

Dietary Fructo-Oligosaccharides Attenuate Early Activation of CD4⁺ T Cells Which Produce both Th1 and Th2 Cytokines in the Intestinal Lymphoid Tissues of a Murine Food Allergy Model

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Keywords

Fructo-oligosaccharides · Food allergy · Antigen-specific CD4⁺ T cells · Mesenteric lymph nodes · Peyer's patches · T helper cells

Abstract

Background: Fructo-oligosaccharides (FOS) are prebiotic agents with immunomodulatory effects involving improvement of the intestinal microbiota and metabolome. In this study, we investigated the cellular mechanisms through which FOS modulate intestinal antigen-specific CD4⁺ T cell responses in food allergy, using OVA23-3 mice. **Methods:** OVA23-3 mice were fed an experimental diet containing either ovalbumin (OVA) or OVA and FOS for 1 week. Body weight and mucosal mast cell protease 1 in the serum were measured as the indicator of intestinal inflammation. Single-cell suspensions were prepared from intestinal and systemic lymphoid tissues for cellular analysis. Cytokine production was measured by ELISA. Activation markers and intracellular cytokines in CD4⁺ T cells were analyzed by flow cytometry. Activated CD4⁺ T cells were purified to examine cytokine production. **Results:** Dietary intake of FOS provided moderate

protection from the intestinal inflammation induced by the OVA-containing diet. FOS significantly reduced food allergy-induced Th2 cytokine responses in intestinal tissues but not in systemic tissues. FOS decreased OVA diet-induced IFN-γ⁺IL-4⁺ double-positive CD4⁺ T cells and early-activated CD45RB^{high}CD69⁺CD4⁺ T cells in the mesenteric lymph nodes. Furthermore, we confirmed that these CD45RB^{high}CD69⁺CD4⁺ T cells are able to produce high levels of IFN-γ and moderate level of IL-4, IL-10, and IL-13. **Conclusions:** Dietary intake of FOS during the development of food allergy attenuates the induction of intestinal Th2 cytokine responses by regulating early activation of naïve CD4⁺ T cells, which produce both Th1 and Th2 cytokines. Our results suggest FOS might be a potential food agent for the prevention of food allergy by modulating oral sensitization to food antigens. © 2017 S. Karger AG, Basel

Introduction

Fructo-oligosaccharides (FOS) are oligosaccharides composed of a glucose unit linked to 2–4 fructose units. FOS are prebiotics that are indigestible in the small in-

testine of humans and animals, and so they reach the large intestine intact where they are then degraded by commensal bacteria. Consequently, administration of FOS not only alters the composition of intestinal microbiota but also generates short-chain fatty acids (SCFAs) such as acetate, propionate, and butyrate, resulting in a reduced colonic pH [1–4]. These alterations in the intestinal environment through the intake of FOS exert several beneficial physiological effects on humans and animals [5].

Previous studies have reported that dietary supplementation with FOS has a variety of immunomodulatory effects, i.e., not limited to the stimulation of mucosal immunoglobulin A responses [4, 6, 7] and including the amelioration of inflammatory diseases like colitis [8, 9] and allergy [10–12]. In fact, using a traditional allergy model in which experimental mice received systemic challenges with an antigen and adjuvant, FOS were reported to ameliorate allergic intestinal inflammation to food antigen, as characterized by the reduction of Th2-associated chemokine receptor (CCR)4-bearing cells in the duodenum [10]. However, it remains to be determined whether FOS modulate immune reactions preceding systemic sensitization with the antigen and adjuvant, which include potentially immune-disrupting effects and/or oral sensitization with the antigen directly. In addition, it remains poorly understood how FOS regulate cellular mechanisms such as antigen-specific CD4⁺ T cell responses during the development of food allergy. To elucidate these issues, we used the OVA23-3 mouse model of food allergy. This transgenic mouse line possesses CD4⁺ T cells that express a T cell receptor specific for ovalbumin (OVA), a major allergen present in egg white. It has been reported that feeding an egg-white-containing diet to these mice *ad libitum* can induce intestinal and systemic Th2 cytokine responses as well as intestinal inflammation with weight loss attributed to IL-4-producing, antigen-specific CD4⁺ T cells [13–16]. In addition, both mesenteric lymph nodes (MLN) and Peyer's patches (PP) are key sites for the generation and maintenance of IL-4-producing CD4⁺ T cells in the development of food allergy [15]. Moreover, by using germ-free OVA23-3 mice, we have shown that antigen-specific CD4⁺ T cell responses to a dietary antigen can be regulated by the intestinal microbiota [17].

In this study, we investigated the effect of dietary intake of FOS on intestinal and systemic antigen-specific CD4⁺ T cell responses in the development of food allergy using OVA23-3 mice.

Materials and Methods

Mice

OVA23-3(+/-) mice were obtained by backcrossing OVA23-3(+/+) mice with BALB/c mice (CLEA Japan, Tokyo, Japan). All mice were maintained at a room temperature of 23–25°C with 40–60% humidity with a 12-h:12-h light-dark cycle, and they were provided with standard chow and sterile deionized water *ad libitum*. All animal experiments were carried out at the animal facility of Nihon University College of Bioresource Sciences, in accordance with the guidelines of Nihon University for the care and use of laboratory animals (approval Nos. AP14B058, AP15B040, AP15B045, and AP16B036).

Female OVA23-3(+/-) mice, 8–12 weeks of age, were randomly divided into 3 experimental groups: a control group, an OVA group, and an OVA-FOS group. Each group was fed an AIN76-formula purified experimental diet. The control group received a diet containing 20% casein, the OVA group a diet containing 20% egg-white protein (as a substitute for casein), and the OVA-FOS group a diet containing 20% egg-white protein in combination with 7.5% FOS (as a substitute for sucrose) (Funabashi Farm, Funabashi, Japan). In some experiments, the FOS group was placed separately, and received a diet containing 20% casein in combination with 7.5% FOS. These diets were supplied for 1 week, with the mice being weighed every 2–3 days and euthanized at day 7 to remove lymphoid tissues for further cellular analysis. Serum samples were collected just before the euthanasia.

Cell Preparation

Single-cell suspensions were prepared from the mesenteric lymph nodes (MLN), Peyer's patches (PP), and spleens (SPL) of each group of mice. Briefly, the MLN and SPL were physically dissociated with slide-glasses in RPMI 1640 medium supplemented with 50 U/mL penicillin, 50 µg/mL streptomycin, 2 mM L-glutamine, and 50 µM 2-mercaptoethanol; this was followed by filtration through a 40-µm cell-strainer. The cell suspensions were treated with BD Pharm Lyse™ lysing buffer (BD Bioscience, Franklin Lakes, NJ, USA) to lyse erythrocytes, and then resuspended in RPMI 1640 medium containing 10% FCS. PP were washed in RPMI 1640 medium, minced into small pieces, and then incubated for 50 min in RPMI 1640 medium containing 10% FCS, 1 mg/mL collagenase D (Roche Diagnostics, Indianapolis, IN, USA), and 20 µg/mL DNase I (Roche Diagnostics). The cell suspensions were filtered through a 70-µm cell-strainer and resuspended in RPMI 1640 medium containing 10% FCS.

Flow Cytometry

Freshly isolated cells were washed and stained in FACS buffer (Dulbecco's modified Eagle's medium containing 1% BSA, 10 mM HEPES, 2 mM sodium pyruvate, 10⁻³ mM EDTA, and 0.01% NaN₃). Nonspecific binding to Fc receptors was blocked with anti-CD16/CD32(93) for 15 min. The cells were then stained with fluorescence-conjugated antibodies for 20 min on ice. All fluorescence-labeled antibodies, dyes, and staining buffer sets were purchased from eBioscience (San Diego, CA, USA) unless otherwise specified. The following antibodies were used: Alexa Fluor 647 or PerCPy5.5 anti-CD4 (RM4-5, BD Biosciences), FITC anti-CD45RB (C363.16A), PE anti-CD69 (H1.2F3, BD Biosciences), and APC anti-CD25 (PC61.5). For the staining of intracellular

molecules, the cells were fixed and permeabilized using the FoxP3 staining buffer set, and then stained with PE-Cy7-conjugated anti-Foxp3 (FJK-16s) or PE anti-CTLA-4 (UC10-4F10-11, BD Biosciences) or their corresponding isotype control antibodies (PE-Cy7-labeled rat IgG2a or PE-labeled hamster IgG) for 30 min on ice. For intracellular cytokine staining, single-cell preparations were stimulated with 50 ng/mL PMA and 500 ng/mL ionomycin in the presence of BD GolgiStop™ (Monensin, BD Biosciences) for 4 h. The cells were stained with FITC anti-CD4 (RM4-5), followed by staining with fixable viability dye 780 to discriminate between live and dead cells. The cells were then fixed and permeabilized using the intracellular fixation and permeabilization buffer set, and stained with antibodies against IFN-γ (XMG1.2), IL-4 (11B11), and IL-13 (eBio13A). Flow-cytometric analyses were performed using a FACSCantoII (BD Biosciences) and an analysis was performed using FlowJo software v7.6.5 (Tree Star, Ashland, OR, USA).

Cell Purification and Sorting

CD4⁺ cells from the MLN of experimental OVA23-3 mice were enriched using anti-CD4 antibody-conjugated immunomagnetic beads in combination with the MACS cell-separation system (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. Enriched CD4⁺ cells were stained with Alexa Fluor 647 anti-CD4, FITC anti-CD45RB, and PE anti-CD69, and sorted into 4 populations based on the expression of CD45RB and CD69 using a FACS Aria II (BD Biosciences). The purity of sorted CD4⁺ cells was >95%.

Cell Culture

Whole cells (2.5×10^6 cells) from the MLN, PP, or SPL of the experimental mice were cultured in 48-well flat-bottom plates in the presence of OVA (5 mg/mL, $2 \times$ Cryst, SEIKAGAKU CO, Tokyo, Japan) for 48 h.

T cells isolated by fluorescence-activated cell sorting (FACS) (1×10^5 cells) were cultured for 48 h in 96-well round-bottom plates in the presence of OVA (5 mg/mL) and mitomycin C-treated, T cell-depleted (Thy1.2⁻) SPL cells (3×10^5 antigen-presenting cells) isolated from nontreated BALB/c mice. Culture supernatants were harvested for subsequent analysis by enzyme-linked immunosorbent assay (ELISA).

ELISA

Cytokine concentration in culture supernatants and the level of mucosal mast cell protease 1 (mMCP1), total IgE, and OVA-specific IgE in the serum were assayed by sandwich ELISA. Measurement of IFN-γ, IL-2, IL-4, and total and OVA-specific IgE was performed as described previously [14, 17, 18]. Purified rat anti-mouse IFN-γ (AN-18), IL-2 (JES6-1A12), and IL-4 (BJD4-1D11), IgE (R35-92) monoclonal antibodies, or OVA were used as capture reagents; biotinylated rat anti-mouse IFN-γ (XMG1.2), IL-2 (JES6-5H4), IL-4 (BJD6-34G2), and IgE (LO-ME-2, Serotec) monoclonal antibodies were used as detection antibodies. All antibodies were purchased from BD Biosciences unless otherwise specified. The level of IL-10 was measured using the BD OptEIA mouse ELISA set, and the levels of IL-13 and mMCP1 were measured using the Mouse ELISA Ready-SET GO! (BD Biosciences and eBiosciences, respectively) according to the manufacturer's instructions.

Statistical Analysis

Statistical significance was calculated using SPSS software (IBM SPSS Statistics 20, IBM Machines Corp., Armonk, NY, USA). Data were analyzed using one-way analysis of variance (ANOVA) followed by the Tukey HSD test or the Dunnett T3 test. Differences were considered statistically significant for values of $p < 0.05$.

Results

Dietary FOS Have a Moderate Protective Effect on Intestinal Inflammation Induced by Food Allergy

To determine the protective effect of FOS on intestinal inflammation caused by food allergy in OVA23-3 mice, we measured the body weight of all experimental mice over the course of each experiment. Consistent with previous reports [13, 15, 16], the mice in the OVA group, which were fed a diet containing 20% egg white, equivalent to approximately 10% OVA, displayed a reduced body weight at day 7 that was significantly lower than that of the control group (Fig. 1a). The OVA-FOS group had slightly more weight after receiving the experimental diet compared to the OVA group ($p = 0.12$, Fig. 1a). We also confirmed that FOS feeding alone did not affect the body weight of the mice (online suppl. Fig. 1A; see www.karger.com/doi/10.1159/000481984 for all online suppl. material).

We next analyzed the level of mMCP1 in the serum, which has been reported as a marker of intestinal mast cell degranulation and intestinal inflammation [19]. No mMCP1 was detected in the serum from the control group but the level was significantly increased in the OVA group (Fig. 1b). The OVA-FOS group showed a slightly lower level of mMCP1 than the OVA group ($p = 0.13$; Fig. 1b). These data suggest that dietary intake of FOS provides a mild protection from intestinal inflammation induced by food allergy.

We also measured the serum level of OVA-specific and total IgE from each experimental group. Although no OVA-specific IgE was detected and the level of total IgE was very low in all groups at this time point (after 1 week of feeding them the experimental diet), the results show that the level of total IgE was slightly lower in the OVA-FOS group than in the OVA group ($p = 0.08$; Fig. 1c).

FOS Supplementation Attenuates Th2 Cytokine Responses in the Intestinal Immune Tissues during the Development of Food Allergy

To investigate the effect of FOS supplementation on intestinal and systemic responses during the develop-

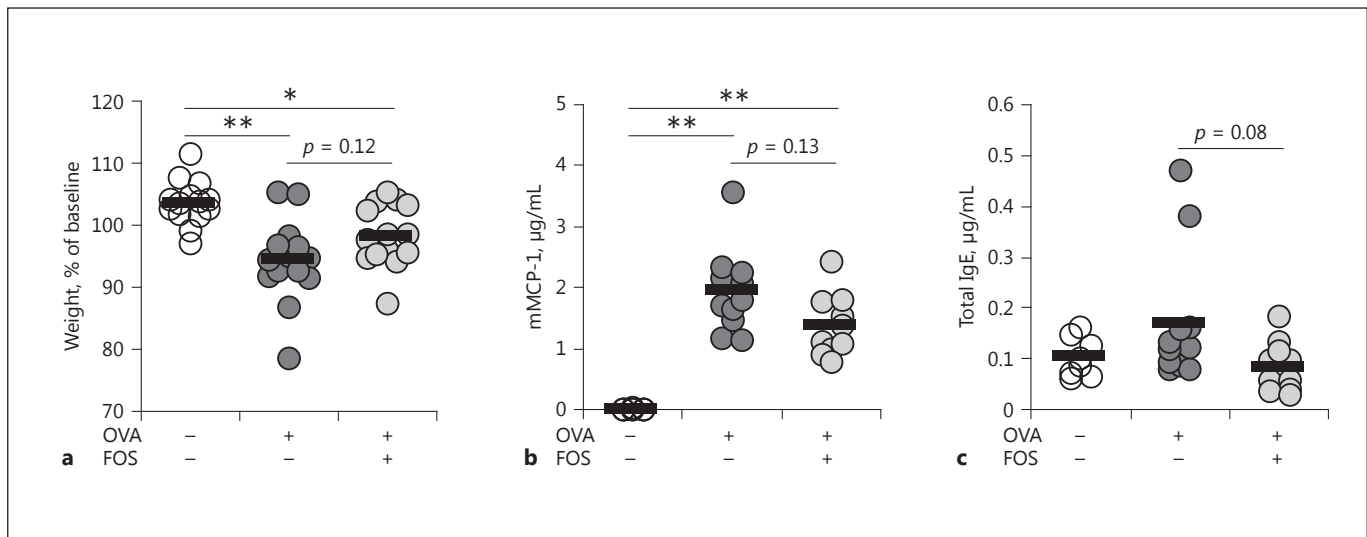


Fig. 1. Dietary FOS have a moderate protective effect on intestinal inflammation caused by food allergy. **a** The body weight of each mouse at day 7 was indicated as a percentage of the initial weight at day 0. **b, c** The level of mMCP-1 (**b**) and total IgE (**c**) in the se-

rum samples collected at day 7 was assayed by ELISA. Data represent the body weight or the concentration of each protein from an individual mouse ($n = 9\text{--}14$ for each group). * $p < 0.05$, ** $p < 0.01$.

ment of food allergy, we measured the cytokine production of single cells prepared from the MLN, PP, or SPL of each experimental group after in vitro stimulation with OVA. Cells isolated from tissues in the OVA group showed significantly higher levels of the Th2 cytokines IL-4 and IL-13, and lower levels of the Th1 cytokines IFN- γ and IL-2 when compared to similar cells from the control group (Fig. 2). This suggests that Th2-dominant cytokine responses were induced in the intestinal and systemic tissues following the consumption of an OVA-containing diet. Remarkably, cells isolated from the intestinal tissues of mice from the OVA-FOS group showed significantly lower IL-4 and IL-13 production than those from the OVA group (Fig. 2), but IFN- γ and IL-2 production was comparable. On the other hand, cells prepared from the SPL, which reflect systemic immunity, showed similar cytokine production in the OVA-FOS and OVA groups (Fig. 2). We also confirmed that FOS-feeding alone had no effect on Th2 cytokines and a minimal effect on the Th1 cytokine responses by the cells from both the intestinal and systemic tissues (online suppl. Fig. 1B). These data suggest that FOS supplementation decreases Th2 cytokine responses in the intestinal immune tissues during the development of food allergy.

FOS Supplementation Decreases the Induction of IFN- γ ⁺IL-4⁺ Double-Positive CD4⁺ T Cells during the Development of Food Allergy

We next performed intracellular cytokine staining to determine the effect of FOS supplementation on the cytokine profile of intestinal and systemic CD4⁺ T cells during the development of food allergy. The results showed that expression of IL-4, IL-13, and IFN- γ in CD4⁺ T cells in the MLN and SPL was significantly higher in both the OVA and OVA-FOS groups than in the control group (Fig. 3a), suggesting that the OVA-containing diet increased the potential of CD4⁺ T cells from the MLN and SPL to produce both Th1 and Th2 cytokines. Notably, the expression of Th2 cytokines in CD4⁺ T cells from the MLN was significantly lower in mice from the OVA-FOS group than in those from the OVA group (Fig. 3a), despite a comparable level of cytokine expression in the CD4⁺ T cells from the SPL. In addition, as a result of a more detailed analysis of IL-4-expressing CD4⁺ T cells, we found that both IFN- γ ⁺IL-4⁺ and IFN- γ ⁺IL-4⁺ CD4⁺ T cells were induced in the MLN and SPL after the consumption of an OVA-containing diet (data not shown; Fig. 3b, c). In particular, the percentage of IFN- γ ⁺IL-4⁺ double-positive but not that of IFN- γ ⁺IL-4⁺ single-positive CD4⁺ T cells was significantly lower in the MLN of the OVA-FOS group than in the OVA group (Fig. 3b, c).

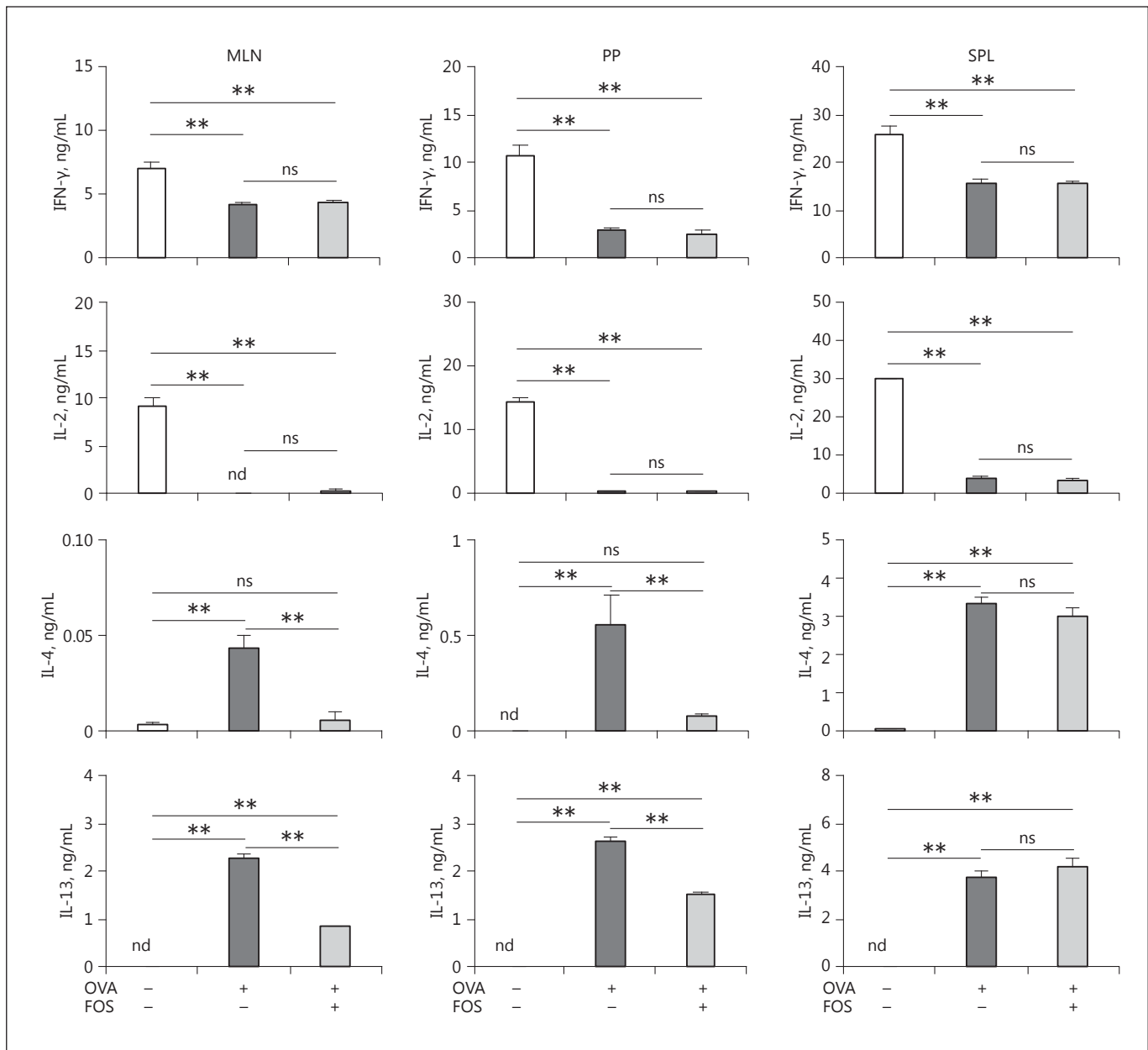


Fig. 2. Dietary FOS decrease food allergy-induced Th2 cytokine responses in the intestinal tissues. Single cells from the MLN, PP, and SPL of each experimental group were stimulated in vitro with 5 mg/mL of OVA for 48 h. The concentration of each cytokine in

the culture supernatants was assayed by ELISA. Data are shown as mean $\pm$ SD, and are representative of 4 independent experiments. ** $p < 0.01$. nd, not detected; ns, not significant.

FOS Supplementation Does Not Promote Regulatory T Cell Responses during the Development of Food Allergy

In order to determine the cellular mechanisms underlying the reduced food allergy-induced cytokine responses in intestinal CD4⁺ T cells resulting from FOS supple-

mentation, we analyzed the expression of regulatory T (Treg) cells and their related molecules. The expression of Foxp3 and CTLA4 in CD4⁺ T cells was higher in all tissues of the OVA and OVA-FOS groups than in the control group, but comparable in the OVA and OVA-FOS groups (Fig. 4a). Next, we measured the amount of anti-

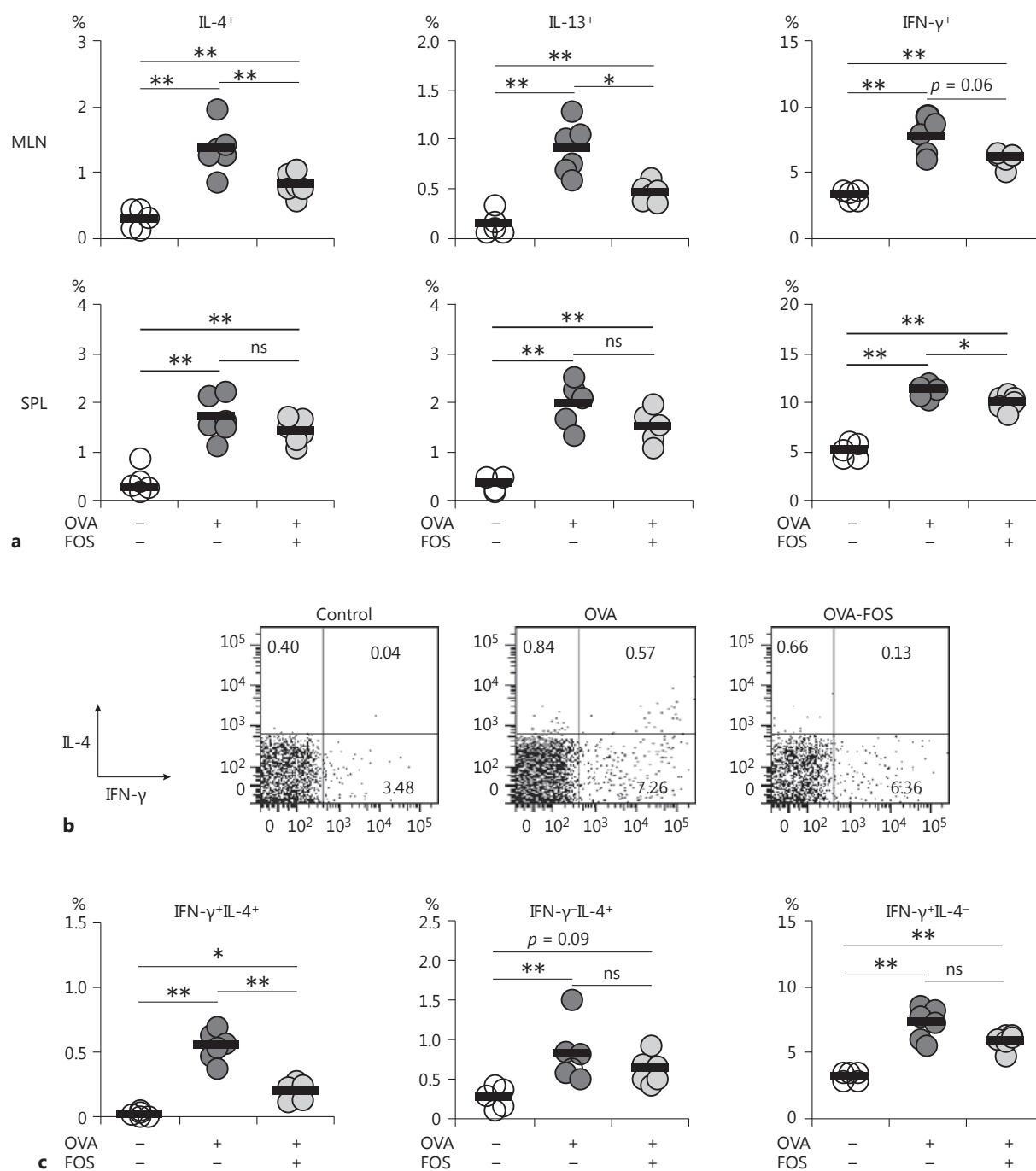


Fig. 3. Dietary FOS decrease the induction of IFN-γ⁺IL-4⁺ double-positive CD4⁺ T cells in MLN during the development of food allergy. MLN and SPL cells from each experimental group were stimulated with PMA/ionomycin and BD GolgiStop[™] for 4 h. The cells were stained with anti-CD4 and fixable viability dye eFluor780 followed by intracellular staining with antibodies

against IL-4, IL-13, and IFN-γ. **a** The percentage of viable CD4⁺ cells expressing each cytokine. **b** Dot plots of IFN-γ and IL-4 expression in MLN CD4⁺ cells. **c** The percentage of viable CD4⁺ cells from the MLN expressing both IFN-γ and IL-4. **a, c** Data are from 5–6 independent experiments. * $p < 0.05$, ** $p < 0.01$. ns, not significant.

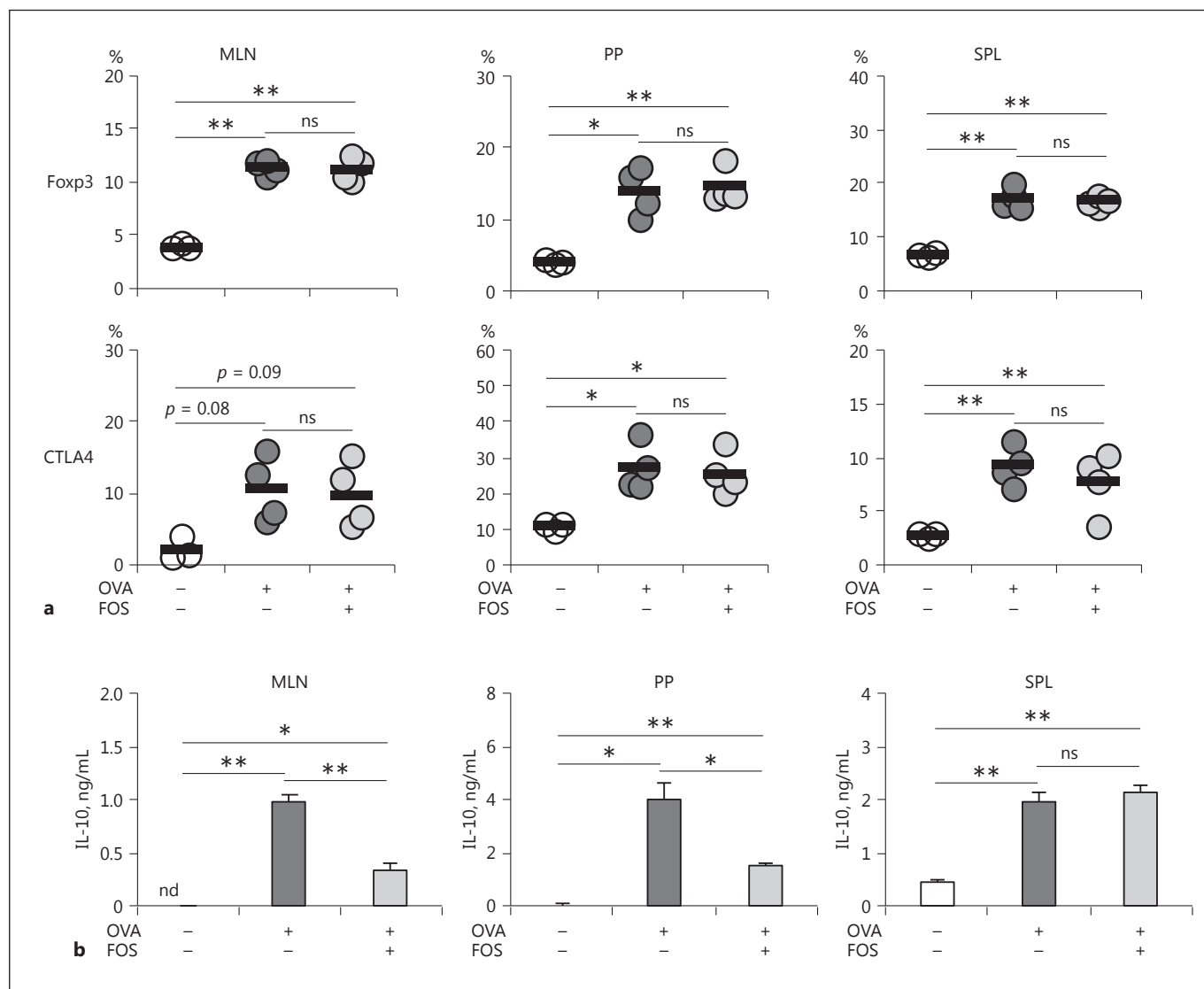


Fig. 4. Dietary FOS do not promote regulatory T cell responses during the development of food allergy. Cells from the MLN, PP, and SPL of each experimental group were stained with anti-CD4, followed by intracellular staining with antibodies against Foxp3 and CTLA-4. **a** The percentage of CD4⁺ cells expressing Foxp3 or CTLA4. Data are representative of 3–4 independent experiments.

b Single cells from the MLN, PP, and SPL of each experimental group were stimulated in vitro with 5 mg/ml of OVA for 48 h. The concentration of IL-10 in the culture supernatants was assayed by ELISA. Data are shown as mean $\pm$ SD and are representative of 4 independent experiments. * $p < 0.05$, ** $p < 0.01$. nd, not detected; ns, not significant.

inflammatory cytokine IL-10 produced by single cells prepared from each monitored tissue type in each experimental group. We observed elevated levels of IL-10 in all tissue preparations from the OVA and OVA-FOS groups compared to the control group (Fig. 4b). However, the production of IL-10 from SPL cells was comparable in the OVA and OVA-FOS groups, and the cytokine production from the intestinal tissues of mice from the OVA-FOS group was significantly lower than in the OVA group

(Fig. 4b). These data suggest that FOS supplementation did not contribute to the expansion or activation of Treg cells in our experimental systems.

FOS Supplementation Decreases the Induction of Early-Activated CD4⁺ T Cells during the Development of Food Allergy

We next tested the possibility that FOS supplementation may affect the process of T cell activation during the

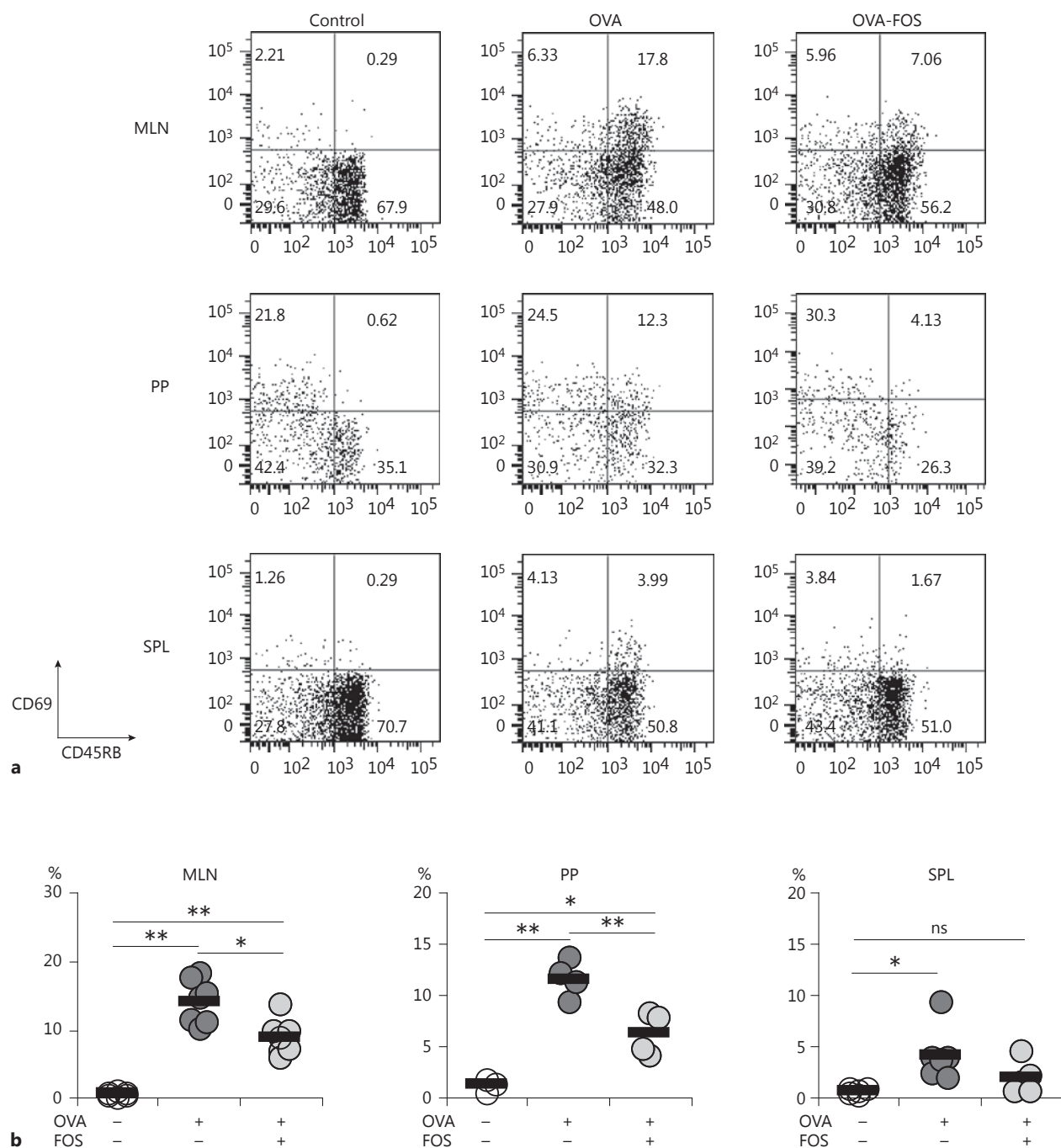


Fig. 5. Dietary FOS decrease the induction of activated CD4⁺ T cells in the intestinal tissues during the development of food allergy. Cells from the MLN, PP, and SPL of each experimental group were stained with anti-CD4, anti-CD45RB, and anti-

CD69. **a** Dot plots of CD45RB and CD69 expression in CD4⁺ cells from each tissue. **b** The percentage of CD4⁺ cells expressing CD45RB^{high}CD69⁺ population. Data are representative of 3–7 independent experiments. * $p < 0.05$, ** $p < 0.01$.

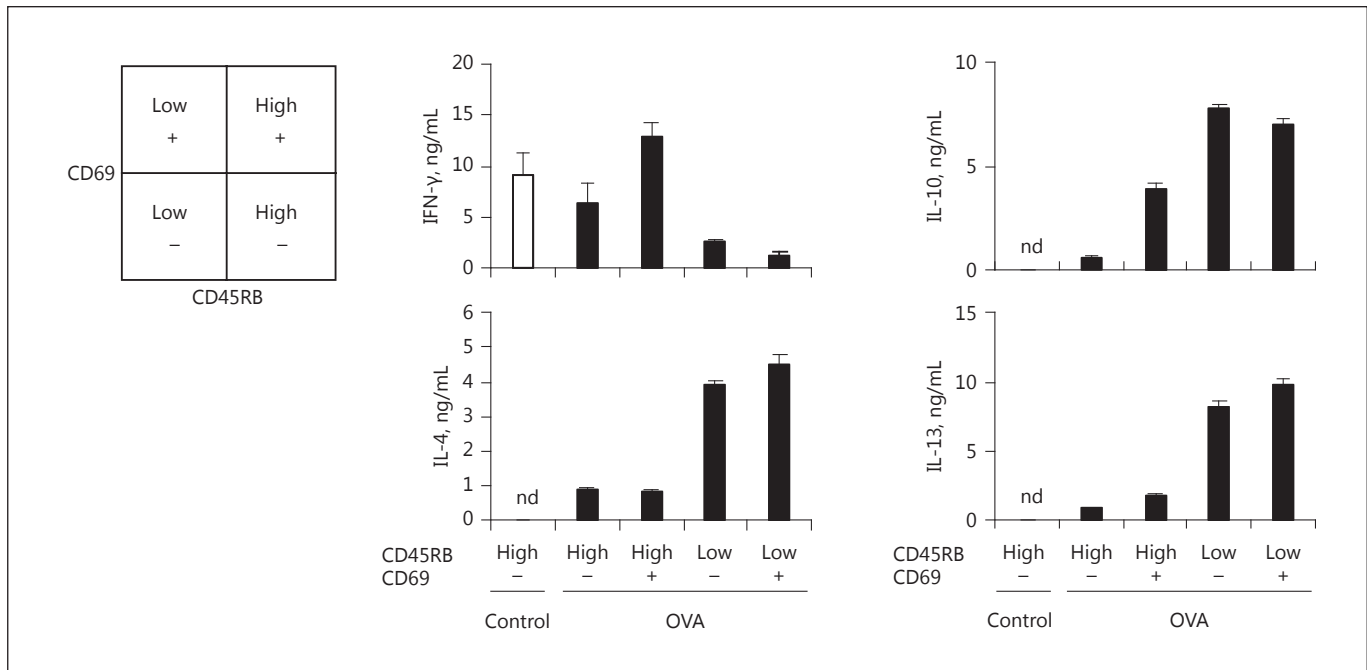


Fig. 6. CD45RB^{high}CD69⁺CD4⁺ T cells induced in food allergy produce both Th1 and Th2 cytokines. MLN CD4⁺ cells from the OVA and control groups were distributed into 4 different populations based on the expression of CD45RB and CD69, using FACS. The sorted CD4⁺ T cells were stimulated in vitro with 5 mg/mL of OVA

in the presence of antigen-presenting cells for 48 h. The levels of cytokines in the supernatants were assayed using ELISA. Data are shown as mean $\pm$ SD and are representative of 3 independent experiments. nd, not detected.

development of food allergy. To this end, we analyzed the cell surface expression of CD45RB and CD69, which have been widely used to define the activated and naïve states of CD4⁺ T cells, respectively. Interestingly, we found that the CD45RB^{high}CD69⁺CD4⁺ population, which represents early-activated CD4⁺ T cells, was present in very low numbers in all tissues from the control group, but was increased in the intestinal tissues and slightly augmented in the SPL after the consumption of an OVA-containing diet (Fig. 5a, b). In the groups fed OVA-containing diets, the proportion of early-activated CD4⁺ T cells was significantly lower in the MLN and PP from the OVA-FOS group than in those from the OVA group (Fig. 5a, b). These data suggest that FOS supplementation attenuates the activation of naïve CD4⁺ T cells in the intestinal tissues during the development of food allergy.

Food Allergy-Induced Activated CD45RB^{high}CD69⁺CD4⁺ T Cells Produce High Levels of Th1 Cytokines and Moderate Levels of Th2 Cytokines

Finally, we wanted to determine the functional properties of the CD45RB^{high}CD69⁺CD4⁺ T cells augmen-

ted in the intestinal tissues of mice fed an OVA-containing diet. To this end, we sorted 4 different populations of CD4⁺ T cells from the MLN of mice in the control or OVA group, based on their levels of CD45RB and CD69. We then stimulated them with OVA in the presence of antigen-presenting cells in vitro. The results show that the cytokine profile of these FACS-purified CD4⁺ T cells was largely associated with the expression level of CD45RB rather with that of CD69; CD45RB^{high} populations had higher levels of the Th1 cytokine IFN- γ and lower levels of the Th2 cytokines IL-4, IL-10, and IL-13 when compared with the CD45RB^{low} populations (Fig. 6). In addition, we found that the CD45RB^{high}CD69⁺ population from the OVA group produced moderate levels of IL-4, IL-10, and IL-13 as well as the highest level of IFN- γ among all of the CD4⁺ populations tested (Fig. 6). Furthermore, these early-activated CD4⁺ T cells showed higher Th2 cytokine production than the naïve CD45RB^{high}CD69⁻ cell population from the control group as well as higher production of IL-10 and IL-13 than the naïve population from the OVA group (Fig. 6).

Discussion

In this study, we showed the cellular mechanisms by which the dietary intake of FOS modulates antigen-specific CD4⁺ T cell responses in food allergy by making use of the OVA23-3 mouse model. It has been reported that simply feeding an egg-white-containing diet to OVA23-3 mice can lead to excessive IL-4 production from OVA-specific CD4⁺ T cells in the MLN, which has an inhibitory influence on the induction of Treg cells, resulting in intestinal inflammation [15, 16]. Here, we demonstrate that the administration of FOS reduces intestinal Th2 cytokine responses and has a mild protective effect against the intestinal inflammation induced by feeding an OVA-containing diet to OVA23-3 mice. As SCFAs metabolized from dietary fibers have been reported to be important inducers of colonic Treg cells [20, 21], we initially hypothesized that dietary intake of FOS could promote the expansion and/or activation of Treg cells through an increase in luminal SCFAs. However, we did not observe any effect of FOS on the number of Treg cells, the amount of their effector molecules, or the production of anti-inflammatory cytokine IL-10 from cell preparations made from any of the tissues assessed. In addition, the pattern of IL-10 production was similar to that of other Th2 cytokines, indicating that, in our experimental system, IL-10 behaved as a Th2 cytokine rather than as a cytokine produced by Treg cells. We also confirmed that the feeding of FOS alone for 1 week did not affect the number of Treg cells, and it activated the CD4⁺ T cells in the intestinal and systemic tissues (online suppl. Fig. 1C, D). These results suggest that FOS supplementation alters intestinal Th2 cytokine responses induced by feeding mice an OVA-containing diet via cellular mechanisms other than the modulation of Treg cells.

Intriguingly, we found that dietary FOS diminished the OVA-induced expansion of IFN- γ - and IL-4-coexpressing CD4⁺ T cells but not that of IL-4 single-positive CD4⁺ T cells in the MLN. IFN- γ ⁺IL-4⁺ double-positive CD4⁺ T cells have reportedly been generated *in vivo* during infection by certain parasites [22, 23], i.e., in strong, Th2-dominated settings, and also *in vitro*, from naïve CD4⁺ T cells stimulated with anti-CD3 and anti-CD28 in the presence of both IFN- γ and IL-4 [24]. These double-positive CD4⁺ T cells express T-bet and GATA3 [22], the key transcription factors for Th1 and Th2 cells, respectively, suggesting combined transcriptional properties. Furthermore, it was reported that these hybrid CD4⁺ T cells cause less immunopathology than Th2 cells in an allergic airway inflammation model, due to competition

between Th1 and Th2 cytokine production [23]. In this regard, it is possible that the inability of dietary FOS to prevent the induction of IL-4 single-positive CD4⁺ T cells might be associated with moderate protection from food allergy-induced weight loss and an increase in the serum level of mMCP1 in OVA23-3 mice. Furthermore, we found that dietary intake of FOS decreased the generation of a unique, CD45RB^{high}CD69⁺ CD4⁺ T cell population in the intestinal tissues after the consumption of an OVA-containing diet. It has been shown that upon antigen stimulation of T cells, CD69 can be rapidly upregulated within the first cycle of cell division and then decreased with additional cycles. In contrast, CD45RB is expressed at high levels on naïve CD4⁺ T cells and downregulated in proliferating CD4⁺ T cells [25]. Thus, it is likely that the CD45RB^{high}CD69⁺ population comprises the early-activated CD4⁺ T cells that have not yet divided, or are within an early proliferating stage. Moreover, we determined the functional properties of the CD45RB^{high}CD69⁺CD4⁺ T cells representing both Th1 and Th2 hybrid cytokine production. Together, our data suggest that dietary supplementation with FOS diminishes the induction of IFN- γ - and IL-4-producing early-activated CD4⁺ T cells during the development of food allergy. Further investigations are needed to determine the function of these hybrid CD4⁺ T cells in the intestinal inflammation of this mouse model of food allergy.

Although it is still unknown how dietary FOS inhibits the induction of these early-activated CD4⁺ T cells during the development of food allergy, we speculate that dietary FOS may modulate the function of antigen-presenting cells, such as dendritic cells, in order to initiate and/or activate CD4⁺ T cell responses in the intestinal tissues. Trompette et al. [26] have recently reported that propionic acid, a metabolite generated from dietary fiber, could increase hematopoiesis and modify dendritic cell function by downregulating costimulatory molecules, resulting in protection from allergic asthma. Since intake of a high-fiber diet has been shown to increase the concentration of SCFAs not only in the intestines but also in the serum [26], it is quite likely that SCFAs could regulate the properties of dendritic cells in the intestinal mucosa as well as in regional lymph nodes, such as the MLN.

We observed that supplementation with FOS had a moderate protective effect on weight loss, an increase in the serum level of mMCP1 and total IgE, and little effect on the systemic Th2 cytokine responses induced by the consumption of an OVA-containing diet. It has been reported that elevated IL-4 production by OVA-specific CD4⁺ T cells can be observed in the MLN of OVA23-3

mice 2 days after they are supplied with an OVA-containing diet [16], whereas FOS might exert their effects through changes in the intestinal microbiota and their metabolites in the latter half of the week that mice are fed the experimental diet, potentially leading to limited prevention of intestinal events caused by allergic food reaction. Thus, it is possible that prophylactic treatment or a longer intervention with FOS could be more efficient in inhibiting the induction of intestinal inflammation and systemic allergic responses.

Taken together, our results demonstrate that the dietary intake of FOS diminishes the early activation of naïve intestinal CD4⁺ T cells, which produce both Th1 and Th2 cytokines in a mouse model of food allergy, providing a moderate protective effect against intestinal inflammation due to food allergy. Importantly, our study shows that the intake of both FOS and a food antigen for a short period (only 1 week) can modify the antigen-specific CD4⁺ T cell responses induced by oral sensitization to food antigens. This is in agreement with a previous report

using a traditional model of food allergy, which showed the effects of supplementation of FOS for 7–8 weeks during a period of allergic sensitization [10]. Our results suggest that FOS may be a potential food agent for the prevention of food allergy. Further studies will determine more effective methods to consume FOS for use as a therapeutic agent.

Acknowledgement

We thank Dr. S. Habu (Tokai University, Japan) for providing the OVA23-3 mice. We also thank Meiji Co. Ltd. for providing the fructo-oligosaccharides (Meiologo-P®). This work was supported by a Grant-in-Aid from the Japan Society for the Promotion of Science (grant Nos. 24580200 and 15K07443).

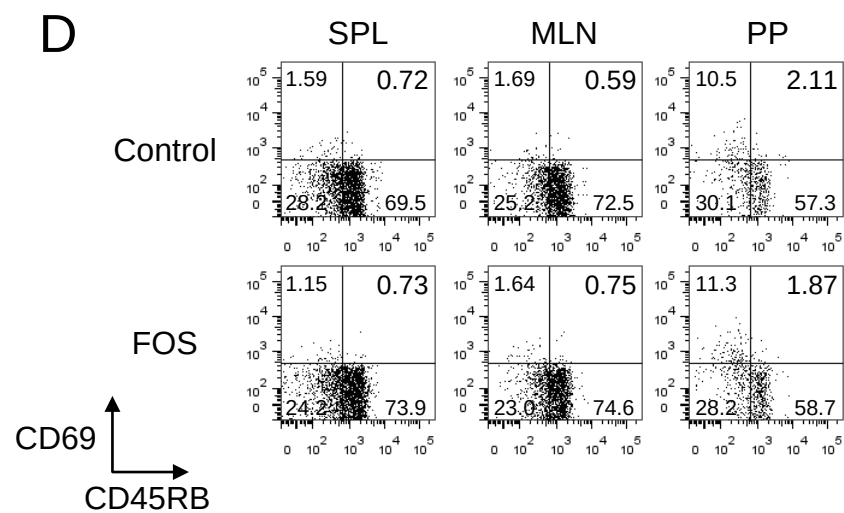
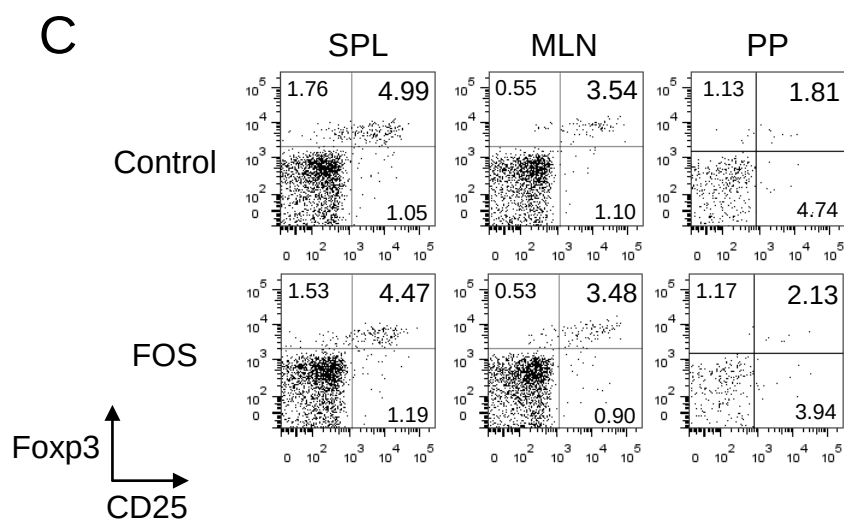
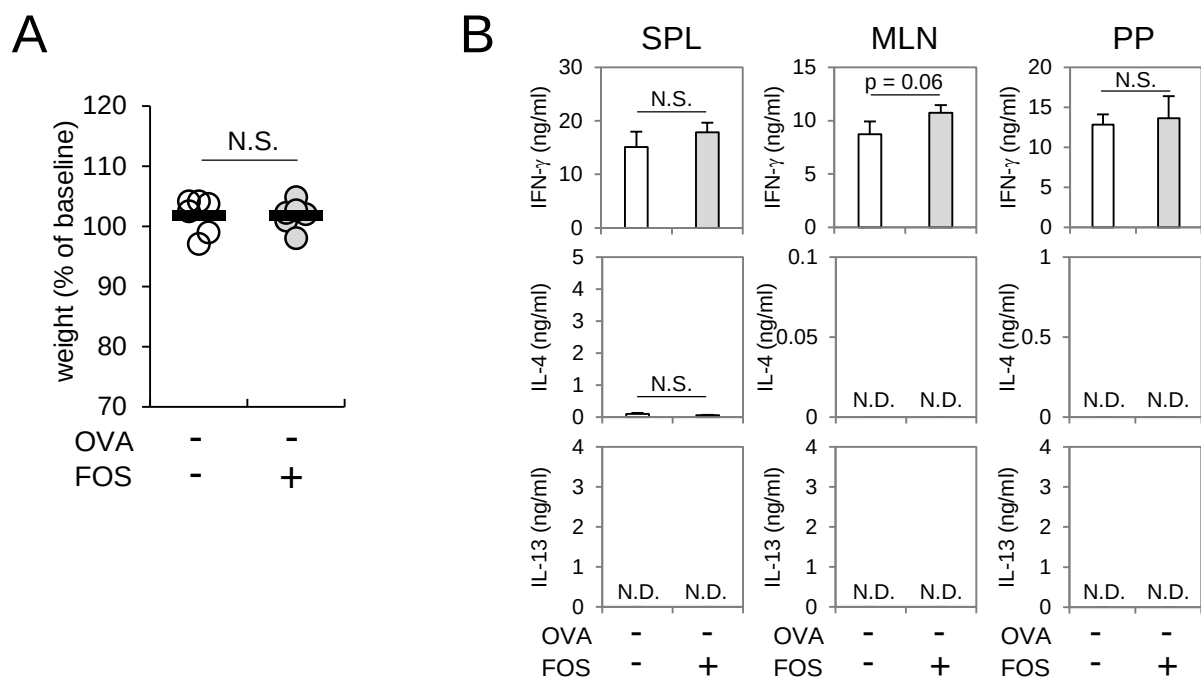
Disclosure Statement

The authors have no conflicts of interest to declare.

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Supplementary Fig. 1 Dietary FOS alone do not affect cytokine responses against *in vitro* stimulation with OVA and the phenotype of CD4⁺ T cells.

(A) The body weight of each mouse at day 7 was indicated as a percentage of the initial weight at day 0. Data represents the body weight from individual mouse from FOS group (received the experimental diet containing 7.5% FOS without OVA) or control group (n = 6 for each group). (B) Single cells from the MLN, PP, and SPL of FOS or control group were stimulated *in vitro* with 5 mg/ml of OVA for 48 h. The concentration of each cytokine in the culture supernatants was assayed by ELISA. Data are shown as mean $\pm$ SD and representative of two independent experiments. (C) Cells from the MLN, PP, and SPL of FOS or control group were stained with anti-CD4 and anti-CD25 followed by intracellular staining with antibodies against Foxp3. Data are shown the representative dot plots of CD25 and Foxp3 expression in CD4⁺ cells from two independent experiments.

(D) Cells from the MLN, PP, and SPL of FOS or control group were stained with anti-CD4, anti-CD45RB and anti-CD69. Data are shown the representative dot plots of CD45RB and CD69 expression in CD4⁺ cells from two independent experiments.