Peritoneal injection of fucoidan suppresses the increase of plasma IgE induced by OVA-sensitization

Yuhki Yanase[a], Takaaki Hiragun[a], Kazue Uchida[a], Kaori Ishii[a], Souichi Oomizu[a, b], Hidenori Suzuki[a], Shoji Mihara[a], Kazumasa Iwamoto[a], Hiroaki Matsuo[c], Nobukazu Onishi[d], Yoshikazu Kameyoshi[a], Michihiro Hide[a, *]

[a]Department of Dermatology, Division of Molecular Medical Science, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3, Kasumi, Minami-ku, Hiroshima 734-8551, Japan, [b]Department of Immunology and Immunopathology, Faculty of Medicine, Kagawa University, Kagawa, 761-0793, Japan, [c]Division of Clinical Pharmacotherapeutics, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3, Kasumi, Minami-ku, Hiroshima 734-8551, Japan, [d]Department of Research and Development, Nishikawa Rubber Co. Ltd, 2-2-8 Misasa-cho, Nishi-ku, Hiroshima 733-8510, Japan.

*Corresponding author. Fax: +81 82 257 5239

E-mail address: ed1h-w1de-road@hiroshima-u.ac.jp (M. Hide)

Abstract

We previously reported that fucoidan, a dietary fiber purified from seaweed, inhibited IgE production by B cells *in vitro*. In this study, we examined the effect of fucoidan on IgE production *in vivo*. The OVA-induced increase of plasma IgE was significantly suppressed when fucoidan was intraperitoneally, but not orally, administered prior to the first immunization with OVA. The production of IL-4 and IFN-γ in response to OVA in spleen cells isolated from OVA-sensitized mice treated with fucoidan *in vivo* was lower than that from mice treated without fucoidan. Moreover, the flow cytometric analysis and ELISpot assay revealed that the administration of fucoidan suppressed a number of IgE-expressing and IgE-secreting B cells respectively. These results indicate that fucoidan inhibits the increase of plasma IgE through the suppression of IgE-producing B cell population, and the effect of fucoidan *in vivo* is crucially dependent on the route and timing of its administration.

Key words

Fucoidan, B cells, T cells, IgE, Ovalbumin (OVA)

Introduction

Elevated serum levels of specific IgE towards common environmental allergens characterize allergic diseases such as asthma, allergic rhinitis, and atopic dermatitis [1, 2]. IgE plays a critical role in type I hypersensitivity reactions, binding to the high-affinity IgE receptor (FcεRI) on mast cells via the Cε3 domain on its Fc fragment. The cross-linking of IgE on mast cells by specific antigens results in the release of inflammatory mediators, such as histamine, enzymes, and cytokines [3]. These factors cause the immediate hypersensitivity reactions, and the late-phase inflammatory reaction involving T cells and eosinophils [4].

Recent studies have shown that the ligation of IgE itself on FcεRI without antigen can cause the release of such inflammatory mediators, morphological changes, such as membrane ruffling, and migration of mast cells [5, 6]. Moreover, it is reported that recombinant humanized anti-IgE, which rapidly reduces serum free IgE concentrations and down-regulates IgE receptors, alleviates symptom of allergic diseases [7, 8]. Therefore, the control of IgE production is an important target for both prevention and treatment of atopic diseases.

Fucoidan, a dietary fiber purified from seaweed, is composed of a polymer of α1→3-linked L-fucose with sulfate groups on some of the fucose residents at the 4 position. It is reported that fucoidan may modulate the function of immune cells including macrophages, natural killer (NK) cells, lymphocytes and neutrophils [9, 10]. However, only few studies of

fucoidan have been conducted concerning the effect on allergy. We previously reported that fucoidan inhibits IgE production from B cells derived from mouse spleen by preventing NF-κB p52-mediated pathways *in vitro* [11].

In this study, we investigated the effects of fucoidan on the increase of plasma IgE and the population of IgE-producing and -secreting B cells in ovalbumin (OVA)-sensitized mice *in vivo*, and the release of IL-4 and IFN-γ from T-cells *in vitro*.

Materials and methods

Animals: Female Balb/c (4 weeks) mice were purchased from Charles River Japan (Yokohama, Japan). They were maintained in a specific pathogen-free environment in closed racks with free access to food and water in the Institute of Laboratory Animal Science, Hiroshima University. This study was carried out in accordance with the Guidelines for Animal Experiments for Laboratory Animal Science, Natural Science Center for Basic Research and Development (N-BARD), Hiroshima University.

Preparation and culture of spleen cells: Spleens were isolated from mice and minced in RPMI 1640 medium (Invitrogen) with 10% fetal calf serum and antibiotics. Lymphocyte suspensions (spleen cells) were obtained with a nylon mesh filter (BD Biosciences, Franklin

Lakes, NJ) and a density separation medium (Lympholyte-M; Cedarlane Laboratories, Hornby, Canada). The isolated spleen cells were distributed onto a 96-well tissue plate and maintained for 3 days at 37℃ in an atmosphere containing 5% CO_2 in the presence of 1 mg/ml OVA with or without fucoidan.

Ovalbumin immunization and administration of fucoidan: Mice were immunized intraperitoneally with OVA (2 μg/mouse) (Sigma) with an alum adjuvant (LSL, Tokyo, Japan). Two, four and six weeks after the first immunization, the mice were given boosters using the same doses of the antigen. From 1 week before the first immunization or indicated time points, mice were administered with fucoidan either orally (10 mg/mouse) or intraperitoneally (100 μg/mouse) 3 times every week. One week after every antigen challenge, plasma was obtained by retro-orbital bleeding. Blood from each mouse was placed in a heparinized plastic tube and centrifuged at 10,000 g for 10 min. After centrifugation, plasma was collected and stored at –80℃ for analysis.

Measurement of IgE, IgG1,IgG2a, IL-4, and IFN-γ in plasma: The concentrations of IgE, IL-4 and IFN-γ in plasma were determined by means of mouse IgE ELISA kit purchased from Bethyl Laboratories (Montgomery, TX), mouse OVA-specific IgE ELISA kit purchased

from Shibayagi (Gunma, Japan), mouse IL-4 ELISA kit purchased from BD Biosciences, or mouse IFN-γ ELISA kit purchased from R&D systems, following the manufacturers' instructions, respectively. The concentrations of total IgG1 [12], OVA-specific IgG1 [13], total IgG2a [12], OVA-specific IgG2a [12] were determined by sandwich ELISA as described previously.

Flow cytometric analysis: For measurement of IgE-expressing B cells, isolated spleen cells were incubated with 0.05 M acetate buffer (pH 4.0) containing 0.085 M NaCl, 0.005 M KCl, and 1% fetal calf serum for 1 min on ice to remove soluble IgE bound to CD23 on B cell surface [14]. The acid-treated cells were incubated with fluorescein isothiocyanate (FITC)-labeled anti-IgE antibody (clone R35-72, BD Biosciences) or FITC-labeled isotype control for 30 min in the presence of phycoerythrin (PE)-labeled anti-CD-19 antibody (clone ID3, BD Biosciences) and then washed with PBS twice before analysis. The stained cells were analyzed by FACSCalibur® (BD Biosciences).

ELISpot assay: The number of IgE secreting B cells was determined by means of ELISpot (AP ELISpot kit, KLP) following the manufactures' instruction. The isolated spleen cells were cultured overnight in a 96-well plate coated with anti-mouse IgE antibody (BD), and then

released-IgE captured on the plate was stained with biotinylated anti-mouse antibody (BD), AP-streptavidein and BCIP/NBT substrate. The number of spots was counted manually under a stereomicroscope.

Results

Effects of fucoidan on the increase of plasma IgE levels in OVA-sensitized mice in vivo

We administered fucoidan either orally or intraperitoneally, to mice being sensitized with OVA, and examined the increase of plasma IgE. As shown in Fig. 1, intraperitoneal injections, but not oral treatments, of fucoidan to OVA-sensitized mice effectively prevented the increase of plasma IgE levels.

Timing of fucoidan administration to mice in the course of OVA-sensitisation

To determine optimal timing of administration for fucoidan to suppress the increase of plasma IgE by OVA sensitization, we commenced administration of fucoidan at several time points; 1 week before (group A), 1 day before (group B), at the same time as (group C), or 1 week after the first immunization with OVA (group D). Group E was immunized with OVA, but not treated with fucodian. Group F was treated neither with OVA nor fucoidan. As shown in Fig. 2a and b, total IgE levels in plasma of OVA-sensitized mice were significantly suppressed by

repetitive injections of fucoidan that were started before the immunization with OVA (group A and B). However, only a small effect of fucoidan was observed when injections were started at the same time as, or after the immunization with OVA (group C and D). OVA-specific IgE levels in plasma of OVA-sensitized mice were also significantly suppressed by repetitive injections of fucoidan started before the first immunization with OVA as well (group A and B) (Fig. 2c). Both total and OVA-specific IgG1 levels in plasma of OVA-sensitized mice were also suppressed by fucoidan (Supplementary figure. 1a), whereas both total and OVA-specific IgG2a levels tended to be higher in mice treated with fucoidan (Supplementary figure. 1b) .

Effects of fucoidan on the release of IL-4 and IFN-γ from spleen cells isolated from OVA-sensitized mice

It is well established that IL-4 and IFN-γ play critical roles in the regulation of IgE levels in plasma. We therefore investigated the effects of fucoidan on the release of cytokines from spleen cells in response to OVA. Spleen cells isolated from OVA-sensitized mice without treatment with fucoidan *in vivo*, were stimulated by 1 mg/ml OVA for 3 days in the presence or absence of 100 μg /ml fucoidan *in vitro*. As shown in Fig.3a, the release of IL-4 and IFN-γ in response to OVA from spleen cells, which were isolated from mice sensitized with OVA *in vivo*, was not affected by the presence of fucoidan *in vitro*.

Next we investigated the effect of injection with fucoidan on the release of cytokines *in vitro* in response to OVA. As shown in Fig. 3b, the release of IL-4 and IFN-γ in response to OVA from spleen cells, which were isolated from OVA-sensitized mice injected with fucoidan *in vivo*, was lower than that from mice sensitized with OVA without injections of fucoidan.

Effects of fucoidan on the population of IgE-producing B cells in OVA-sensitized mice

To study whether fucoidan inhibited the differentiation of B cells to produce IgE *in vivo*, we performed FACS analysis and ELISpot assay to measure the number of IgE-expressing and -secreting B cells. For FACS analysis, cells were treated with acetate buffer to remove soluble IgE bound to CD23 prior to the staining of isolated spleen cells with antibodies (see the Materials and Methods section). As shown in Fig. 4a, b, the administration of fucoidan significantly suppressed the increase of IgE-expressing and -secreting B cells *in vivo*.

Discussion

We have demonstrated that the increase of plasma IgE levels was significantly suppressed by repetitive injections of fucoidan initiated before the immunization with OVA. The analysis by flow cytometry and ELISpot showed that the administration of fucoidan suppressed the number of IgE-expressing and –secreting B cells *in vivo*.

When fucoidan was not injected before the immunization with OVA, the effect of fucoidan was modest. We previously reported that the production of IgE and its class switching in B cells were inhibited when B cells were exposed to fucoidan within 48 hr, but not at 72 hr from the beginning of the culture with IL-4 and anti-CD40 antibodies *in vitro* [11]. These results indicated that once B cells are engaged to produce IgE by completing immunoglobulin class switching, fucoidan may no longer prevent IgE production. Thus, the presence of fucoidan during immunization with OVA might be important to suppress the increase of plasma IgE *in vivo*, as well as *in vitro*. In fact, the flow cytometry analysis and ELISpot assay of spleen cells in mice immunized with OVA in the presence or the absence of fucoidan has demonstrated that the increase of IgE-expressing and -secreting B cells was substantially suppressed by fucoidan. These results suggest that intraperitoneal injections of fucoidan into OVA-sensitized mice suppress the increase of plasma IgE, presumably through the inhibition of IgE class switching of B cells.

The release of IL-4 and IFN-γ in response to OVA from spleen cells of mice sensitized with OVA preceded by injections of fucoidan was lower than that from cells of mice without injections of fucoidan. This result suggests that fucoidan may suppress the increase and/or functions of OVA-specific T cells, *in vivo*. However, the release of IL-4 and IFN-γ in response to OVA from spleen cells isolated from OVA sensitized mice without *in vivo* treatments with

fucoidan was not inhibited by the presence of fucoidan *in vitro*. Therefore, fucoidan may suppress an upstream mechanism of T cell activation, such as antigen presentation by dendritic cells, or the differentiation of Th0 cells into Th1 and/or Th2 cells *in vivo* rather than the function of effector T cells.

The reason why oral treatment of OVA-sensitized mice with fucoidan did not prevent the increase of serum IgE is a matter of discussion. The amount of fucoidan absorbed via the gastrointestinal tract may be lower than that injected intraperioneally. However, Irhimeh *et al*. detected 4.0 mg/l fucoidan by enzyme-linked immunosorbent assay (ELISA) in sera of six persons, who ate 300 mg fucoidan over a period of 12 days [15]. This result indicated that more than 0.5% of orally administered fucoidan was transferred to the blood, suggesting that oral treatment with 10 mg fucoidan per mouse with 2 ml blood circulation conveys 25 μg/ml fucoidan into the plasma, which is enough to suppress the activation of B cells. Another possibility is that fucoidan may be digested into an inactive form in gastrointestinal tract. Further study of plasma concentrations of fucoidan and its biological activities are necessary to evaluate the usability of oral administration of fucoidan.

In conclusion, fucoidan may suppress the increase of plasma IgE level in response to OVA injections, through the inhibition of IgE production and class switching in B cells *in vivo*. Further studies of molecular actions of fucoidan may allow us to use fucoidan as a valuable

supplement or medicine for the prevention of IgE-mediated allergy.

Acknowledgments

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Figure legends

Fig. 1. Effects of fucoidan on the increase of plasma IgE induced by OVA sensitization in *vivo*. OVA-sensitized mice received fucoidan either by intraperitoneal injections or oral treatments. The administrations of fucoidan were started 1 week before the first immunization with OVA. The levels of serum IgE were determined as described in the Materials and Methods section. Data are means ± SEM (n=4) in one representative experiment. Similar results were obtained by three independent experiments. The difference among groups at each time points were tested using a one-way ANOVA followed by Dunnett's test and considered significant with $P<0.01$(**).

Fig. 2. Effects of time to start fucoidan administrations on the increase of plasma IgE in the course of OVA-sensitization. (a) During the course of OVA-sensitization, mice were injected with fucoidan intraperitoneally from 1 week before (group A), 1 day before (group B), at the same time as (group C), or 1 week after the first immunization with OVA (group D). Group E was immunized with OVA without treatment with fucoidan. Group F was neither with OVA nor fucoidan. (b) The level of total plasma IgE in mice 7 weeks after the first immunization with OVA. (c) The level of OVA-specific IgE in plasma of mice 7 weeks after the first immunization

with OVA. Data are means ± SEM (n=8) in one representative experiment. Similar results were obtained by three independent experiments. Differences among groups at each time points were tested using a one-way ANOVA followed by Dunnett's test and considered significant with P<0.01(**). ND: not detected.

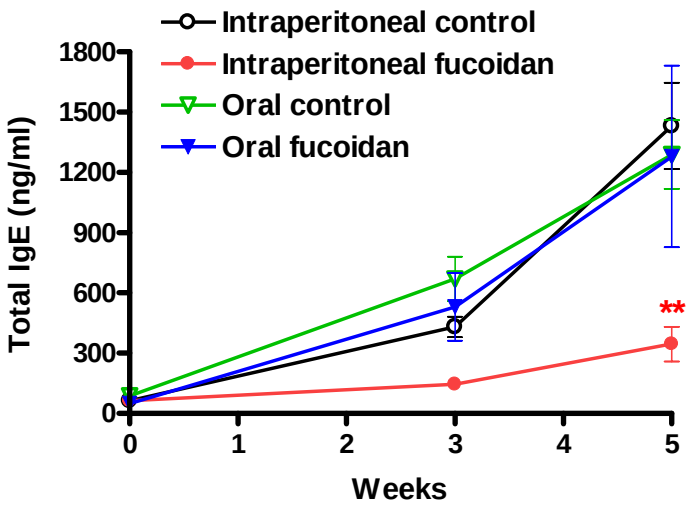
Fig. 3. Effects of fucoidan on the release of IL-4 and IFN-γ from spleen cells of mice in response to OVA. (a) Spleen cells were isolated from OVA-sensitized mice and stimulated with 1 mg/ml OVA for 3 days with or without 100 μg/ml fucoidan *in vitro*. (b) Spleen cells of OVA sensitized mice injected with OVA with or without fucoidan *in vivo*, and stimulated with 1 mg/ml OVA *in vitro* for 3 days in the absence of fucoidan *in vitro*. The concentrations of IL-4 and IFN-γ in culture medium were measured by means of ELISA. Data are means ± SEM in four clusters of culture in one representative result from three (a) or five (b) separate experiments. The difference among each points were tested using a one-way ANOVA followed by Dunnett's test and considered significant with P<0.01(**). NS: not significant (P>0.05), ND: not detected.

Fig. 4. FACS analysis of IgE expression on the surface of B cells and ELISpot assay of IgE-secreting B cells. (a)Spleen cells were isolated from control mice (group F, right), mice

sensitized by OVA with (group A, middle) or without (group E, left) injections of fucoidan, treated with the acetic acid buffer to remove soluble IgE bound to CD23, and then incubated with FITC-labeled anti-IgE or FITC-labeled isotype control antibodies and PE-labeled anti-CD19 antibody for 30 min at room temperature. Dot plots of spleen cells labeled with FITC and PE were shown. Vertical and horizontal axes show the intensity of PE and FITC, respectively. The numbers in graphs indicate the percentage of cells in each region separated by cross bars in spleen cells. Similar results were obtained by three independent experiments. (b) Spleen cells were isolated from control mice (group F, right), mice sensitized by OVA with (group A, middle) or without (group E, left) injections of fucoidan, and cultured overnight in plate coated with capture antibody. Released IgE bound to the membrane were stained with biotinylated anti-mouse antibody, AP-streptavidein and BCIP/NBT substrate. The number of spots was counted under a stereomicroscope. Similar results were obtained by three independent experiments. Differences among groups at each time points were tested using a one-way ANOVA followed by Dunnett's test and considered significant with $P<0.001$(***).

S**upplementary Figure 1.** *Effects of time to start fucoidan administrations on the increase of plasma IgG1 and IgG2 in the course of OVA-sensitization*. During the course of OVA-sensitization, mice were injected with fucoidan intraperitoneally from 1 week before

(group A), 1 day before (group B), at the same time as (group C), or 1 week after the first immunization with OVA (group D). Group E was immunized with OVA without treatment with fucoidan. Group F was neither with OVA nor fucoidan. (a) The level of total and OVA-specific plasma IgG1 in mice 7 weeks after the first immunization with OVA. (b) The level of Total and OVA specific IgG2a in plasma of mice 7 weeks after the first immunization with OVA. Data are means ± SEM (n=6). Differences among groups at each time points were tested using a one-way ANOVA followed by Dunnett's test and considered significant with $P<0.05$(*) and $P<0.01$(**). ND: not detected.


Intraperitoneal control
Intraperitoneal fucoidan
Oral control
Oral fucoidan
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1200
900
600
300
0
0
1
2
3
4
5
Weeks
**

Fig. 1.

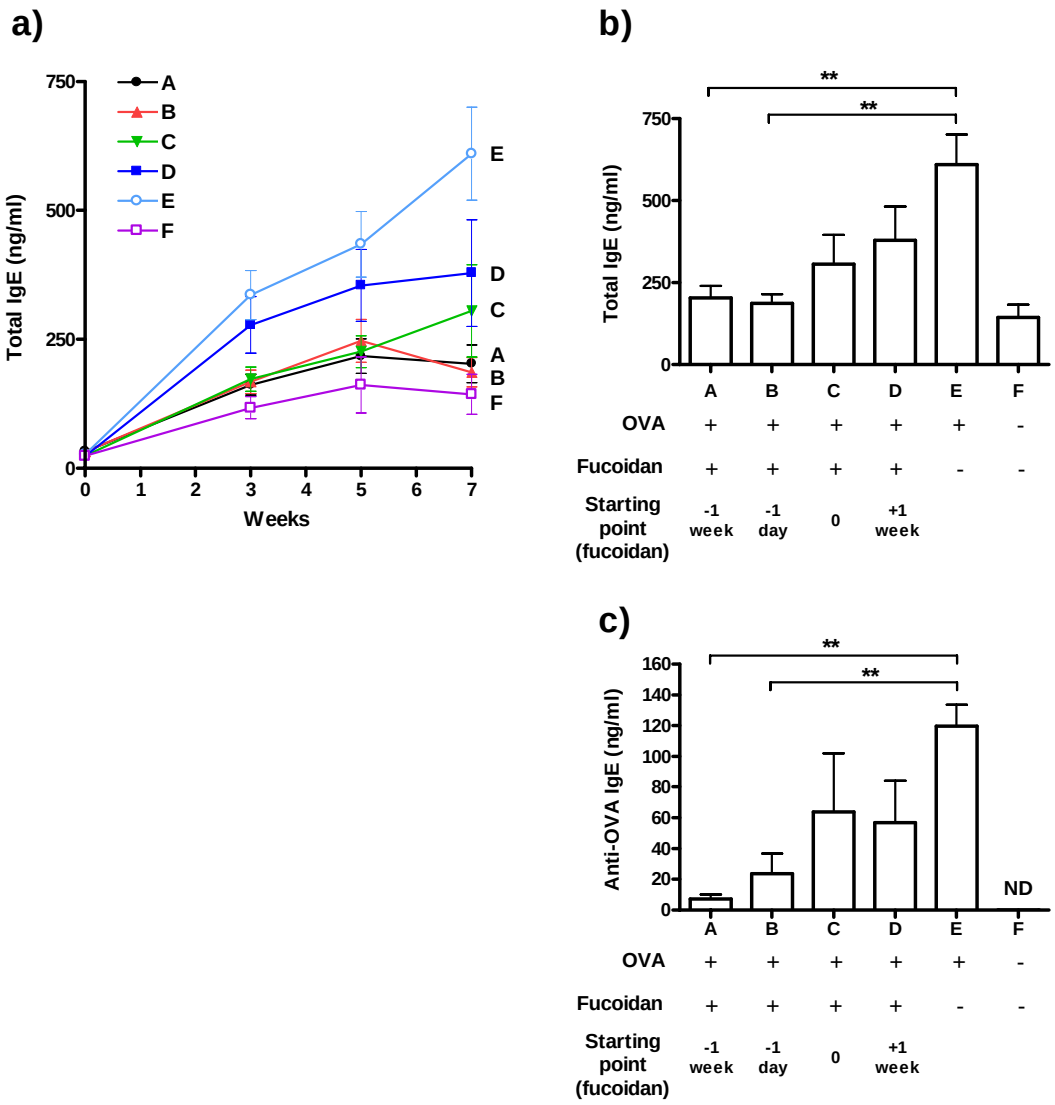
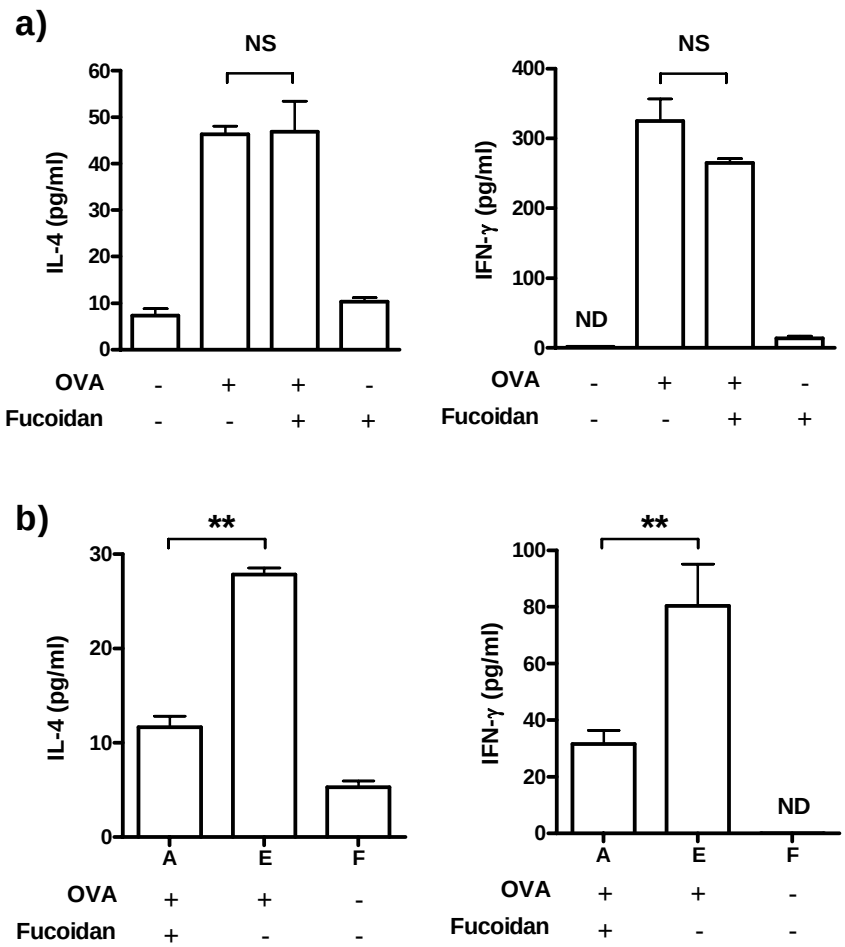

a)
Total IgE (ng/ml)
750
500
250
0
0 1 2 3 4 5 6 7
Weeks
A
B
C
D
E
F
b)
Total IgE (ng/ml)
**
**
750
500
250
0
A B C D E F
OVA + + + + + -
Fucoidan + + + + - -
Starting point (fucoidan) -1 week -1 day 0 +1 week
c)
Anti-OVA IgE (ng/ml)
**
**
160
140
120
100
80
60
40
20
0
ND
A B C D E F
OVA + + + + + -
Fucoidan + + + + - -
Starting point (fucoidan) -1 week -1 day 0 +1 week

Fig. 2.


a)
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IL-4 (pg/ml)
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50
40
30
20
10
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OVA - + + -
Fucoidan - - + +
NS
IFN-γ (pg/ml)
400
300
200
100
ND
0
OVA - + + -
Fucoidan - - + +
b)
**
IL-4 (pg/ml)
30
20
10
0
A E F
OVA + + -
Fucoidan + - -
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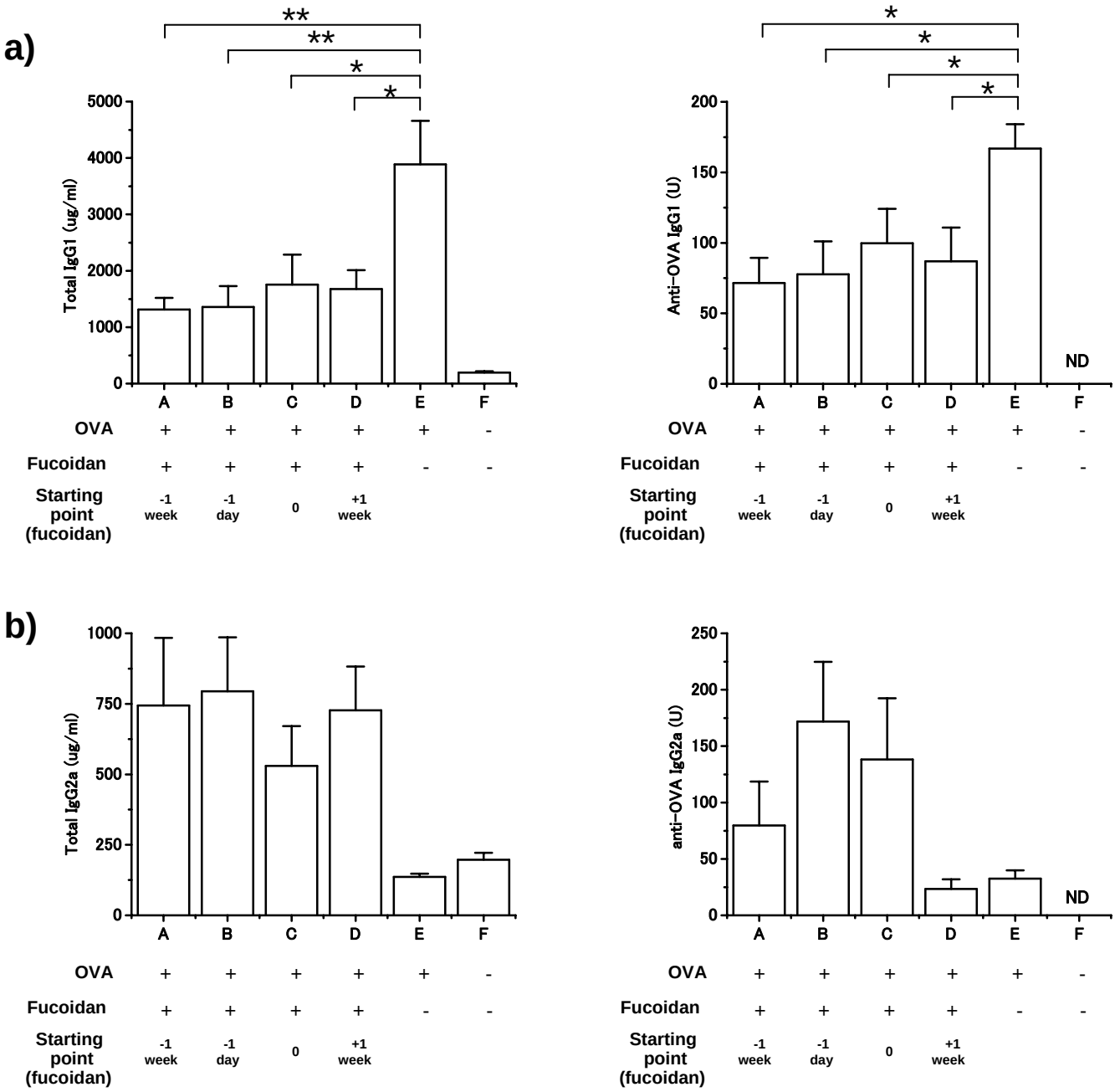
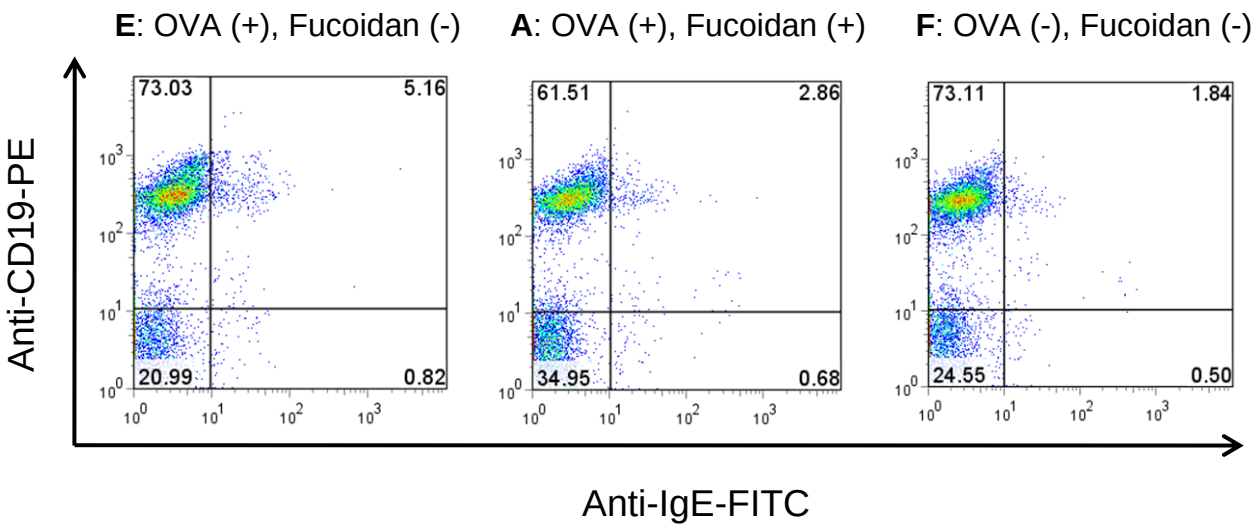
Fig. 3.

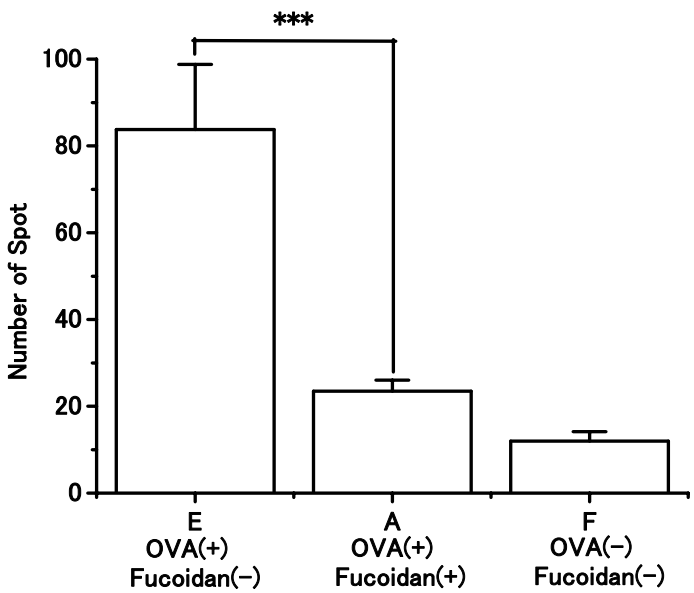


Supplementary figure 1.

a)


E: OVA (+), Fucoidan (-)
A: OVA (+), Fucoidan (+)
F: OVA (-), Fucoidan (-)
73.03
5.16
20.99
0.82
61.51
2.86
34.95
0.68
73.11
1.84
24.55
0.50
Anti-CD19-PE
Anti-IgE-FITC

b)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Number of Spot
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80
60
40
20
0
E
OVA(+)
Fucoidan(−)
A
OVA(+)
Fucoidan(+)
F
OVA(−)
Fucoidan(−)

Fig. 4.