A sulfated polysaccharide, fucoidan, enhances the immunomodulatory effects of lactic acid bacteria

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Abstract. Fucoidan, a sulfated polysaccharide contained in brown algae, has a variety of immunomodulatory effects, including antitumor and antiviral effects. On the other hand, lactic acid bacteria (LAB) also have immunomodulatory effects such as anti-allergic effects. In this study, we demonstrated that fucoidan enhances the probiotic effects of LAB on immune functions. By using Peyer's patch cells and spleen cells *in vitro*, fucoidan amplified interferon (IFN)-γ production in response to a strain of LAB, *Tetragenococcus halophilus* KK221, and this activity was abolished by desulfation of fucoidan. Moreover, this IFN-γ response was abolished by interleukin (IL)-12 neutralization. These results indicate that fucoidan enhanced IL-12 production in response to KK221, resulting in promoting IFN-γ production. In an *in vivo* study, Th1/Th2 immunobalance was most improved by oral administration of both fucoidan and KK221 to ovalbumin-immunized mice. These findings suggest that fucoidan can enhance a variety of beneficial effects of LAB on immune functions.

Introduction

Brown algae seaweeds contain fucoidan, a complex sulfated polysaccharide, and it has a variety of biological activities, including anti-inflammatory, antiviral, and antitumor effects (1-5). In an *in vitro* analysis, fucoidan was reported to enhance phagocytic activity of macrophages and B cell blastogenesis, but did not change the release of NO_2 by macrophages (2). These effects led to the augmentation of natural killer (NK) cell activity and T cell cytotoxicity, resulting in an antiviral action (2). In another report, oral administration of fucoidan to T cell receptor transgenic (DO11.10-Tg) mice promoted antitumor activities by enhancing T helper type 1 (Th1) immune responses and resultant T cell-mediated NK cell activation (6). It has also been shown that treatment of dendritic cells (DCs) with fucoidan enhances interferon (IFN)-γ production by $CD8^+$ T cells and induces T cell cytotoxicity against antigen-expressing human cancer cells (7). These reports suggest that fucoidan enhances the antigen-presenting activity of DCs and macrophages, thereby inducing upregulation of $CD8^+$ T cell cytotoxicity or NK cell activity, resulting in activation of anti-infectious and antitumor responses.

Recently, many reports have revealed that orally administered lactic acid bacteria (LAB) are protective against immune (8-13) and infectious (14-19) diseases. Th cells are key components of acquired immune responses, and are classified into several subsets such as Th1, Th2, Th17, and regulatory T cells (20). Because Th2 cell development preferentially initiates allergic responses, foods that support Th1 development are considered a useful dietary supplement when taken as a precaution against allergy development (21). Some LAB influence the production of a variety of cytokines from DCs and macrophages (22,23), notably interleukin-12 (IL-12), which induces Th1 cell development.

Certain strains of LAB have been demonstrated to induce IL-12 production from DCs and macrophages, converting a Th2 response into a Th1-dominated response, resulting in suppression of antigen-specific immunoglobulin E (IgE) production in mice (8-10). We have previously reported anti-allergic effects of *Tetragenococcus halophilus* KK221 (also called strain Th221 in our previous reports), which was isolated from soy sauce brewing (10,13). This strain induces a profound production of IL-12 from macrophages, and oral administration of KK221 has been shown to suppress allergic symptoms in mice and humans (10,13). As demonstrated in these reports, some strains of LAB activate DCs and macrophages to produce a variety of cytokines, and influence the differentiation of naïve T cells into Th1 cells, leading to anti-allergic effects.

As described above, although fucoidan and LAB target different cells and signaling pathways, both have multiple effects on immune function. In this study, we evaluated the synergistic effects of fucoidan and KK221, focusing on cytokine production *in vitro* and Th1/Th2 immunobalance *in vivo*.

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Materials and methods

Bacteria. *Tetragenococcus halophilus* KK221 was grown by stationary culture in MRS medium (BD Diagnostics, Franklin Lakes, NJ) containing 10% (w/v) NaCl at 30˚C for 24-48 h. After completion of the culture, bacteria were heat-killed at 95˚C for 10 min, washed twice with saline, and used for *in vitro* and *in vivo* assessment. To label KK221 with fluorescein isothiocyanate (FITC), heat-killed KK221 was incubated with 100 μg/ml FITC isomer-I (Dojindo Laboratories, Kumamoto, Japan). Thereafter, KK221 was washed 3 times with saline, and used for phagocytosis assessment.

Fucoidan. Fucoidan was isolated from the dried sporophyll of *Undaria piaantifida* as previously described (24). Desulfated fucoidan was prepared from fucoidan following a protocol published previously (25). Low molecular-weight fucoidan (LM-fucoidan) was prepared by treatment with acid, as follows: hydrolysis of fucoidan (10 g) was achieved by dissolution in 1000 ml of 2 mM HCl at high temperature using an autoclave apparatus (120˚C, 3 h). This solution was neutralized by 1 M NaOH. The neutralized fucoidan was fractionated using ultrafiltration with 50 and 3 kDa molecular weight cut-off membranes. LM-fucoidan, in the fraction between 50 and 3 kDa, was obtained and freeze-dried. Positive identification of LM-fucoidan in this fraction was achieved by gel filtration using (in series) Toyopearl HW-65F (8 mm x 1,000 mm) and Toyopearl HW-40F (8 mm x 1,000 mm) columns purchased from Toyosoda (Tokyo, Japan). The sample (20 μl) was injected, eluted with 0.05% formic acid solution at a flow rate of 1 ml/min at room temperature, and detected with a differential refractometer.

Reagents. Fucose, a carbohydrate component of fucoidan, was purchased from Wako (Osaka, Japan). Anti-IL-12 monoclonal antibody (mAb) and control Ab (rat IgG2a) were purchased from eBioscience (San Diego, CA). Ovalbumin (OVA, Grade V) was purchased from Sigma (St. Louis, MO).

Animals. For *in vitro* and *in vivo* experiments, male BALB/c mice were purchased from Charles River Co. (Atsugi, Japan). These experiments were approved and supervised by the local Ethics Committee according to the Guidelines of the Japanese Association for Laboratory Animal Science (published in 1987) and the Guidelines for Animal Experiments of the Research and Development Division, Kikkoman Corporation.

Cell preparation. Mouse peritoneal macrophages were prepared from male BALB/c mice (6-10-weeks-old) after stimulation by an intraperitoneal injection of fluid thioglycolate medium (2 ml, BD Diagnostics). Cell suspensions containing DCs were prepared from spleens, mesenteric lymph nodes, and Peyer's patches of male BALB/c mice (6-10-weeks-old) by digestion of each organ with type I collagenase.

In vitro cytokine production assay. Murine macrophages ($2x10^5$) were stimulated with heat-killed KK221 ($2x10^5$) for 24 h. Cells ($5x10^5$) from spleens, mesenteric lymph nodes, or Peyer's patches were cultured with $5x10^5$ KK221 in RPMI-1640 (Gibco, Eggenstein, Germany) containing 10% fetal bovine serum (FBS, Nichirei, Tokyo, Japan) for 48 h at 37˚C. Culture supernatants were collected, and cytokine levels were measured by enzyme-linked immunosorbent assay (ELISA).

Assay for phagocytosis of bacteria. Murine macrophages ($2x10^5$) were pre-treated in the presence or absence of fucoidan for 2 h at 37˚C. Thereafter, fucoidan-treated or untreated macrophages were incubated with FITC-labeled, heat-killed KK221 ($2x10^5$) for 4 h at 37˚C. After incubation, macrophages were collected using trypsin (Sigma, St. Louis, MO), and the ratio of $FITC^+$ cells was analyzed by flow cytometry.

In vivo experiments. For the OVA-immunization test, 6-week-old male BALB/c mice (n=6 per group) were injected intraperitoneally on Days 0 and 14 with 20 μg of OVA and 2 mg of $Al(OH)_3$ (Sigma) as adjuvant in a total volume of 0.2 ml. The mice received daily oral administration of saline, fucoidan (5 mg/day), KK221 ($4x10^8$/day), and fucoidan/KK221 (1 mg/day and $4x10^8$/day, respectively) on days 0-20. Sera were collected on Day 21, and OVA-specific IgE levels were measured by ELISA. Sera were also collected from 3 naïve mice as OVA-untreated control. On Day 21, spleen cells ($1x10^7$ cells/well) were incubated in culture medium (RPMI-1640 containing 10% FBS) with 100 μg/ml OVA in a 24-well flat-bottom plate at 37˚C for 72 h. IFN-γ and IL-4 concentrations in each supernatant were quantified by ELISA.

ELISA. IL-12p70, IFN-γ, IL-4, IL-6 and TNF-α concentrations in culture medium were determined using Mouse OptEIA™ ELISA Sets (BD Biosciences, San Jose, CA), following the manufacturer's instructions. OVA-specific IgE levels were determined by ELISA as previously described (26).

Statistical analysis. Data are presented as the mean ± SD. In *in vitro* tests, statistical analyses were carried out using the Student's t-test, and probability $P<0.05$ were considered to be significant. In the *in vivo* analysis, statistical comparisons were made by one-way analysis of variance (ANOVA), followed by a Duncan multiple-comparison test. Differences were considered to be significant when $P<0.05$.

Results

Cytokine responses of macrophages to fucoidan and LAB strain, KK221. We have previously reported that KK221 induces a high level of IL-12 production by macrophages (10). We therefore evaluated the effect of fucoidan on IL-12 induction by KK221. Peritoneal macrophages stimulated with fucoidan produced considerably less IL-12 than those stimulated with KK221, while co-stimulation with fucoidan and KK221 enhanced IL-12 production in a fucoidan dose-dependent manner (Fig. 1A). Although IL-6 and TNF-α were secreted by peritoneal macrophages in response to both fucoidan and KK221 individually, this IL6/TNF-α response was potentiated by co-stimulation with fucoidan and KK221 (Fig. 1B and C). Fucose, a carbohydrate component of fucoidan, did not affect cytokine production in response to KK221 (Fig. 1A-C). These results indicate that fucoidan and KK221 synergistically enhance the cytokine responses

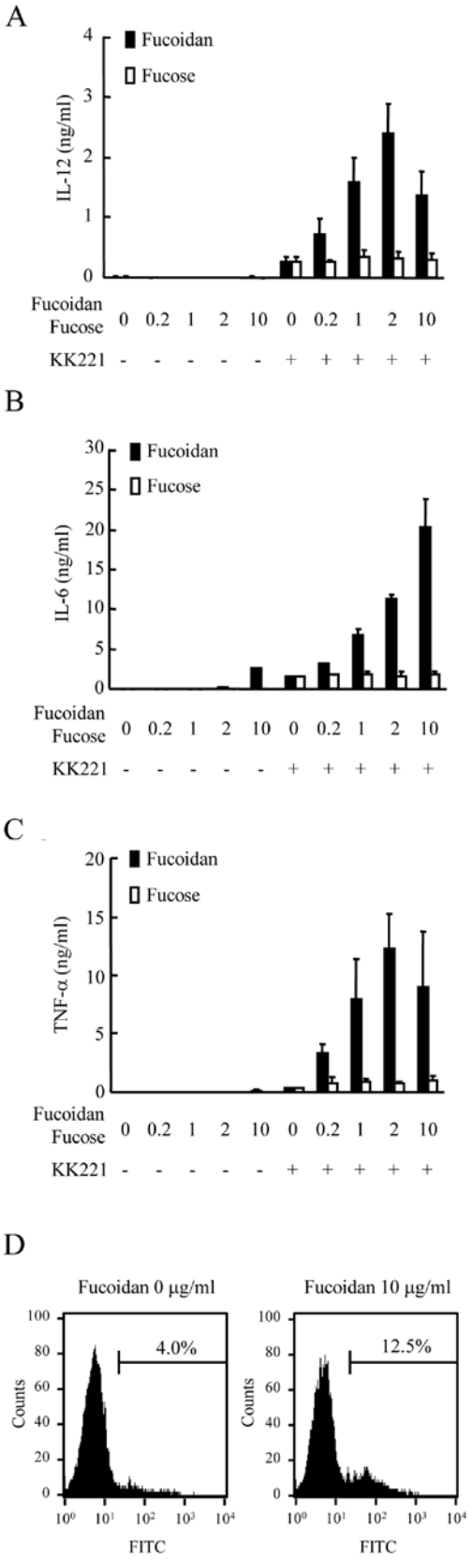

Figure 1. Mouse peritoneal macrophages from BALB/c mice were stimulated with KK221 and/or fucoidan (0-10 μg/ml) for 24 h. (A) IL-12, (B) IL-6 and (C) TNF-α concentrations in the culture supernatants were measured by ELISA. Results are shown as the mean ± SD of triplicates from a single experiment, representative of 3 independent experiments. (D) Mouse peritoneal macrophages were incubated with FITC-labeled KK221 pre-treated with 0 or 10 μg/ml fucoidan. Values in the figures represent the ratio of FITC$^+$ cells. Data are representative of 3 independent experiments.

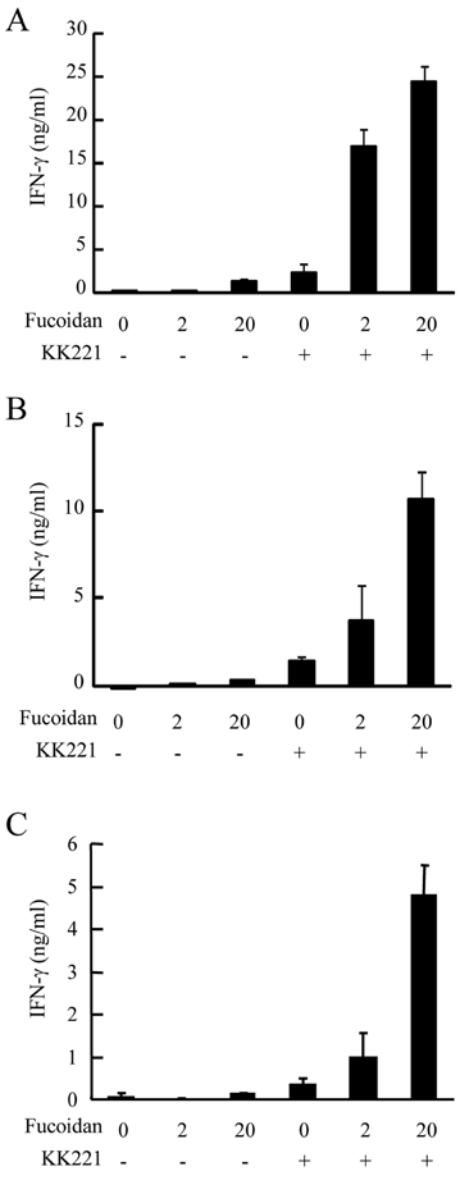

Figure 2. (A) Spleen cells, (B) mesenteric lymph node cells, or (C) Peyer's patch cells from BALB/c mice were cultured with KK221 and/or fucoidan (0-20 μg/ml) at 37˚C for 48 h. IFN-γ concentration in the culture supernatants was measured by ELISA. Results are shown as the mean ± SD of triplicates within a single experiment, which is representative of 3 independent experiments.

of peritoneal macrophages. To analyze KK221 phagocytosis by macrophages, peritoneal macrophages were incubated with FITC-labeled KK221 in the presence of fucoidan. We found that fucoidan supplementation increased the ratio of FITC$^+$ macrophages (Fig. 1D), indicating that fucoidan upregulates KK221 phagocytosis by macrophages.

Cytokine responses of cells from spleens, mesenteric lymph nodes and Peyer's patches to fucoidan and KK221. To evaluate this synergistic effect on cytokine responses by other cells, cell suspensions containing DCs were prepared from mouse spleens, mesenteric lymph nodes, and Peyer's patches of mice. In cells from each tissue, fucoidan and KK221 stimulation caused the synergistic upregulation of IFN-γ production in a fucoidan dose-dependent manner (Fig. 2). Production of

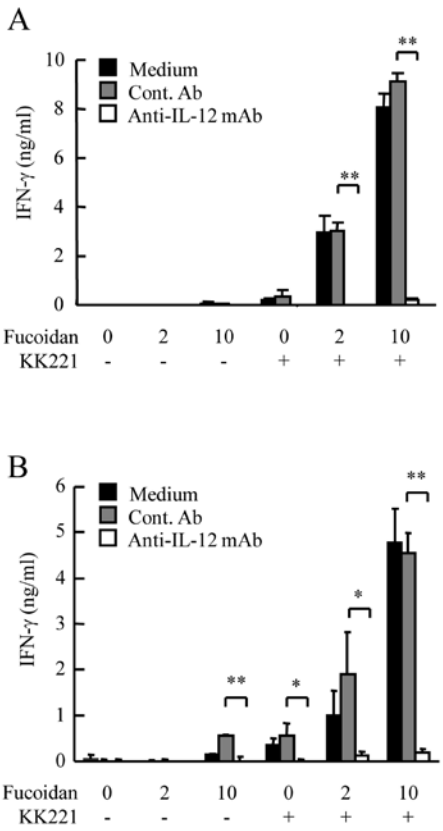

Figure 3. (A) Cells from the spleens or (B) Peyer's patches of BALB/c mice were cultured with KK221 and/or fucoidan (0-10 μg/ml) in the presence of control antibody (Cont. Ab) or anti-IL-12 mAb at 37˚C for 48 h. IFN-γ concentration in the culture supernatants was measured by ELISA. Results are shown as the mean ± SD. Data are representative of 3 independent experiments *P<0.05, **P<0.01, significant differences between the indicated columns..

other cytokines such as IL-6 and IL-10 were also enhanced by stimulation with fucoidan and KK221 (data not shown). These results indicate that fucoidan upregulates cytokine responses in tissue DCs as well as peritoneal macrophages, resulting in enhancement of IFN-γ production by T cells and other cells.

IL-12 neutralization assay. IL-12 was upregulated by co-stimulation with KK221 and fucoidan (Fig. 1A). IL-12 is known to promote IFN-γ production and to enhance Th1 immune responses (27). To investigate whether the synergistic enhancement of IL-12 production by fucoidan and KK221 causes concomitant upregulation of IFN-γ, we added anti-IL-12 mAb or control Ab to the cell culture medium. IFN-γ production from spleen cells in response to either KK221 or fucoidan/KK221 was significantly reduced by anti-IL-12 mAb (Fig. 3A). IFN-γ production from Peyer's patch cells stimulated with fucoidan, KK221, or fucoidan/KK221 was also abolished by anti-IL-12 mAb (Fig. 3B). These results indicate that fucoidan and KK221 stimulate IL-12 production from DCs that then drives IFN-γ production by T cells.

Desulfation of fucoidan. Fucoidan is a polysaccharide containing sulfate groups. In this experiment, we tried to determine which moiety of fucoidan mediates the enhancement of cytokine responses. Desulfated fucoidan contained only 1.2% (w/w) sulfate, compared to 26.7% (w/w) in normal fucoidan (Table I). Using acid-hydrolysis, we prepared LM-fucoidan (MW range: 2-206 kDa; MW of peak polysaccharide: 20 kDa) from normal fucoidan (MW range: 3-400 kDa; MW of peak polysaccharide: 67 kDa) (Fig. 4A). LM-fucoidan contained 12.6% (w/w) sulfate (Table I), indicating that the sulfate group content was partially reduced by hydrolytic reduction

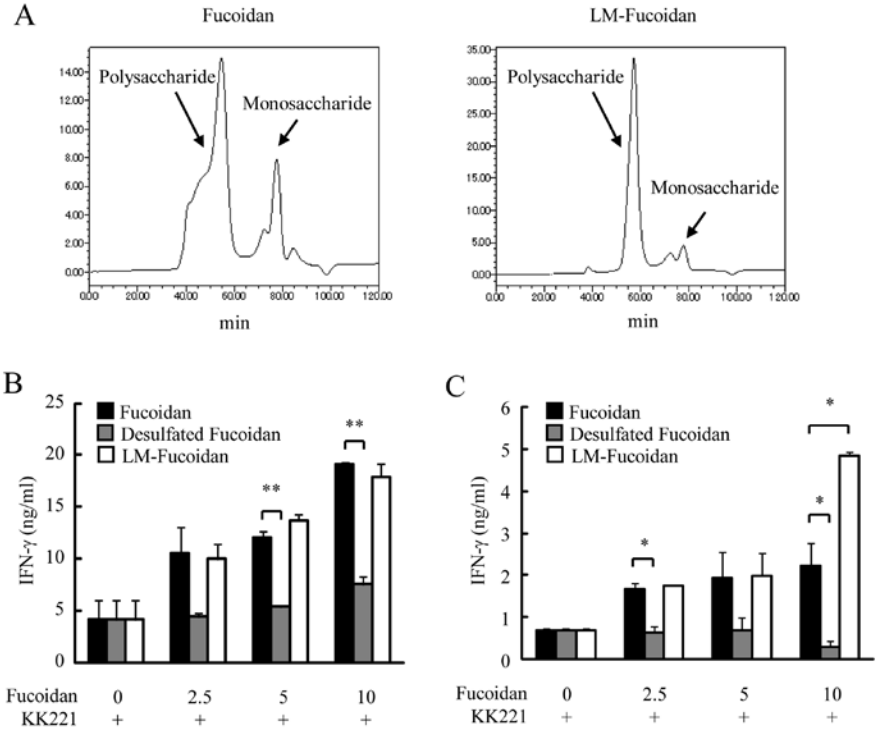

Figure 4. (A) Chromatogram of fucoidan and low molecular-weight fucoidan (LM-fucoidan). (B) Spleen cells, or (C) Peyer's patch cells from BALB/c mice were cultured with KK221 and either fucoidan, desulfated fucoidan or LM-fucoidan (0-10 μg/ml) at 37˚C for 48 h. IFN-γ concentration in the culture supernatants was measured by ELISA. Results are shown as the mean ± SD. Data are representative of 3 independent experiments.*P<0.05, **P<0.01.

Table I. Ratio of sulfur and sulfate group in fucoidan, desulfated fucoidan, and low molecular-weight fucoidan (LM-fucoidan).

Sample name	Sulfur (% w/w)	Sulfate group (% w/w)
Fucoidan	8.9	26.7
Desulfated fucoidan	0.4	1.2
LM-fucoidan	4.2	12.6

of fucoidan molecular weight. In combination with KK221, desulfated fucoidan failed to enhance IFN-γ production from cultured spleen and Peyer's patch cells, whereas LM-fucoidan activity was not different to untreated fucoidan (Fig. 4B). These results indicate that the fucoidan sulfate group is essential for upregulating cytokine responses.

Effects of fucoidan and KK221 on Th1/Th2 immunobalance in vivo. To evaluate whether the synergistic effects of fucoidan and KK221 translated to the *in vivo* situation, antigen-specific responses were tested using OVA-immunized mice. It has been reported that IgE production is suppressed when fucoidan is intraperitoneally, but not orally, administered prior to the first immunization with OVA (4). Serum OVA-specific IgE was enhanced by intraperitoneal injection of OVA/Alum indicating that systemic immune responses to the antigen were induced. Oral administration of fucoidan alone had no effect on the serum OVA-specific IgE level, whereas this was significantly suppressed in mice fed either KK221 or fucoidan/KK221 (Fig. 5A). We have previously described this suppression of OVA-specific IgE production by oral administration of KK221 (10). To evaluate Th1/Th2 immunobalance, IFN-γ (secreted by Th1 cells) and IL-4 (secreted by Th2 cells) were quantified by stimulating spleen cells from each mouse with OVA. IFN-γ production was significantly upregulated only in the fucoidan/KK221-treated group, whereas IL-4 production was significantly suppressed in this group (Fig. 5B). Together, these results demonstrate that, both *in vitro* and *in vivo*, co-administration of fucoidan and KK221 dramatically enhances Th1 immune responses and suppresses allergic responses (Figs. 1 and 2).

Discussion

In this study, we demonstrated that fucoidan enhanced the anti-allergic effect of a probiotic strain of LAB, *Tetragenococcus halophilus* KK221, by stimulating cytokine production from antigen-presenting cells (APCs), and that fucoidan needs sulfate groups, but not the full length of its polysaccharide, to exert this activity. In our *in vivo* study, fucoidan enhanced the effect of KK221 administration on improvement of Th1/Th2 immunobalance. Moreover, one of the mechanisms contributing to these effects was the promotion of KK221 phagocytosis by macrophages and/or dendritic cells. Enhancement of macrophage phagocytic activity by fucoidan or other sulfated polysaccharides has been demonstrated in several reports (2,28,29), and could be involved in the synergistic activation of *in vitro* cytokine production and *in vivo* anti-allergic immune responses that we observed in response to fucoidan and KK221.

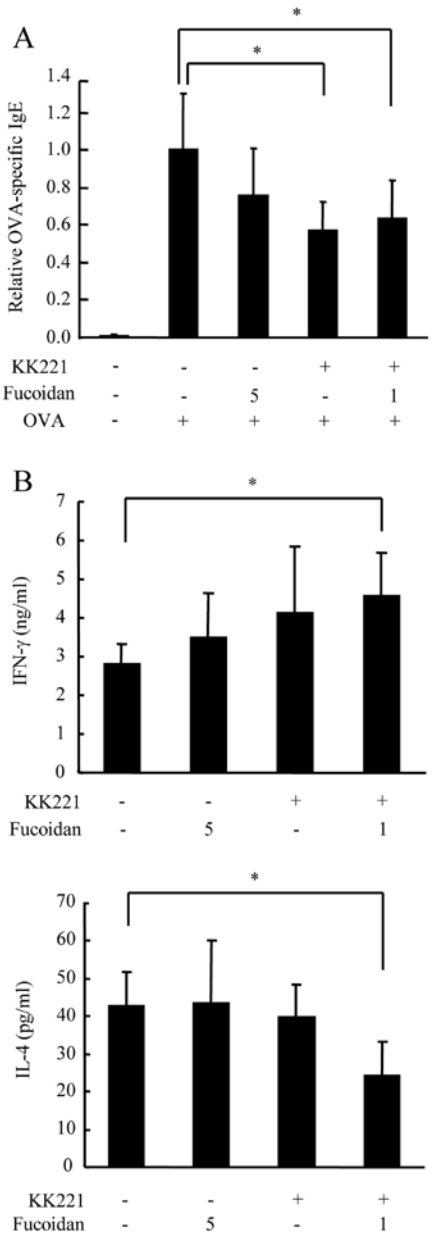

Figure 5. Suppression of serum OVA-specific IgE levels and improvement of Th1/Th2 immunobalance by oral administration of fucoidan and KK221. Saline, fucoidan (5 mg/day), KK221 ($4x10^8$/day), fucoidan + KK221 (1 mg/day and $4x10^8$/day, respectively) were orally administered during the test period. OVA/Alum was injected intraperitoneally on Days 0 and 14. Serum was collected from each mouse and from 3 non-immunized mice on Day 21. (A) OVA-specific IgE in serum was quantified by ELISA. Results are shown as values relative to the OVA-immunized control group. Data are shown as the mean ± SD. *Significant differences between the indicated columns. (B) Spleens were collected from OVA-immunized mice on Day 21. Spleen cells were cultured in the presence of OVA (100 μg/ml) for 3 days. IFN-γ and IL-4 concentrations in culture supernatants were quantified by ELISA. Data are shown as the mean ± SD. *Significant difference between the indicated columns.

Several reports have demonstrated that the sulfate content of fucoidan is important for its anticancer (30) and antiviral (31) activities. Sulfated fucans were responsible for the antiviral activity of certain fractions isolated from a water extract of brown seaweed (31). In our study, we show that degradation of the sulfate groups abolishes the synergistic enhancement of cytokine production in response to KK221. This role of

sulfate groups has precedents in other polysaccharides. A polysaccharide of *Grifola frondosa mycelia* enhances antitumor effects and peritoneal macrophage phagocytosis by chemical sulfation (28), and a sulfated derivative of β-glucan isolated from sporophytes of *Laminaria digitata* stimulates phagocytic and NK cell activity (29). In the case of other types of β-glucan, we confirmed that those from *Lentinula edodes* and S*accharomyces cerevisiae* (Wako, Osaka, Japan) failed to enhance cytokine production by peritoneal macrophages in response to LAB (data not shown). These data indicate that polysaccharide sulfate groups are responsible for some of their immunological functions. Although decreasing the MW of fucoidan by hydrolysis did not change its effects on cytokine production in our experiment, a recent report has shown that antitumor activity of fucoidan is significantly enhanced by lowering its MW (32). However, this only occurred following depolymerization in mild conditions (boiling for 5 min), whereas our hydrolysis conditions (120˚C for 3 h) were much harsher, resulting in desulfation. Therefore, if we were to reduce the MW of fucoidan without causing desulfation, we might expect that its bioactivities (e.g. antiviral or antitumor activity) would be similarly enhanced.

Activation of innate immunity is a critical step in the development of acquired immunity (33), and is mediated by Toll-like receptors (TLRs) (34). Various bacterial components are recognized by TLRs which, when engaged, induce APCs to secrete various cytokines and chemokines. The TLR family is composed of at least 11 members, with each member recognizing different molecular structures or classes of microorganisms. TLR2 and TLR4 recognize cell wall components of gram-positive bacteria such as lipoteichoic acid and peptidoglycan, and TLR9 recognizes bacterial CpG-DNA (34). TLR2 and TLR4 are expressed at the cell surface, while TLR9 is expressed in endosomes (34). Recently, the specific TLRs involved in LAB recognition and cytokine production by DCs and macrophages have been identified (35-38). Rigaux *et al* have reported that IL-12p40 secretion by mouse DCs in response to *L. plantarum* NCIMB8826 was dependent on TLR2- and TLR9-mediated signaling (37). A second report showed that a CpG oligonucleotide from some LAB activates the TLR9 signaling pathway (38). Endosomal TLR9 recognition of bacterial oligonucleotide may occur after phagocytosis and digestion of bacteria. We revealed here that fucoidan enhances KK221 phagocytosis by macrophages, which is likely to activate the TLR signaling pathway via endosomal TLRs, especially TLR9, leading to enhanced IL-12 production and Th1 cell activation. This mechanism neatly explains the synergistic effect of fucoidan and KK221 in improving Th1/Th2 immunobalance.

It has been shown that oral administration of LAB induces $CD4^+CD25^+Foxp3^+$ regulatory T cells, which suppress allergic disorders such as asthma or food allergy (39,40). In Peyer's patches and spleen, these cells cause suppression of IgE production (41). Although we have focused here on activation of Th1 immune responses by oral administration of KK221 and fucoidan, induction of regulatory T cell differentiation could also lead to improvement of allergic symptoms. Besides being anti-allergic, KK221 also has an anti-inflammatory effect against dextran sulfate sodium-induced experimental colitis (unpublished observation), and other probiotic strains have anti-infectious effects against pathogenic bacteria, such as *Listeria monocytogenes or Escherichia coli* (14,15), and viruses (16-19). These observations indicate that oral administration of probiotic LAB contributes to the maintenance of immune homeostasis and augments the systemic immune system through gut-associated lymphoid tissues (GALT), a major site of host encounter with exogenous antigens and pathogens. Because fucoidan enhances KK221-induced APC cytokine responses *in vitro*, fucoidan might also be expected to augment these various other probiotic effects. We are currently investigating whether these synergistic effects of LAB and fucoidan are induced in mouse models of autoimmune diseases, inflammatory bowel diseases, and infection by pathogenic bacteria or viruses. We also aim to identify which subset of DCs or macrophages responds to fucoidan and contributes to these effects in lamina propria or Peyer's patches in the small intestine.

Acknowledgements

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