *in vivo*. We also tried to confirm that the induction of the Th1 cytokines by the bacteria was mediated by NF- κ B, p38 and JNK signaling pathways.

2. Materials and methods

2.1. Animals

Male Balb/c or C57BL/6 mice at 8 weeks of age were purchased from Charles River Korea (Orient, Korea) and maintained with rodent chow and water *ad libitum* in a temperature and humidity controlled pathogen free animal facility at Kyunghee University. Mice were cared for according to the Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington, DC, USA). All experiments were approved by our Institutional Ethical Committee for animal welfare.

2.2. Preparation of microorganisms

LMM (KCTC 3100) was obtained from the Korean Collection for Type Cultures. The microorganism was cultured in MRS broth (DIFCO, Detroit, MI, USA) for 16 h at 30 °C and collected by centrifugation, and washed several times with sterile distilled water. Then it was stored frozen at –80 °C and lyophilized. For the *in vitro* experiment, the organism was killed by heating at 120 °C for 30 min and lyophilized.

2.3. Cell line

Mouse macrophage RAW 264.7 cells were cultured at 37 °C in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS and 1% antibiotic–antimycotic in a humidified atmosphere of 5% CO₂.

2.4. *In vitro* stimulation of splenocytes with LMM

To study the effects of LMM on the *in vitro* cytokine production and transcription in splenocytes, spleen was obtained from Balb/c mice and cells were prepared by disrupting the spleen between glass slides in complete medium (RPMI 1640 with 10% FBS, 1% antibiotic–antimycotic). After a 10 min centrifugation at 1000 rpm to separate cells from debris, the cells were washed, followed by lysis of erythrocytes. Cells were resuspended and stimulated with heat-killed lactic acid bacteria for 24 h. Supernatant was harvested for ELISA and the cell pellet was used for real time RT-PCR. For the analysis of IL-12 mediated IFN- γ production, anti-IL-12 antibodies (Pharmingen, San Diego, CA, USA) were pre-treated 30 min before stimulation with LMM. To evaluate the secretion profile of IFN- γ and IL-4 in anti-CD3 or anti-CD3 plus recombinant IL-4 (rIL-4), splenocytes were activated with 2 μ g/ml anti-CD3e (Pharmingen) and 5 ng/ml rIL-4 (Pharmingen), and cell supernatants were harvested at indicated time points.

2.5. Preparation of peritoneal macrophages

Peritoneal macrophages were obtained from C57BL/6 mice 3 days after intraperitoneal injection of 2 ml of sterile thioglycolate medium (Difco). Cells were centrifuged, followed by RBC lysis. Then the cell pellet was resuspended in complete DMEM and seeded at a density of 5×10^5 /ml. After 90 min, non-adherent cells

were removed. For inhibition studies, cells were pre-treated with various doses of sulfasalazine (Sigma, St. Louis, MO, USA) dissolved in NH₄OH, SB203580 (Promega, Madison, WI, USA), U0126 (Sigma) and SP600125 (Sigma) all dissolved in dimethyl sulfoxide (DMSO) for 30 min before stimulation with the bacteria for another 24 h.

2.6. OVA immunization and oral treatment of LMM

Mice were sensitized by three intraperitoneal injections of 1 μ g of ovalbumin (OVA) (Grade V, Sigma) dissolved in a mixture of the equal amount of Al(OH)₃ gel (alum) and PBS in 7 day intervals, followed by intranasal dropping of 20 μ l of 0.01% OVA solution on days 23, 25, 27 after the 1st immunization. Normal mice were sensitized with the equal volume of PBS.

Mice were given 2×10^7 colony forming units (CFU) of live lactic acid bacteria in 9% skim milk by oral gavage three times a week. The experimental procedures were divided into two settings. For pre-sensitization application, oral administration began 1 week before the first OVA sensitization and was completed during the experiment for a total of 5 weeks. In order to perform the evaluation of post-sensitization effect, the treatment was applied 2 weeks after the first sensitization and continued for 2 weeks. Normal and control mice were sham treated with 9% skim milk. Mice were killed after intranasal OVA challenge and serum and splenocytes were obtained. The procedures were summarized in Fig. 1.

2.7. Cell culture for OVA-Induced cytokine production *ex vivo*

At the end of experiment, splenocytes from sensitized mice were obtained and the cells were resuspended in complete medium and adjusted to 5×10^6 cells/ml in 24 well plates and cultured for 72 h with OVA (1 mg/ml) at 37 °C in 5% CO₂ incubator.

2.8. Real-time RT-PCR

Total RNA was isolated with an RNeasy Mini Kit (QIAGEN) and cDNA was reverse-transcribed by a Superscript II reverse transcriptase (Invitrogen). Oligonucleotide primers were designed using the Primer Express software (Applied Biosystems, Foster City, CA). GAPDH gene was used as an endogenous control to normalize the expression of target genes. Quantitative real-time PCR was performed in triplicate using a 96-well optic tray on an ABI PRISM 7300 sequence detector (Applied Biosystems). The negative controls lacking template RNA were included in each experiment. The expressions of each mRNA were quantified by relating the PCR threshold cycle obtained from the samples to amplicon-specific standard curves.

2.9. Measurement of cytokines and IgE

IL-4, IL-12, IFN- γ and total IgE were measured using a commercial OptEIA set (BD Pharmingen). To measure OVA-specific IgE, 96-well plates were coated overnight at 4 °C with purified rat anti-mouse IgE Ab diluted to 2 μ g/ml in PBS. After washing, the plates were blocked with 10% FBS in PBS for 1 h at room temperature. Diluted samples were incubated for 2 h at RT. After washing biotinylated OVA was added and incubated for 2 h at RT. OVA was biotinylated using Biotin N hydroxysuccinimide ester-water soluble (Vector Laboratories, CA, USA). After washing, Avidin-HRP was added and incubated for 30 min at RT. After washing, plates were incubated with TMB and the stop solution was added. The optical densities were measured at 450–570 nm.

2.10. Western blot analysis

RAW264.7 cells were stimulated with lactic acid bacteria for the indicated times in serum free media and washed twice with

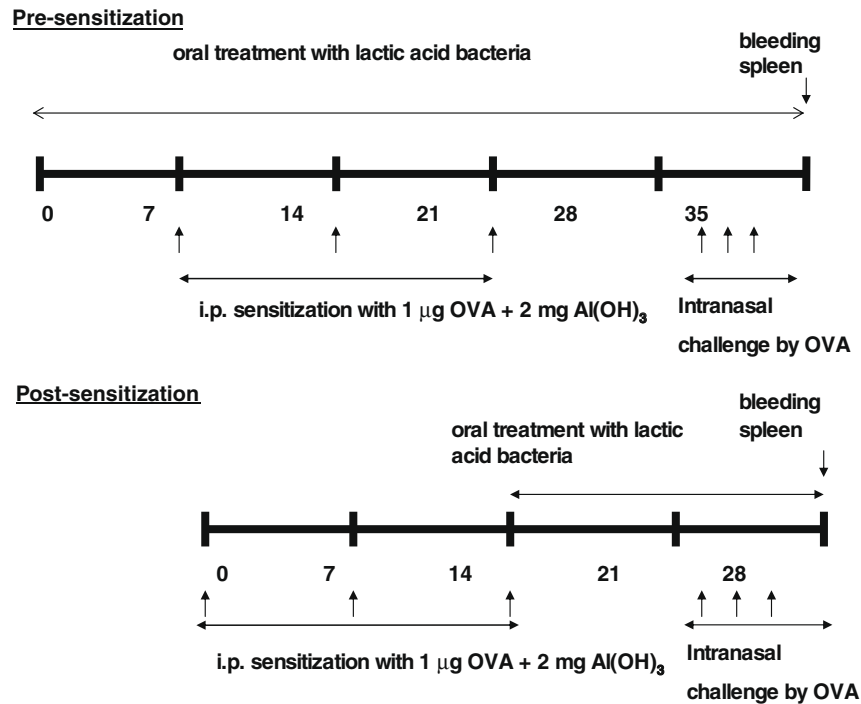


Fig. 1. Experimental procedures. Day 0 is defined as the start of the experiment. Eight week old Balb/c mice were orally treated with lactic acid bacteria before (pre-sensitization) or after (post-sensitization) sensitization with OVA developed. Spleens and blood samples were taken after intranasal challenge by OVA.

ice-cold PBS and lysed on ice for 20 min using PRO-PREP protein extraction solution (iNtRon, Korea). Lysates were centrifuged at 12,000g at 4 °C for 15 min and the supernatants were obtained. Protein concentration was determined using the Bradford's method. Samples were separated on 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat milk in TBS-T for 1 h and then incubated with polyclonal antibody for p38, SAPK/JNK, ERK1/2, phospho-p38, phospho-SAPK/JNK, phospho-ERK1/2 (Cell Signaling Technology, MA, USA), IκBα and β-actin (Santa Cruz Biotechnology, CA, USA) overnight at 4 °C. After washing three times with TBS-T, the membranes were incubated with horse radish peroxidase-conjugated goat anti-mouse IgG (Santa Cruz) for 1 h. Band detection was conducted using an enhanced chemiluminescence (ECL) detection system (Amersham Biosciences, Sweden).

2.11. Statistical analysis

Comparisons between groups were determined by ANOVA followed by Dunnet's post hoc test. Calculations were carried out using SPSS version 12. *P* values less than 0.05 were considered significant.

3. Results

3.1. Effect of LMM on the expression of IFN-γ and IL-12 in mouse splenocytes in vitro

The ability of IL-12 production is proposed as an important criterion for the selection of Th1-promoting lactic acid bacteria since the expression of IFN-γ by lactic acid bacteria depends on the presence of APC [31–33]. We examined the capacity of heat-killed LMM to induce type 1 cytokines in the whole splenocytes in vitro. Cell culture supernatants and mRNA were obtained after incubation with various concentrations of lactic acid bacteria for 24 h. LMM was a good inducer of IFN-γ and IL-12 as determined by real-time

RT-PCR and ELISA (Fig. 2A and B). We also tried to measure whether LMM-induced IL-12 was necessary for the production of IFN-γ. Addition of neutralizing anti-IL-12 antibodies caused a reduction in IFN-γ production, showing that the induction of IFN-γ depends on the production of IL-12 by LMM (Fig. 2C).

3.2. Effect of LMM on the production of IFN-γ and IL-4 in splenocytes before or after stimulation with anti-CD3 plus rIL-4 in vitro

Since the induction of Th1 cytokines by LMM was demonstrated, we wanted to know whether early or later exposure to the bacteria was able to skew the TCR/CD3 stimulated IFN-γ and IL-4 production in the presence of rIL-4. First, we measured the time course kinetics of IFN-γ and IL-4 produced by splenocytes stimulated with lactic acid bacteria, soluble anti-CD3 or anti-CD3 plus rIL-4. IFN-γ production in response to anti-CD3 or anti-CD3 plus rIL-4 resulted in a peak on day 2 and then gradually declined while lactic acid bacteria induced a time dependent increase (Fig. 3A). Initially we expected that exogenous rIL-4 might interfere with IFN-γ production because it is a negative modulator of IL-12 receptor on T cells [34]. On the contrary, it rather contributed to the enhanced IFN-γ secretion. rIL-4, in this setting, seemed to act as additional growth factor for the whole splenocytes during the early culture period [35]. The large amount of IL-4 detected in anti-CD3 plus rIL-4 treated cells must be the remnant of the exogenous rIL-4 as its levels declined over time (Fig. 3B). LMM did not produce detectable IL-4.

Next, we treated cells with LMM 24 h before or after stimulation with anti-CD3 plus rIL-4 and measured each cytokine on days 1, 2, 3 and 6. Pre-treatment with LMM augmented IFN-γ production in a time dependent manner, but post-treatment failed to do so (Table 1). On the other hand, the IL-4 levels in cells from both treatments gradually declined, as observed with those in cells treated with anti-CD3 plus rIL-4 alone. Interestingly, the one from pre-treatment with LMM one day after the addition of rIL-4 (2887 ± 41 pg/ml) almost doubled compared with those from cells

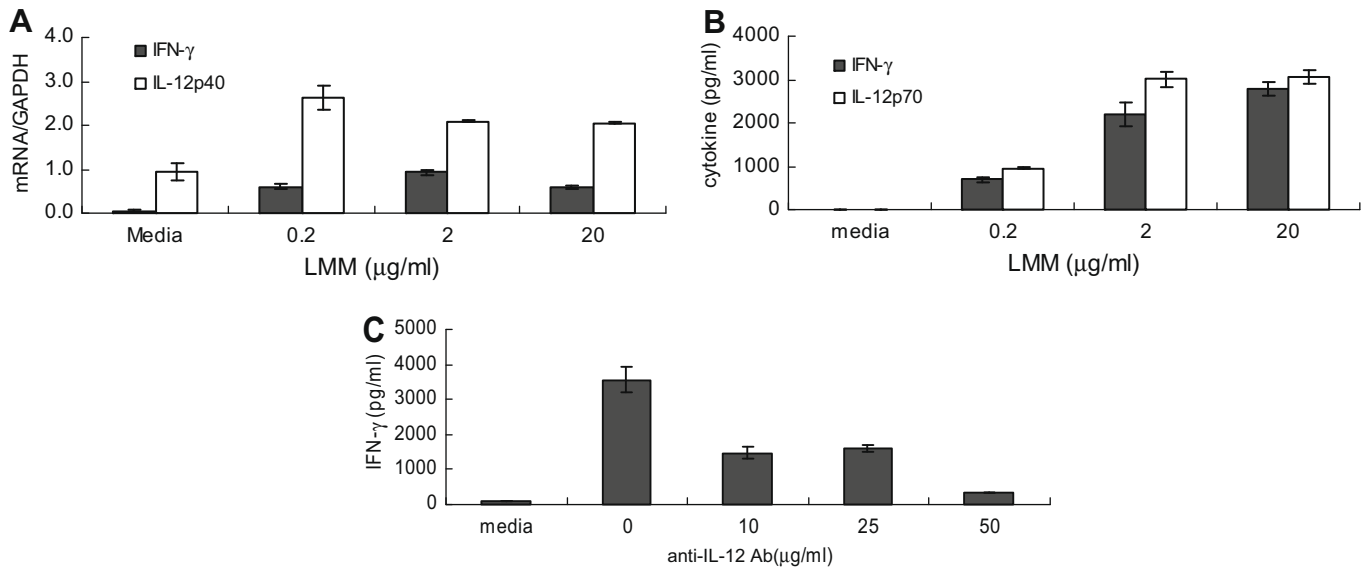


Fig. 2. Expression of IFN- γ and IL-12 by *Leuconostoc mesenteroides* subsp. *mesenteroides* (LMM) in mouse splenocytes. Splenocytes from Balb/c mice were cultured with heat-killed LMM for 24 h (A and B). Anti-IL-12 antibodies were pre-treated 30 min before stimulation with LMM and cultured for 24 h. (A) mRNA expression was performed by real-time RT-PCR. (B and C) Cytokine production was measured by ELISA. Data are means $\pm$ SD of three independent experiments.

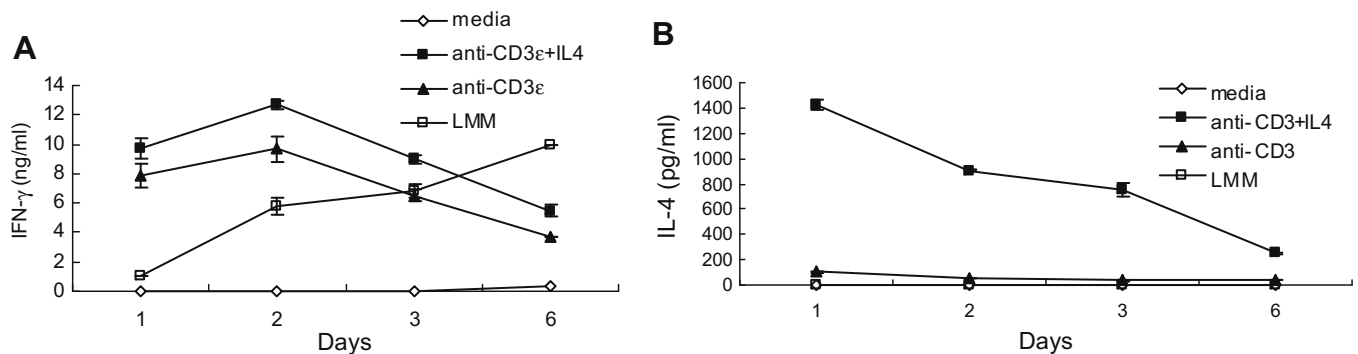


Fig. 3. Secretion pattern of IFN- γ and IL-4 by LMM in response to anti-CD3 plus rIL-4. Splenocytes were stimulated with heat-killed LMM (2 μ g/ml), soluble anti-CD3 (2 μ g/ml), or soluble anti-CD3 plus rIL-4 (5 ng/ml) and harvested on day 1, 2, 3 and 6. IFN- γ and IL-4 were measured by ELISA. Data are means $\pm$ SD of three independent experiments.

Table 1

Differential effects of early or late exposure to *L. mesenteroides* subsp. *mesenteroides* (LMM) on the secretion of IFN- γ and IL-4 in response to anti-CD3 plus IL-4.

Day	IFN- γ (ng/ml)			IL-4 (pg/ml)		
	LMM (-)	Pre-treatment ^a	Post-treatment ^b	LMM (-)	Pre-treatment ^a	Post-treatment ^b
1	9.7 $\pm$ 0.7	2.3 $\pm$ 0.1	8.9 $\pm$ 0.3	1423 $\pm$ 41	ND	1474 $\pm$ 47
2	12.7 $\pm$ 0.2	34.9 $\pm$ 2.3	12.6 $\pm$ 2.2	904 $\pm$ 10	2887 $\pm$ 41	800 $\pm$ 15
3	8.9 $\pm$ 0.3	43.1 $\pm$ 3.4	10.1 $\pm$ 1.6	746 $\pm$ 53	933 $\pm$ 15	576 $\pm$ 15
6	5.4 $\pm$ 0.4	46.5 $\pm$ 5.3	6.0 $\pm$ 1.0	251 $\pm$ 3	542 $\pm$ 10	119 $\pm$ 8

Data are means $\pm$ SD of three independent assays.

^a Splenocytes were pre-stimulated with lactic acid bacteria for 24 followed by stimulation with anti-CD3 plus IL-4.

^b Splenocytes were stimulated with anti-CD3 plus IL-4 for 24 h followed by lactic acid bacteria.

with or without LMM at post-treatment under the same condition (1474 $\pm$ 47 pg/ml, 1423 $\pm$ 41 pg/ml, respectively). The enhanced production of IL-4 and IFN- γ during an early period of T cell activation indicates that lactic acid bacteria positively affected the TCR/CD3 triggered cell activation without being biased toward the Th1/Th2 phenotype. In spite of that, the continuous production of IFN- γ by LMM promoted the differentiation of Th1 cells. However, it was not clear why post-treatment with LMM did not affect the on-going cytokine production in response to anti-CD3 plus rIL-4.

3.3. Effects of pre-sensitization or post-sensitization treatment of LMM on OVA induced allergy in mice

The OVA sensitization allergy model in mice is typical of Th2 response, characterized by increased serum IgE levels and antigen specific IL-4 production concomitantly with a decrease in IFN- γ . In order to assess whether in vivo treatment of LMM was able to affect the Th2 dominated state, the microorganism was given mice by oral gavage before or after allergen sensitization developed.

Both pre- and post-sensitization treatments increased IFN- γ production in OVA restimulated splenocytes from OVA sensitized mice (Figs. 4 and 5). These results were accompanied by a reduction in serum total and OVA-specific IgE levels, indicative of the suppression of systemic Th2 response. A significant reduction in IL-4 from OVA restimulated cells was observed only in the pre-sensitization group but not in the post-sensitization group. It seems that the inhibition of serum IgE by LMM is more closely associated with an increase in IFN- γ than with effects exerted by IL-4.

3.4. Effect of NF- κ B and MAPK on the induction of IL-12 by LMM

Microbes initiate a variety of inflammatory responses through activation of transcription factors including MAPK and NF- κ B after they make contact with the pattern recognition molecules such as Toll-like receptors on the macrophages. In an attempt to determine whether LMM must activate the NF- κ B and MAPK pathways to

produce IL-12, peritoneal macrophages were treated with various concentrations of sulfasalazine (NF- κ B inhibitor), SB203580 (p38 inhibitor), U0126 (ERK inhibitor) and SP600125 (JNK inhibitor) 30 min before the beginning of stimulation. Sulfasalazine, SB203580 and SP600125 all inhibited LMM-induced IL-12 production but U0126 did not show a reduction except for the high dose. These findings show that NF- κ B, p38 and JNK participated in the IL-12 production by LMM. In particular, SP600125 tested at all doses caused more potent inhibition than the others, suggesting that the JNK pathway might play a dominant role in IL-12 production (Fig. 6A and B). In order to identify the LMM-induced NF- κ B and MAPK activation, we used RAW264.7 mouse macrophage cell lines and stimulated the cells with the bacteria for different time points. Phosphorylation of p38, JNK, and ERK1/2 was detected using Western blotting analysis (Fig. 6C). LMM also caused degradation of I κ B α , which indicates the translocation of NF- κ B to the nucleus. Therefore, we confirmed that the induction of IL-12 by

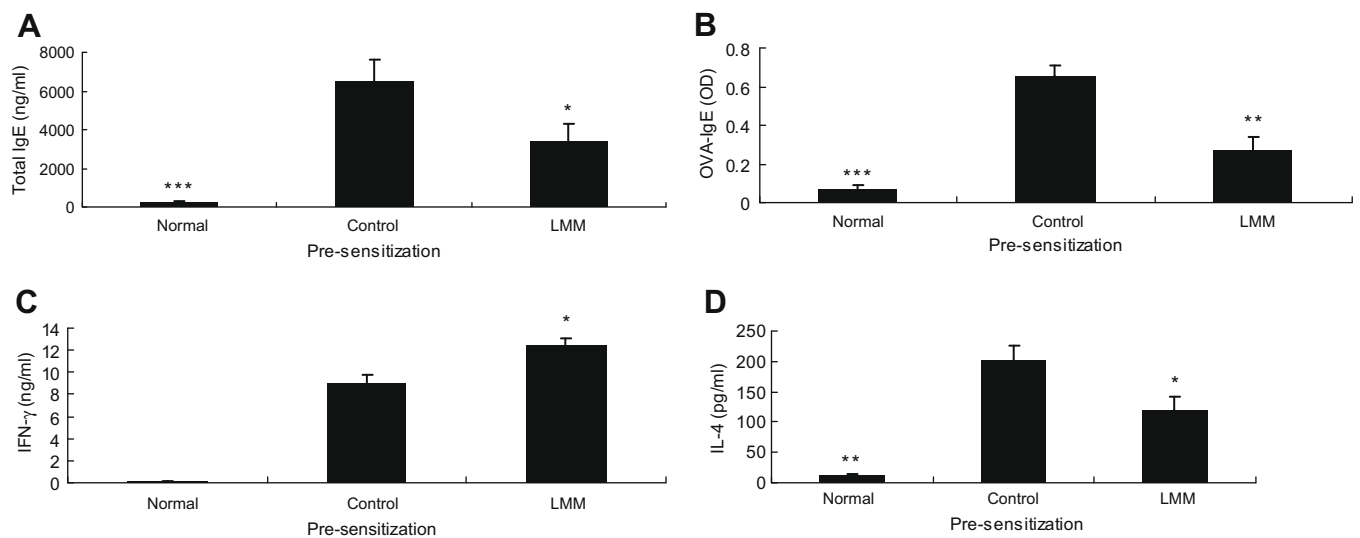


Fig. 4. Pre-treatment with LMM increases OVA-stimulated IFN- γ and inhibits serum IgE levels and OVA-stimulated IL-4. LMM was given to Balb/c mice by oral gavage, beginning 1 week before OVA sensitization as depicted in Fig. 1. The concentrations of total IgE and OVA-specific IgE in serum were individually assayed instead of pooling serum. Splenocytes from OVA sensitized mice were obtained and stimulated with OVA for 72 h. Levels of total IgE in serum and cytokines from splenocytes were calculated as a reference to standard protein. Levels of OVA-specific IgE in serum were displayed as optical density. Data are means $\pm$ SD from two independent experiments. * P < 0.05, ** P < 0.01, *** P < 0.001, compared with control (ANOVA followed by Dunnet's post hoc test). n = 7/group.

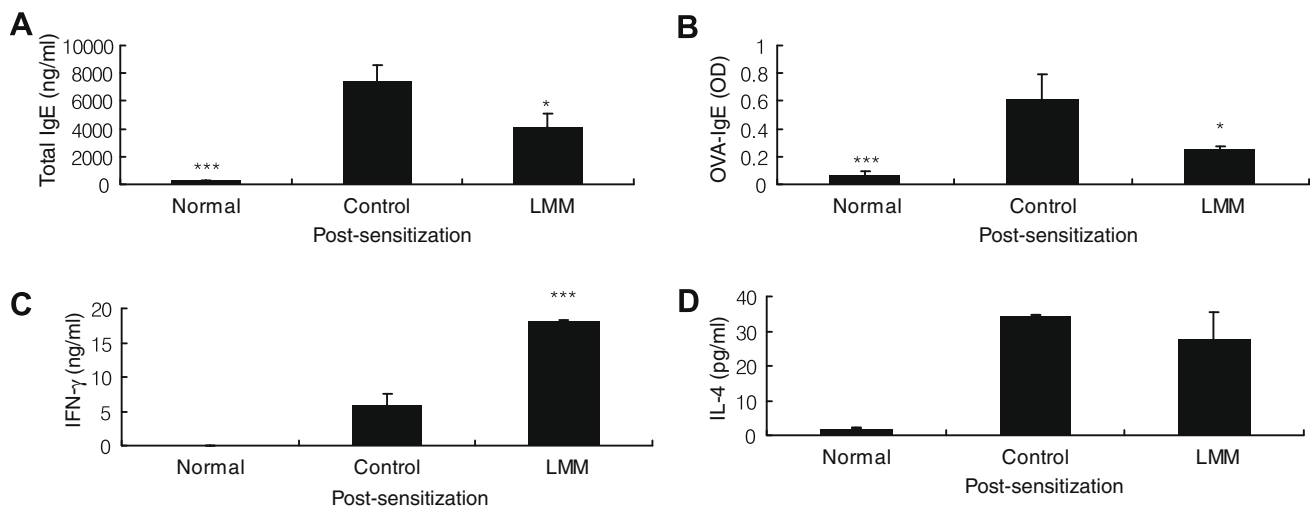


Fig. 5. Post-treatment with LMM increases OVA-stimulated IFN- γ and inhibits serum IgE levels with no effect on OVA-stimulated IL-4. LMM was orally given, starting 2 weeks after OVA sensitization as depicted in Fig. 1. Cytokines and serum IgE were measured as described in the legend of Fig. 4. Data are means $\pm$ SD from two independent experiments. * P < 0.05, ** P < 0.01, *** P < 0.001, compared with control (ANOVA followed by Dunnet's post hoc test). n = 7/group.

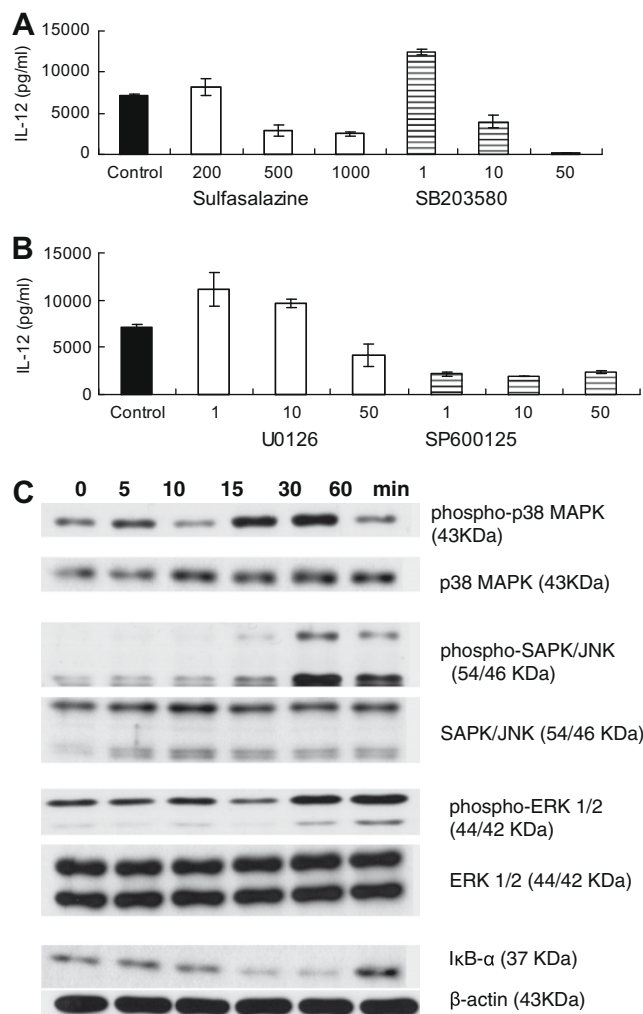


Fig. 6. The requirement of NF- κ B, p38 and JNK in LMM-induced IL-12 production. (A and B) Peritoneal macrophages from C57BL/6 mice were pre-treated with various concentrations (μ M) of sulfasalazine, SB203580, U0126, SP600125 for 30 min before the bacteria treatment and cultured for another 24 h. Control cells (0 μ g/ml) were treated with NH_4OH or DMSO. Supernatants were harvested and IL-12 was measured by ELISA. (C) RAW 264.7 macrophage cell lines were stimulated with LMM (2 μ g/ml) for 0, 5, 10, 15, 30 and 60 min in serum free media and cell lysates were obtained for Western blotting. One of four independent experiments was shown.

LMM used the activation of p38, JNK and NF- κ B signaling pathways.

4. Discussion

In this study, we observed that *L. mesenteroides* subsp. *mesenteroides* in vitro and in vivo had the ability to augment IFN- γ in the Th2-like experimental settings. LMM is hetero-fermentative lactic acid bacteria, being one of the major bacteria in the early kimchi fermentation process and used as the start culture for some commercial kimchi [29]. As recent studies on the relationship between the prevalence of allergic disease and exposure to microbial stimuli have brought more attention to the potential beneficial effects of probiotic lactic acid bacteria, we have become interested in the use of LMM as a Th1-inducing agent in the presence of pro-Th2 stimuli.

IL-12 production is largely produced by macrophages during contact with microbes or further promoted by CD40 ligand on activated T cells [36,37]. Incubation with LMM induced the expression of IFN- γ in mouse splenocytes, paralleled by that of IL-12, which

directly mediates the synthesis of IFN- γ . IFN- γ production does not occur as a result of direct contact with microbes but in response to either antigen on the APC or IL-12 mediated signaling [38]. This means that, in our in vitro culture, the production of IFN- γ by LMM was largely due to the latter mechanism since abrogation of IL-12 in the culture reduced IFN- γ production by LMM.

As shown by several studies and ours, bacteria stimulated splenocytes or human peripheral blood mononuclear cells in vitro produced a large amount of IFN- γ with low or undetectable IL-4 [22,26,39]. Other investigations reported that oral consumption of lactic acid bacteria did not affect Th2 cytokine production [32,40]. On the contrary, a number of studies using antigen primed mice fed with lactic acid bacteria showed increased levels of IFN- γ with reduced levels of IL-4 and IgE [41,42]. It is not clear whether their downregulatory effects on Th2 responses occur as a result of the enhanced Th1 cytokine production or independently. At a cellular level, early exposure to LMM in splenocytes prolonged the production of IFN- γ in response to anti-CD3 plus rIL-4, which otherwise would peak on day 2 and gradually decline thereafter. Under this condition, IL-4 was also increased at early time points, although treatment with LMM alone did not produce detectable IL-4 in vitro. Our initial hypothesis was that, as T cells became active, there would be a mixed group of Th1 and Th2 cells, and addition of rIL-4 would lead to a higher ratio of Th2/Th1 cells. A general Th2 polarization culture system uses CD4 T cells supplemented with rIL-4 and anti-IL-12 antibodies, but given the nature of bacteria, the presence of IL-12 producers was necessary. In this regard, we used the whole splenocytes and did not remove IL-12 in the media. In our setting, the use of rIL-4 did not reduce IFN- γ secretion but rather promoted it. This is somewhat in keeping with a previous report demonstrating that IL-4 can enhance IFN- γ production in the presence of signals from APC [43]. Our data showed that LMM did not negatively affect IL-4 production but enhanced its peak response during early T cell activation. Based on these observations, it was clear that LMM was able to increase anti-CD3 induced IL-4 production, not to mention IFN- γ . Interestingly enough, late exposure to the bacteria did not cause a parallel pattern in cytokine production. Perhaps in the in vitro culture condition, once the TCR dependent signaling pathway initiates IFN- γ production, other stimuli such as bacteria cannot interfere with this process up to a certain point in time. Further studies are required to examine such mechanism in more detail.

Using the in vivo OVA allergy model, we found that oral application of LMM increased IFN- γ production in OVA restimulated splenocytes and systemic suppression of IgE production regardless of at which time OVA was sensitized. It is not clear by what mechanism the inhibition of serum IgE occurred in lactic acid bacteria treated group. This could be explained by the downregulation of IL-4, but our post-sensitization treatment did not show a parallel reduction in antigen specific IL-4 production. It should be pointed here that in the post-sensitization treatment, the duration of oral administration of bacteria lasted only for 2 weeks, too short to give a full signal to downregulate the overexpression of IL-4. Even so, this cannot provide an explanation for the suppressed serum IgE levels. In fact, rIFN- γ or strong inducers of IFN- γ such as specific bacteria DNA sequences, were reported to directly inhibit IgE generation in the presence of IL-4 [1,44]. Therefore, supported by others, it can be assumed that the enhanced IFN-production by lactic acid bacteria may be directly responsible for IgE suppression with or without IL-4 downregulation [45,46].

It is generally believed that several downstream signaling molecules are required for the production of IL-12 in macrophages. Under resting conditions, NF- κ B is kept by I κ B in the cytoplasm and various stimuli cause degradation of I κ B and allow NF- κ B to migrate to the nucleus and activate IL-12 expression [47]. Like NF- κ B signaling, p38, JNK and ERK, the major MAPK, are also involved

in the production of inflammatory cytokines in response to microorganism [48]. The use of pharmacological inhibitors enabled us to confirm the requirement of NF- κ B, p38 and JNK in LMM-induced IL-12 production in peritoneal macrophages. Although the activation of ERK1/2 was identified, this signaling was not used for the production of IL-12 by LMM. It can be interpreted that the IL-12 induction by LMM through the NF- κ B, p38 and JNK signaling pathways in macrophages provides continuous signals for the production of IFN- γ regardless of the amount of IL-4.

In conclusion, our data presented that the upregulation of IFN- γ by LMM, mediated by IL-12 production through NF- κ B, p38 and JNK, was maintained in the presence of IL-4 and OVA induced allergy in mice. These observations suggest that LMM can be a useful microorganism in influencing the Th1/Th2 imbalance and contributing to the induction of Th1 cytokine.

Conflicts of interest

There are no conflicts of interest and no source of funding.

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