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Dietary Supplementation with Nondigestible Oligosaccharides Reduces Allergic Symptoms and Supports Low Dose Oral Immunotherapy in a Peanut Allergy Mouse Model

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Scope: A major downside of oral immunotherapy (OIT) for food allergy is the risk of severe side effects. Non-digestible short- and long-chain fructo-oligosaccharides (scFOS/lcFOS) reduce allergy development in murine models. Therefore, it is hypothesized that scFOS/lcFOS can also support the efficacy of OIT in a peanut allergy model.

Methods and Results: After sensitization to peanut extract (PE) using cholera toxin, C3H/HeOuJ mice are fed a 1% scFOS/lcFOS or control diet and receive OIT (1.5 or 15 mg PE). Hereafter, mice are exposed to PE via different routes to determine the safety and efficacy of treatment in clinical outcomes, PE-specific antibody production, and numbers of various immune cells. scFOS/lcFOS increases short-chain fatty acid levels in the caecum and reduce the acute allergic skin response and drop in body temperature after PE exposure. Interestingly, 15 mg and 1.5 mg OIT with scFOS/lcFOS induce protection against anaphylaxis, whereas 1.5 mg OIT alone does not. OIT, with or without scFOS/lcFOS, induces PE-specific immunoglobulin (Ig) IgG and IgA levels and increases CD103+ dendritic cells in the mesenteric lymph nodes.

Conclusions: scFOS/lcFOS and scFOS/lcFOS combined with low dose OIT are able to protect against a peanut-allergic anaphylactic response.

1. Introduction

Peanut allergy affects 1% to 3% of children in Western countries and its prevalence has tripled over the past 10–15 years in the United States.[1] Unfortunately, there is still a lack of effective and safe treatments. Food allergen–specific immunotherapy has the potential to desensitize or even tolerize patients, and is therefore widely studied.[2–5] In particular, oral immunotherapy (OIT) can induce sustained unresponsiveness, by utilizing the pathways underlying oral tolerance and, hereby restoring the nonresponsiveness to the allergen.[6] Unfortunately, the high allergen dose and dose escalations used with OIT can cause adverse effects in many subjects.[7–9] Moreover, long-term adherence to this therapy is difficult, whereas permanent tolerance without continuing exposure seems limited.[10] As a result of these limitations, it is still recommended that peanut OIT should be restricted to a clinical trial setting.[10–12] Therefore, it is essential to develop new OIT concepts to reduce side effects of OIT whereas keeping the efficacy intact or even improve the efficacy.

Dietary nondigestible oligosaccharides, mimic the immunomodulatory effects of human milk oligosaccharides in breast-fed infants and have been shown to prevent atopic dermatitis, food allergy, and allergic asthma.[13] We examined whether a diet containing short- and long-chain fructo-oligosaccharides (scFOS/lcFOS), could support OIT and lead to a safer and more effective protocol. Nondigestible

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oligosaccharides, whether or not in combination with a bacterial strain, have a protective effect against allergic sensitization in mice.[14,15] In infants, administrating pre- and probiotics induced a Bifidobacteria- and lactobacilli-predominating gut microbiota, and reduced the occurrence of allergic manifestations.[16,17] Furthermore, synbiotics induced desensitizing and immunomodulatory effects when administered after sensitization in a mouse model.[18] Therefore, a dietary intervention with scFOS/lcFOS may be a new therapeutic strategy for food allergy.

In previous studies by our group, we have shown that OIT increases the levels of immunoglobulin (Ig) IgA, IgG1, and IgG2a in peanut allergic mice, and that OIT alone did not show effects on T-cell responses.[19] OIT aims to induce oral tolerance to the offending food, by redirecting the allergic immune response and protect against an inappropriate reaction after allergen exposure. This probably entails various mechanisms involved, including changes in the humoral (IgA and IgG4) responses, also suppression of allergen-specific B- and T-cell effector responses, less activation of basophils and mast cells, and switching from T-helper (TH) TH_2 to TH_1 response with accompanying induction of specific regulatory T cells (Treg).[20] In humans, an increase in Treg-cell numbers after OIT in peanut allergic patients is not always found, which may explain a lack of sustained oral tolerance.[7] Therefore, improving OIT to induce a sustainable (or more effective) tolerogenic immune response on both the humoral and cellular mucosal compartment would be of great importance.

In the present study, it is investigated whether the scFOS/lcFOS-supplemented diet has potential to reduce allergic responses in peanut-allergic mice. Moreover, it is investigated whether scFOS/lcFOS could support OIT. To study this, the scFOS/lcFOS diet was given after sensitization and combined with two doses of OIT. To investigate the effects of scFOS/lcFOS and OIT on the immune system, cellular and humoral responses are measured on three time points during the study.

2. Experimental Section

2.1. Mice

Female, specific-pathogen free, 5 to 6 weeks old C3H/HeOuJ mice (total $n = 123$, $n = 5/6$ per group) were purchased from Charles River Laboratories (Erkrath, Mettmann, Germany). The mice were maintained on a 12 h light/dark cycle, in filter-topped macrolon cages. Food pellets and drinking water were available ad libitum. This study was carried out in accordance with the recommendations of the principles of good laboratory animal care following the European Directive for the protection of animals used for scientific purposes. The protocol was approved by an independent ethics committee for animal experimentation (the Ethical Committee of Animal Research of Utrecht University, Utrecht, the Netherlands, registered by DEC2014.III.03.032).

2.2. Reagents

Raw peanuts (provided by Intersnack Nederland BV, Doetinchem, the Netherlands) were used to prepare peanut protein extract (PE) as described by Koppelman et al.[21] Protein content was checked by BCA analysis (Pierce, Waltham, MA); the extract contained 30 mg/ml protein. Cholera toxin (CT) was obtained from List Biological Laboratories (Inc, Campbell, Santa Clara, CA).

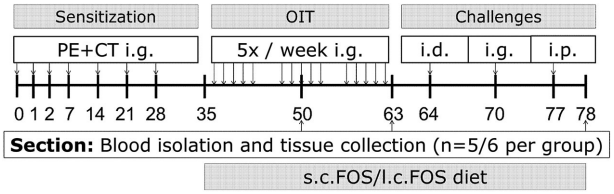

Figure 1. Schematic overview of the experimental set-up. CT, cholera toxin; i.d., intradermal; i.g., intragastric; i.p., intraperitoneal; OIT, oral immunotherapy; PE, peanut extract.

2.3. Diets

Semi-purified peanut protein-free AIN-93G-based diets were composed and nondigestible oligosaccharides were added by Ssniff Spezialdiäten (Soest, Germany). These nondigestible oligosaccharide diets consisted of 1% w/w of a 9:1 mixture of short-chain fructo-oligosaccharides (scFOS: oligofructose; Raftilose P95, Orafti, Wijchen, the Netherlands; >95% degree of polymerization [DP] < 6) and long-chain fructo-oligosaccharides (lcFOS: long chain inulin; Raftiline HP, Orafti, Wijchen, the Netherlands; average DP 23 or higher, <1% DP < 5) derived from chicory inulin (Raftiline HP, Orafti, Wijchen, the Netherlands).

2.4. Oral Sensitization, Immunotherapy, Dietary Interventions, and Challenges

After acclimatization and random allocation, mice were sensitized intragastrically (i.g.) to PE (6 mg in 200 µL PBS) or PBS (sham-sensitization), using CT (15 µg/mouse) as an adjuvant (day 0, 1, 2, 7, 14, 21, and 28, **Figure 1**) according to the method described by van Wijk et al.[22] After sensitization (from day 35), selected groups were fed an scFOS/lcFOS-supplemented diet, the rest of the groups remained on control diet. From day 42, the mice were treated i.g. (OIT) with 1.5 or 15 mg PE in 500 µL PBS, or PBS (PE-sensitized control animals) for five times/week, for three weeks (day 42–60).

On day 64, prior to intradermal (i.d.) injection with PE in both ear pinnae, mice were anesthetized using inhalation of isoflurane. All mice were injected i.d. in both ear pinnae with 1 µg PE in 20 µL PBS to induce an acute allergic skin response. Ear thickness was measured with a digital micrometer (Mitutoyo, Veenendaal, the Netherlands). Ear thickness was measured in both ears before and 1 h after the injection. Mean basal ear thickness of both ears (µm) was subtracted from the mean ear thickness after challenge to determine Δ ear swelling as a measure for the acute allergic skin response. On day 70, an i.g. challenge (using 15 mg PE in 500 µL PBS) was performed and blood was collected after 30 min to measure murine mast cell protease-1 (MMCP-1), as a marker for mast cell degranulation. Mice were challenged intraperitoneally (i.p.) on day 77 (using 100 µg PE in 200 µL PBS) to measure drop in body temperature and anaphylactic shock symptom scores. Body temperature was measured every 10 min

after the i.p. challenge using a rectal thermometer and clinical symptoms were scored after 40 min, according to the method described by Li et al.[23]

Both allergic and immunologic parameters were studied on three different time points to investigate potential underlying mechanisms. We hypothesized that the most interesting differences could occur during or after immunotherapy or after the challenges. Therefore, at day 50, 63, and 78, mice were killed by cervical dislocation and blood and organs were collected.

2.5. Short Chain Fatty Acids

Caecal content was collected and stored at −80 °C until measurement. After homogenizing and diluting the samples (1:10), SCFA were captured using a Shimadzu GC2010 gas chromatograph (Shimadzu Corporation, Kyoto, Japan), equipped with a flame ionization detector. Concentrations of acetic, propionic, valeric, and butyric acid were determined by means of gas chromatography as described by de Theije et al.,[24] using 2-ethylbutyric acid as internal standard.

2.6. Basophil Activation Assay

To measure basophil activation, blood was taken from the mice on (day 68) and stimulated and analyzed according to Torrero et al.[25] In summary, whole blood was collected in heparinized tubes and diluted 1:1 in RPMI 1640 Medium (Gibco, Invitrogen, Carlsbad, CA). Blood was incubated with anti-mouse IgE at 0.125 μg/mL (R35-72, BD Biosciences, Franklin Lakes, NJ), PE at 20 μg/mL or medium for 90 min at 37 °C in 5% CO_2. Activation was stopped with PBS containing EDTA. After washing, red blood cells were lysed, and cells were fixed using the Whole Blood Lysing Reagents (Beckman Coulter, Fullerton, CA). Cells were incubated with anti-CD16/CD32 (clone 2.4G2) to block the FcR, then stained with the following fluorescent-labeled antibodies for 30 min at 4 °C in the dark: anti-IgE-FITC (1:100, clone 23G3), anti-CD49b-APC (1:200, clone CX5), anti-CD4-PE (1:200, clone RM4-5), and anti-CD200R-Percpefluor 710 (1:200, clone OX110), and anti-CD19-PE (1:200, clone 6D5) from eBioscience (Breda, the Netherlands). Analysis of the samples was performed on the BD Accuri™ C6 flow cytometer, analysis with BD sampler software (BD Biosciences).

2.7. Serum Levels of MMCP-1 and PE-Specific IgE, IgA, IgG1, and IgG2a

On day 35, 50, 64, 70, and 78 blood samples were collected via cheek puncture and after centrifugation (10 000 rpm for 7 min at RT) sera were stored at −20 °C until further analysis.

ELISA was used to determine PE-specific IgA, IgE, IgG1, and IgG2a levels in serum, as previously described by van Wijk et al.[22] An Asys expert 96 plate reader (Biochrom, Cambourne, UK) was used to measure the absorbance 405 nm (IgG1 and IgG2a) or 450 nm (IgE and IgA). As a standard, a positive pool serum derived from PE-sensitized mice was used as reference value to determine antibody concentrations in arbitrary units.

MMCP-1 in serum obtained 30 min after i.g. challenge, was determined by using an MMCP-1 Sandwich ELISA kit (eBioscience Mouse MCPT-1 ELISA Ready-SET-Go Kit).

2.8. ELISPOT Analysis

For the PE-IgG1- and IgA-specific lymphocyte ELISPOT assay, Immubulon-P transfer membranes were coated with PE 10 μg mL^{-1} in PBS/Tween overnight (4 °C on shaker). After washing the membranes, splenic-cell culture (0.5×10^6 cells/well) was incubated in the wells for 4 h (37 °C, 5% CO_2). After washing, membranes were incubated with conjugated goat anti-mouse IgG1-, or IgA-AP antibody overnight (4 °C on shaker). The chromogen substrates used to develop spot color consisted of a nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate toluidine mix. Membranes were incubated for ±15 min (depending on the color) with the substrate. Color development was stopped by washing under running tap water. After drying at room temperature, spots were counted.

2.9. Lymphocyte Isolation from Mesenteric Lymph Nodes

Lymphocytes from the mesenteric lymph nodes (MLN) were obtained by squeezing the organs through a 70 μm strainer, after which single cells were washed once.

2.10. T-Cell and Dendritic Cell Analysis Using Flow Cytometry

Single cell suspensions of MLN were used to analyze dendritic cell (DC) and T-cell subsets by flow cytometry. A total of $5–10 \times 10^5$ cells per well were plated in fluorescence-activated cell sorting buffer (PBS containing 0.25% BSA, 0.05% NaN_3, 0.5mM EDTA). For the DC staining, cells were first stained with LIVE/DEAD fixable near-IR stain (Molecular Probes, Invitrogen, Carlsbad, CA). Cells were incubated with anti-CD16/CD32 (clone 2.4G2, BD Biosciences), then stained with fluorescent-labeled antibodies and stored (4 °C in the dark) until measurement. Cells stained for extracellular markers were fixed using 0.4% paraformaldehyde and cells stained for intracellular forkhead box protein 3 (FoxP3)-APC were permeabilized and fixed using the buffer set (eBioscience). Antibody concentrations were titrated beforehand.

The following antibodies were used: anti-CD4-FITC (1:200, clone RM4-5), anti-FoxP3-APC (1:40, clone FJK-16s), anti-CD25-PE (1:200, clone PC61.5), anti-CD69-APC (1:200, clone H1.2F3), anti-CXCR3-PE (1:100, clone CXCR3-173), anti-CD103 (1:200, clone 2E7), anti-CD11b-FITC (1:200, clone M1/70), anti-CD11c-APC (1:200, clone N418) from eBioscience, anti-CD4-PerCp (1:200, clone RM4-5) from BD Pharmingen, and anti-MHCII-PerCP (1:400, clone M5/114.15.2) and anti-CD64-Briljant violet 421 (1:200, clone X54-5/7.1) from Biolegend.

Analysis of the samples was performed on the BD Accuri™ C6 flow cytometer, analysis with BD sampler software (BD Biosciences) or on the BD canto II, analysis with FlowJo® (FlowJo, LLC, Ashland, OR). Based on forward/sideward scatter properties aggregated cells were excluded. Cut-off gates for positivity were established using the fluorescence-minus-one (FMO) technique.

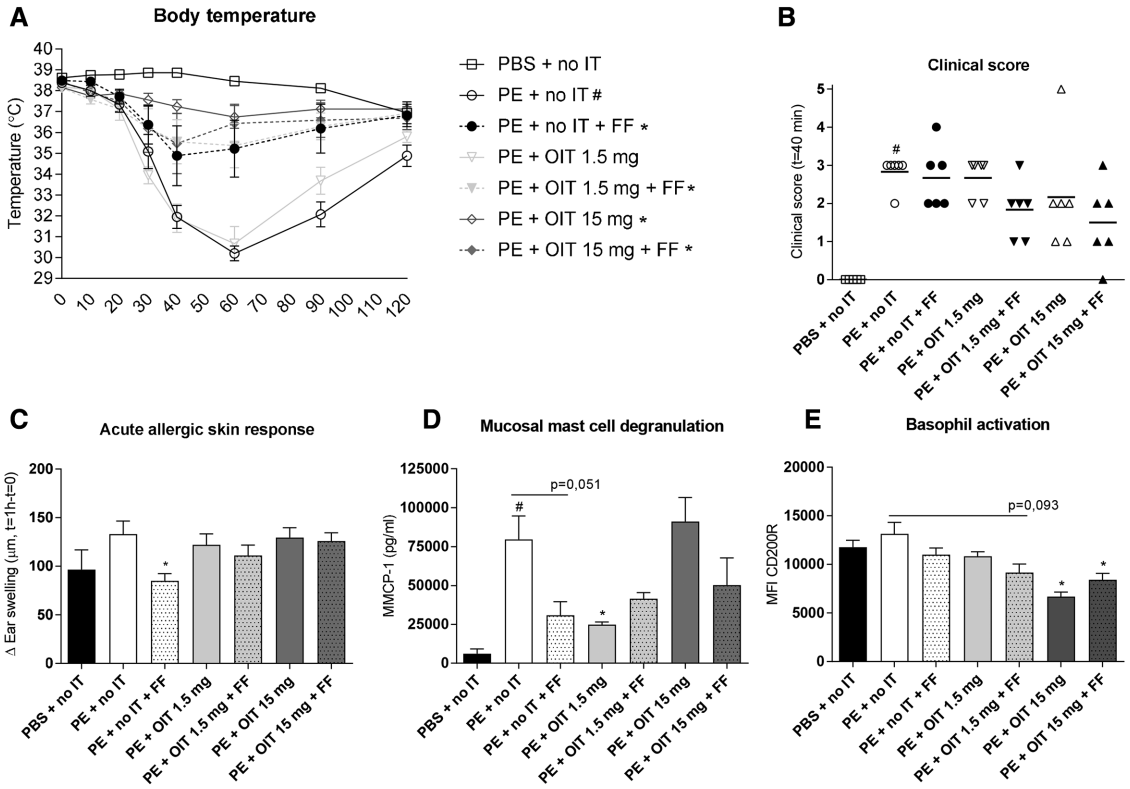

Figure 2. Allergic manifestations evaluated in PE-sensitized mice after having received OIT or OIT + scFOS/lcFOS (FF). A) Change in body temperature after intraperitoneal challenge on day 77. B) Anaphylactic shock symptom scores determined 40 min after intraperitoneal challenge on day 77. C) Acute allergic skin response measured as Δ ear swelling 1 h after intradermal challenge on day 64. D) Concentrations of MMCP-1 in serum collected 30 min after intragastric challenge on day 70. E) Peripheral blood basophil activation was measured after whole blood stimulation with αIgE on day 68. Data are represented as mean ± SEM $n = 5/6$ mice/group. Statistical analysis was performed using repeated measures two-way ANOVA and Bonferroni's post hoc test (body temperature), one-way ANOVA, and Bonferroni's post hoc test to compare preselected combinations (ear swelling, MMCP-1, and basophil activation) or a Kruskal–Wallis test with Dunn's post hoc test (clinical score). $^{\#}p < 0.05$; compared to sham-sensitized control; $^{*}p < 0.05$ compared to PE-sensitized control. FF, scFOS/lcFOS dietary supplementation; i.p., intraperitoneal; IT, immunotherapy; MMCP-1, mast cell protease-1; OIT, oral immunotherapy; PE, peanut extract.

2.11. Data Analysis and Statistics

GraphPad Prism 6.00 software for Macintosh (GraphPad Software, San Diego, CA) was used for all statistical analyses. Body temperature was statistically analysed using repeated measures two-way ANOVA and Bonferroni's post hoc test. The acute allergic skin response and MMCP-1 levels were statistically analyzed by one-way ANOVA followed by Bonferroni's post hoc test to compare preselected combinations. Antibody levels were log-transformed prior to testing, and statistical difference compared to the PE-sensitized control treatment was analyzed each day by a one-way ANOVA and Bonferroni's post hoc test. The antibody-secreting cell numbers, flow cytometry data, and SCFA content were analyzed by one-way ANOVA per day and Bonferroni's post hoc test to compare preselected combinations. Anaphylaxis symptom scores and basophil activation were analyzed using Kruskal–Wallis test for nonparametric data with Dunn's post hoc test. All data are presented as mean ± SEM of 5/6 mice per group and results were considered statistically significant when $p < 0.05$.

3. Results

3.1. scFOS/lcFOS-Induced Protection Against Anaphylaxis

The therapeutic effect of scFOS/lcFOS, OIT, and OIT complemented with scFOS/lcFOS was examined by analyzing allergic responses after a challenge with high PE exposure (**Figure 2**). The systemic challenge with PE elicited an anaphylactic response, characterized by a sharp drop in body temperature and high clinical symptom scores in PE-sensitized control mice compared to the sham-sensitized control mice (Figure 2A,B). Treatment with scFOS/lcFOS alone, 1.5 mg OIT plus scFOS/lcFOS, or 15 mg OIT with or without scFOS/lcFOS decreased the drop in body temperature compared to the PE-sensitized control mice, whereas 1.5 mg OIT alone did not (Figure 2A).

Of all treatments, only scFOS/lcFOS treatment reduced acute allergic skin responses compared to the PE-sensitized control mice (Figure 2C).

To test whether any treatment influenced effector cell responses, serum MMCP-1 levels and activation status of

peripheral blood basophils were assessed. MMCP-1 levels were increased in PE-sensitized control mice when compared to sham-sensitized control mice. A total of 1.5 mg OIT reduced the MMCP-1 levels compared to the PE-sensitized control mice, whereas 15 mg OIT did not (Figure 2D). Stimulation with αIgE increased the basophil activation, but activation was lower when mice were treated with 15 mg OIT, indicating a reduction of the effector cell response with this treatment (Figure 2E).

3.2. OIT-Induced Allergen-Specific IgG and IgA

PE-sensitized mice showed enhanced serum levels of PE-specific IgE, IgA, and IgG1 and IgG2a compared to the sham-sensitized mice (**Figure 3**). Remarkably, all OIT treatments caused a further increase of IgA, IgG1, and IgG2a levels in serum compared to PE-sensitized control mice (from day 50, Figure 3B–D), whereas only 15 mg OIT resulted in an increase of PE-specific IgE levels (from day 63, Figure 3A). Compared to the control diet, scFOS/lcFOS led to an increase in levels of IgA, IgG1, and IgG2a, but only from day 70 until the end of the study (Figure 3B–D). OIT also resulted in increased numbers of IgA- and IgG1-secreting cells compared to PE-sensitized control mice (day 50, Figure 3E,F).

3.3. OIT Induces CD103+ DCs and Treg Cells in the MLN

OIT, with or without scFOS/lcFOS, increased the percentage of CD103+ DC (day 50 and 63, **Figure 4**A,B) and Treg cells (Figure 4C) in the MLN compared to the PE-sensitized control group on day 50.

Treg percentages were at the same level in the scFOS/lcFOS-only-treated group than in OIT-treated groups, while CD103+CD11b+ DC were decreased by scFOS/lcFOS-only-treatment (Figure 4A–C). Treatment with 1.5 mg OIT increased percentages of TH_1 cells compared to the PE-sensitized control mice (day 50), whereas treatment with 15 mg OIT did not show this effect (Figure 4D).

3.4. scFOS/lcFOS Causes a Shift in SCFA Levels in Caecum

On day 50, caecal SCFA levels were not different between groups (data not shown). On day 63, the levels of acetic, butyric, propionic, and valeric acid (mmol/L) were increased in the treatment group receiving 15 mg OIT plus scFOS/lcFOS (**Figure 5**B–E), which was reflected in the increase of total SCFA levels (Figure 5A). The scFOS/lcFOS diet induced butyric acid formation in the PE-sensitized control mice (day 63) and in the 1.5 mg OIT-treated mice (day 78) compared to the control diet (Figure 5B).

4. Discussion

Randomized controlled trials done in peanut allergic patients showed that OIT is able to induce unresponsiveness to a high daily dose of peanut,[5,7,8,26] particularly in young patients.[27] Unfortunately, OIT also caused adverse effects in many subjects at some point during the treatment period,[9] and therefore OIT is still not recommended for wide use.[28] We used a peanut allergy mouse model to investigate the effects of scFOS/lcFOS, OIT, or a combination of these two treatments on allergic disease. Our data shows that scFOS/lcFOS alone has protective effects against acute allergic skin response and anaphylactic drop in body temperature. In addition, we show that scFOS/lcFOS improves the low dose OIT with regard to anaphylactic drop in body temperature and increased the number of activated TH_1 cells in the spleen. Moreover, OIT induces antigen-specific IgA and IgG levels and altered DC differentiation.

The allergy-modifying effect of scFOS/lcFOS has been demonstrated before. For instance, it has been shown that administration of scFOS/lcFOS by itself reduced challenge-induced MMCP-1 levels in ovalbumin-allergic mice.[18] This effect of scFOS/lcFOS was enforced by co-administration of *Bifidobacterium breve* M-16V suggesting that microbial activity, for example, production of SCFA, may be involved in the protective activity of oligosaccharides.[18] In a randomized trial it has been shown that OIT combined with a probiotic, effectively induced unresponsiveness in seven out of nine peanut allergic patients.[29] However, since no OIT only control group was included it is difficult to conclude whether the diet improved the OIT effect[29] We here demonstrated that scFOS/lcFOS alone lowers the acute allergic skin response and protects against the drop in body temperature. Notably, administration of the oligosaccharide mixture did not elicit an increase of peanut-specific antibody levels before the challenges, as the specific OIT treatment does. This suggests that the protective effect of oligosaccharides is not antigen-specific. In contrast, the protective effect of scFOS/lcFOS alone is absent in a mouse model for cow's milk allergy,[30] therefore arguing against an antigen-independent effect of scFOS/lcFOS. Further research is warranted to elucidate the direct protective effect of scFOS/lcFOS on the acute allergic skin response and the contribution of the antigen itself to this protection.

In previous studies, we have already shown that 15 mg OIT was able to protect against allergic symptoms after PE challenge, measured as MMCP-1 levels.[19] However, in this study 15 mg OIT was not able to lower the MMCP-1 levels after i.g. challenge, while it did protect against anaphylaxis after the i.p. challenge (body temperature drop). Interestingly, the increase in antigen-specific IgE levels, constantly seen after antigen challenge in the control groups, is reduced in groups receiving 1.5 mg OIT but is still high in the 15 mg OIT group. We hypothesized that the lasting elevated levels of IgE in this high dose group could explain the increased MMCP-1 levels, measured after i.g. challenge in the same groups. This would be comparable to the amplification loop of the IgE-dependent upregulation of mast-cell FcεRI surface expression in patients who develop increased concentrations of IgE.[31]

Although the mechanisms underlying the effects of nondigestible oligosaccharides on the development of allergy are not clear yet, it is hypothesized that nondigestible oligosaccharides can modify the immunological environment in the gastrointestinal tract and additionally have a direct effect on immune cells, possibly via the interaction with specific sugar receptors.[32–36] This environmental modification may result from an increase of SCFA, as we observed here by analyzing the caecal SCFA content. In mice, high-fiber feeding alters gut microbial ecology and increased the release of SCFAs, particularly acetate

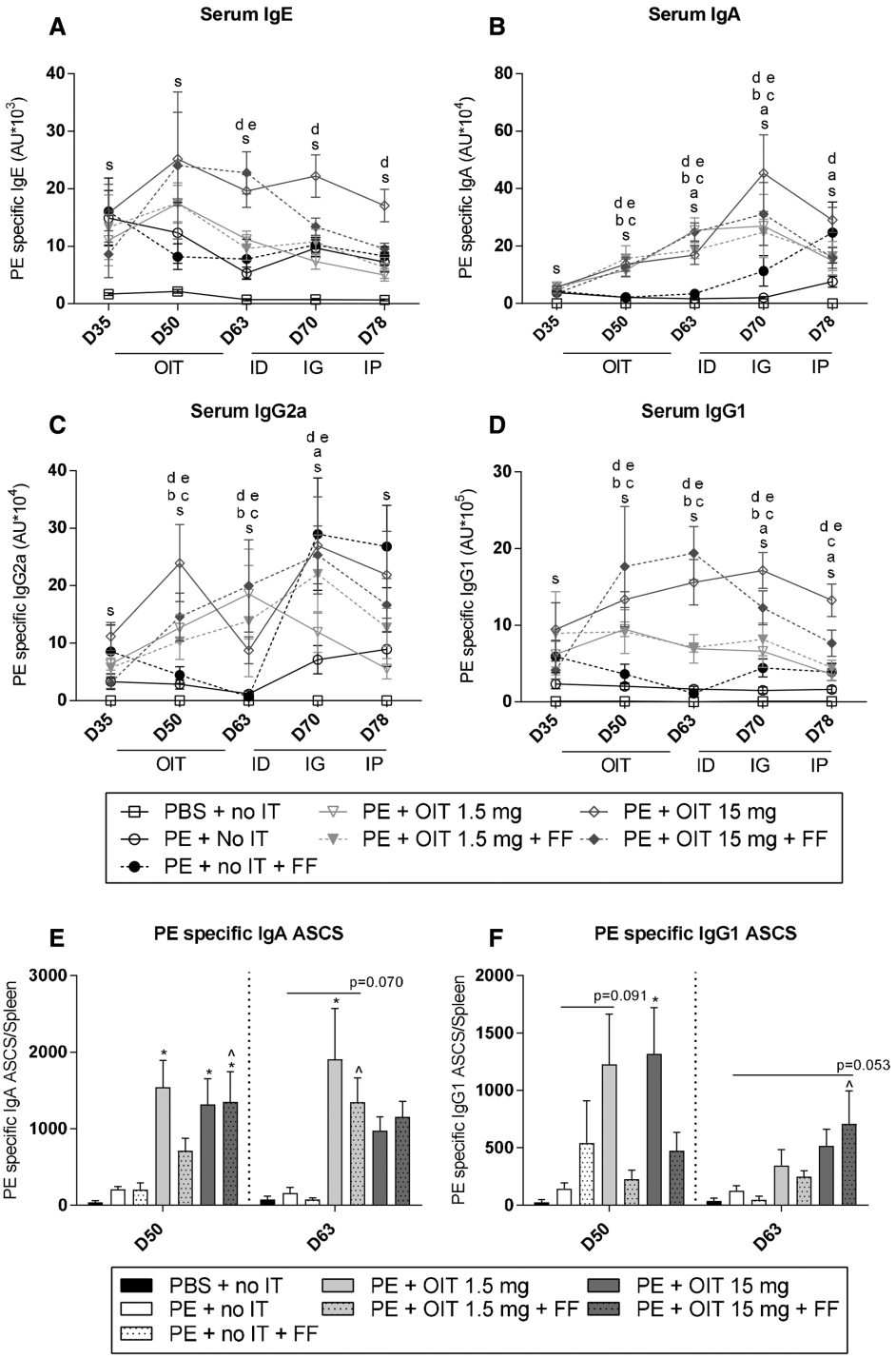

Figure 3. PE-specific IgE, IgA, IgG1, and IgG2a levels in serum determined by ELISA and IgA and IgG1 production by splenocytes determined by ELISPOT. Blood was taken on day 35, 50, 63, 70, and 78. A,B) Allergen-specific IgE and IgA measured in serum by ELISA. C,D) Allergen-specific IgG2a and IgG1 measured in serum by ELISA. E,F) Number of allergen-specific IgA and IgG1 antigen secreting splenocytes (ASCS) per spleen. Data are represented as mean ± SEM $n = 5/6$ mice/group. Statistical analysis of the antibody levels was performed on each individual time point, after log transformation, using one-way ANOVA and Bonferroni's post hoc test for multiple comparisons. All treatment groups were compared to the sensitized control group and significant differences were indicated with letters: Letters used: s for sham-sensitized control; a for no OIT plus scFOS/lcFOS; b for 1.5 mg OIT; c for 1.5 mg OIT plus scFOS/lcFOS; d for 15 mg OIT; and e for 15 mg OIT plus scFOS/lcFOS when $p < 0.05$. Statistical analysis for the ELISPOT results was performed on each time point using one-way ANOVA and Bonferroni's post hoc test for multiple comparisons. $^{\#}p < 0.05$ compared to sham-sensitized control; $^{*}p < 0.05$ compared to PE-sensitized control; $^{\wedge}p < 0.05$ compared to scFOS/lcFOS control. FF, scFOS/lcFOS dietary supplementation; id, intradermal challenge; ig, intragastric challenge; ip, intraperitoneal challenge; OIT, oral immunotherapy; PE, peanut extract.

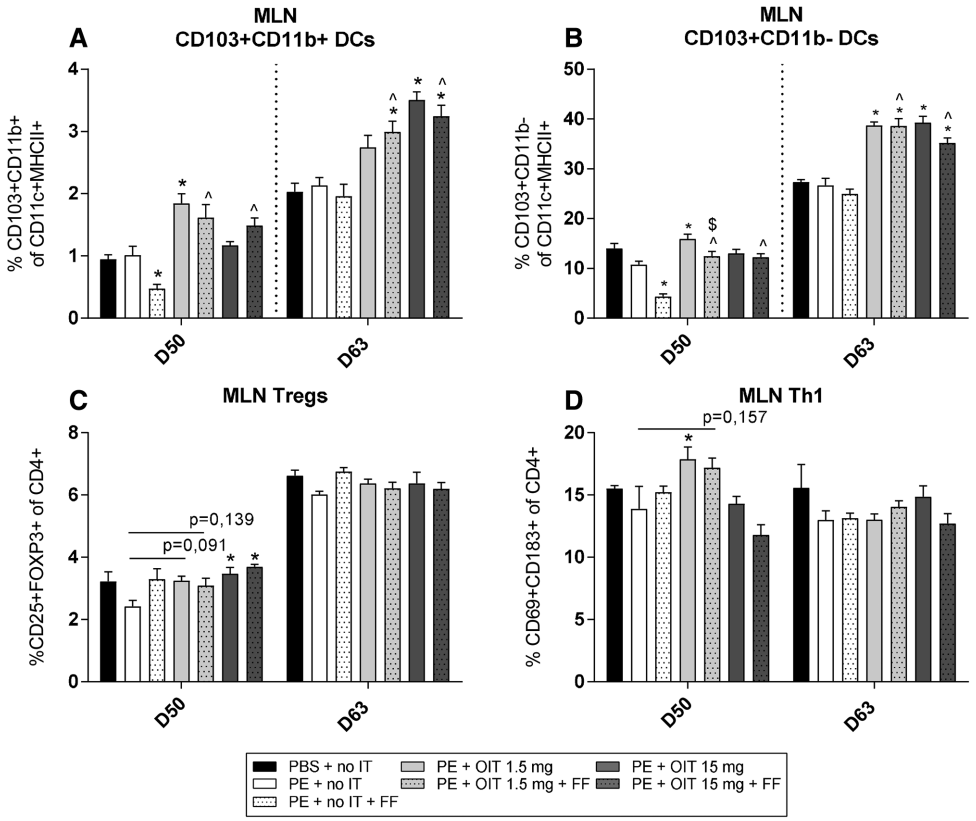

Figure 4. Flow cytometric analysis of DC and T-cell populations in the MLN. Cells were gated based on FSC–SSC properties and the fluorescence-minus-one (FMO) technique. For DC, the live cells were gated on: CD64 negative, MHC Class II positive and CD11c positive. The CD11c/MHCII positive population was further characterized on the basis of CD103 and CD11b expression. A,B) Percentage of CD103+CD11b+ DC and percentage of CD103+CD11b-DC. C) Percentage of regulatory T cells (CD25+FoxP3+ of CD4+) and D) activated TH_1 cells (CXCR3+CD69+ of CD4+). All data are represented as mean ± SEM $n = 5/6$ mice/group. Statistical analysis was performed for each time point using a one-way ANOVA and Bonferroni's post hoc test. $^*p < 0.05$ compared to PE-sensitized control; $^\wedge p < 0.05$; compared to scFOS/lcFOS control; $^\$p < 0.05$ compared to the OIT control diet group. FF, scFOS/lcFOS dietary supplementation; IT, immunotherapy; OIT, oral immunotherapy; PE, peanut extract.

and butyrate, which may contribute to protection against food allergy.[37] On day 63, mice treated with 15 mg OIT combined with scFOS/lcFOS exhibited increased SCFA levels compared to the scFOS/lcFOS-treated control group. We hypothesize that the presence of protein may influence the SCFA production. For instance, the microbiota in the colon can ferment both carbohydrates and proteins into SCFA, if proteins are not fully digested in the upper part of the digestive tract.[38] Both dietary- and bacterial composition in the colon influence the type and quantity of the produced SCFA.[39] Moreover, from our results we did not obtain a complete picture of oligosaccharide fermentation, because SCFA measurements were limited to three time points. Infants that were administered prebiotic formula containing scGOS/lcFOS gained Bifidobacteria and their intestinal SCFA pattern contained a higher proportion of acetate and a lower proportion of propionate.[40] Specifically butyric acid can influence epithelial cells and regulate their gene expression, proliferation, and differentiation.[41] In addition, mice fed with SCFAs had increased numbers of IL-10-producing FoxP3+ T cells in the colon,[42] and butyric acid seemed to be potent in inducing Treg cell differentiation.[43,44] Future studies will have to reveal how oligosaccharides improve protection against anaphylactic responses and what the role of the high levels of SCFA is.

In clinical studies using peanut OIT, binding of peanut-specific IgE to the allergen was reduced during treatment, whereas the concentration of peanut-specific IgG4 was increased.[26] This indicates that in humans functionally "blocking" IgG4 antibodies develop during immunotherapy since a reduction in IgE binding occurs at the same epitopes that binds IgG4.[26] In mice, IgG antibodies may also be able to block systemic anaphylaxis induced by specific allergen.[45,46] In our model, OIT significantly induced IgA, IgG1, and IgG2a in serum. This is consistent with the theory that antibodies of the IgG and IgA class develop during immunotherapy and might be functionally inhibitory.[45,47,48] In comparison, Leonard et al., who studied OIT in an OVA allergy mouse model, have shown the same desensitization effects accompanied by a boost in IgA.[49] The increase in IgG and IgA may, partly explain the induced protection by OIT in the present study.

In summary, we have shown in a mouse model for peanut allergy, that a prebiotic diet containing scFOS/lcFOS alters the SCFA composition in the caecum and protects against acute allergic skin responses and anaphylaxis. We argue that the oligosaccharide effect is not antigen-specific, nevertheless, the amount of Tregs is restored by oligosaccharide treatment. We additionally found that scFOS/lcFOS improves the efficacy of the lower dose

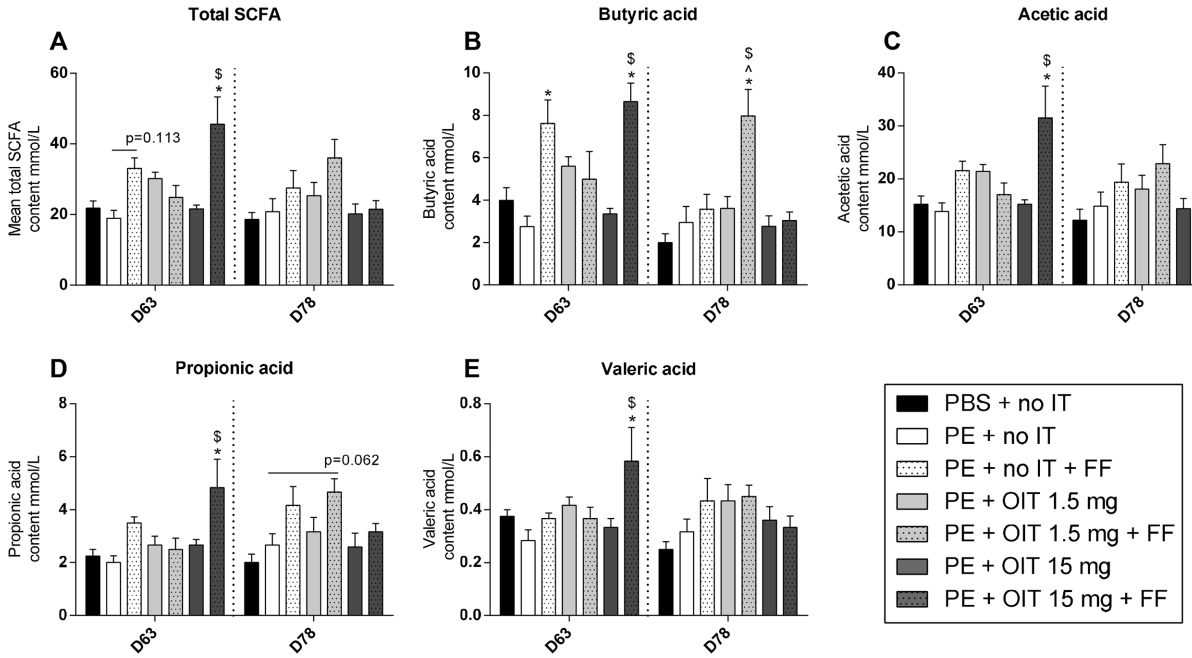

Figure 5. Levels of total and individual short chain fatty acids (SCFA). A) Mean total SCFA content (mmol L^{-1}). B–E) Butyric, acetic, propionic, and valeric acid content (mmol L^{-1}). All data are represented as mean ± SEM $n = 5/6$ mice/group. Statistical analysis was performed for each time point using one-way ANOVA and Bonferroni's post hoc test for multiple comparisons to compare preselected combinations. $*p < 0.05$ compared to PE-sensitized control; $^{\wedge}p < 0.05$ compared to scFOS/lcFOS control; $^{\$}p < 0.05$ compared to the OIT alone group. FF, scFOS/lcFOS dietary supplementation; IT, immunotherapy; OIT, oral immunotherapy; PE, peanut extract; SCFA, short-chain fatty acids.

OIT, including a lower anaphylactic response after allergen challenge. We show that this combination is associated with a boost of antigen-specific serum IgA and IgG levels as well as increasing amounts of $CD103^{+}$ DC and Tregs. These results indicate that the induction of antibodies could be a component of the underlying mechanism of specific protection of OIT, and studies are ongoing to further clarify this. The option to use a lower dose OIT, due to the support of a prebiotic diet, could increase adherence to therapy in clinical trials. Clearly, clinical studies are essential to assess whether scFOS/lcFOS enables the use of a lower, presumably safer dose of OIT for peanut allergy in human patients. In all, we envision that oligosaccharides induce protection against allergen challenges and OIT, which induces allergen-specific mechanisms that eventually may restore tolerance. Hence, the combination of oligosaccharides and OIT may indeed improve the efficacy and safety of OIT.

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L.W., J.S., and R.P. designed the experimental setup, wrote the animal applications, performed the literature search, data collection, analysis and interpretation, created the figures, and wrote the manuscript. M.B., G.G., M.V., B.E., and J.S. contributed to the data collection, M.B., G.G., M.V., J.S., R.P., B.E., L.K., and J.G. contributed to analysis and interpretation of data and manuscript writing. The authors would like to thank Manon van Roest, Laura van der Tuijn, Marjolein Oostserveen-van der Doelen, Esther van Zomeren, and Friederike Sonnet for their technical assistance.

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Conflict of Interest

None of the authors have a competing financial interest in relation to the presented work; L.K. is employed by Nutricia Research, and B.E. and J.G. are partly employed by Nutricia Research B.V. Utrecht, The Netherlands.

Keywords

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