

ORIGINAL PAPER

Levan (β -2, 6-fructan), a major fraction of fermented soybean mucilage, displays immunostimulating properties via Toll-like receptor 4 signalling: induction of interleukin-12 production and suppression of T-helper type 2 response and immunoglobulin E production

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Clinical and Experimental Allergy

Summary

Background Products from the fermentation process of soybeans by *Bacillus subtilis* (natto) have been shown to possess anti-tumour and immunomodulatory activities. However, the formulations previously examined were not chemically pure, and this is a major limitation for elucidation of the molecular mechanisms for their activities.

Objective In order to determine which components in soybean mucilage exert immunostimulatory activities, we examined the activities of their purified forms *in vitro* and *in vivo* in mice.

Methods *B. subtilis* (natto) and fractions including levan and poly- γ -glutamic acid (γ -PGA) from fermented soybean mucilage were prepared. Levels of cytokine production by mouse macrophage cells after treatment with the fractions were measured by means of ELISA. *In vivo* effect of levan delivered intragastrically on ovalbumin (OVA)-specific T-helper type 2 (Th2) response with IgE production was examined in BALB/c mice that had been immunized intraperitoneally with OVA.

Results Levan but neither γ -PGA nor killed *B. subtilis* (natto) was found to exert strong activity to induce production of IL-12 p40 and TNF- α by macrophage cell lines *in vitro*.

Results of experiments using Toll-like receptor (TLR) 4-deficient mice and TLR4-transfected human cell line indicated that TLR4 is involved in pattern recognition of levan. Oral administration of levan *in vivo* significantly reduced the serum levels of OVA-specific IgE and Th2 response to OVA in mice immunized with OVA.

Conclusion Levan is an immunostimulatory moiety in products from the fermentation process of *B. subtilis* (natto) and may be useful for prevention of allergic disorders with IgE production.

Keywords allergy, cytokines, IgE, levan, Th1, Th2

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Introduction

Soybean products are consumed in various forms, including fermented soybean (natto), soybean curd (tofu) and fermented soybean paste (miso) [1]. Isoflavones, a group of phytoestrogens, in soybean products are thought to have anti-carcinogenic effects on hormone-related cancers such as breast cancer and prostate cancer [2, 3]. Products obtained from the fermentation process of soybeans by *Bacillus subtilis* (natto) have been shown to possess anti-tumour and immunomodulatory activities [4, 5]. Results of

some studies have demonstrated that *B. subtilis* (natto) and spores of *B. subtilis* (natto) worked as probiotics by promoting growth and viability of beneficial lactic acid bacteria in the intestinal tracts of humans and some animals [6, 7]. However, little is known about the immunological functions of components in the product obtained from the fermentation process of soybeans by *B. subtilis* (natto).

Many polysaccharides produced by living organisms have been reported to possess anti-tumour and immunomodulatory activities [8–10]. β -(1,3)-glucans are major structural components of fungal cell walls that modulate

innate immunity in part by macrophage activation in mammals [11]. High-molecular-weight levan is a fructose polymer consisting of a backbone of β -(2,6)-linked fructoses and β -(2,1)-linked branches, which are produced by many microorganisms [12]. It has been reported that levan produced by *Aerobacter levanicum* [13, 14] and *Zymomonas mobilis* [15, 16] displayed anti-tumour and immunomodulatory effects. Levan is also produced by *B. subtilis* (natto), which is used to make fermented soybeans (natto) [17]. Levan has been shown to occupy about 20% of the mucilage of natto, suggesting that levan might be related to the immunostimulatory activity of natto.

The innate immune system represents the first line of defense against microorganisms as it promptly initiates a local inflammatory reaction. The recognition of different pathogen components is mediated by Toll-like receptors (TLRs), which recognize the microbial non-self and mediate activation of the innate immune system [18]. On the basis of their homology to the *Drosophila* Toll protein, 11 TLRs have so far been identified in mammals and the specific ligands of some of them have been defined [19]. TLR4, one of the identified TLRs, has been reported to function as a receptor for lipopolysaccharide (LPS), an integral component of the outer membrane of Gram-negative bacteria [20]. TLR2 is essential for recognizing lipopeptides and lipoproteins from several microorganisms and also peptidoglycans derived from Gram-positive bacteria [21]. TLR3, TLR5, TLR7 and TLR9 recognize double-strand RNA, bacterial flagellin, imidazoquinolines and bacterial DNA, respectively [19]. Zymosan, which consists of yeast cell particles, is also one of the strong macrophage activators containing (1,3)- β -D-glucan [22, 23]. It has been suggested that signals from zymosan are transmitted through heterodimers of TLR2 and TLR6 [24, 25]. We previously found that AC-1, an extracellular polysaccharide produced by *Acetobacter xylinum*, which is composed of (1,4)- β -D-glucan with branches of glucosyl residues, showed strong activities to induce production of IL-12 p40 via TLR4 signalling and to suppress T-helper type 2 (Th2) response with IgE production following immunization with ovalbumin (OVA) [26, 27]. IL-12 has the ability to stimulate NK cells and Th1 cells to produce IFN- γ , resulting in inhibition of Th2 type immune responses [28]. Moreover, IL-12 prevents the development of Th2 response independently of IFN- γ [29]. Thus, potent IL-12 inducers such as high-molecular-weight polysaccharides may be useful for the prevention of allergic disorders with IgE production.

In the present study, we found that levan extracted from fermented soybeans was a potent inducer of IL-12 p40 and TNF- α production by macrophages *in vitro* via TLR4. Furthermore, we showed that levan, a potent IL-12 inducer, suppressed allergic inflammation with IgE production, thus offering an approach for the prevention of allergic disorders.

Materials and methods

Animals

C3H/HeN (TLR4^{+/+}) mice, C3H/HeJ (TLR4^{-/-}) mice, BALB/c mice and C57BL/6 mice (SLC, Shizuoka, Japan) were used in the experiments at 6–10 weeks of age. TLR2-deficient (TLR2^{-/-}) mice were kindly provided by S. Akira (Osaka University, Japan) [30]. These mice were bred at our institute under specific pathogen-free conditions. All experiments were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* prepared by the National Academy of Sciences.

Purification of levan and poly- γ -glutamic acid from natto mucilage

Natto mucilage was extracted from commercial natto (Honegenki natto, Mizkan Group Co., Ltd, Handa, Japan) by a method similar to that described by Tanimoto et al. [31]. Briefly, 5% sodium chloride solution (2 L) was added to 1 kg of natto, and natto beans were filtered with gauze and removed from the slurry. The filtrate was centrifuged at 4 °C, 12 000 g for 30 min. The supernatant was poured slowly into two volumes of absolute ethanol. Precipitated natto mucilage was collected and dissolved with distilled water. Then, 10% aqueous cetyltrimethyl ammonium bromide (CTAB) solution was added to the aqueous natto mucilage solution until no more precipitate was formed. The above mixture was incubated at 37 °C overnight with agitation to allow complete formation of poly- γ -glutamic acid (γ -PGA)-CTAB complex. The insoluble γ -PGA-CTAB complex as collected by centrifugation and used for further purification of γ -PGA, and the supernatant was used for further purification of levan. The above supernatant was precipitated with absolute ethanol and re-dissolved in distilled water three times. Finally, the precipitate was dialysed against distilled water for 24 h and freeze dried to obtain pure levan. The insoluble γ -PGA-CTAB complex was redissolved in 2 M sodium chloride solution. After dialysis against running water, the γ -PGA was precipitated with ethanol, dissolved in water, and dialysed against distilled water and lyophilized to give pure γ -PGA.

Microorganisms and culture conditions

The vitamin K₂ (VK₂) *B. subtilis* (natto) strain [32] used for fermentation of commercial Honegenki natto, mutant levansucrase-defective (*sacB22*) *B. subtilis* strain (Japan patent no. 2004-016130) and mutant defective γ -PGA (*pgsBCA*) *B. subtilis* strain was produced by Mizkan Group Co. Ltd based on the method described previously [33] (unpublished data). The bacteria were cultured by the previously described method [32]. VK₂ *B. subtilis* (natto)

spores were cultured in a spore formation medium described elsewhere [34].

Cell preparation

All cell lines were grown in tissue culture flasks at 37 °C in 5% CO₂/95% air. Two mouse macrophage cell lines, J744.1 and RAW264.7, and a human embryonic kidney cell line, HEK 293 human embryonic kidney 293 (HEK 293), were obtained from the Institute of Physical and Chemical Research Cell Bank (Tsukuba, Japan) and maintained in Dulbecco's minimum essential medium with 10% fetal bovine serum (FBS) (JRH Biosciences, Inc., Enxeta, KS, USA). Adherent cells from the peritoneal cavity of C3H/HeN, C3H/HeJ, C57BL/6 (TLR2^{+/-}), or TLR2^{-/-} mice were used as a source of mouse macrophages. Briefly, peritoneal exudate cells (PEC) were suspended in RPMI containing 10% FBS and cultured in plastic plates for 2 h at 37 °C. After non-adherent cells had been removed, fresh complete medium was added to the adherent cells with or without levan or control reagents in the presence of IFN- γ (10 ng/mL).

Plasmids

Murine TLRs tagged with p3xFlag at the carboxyl terminus were generated by PCR and ligated into the expression plasmid CMV14 (Sigma-Aldrich, Inc., St. Louis, MO, USA) as described previously [27]. A mouse CD14 expression plasmid, pcDNA3.1(+)-mCD14, and an nuclear factor (NF)- κ B responsive reporter, pGL3-NF- κ BLuc, have been previously described [35].

Luciferase assay

HEK 293 cells were seeded in 3.5 cm dishes at a density of 1×10^6 cells/dish 1 day before transfection. HEK 293 cells were transiently transfected with 0.1 μ g of pGL3-NF- κ B/Luc (a luciferase reporter construct containing the consensus NF- κ B binding sequence), 0.4 μ g of pSV- β -galactosidase as an internal control (Promega, Madison, WI, USA), and 1.0 μ g mTLRs/Flag using lipofectamineTM (Invitrogen Co, Carlsbad, CA, USA). Forty-eight hours after transfection, the HEK 293 cells were stimulated with levan for 8 h. The cells were harvested, washed and lysed in 200 μ L lysis buffer, and the luciferase activity was measured using a luminometer (Wallac 1420, Perkin Elmer, Inc., Boston, MA, USA) with a Steady-Glo Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions.

Cytokine measurement

J744.1 or RAW264.7 cells (5×10^5 cells/mL) were stimulated with levan and other fractions of natto, and the levels of IL-12 p40 and TNF- α production were determined using an ELISA 24 h after incubation. Adherent

cells of PEC from C3H/HeN and C3H/HeJ or TLR2^{+/-} and TLR2^{-/-} mice were incubated for 24 h with levan, LPS (*Escherichia coli* O55, Sigma), synthetic lipoprotein (Bachem AG, Bubendorf, Switzerland), or synthetic lipid A (Ono-4007, Osaka, Japan) in the presence or absence of IFN- γ , and the culture supernatant was then collected. ELISA for IL-12 p40 and TNF- α were performed using commercially available kits from Genzyme (Cambridge, MA, USA).

Immunization and challenge

Mice were intraperitoneally immunized with 50 μ g chicken OVA (Grade V; Sigma) absorbed in 100 μ L of alum on days 0 and 12. The mice were challenged intragastrically with a suspension of 100 μ L levan (10 mg/mL) or phosphate-buffered saline (PBS) on days 1, 4, 9 and 14. The most effective schedule and dose of levan administration were determined by our preliminary experiments. Spleens and sera were obtained on day 29 for further analysis.

Measurement of cytokine production of spleen cells

Spleen T cells were collected, cultured *in vitro* with OVA (1 μ g/mL) for 48 h, and the amounts of IL-4 and IFN- γ secreted by spleen T cells were determined by ELISA as described previously [27].

Measurement of ovalbumin-specific immunoglobulins E, G1 and G2a levels

Levels of OVA-specific IgE, IgG1 and IgG2a were determined by ELISA as described previously [27].

Statistical analysis

The statistical significance of the data was determined by Student's *t*-test. A *P*-value of less than 0.05 was taken as significant.

Results

Levan fraction in fermented soybean (natto) mucilage stimulated mouse macrophages to secrete tumour necrosis factor- α and interleukin-12 p40

We screened various fractions isolated from natto by measuring levels of cytokine production in the mouse macrophage cell lines RAW264.7 and J774.1. As shown in Fig. 1a, autoclaved *B. subtilis* (natto) (*B. S. (natto)*), autoclaved spores of *B. subtilis* (natto) and mucilage were able to stimulate RAW264.7 or J774.1 cells to produce TNF- α and IL-12 p40, respectively. Mucilage of natto is mainly composed of about 20% levan and 80% γ -PGA. Levan showed stronger activity in up-regulating the levels of both TNF- α and IL-12 p40, whereas γ -PGA showed weak activity (Fig. 1b). To further confirm which of the

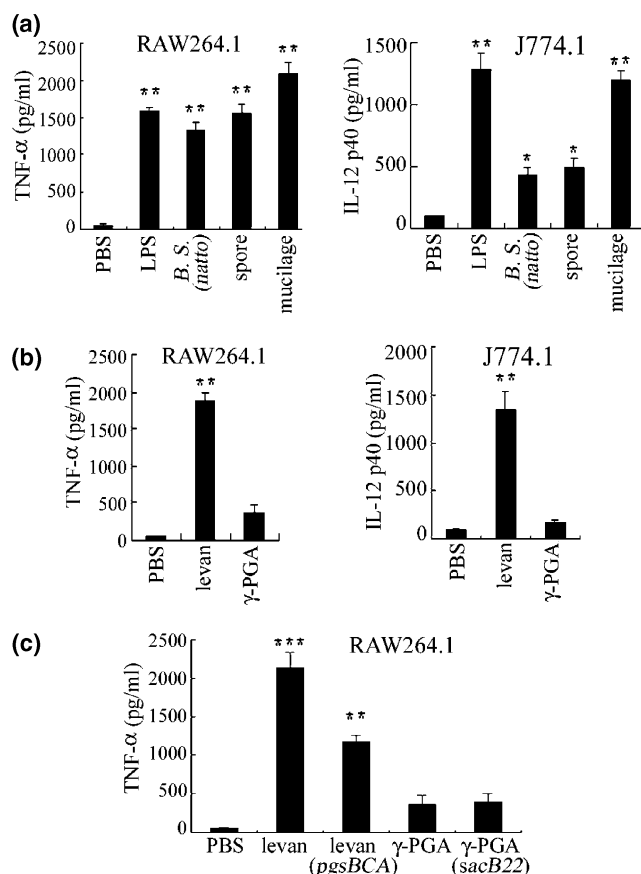


Fig. 1. Production of TNF- α and IL-12 p40 by macrophage cell lines in response to various fractions of natto. RAW 264.7 and J774.1 cells were stimulated with 10 μ g/mL indicated fractions of natto (a), fractions of natto mucilage (b), levan and poly- γ -glutamic acid extracted from natto mucilage or culture medium of mutant *Bacillus subtilis* strains for 24 h (c). Concentrations of TNF- α and IL-12 p40 in the culture supernatants were measured by ELISA. Three independent experiments showed similar results, and data are expressed as means $\pm$ SD of triplicate cultures in a representative experiment. * P < 0.05, ** P < 0.01, *** P < 0.001, significantly different from the value for phosphate-buffered saline.

two fractions contributed to the activity of mucilage, we used levansucrase-defective (*sacB22*) and γ -PGA synthetic enzyme-defective (*pgsBCA*) mutant *B. Subtilis* strains, which can only produce γ -PGA or levan, respectively. γ -PGA produced by *sacB22B* mutant strain exhibited only a marginal ability to induce TNF- α production by RAW264.7 cells (Fig. 1c). Although not as strong in activity as levan extracted from natto, levan produced by *pgsBCA* mutant strain induced TNF- α production by RAW264.7 cells (Fig. 1c). The difference in the response to both levans may be because of the quantitative difference in levan produced by different strains of *B. subtilis*. These results indicated that levan is mainly responsible for the immunostimulatory activity of mucilage.

Levan has been reported to be highly susceptible to hydrolysis by acid [12]. As shown in Fig. 2a, the ability of levan to induce IL-12 p40 production by J774.1 cells was

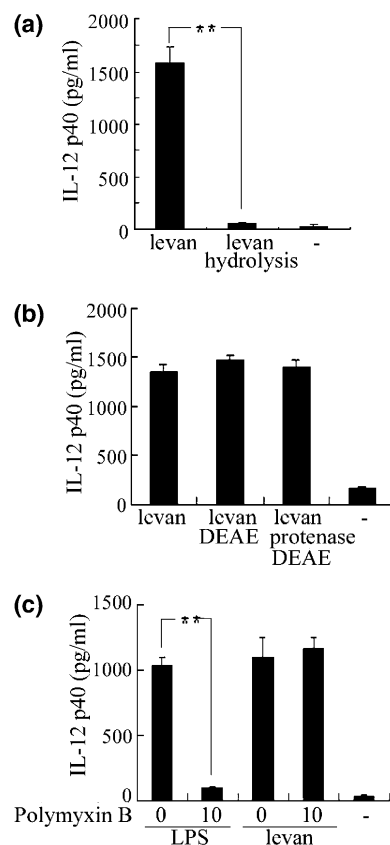


Fig. 2. Effect of treatment with HCl hydrolysis, protease or polymyxin B on levan-induced IL-12 p40 production by J774.1 cells. J774.1 cells were incubated with levan (10 μ g/mL) pretreated with 1 M HCl at 80 $^{\circ}$ C for 3 h (a), 1.0 mg/mL actinase E at 37 $^{\circ}$ C for 24 h (b), and with or without pretreatment with polymyxin B (10 μ g/mL) (c) for 24 h, and concentrations of IL-12 p40 in the culture supernatants were measured by ELISA. Three independent experiments showed similar results, and data are expressed as means $\pm$ SD of triplicate cultures in a representative experiment. ** P < 0.01, significantly different from the value for phosphate-buffered saline.

abolished by HCl treatment, which degrades levan into fructose. To further purify the polysaccharides from levan, we treated levan with protease and subjected them to ion exchange chromatography. Protease-treated products of levan exhibited almost the same level of activity to induce IL-12 p40 production by J774.1 cells as those of non-treated levan (Fig. 2b). Thus, the protein moiety of levan is not required for the activity of levan. As *B. subtilis (natto)* is a Gram-positive bacterium that does not have LPS as a cell wall component, it is unlikely that extracellular products are contaminated by endotoxin. To exclude the possibility of contamination of LPS during the process of purification, we examined the effect of treatment of levan with polymyxin B, which neutralizes LPS activities, on production of IL-12 p40 by J774.1 macrophages. As shown in Fig. 2c, polymyxin B inhibited the activity of LPS (100 ng/mL) but not the activity of levan. Thus, the possibility that induction of the activity of levan is because of contamination by LPS was ruled out.

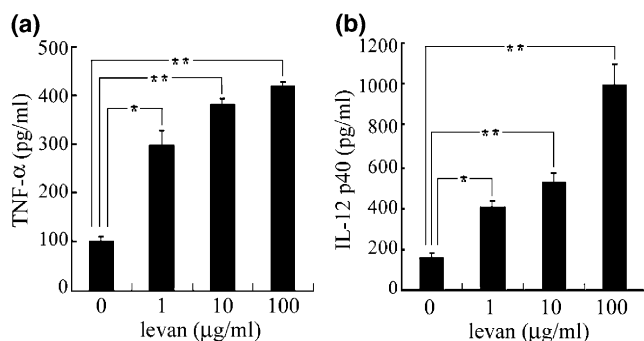


Fig. 3. Effect of levan on induction of IL-12 p40 and TNF- α production from primary culture of macrophages of naive mice. Adherent cells collected from the peritoneal exudate cell (a) and spleen (b) of BALB/c mice were stimulated with levan at concentrations of 1, 10 and 100 $\mu\text{g}/\text{mL}$ for 24 h, and concentrations of TNF- α and IL-12 p40 in the culture supernatants were measured by ELISA. Three independent experiments showed similar results, and data are expressed as means $\pm$ SD of triplicate cultures in a representative experiment. * $P < 0.05$, ** $P < 0.01$, significantly different from the value for phosphate-buffered saline.

Next, an experiment was carried out to determine whether levan could induce production of TNF- α and IL-12 p40 by primary culture of macrophages from naive mice. As shown in Figs 3a and b, levan induced high levels of TNF- α and IL-12 p40 production by peritoneal adherent cells and spleen adherent cells from BALB/c mice, respectively, in a dose-dependent manner.

Toll-like receptor 4 is involved in tumour necrosis factor- α production by peritoneal exudate cell adherent cells stimulated with levan

We examined the involvement of TLR in the recognition of levan by using TLR-deficient mice. The results indicated that the peritoneal adherent cells of TLR4^{+/+} mice produced TNF- α in response to *in vitro* stimulation with levan, a ligand for TLR4 (synthetic lipid A), or a ligand for TLR2 (synthetic lipoprotein) (Fig. 4a). On the other hand, those from TLR4^{-/-} mice responded to lipoprotein to produce a high level of TNF- α , but did not respond to synthetic lipid A or levan, indicating that TLR4 is involved in levan-mediated TNF- α production by the peritoneal adherent cells. The peritoneal adherent cells from TLR2^{-/-} mice did not respond to lipoprotein to produce TNF- α , whereas those from TLR2^{-/-} mice produced TNF- α in response to levan as well as lipid A, albeit to a lesser degree compared with those from TLR2^{+/+} mice (Fig. 4b). These results suggest that TLR2 is not important in levan-mediated cellular activation.

Expression of mouse Toll-like receptor 4 conferred levan-mediated nuclear factor- κ B activation in a human cell line

To further confirm the involvement of TLR in levan-mediated cellular activation, we examined the activity of

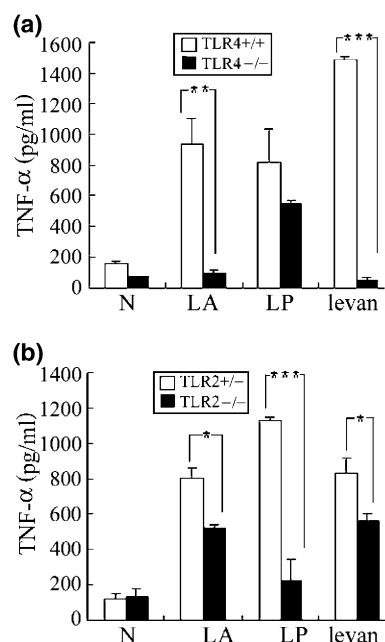


Fig. 4. Levan-induced production of TNF- α by macrophages from the peritoneal exudate cell (PEC) of Toll-like receptor (TLR)4- or TLR2-deficient mice. Adherent cells from the PEC of C3H/HeN (TLR4^{+/+}) or C3H/HeJ (TLR4^{-/-}) mice (a) or TLR2^{+/+} or TLR2^{-/-} mice (b) were stimulated with levan (1 $\mu\text{g}/\text{mL}$), lipoprotein (LP) (0.1 $\mu\text{g}/\text{mL}$) and lipid A (LA) (0.1 $\mu\text{g}/\text{mL}$) in the presence of IFN- γ (10 ng/mL), and the culture supernatants were then collected. TNF- α levels in the culture supernatants were determined by ELISA. Three independent experiments showed similar results, and data are expressed as means $\pm$ SD of triplicate cultures in a representative experiment. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, significantly different from the value for control mice.

levan in a TLR-transfected human cell line. As shown in Fig. 5a, levan significantly induced NF- κ B activation in TLR4-transfected HEK 293 cells. Polymyxin B-treated levan also induced NF- κ B activation in the TLR4-transfected HEK 293 cells, again confirming that contamination of LPS is not involved in the levan activities (Fig. 5a). On the other hand, cells expressing other TLRs, including TLR2 or TLR6, did not respond to levan (Fig. 5b). We confirmed that TLR6 formed a signalling complex with TLR2 and coexpression of TLR2 with TLR6 conferred the cells with responsiveness against zymosan (Fig. 5b). These findings suggest that TLR4, but not TLR2, is at least partially involved in levan-mediated cellular activation.

Effects of oral administration of levan on T-helper type 2 response with immunoglobulin E production to ovalbumin

To determine the role of levan in modulation of immune response *in vivo*, we examined the effect of oral administration of levan on IgE production in mice immunized with OVA. The levan-treated mice had lower OVA-specific IgE level in serum than PBS-treated mice after OVA sensitization. However, there was no significant difference

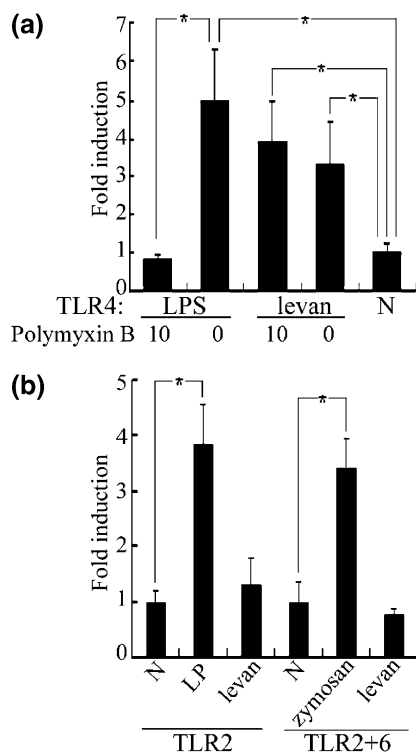


Fig. 5. Reconstitution of responses of HEK 293 cells to levan after transfection with murine Toll-like receptor (TLR)4. (a) TLR4-transfected HEK293 cells were treated with levan (10 μ g/mL) or lipopolysaccharide (0.1 μ g/mL) with or without pretreatment with polymyxin B (10 μ g/mL) for 8 h, and luciferase activity was measured. (b) HEK 293 cells were transfected with TLR2 alone or a complex of TLR2 and TLR6 expression constructs and stimulated with 10 μ g/mL levan, 0.1 μ g/mL lipoprotein LP or 100 μ g/mL zymosan, and luciferase activity was measured. Three independent experiments showed similar results, and data from a representative experiment are shown (* P < 0.05).

in the level of OVA-specific IgG1 or IgG2a between the two groups of mice (Fig. 6b).

We next separated T cells from spleens of mice treated with levan or PBS on day 29 after OVA immunization. As shown in Fig. 6c, T cells from PBS-treated mice sensitized with OVA produced IL-4 in response to OVA, whereas the level of IL-4 production was significantly reduced in levan-treated mice sensitized with OVA. There was no difference between the levels of IFN- γ production in response to OVA in levan- and PBS-treated mice. Thus, Th2 responses to OVA were suppressed by oral administration of levan in mice immunized with OVA.

Discussion

We identified levan as an active substance derived from fermented soybean mucilage produced by *B. subtilis* (*natto*) that shows activity to induce production of TNF- α and IL-12 p40 by macrophages. High-molecular-weight levan is a fructose polymer consisting of a backbone of β -(2,6)-linked fructoses and β -(2,1)-linked branches,

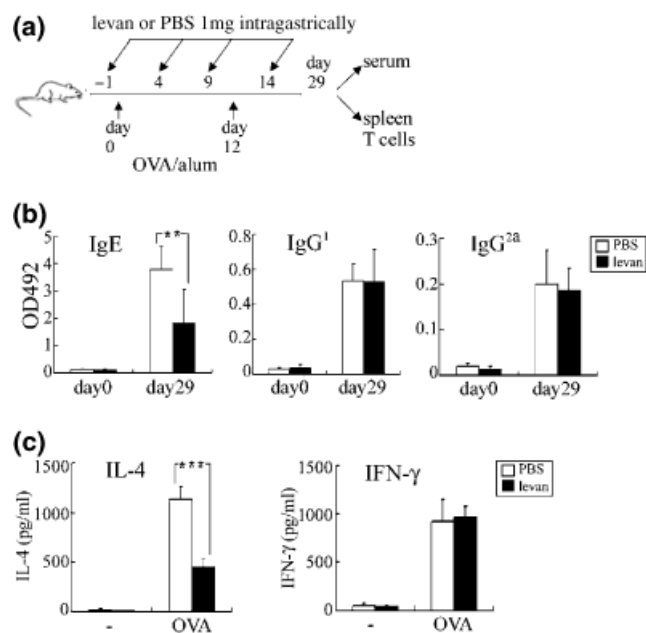


Fig. 6. Immunization and challenge. (a) Immunization protocol. Levels of serum ovalbumin (OVA)-specific IgE, IgG1 and IgG2a; (b) levels of IL-4 and IFN- γ secreted by spleen T cells with or without re-stimulation *in vitro* with 1 μ g/mL OVA for 48 h; (c) from mice orally given levan (black bars) or phosphate-buffered saline (PBS) (white bars) were measured by ELISA. Three independent experiments showed similar results, and data are expressed as means $\pm$ SD of five mice in a representative experiment. ** P < 0.01, *** P < 0.001, significantly different from the value for PBS.

which are produced by many microorganisms [12]. Compared with other ingredients of fermented soybean, including γ -PGA, levan was found to exhibit stronger activity in inducing production of IL-12 p40 and TNF- α by macrophage cell lines *in vitro*. The levan produced by a γ -PGA synthetic enzyme-defective (*pqsBCA*) *B. subtilis* mutant strain showed similar ability in activating macrophage cell line *in vitro* as levan extracted from natto did. In addition, degradation of levan into mono-fructose by HCl treatment abolished the ability of levan to induce IL-12 p40 production by J774.1 cells. These results indicate that levan is an immunostimulatory moiety in products from the fermentation process of *B. subtilis* (*natto*). Production of TNF- α by peritoneal adherent cells from TLR4-deficient mice in response to levan was significantly impaired, and the biological response of HEK 293 cells to levan was reconstituted by transfection with murine TLR4. These results suggest that TLR4 is at least partly involved in cellular response to levan.

TLRs recognize molecular patterns displayed by microorganisms [18]. Stimulation of different TLRs induces distinct patterns of gene expression, which not only leads to the activation of innate immunity but also instructs the development of antigen-specific acquired immunity [18, 36]. The activity of levan was not affected by treatment with protease, thus ruling out the possibility of contamination by the biologically active protein moiety

in levan. Furthermore, the cellular response to levan is not because of LPS contamination, because polymyxin B did not inhibit the activity of levan but did inhibit the activity of LPS. We have previously reported that TLR4 is involved in pattern recognition of AC-1, a (1,4)- β -D-glucan with branches of glucosyl residues, produced by *A. xylinum* [26, 27]. AC-1 is composed of β -(1,4)-glucan with two branches of glucosyl residues *O*- β -D-Glc-(1-6)-D-Glc-(1-6)- β -D-Glc-(1-6)-D-Gal-(1-6)-D-Gal and D-mannose and D-glucuronic [27]. Levan is a fructose polymer consisting of the backbone of β -(2,6)-linked D-fructofuranosyl residues and β -(2,1)-linked D-fructofuranosyl branches [12]. The common structure in (1,4)- β -D-glucan (AC-1) or (2,6)- β -D-fructan (levan) that is necessary for recognition by TLR4 is currently unknown. When AC-1 was treated with β -(1,4)-endoglucanase, which degrades polysaccharides possessing β -(1,4)-glucan backbones, the ability of AC-1 to induce production of TNF- α by macrophages was completely inhibited, suggesting that β -(1,4)-glucan backbone is essential for the stimulatory activity of AC-1. Similarly, when levan was degraded into mono-fructose by hydrolysis with acid, the immunostimulatory ability of levan was completely abolished, suggesting that the β -(2,6)-fructan backbone is essential for the stimulatory activity of levan. Further investigation is required to clarify the pattern recognition of the structure of these polysaccharides by TLR4.

It is notable that levan modulates immune responses not only *in vitro* but also *in vivo*. In the present study, we found that oral administration of levan to mice significantly inhibited the production of OVA-specific IgE following OVA immunization, but apparent augmentation of OVA-specific IgG2a was not accompanied. IgE production is mainly because of Th2 response producing IL-4, whereas IgG2a production depends on Th1 response producing IFN- γ [37, 38]. Consistent with the results of Ig production, the level of IL-4 production by T cells was significantly reduced, but no significant change with the level of IFN- γ production in response to OVA was detected in levan-treated mice sensitized with OVA. As Th1 cells inhibit Th2 response in some systems, particularly in infectious disease models, enhanced Th1 responses may play a beneficial role in allergic disease by down-modulating the effects of Th2 cells [39]. However, in our case, the development of a Th1 response is unlikely to down-modulate Th2 response with IgE production. IL-12 is known to prevent the development of Th2 response not only dependently of IFN- γ , but also independently of IFN- γ [29, 40]. IL-12 induced by levan administration may inhibit IL-4-induced IgE synthesis *in vitro* without enhanced IFN- γ production. Endogenous IL-12 has been shown to inhibit anti-IgD-induced IgE production, parasite antigen-induced IgE production and allergen-specific IgE production *in vivo* [41]. The presence of IL-12 during initial antigen presentation has been shown to inhibit Th2

responses, whereas delayed treatment with IL-12 showed limited inhibitory effects on Th2 responses [42]. This has recently been confirmed by Kips et al. in a murine *in vivo* model of allergic airway diseases. They showed that administration of IL-12 during initial antigen presentation suppressed allergen-specific IgE production, but administration of IL-12 during subsequent antigen exposure failed to suppress allergen-specific IgE production [43]. We have also revealed that levan administration at initial antigen exposure, but not at a later stage of antigen exposure, suppressed OVA-specific IgE production (data not shown). Therefore, it is possible that endogenously induced IL-12 at the induction stage of antigen sensitization might act on T cells primarily stimulated with antigens. Levan produced in the fermentation process of *B. subtilis* (natto) may have prophylactic applications for the controlling allergic diseases with dominant Th2 responses.

In conclusion, we have found that a soluble β -(2,6)-fructan with branches of β -(2,1)-linked fructose residues shows strong activities to induce production of IL-12 p40 and TNF- α *in vitro* and inhibit Th2 response with IgE production *in vivo*. TLR4 is required for cellular responses to levan. Levan may be useful for prevention of allergic responses.

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