

Supplementing Pregnant Mice with a Specific Mixture of Nondigestible Oligosaccharides Reduces Symptoms of Allergic Asthma in Male Offspring^{1–3}

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Abstract

Background: Previously, maternal supplementation with short-chain galacto- and long-chain fructo-oligosaccharides (scGOS/lcFOS; ratio 9:1) was shown to affect maternal and fetal immune status in mice.

Objective: This study was designed to test the long-term effects of supplementation of mice with scGOS/lcFOS before and during pregnancy on the immune response in the offspring, using an ovalbumin (OVA)-induced model for experimental allergic asthma.

Methods: Female Balb/c mice were fed a control diet or a diet supplemented with 3% scGOS/lcFOS and mated to C57BL/6 males. All dams were fed the control diet after delivery. At 6 wk, male offspring received an intraperitoneal injection of aluminum hydroxide and OVA (control and scGOS/lcFOS group) or saline (sham group). The acute allergic skin response (ASR) after intradermal challenge with OVA or saline was measured at 8 wk. After 3 airway challenges with nebulized OVA or saline, lung function was measured.

Results: The scGOS/lcFOS group had a significantly lower acute ASR ($85 \pm 9 \mu\text{m}$) than the control group ($124 \pm 9 \mu\text{m}$; $P = 0.01$). Lower lung resistance from a response to methacholine challenge was seen in the scGOS/lcFOS group. OVA-specific immunoglobulin (Ig)E concentrations in the control group [93 ± 45 arbitrary unit (AU)] and the scGOS/lcFOS group (67 ± 45 AU) were higher than in the sham group (11 ± 2 AU). OVA specific IgG2a concentrations in the scGOS/lcFOS (146 ± 24 AU) were higher than in the sham group (2 ± 0.3 AU) and control group (18 ± 3.5 AU; $P < 0.05$). Finally, the scGOS/lcFOS group had a higher percentage of regulatory T cells ($1.11\% \pm 0.07\%$) than the sham group ($0.14\% \pm 0.03\%$) and the control group ($0.11\% \pm 0.02\%$; $P < 0.05$).

Conclusion: Maternal supplementation with scGOS/lcFOS during pregnancy leads to a significant decrease in allergic symptoms in the offspring. *J Nutr* doi: 10.3945/jn.114.197707.

Keywords: programming, oligosaccharides, maternal dietary intervention, allergic asthma, pregnancy

Introduction

The development of the immune system starts during pregnancy, and, because many nutrients are thought to have immune modulatory functions, this makes maternal diet important to the

fetus (1). In addition, nutritive allergens may be transferred through the placenta into the cord blood, resulting in prenatal allergen exposure (2). After birth, the immune system of infants is not fully developed, although it is competent to react to infections and immunizations. The first postnatal year is important for developmental programming of the immune system (3). The quality of breast milk is important at this stage, because many immune modulatory agents are present, for example, secretory IgA, lactoferrin, oligosaccharides, lymphocytes, cytokines, and other proteins (4). Disadvantageous factors that affect early-life programming, such as suboptimal maternal diet, can result in increased risks of heart disorders and metabolic and immune diseases later in life (5). Maternal diet can also affect early-life programming in a beneficial way. Previous results from our laboratory indicate that a maternal diet supplemented with essential polyunsaturated FAs given

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³ Supplemental Figures 1 and 2 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at <http://jn.nutrition.org>.

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during pregnancy and/or lactation can attenuate the allergic response and enhance the response to influenza vaccination in the offspring (6). Thus, it is shown that maternal dietary supplementation could induce advantageous long-term effects in the offspring.

Another type of dietary supplement that has gathered more scientific attention in recent years is prebiotics. Prebiotics are believed to be able to reach the lumen of the intestine intact and to interact with the gut microbiota to change their composition and/or activity but also to interact directly with immune cells (7–9). A specific mixture of short-chain galacto- and long-chain fructo-oligosaccharides (scGOS/lcFOS; ratio 9:1)⁸ is shown to decrease the development of allergic symptoms in murine models for cow-milk allergy and ovalbumin (OVA)-induced hyperresponsiveness (10, 11). Furthermore, an improved vaccine-specific immune response is observed in mice supplemented with scGOS/lcFOS (12). The induced beneficial effects of supplementation with specific mixtures of oligosaccharides in allergy and vaccination models are partly mediated by regulatory T cells (Tregs), because depletion of these cells abrogated the observed beneficial effects (13–15).

To date, little is known about prenatal immune modulation through maternal supplementation with oligosaccharides. Earlier studies have shown that nondigestible oligosaccharide supplementation during pregnancy changes the microbiota composition in murine offspring and infants (16, 17), and supplementation with FOSs during pregnancy and lactation can decrease the severity of atopic dermatitis-like skin lesions in offspring of NC/Nga mice (18). However, in a model for contact hypersensitivity this beneficial effect is not observed (19), indicating that maternal dietary supplementation may differentially affect the development of symptoms in different types of allergic reactions in the offspring.

scGOS/lcFOS supplementation during pregnancy changes various immune markers in mother and fetus (20). Although scGOS/lcFOS supplementation beneficially affects T helper 1 (Th1)-driven delayed-type hypersensitivity response to the influenza vaccine Influvac in virgin mice, it does not affect the delayed-type hypersensitivity response in pregnant mice (20). Furthermore, a significantly higher percentage of alternatively activated macrophages is observed in the placentas of supplemented dams, and higher levels of *Ii10* mRNA expression are found in spleens of supplemented dams and in fetal spleens, which may affect subsequent regulatory responses of the offspring later in life (20). Altogether, these studies suggest that maternal supplementation with scGOS/lcFOS may influence immune development in the offspring, and we therefore hypothesized that maternal supplementation with scGOS/lcFOS would be able to reduce the induction of allergic asthma in offspring of supplemented mice. In the present study, healthy mice were supplemented with scGOS/lcFOS diet during pregnancy, and the putative long-term effects on immune development were studied with an OVA-induced allergic asthma model.

Methods

Animals. Eight-week-old Balb/cOlaHsd female and C57BL/6JOLA Hsd male mice were obtained from Harlan and housed at constant temperature (20°C) and humidity (40–60%) at a 12/12 h light/dark cycle in

the animal facility of Utrecht University. Animal care and use were performed in accordance with the guidelines of the Dutch Committee of Animal Experiments and with approval of the Utrecht University Ethical Committee. On arrival, mice were fed the AIN93G control diet. Two weeks before mating, female mice were randomly assigned to one of the experimental groups and were fed either the AIN93G control diet or a diet supplemented with oligosaccharides (described in “Diets”). The mating was performed with C57BL/6 male mice. When the pups were born, the dams were switched back to the AIN93G control diet. There were 3 dams per experimental group, and pups were randomly allocated to one of the dams 2 d after birth. After weaning, the male offspring received the AIN93 control diet, and the suboptimal sensitization protocol of the OVA-induced asthma model was started after the offspring were allocated to 1 of 3 experimental groups: sham group (nonsensitized offspring of dams fed the control diet during pregnancy; $n = 4$), control group (sensitized offspring of dams fed the control diet during pregnancy; $n = 4$), or GOS/FOS group (sensitized offspring of dams fed diet supplemented with scGOS/lcFOS during pregnancy; $n = 6$). We did not include a nonsensitized GOS/FOS group. In previous experiments, mean values of the clinically relevant *in vivo* parameters measured in nonsensitized groups (supplemented or nonsupplemented) never differed significantly (A Hogenkamp, S Thijssen, N van Vlies, J Garssen, unpublished data, 2010).

Diets. Both the control and the experimental diets were AIN93 based and consisted of 20% casein, 37% cornstarch, 13% maltodextrin, 10% dextrose, 5% Vitacel L600–20 (cellulose fiber), 7% fat, and a 5% mixture of vitamins and minerals, as described by Reeves et al. (21). The diets were obtained from Research Diet Services (Wijk bij Duurstede, The Netherlands). The experimental diet was supplemented with a mixture of scGOSs (Vivinal GOS; Friesland Domo, Zwolle, The Netherlands) and lcFOSs (Raftiline HP; Orafit, Wijchen, The Netherlands) in a ratio of 9:1 [Immunofortis (12)] at a dose of 3%. The scGOS/lcFOS replaced 3% of the total carbohydrates present in the diet, resulting in a comparable overall carbohydrate composition for both diets. scGOSs were obtained by enzymatic elongation of lactose with galactose by β -galactosidase, with a degree of polymerization of 3–8, and lcFOSs were derived from chicory inulin with a mean degree of polymerization of > 23 .

OVA-induced allergy model. All offspring were sensitized once to OVA at 6 wk of age by intraperitoneal injection of 10 μ g of OVA (OVA grade V; Sigma-Aldrich, Zwijndrecht, The Netherlands) adsorbed into 22.5 mg of aluminum hydroxide (Alumimject; Pierce, Perbio Science Nederland, Etten-Leur, The Netherlands) in 100 μ L of saline. The sham group received 22.5 mg of aluminum hydroxide in 100 μ L of saline. The acute allergic skin response (ASR) of the offspring was measured at 8 wk of age, by subcutaneous injection of 25 μ L of OVA 0.5 g/L in the pinnae of 1 ear. The other ear was injected with 25 μ L of saline. In the sham group, the mice were injected with saline in both ears. Ear thickness, as a measure of the allergic response, was measured in duplicate before antigen challenge and 1 h afterward, with a digital micrometer (Mitutoyo). The acute ASR was calculated by subtracting the basal ear thickness from the value at 1 h after challenge, correcting for the ear swelling that occurred as a result of subcutaneous injection of saline. In the scGOS/lcFOS group, 1 mouse was excluded because of a small wound on its ear, which made it impossible to perform a reliable ear-swelling measurement. One week later the offspring were exposed to 3 OVA challenges (10 g/L nebulized OVA dissolved in saline) for 20 min with an interval of 2 d between the challenges. Mice in the sham group were exposed to nebulized saline.

In vivo airway hyperresponsiveness to methacholine. Airway responsiveness, as measured by lung resistance after exposure to increasing concentrations of methacholine, was measured at 10 wk of age by using EMKA equipment and iox2 software (EMKA Technologies, Paris, France). Mice were anesthetized by an intramuscular injection of a mixture of 32.9 g/L ketamine (AST Farma, Oudewater, The Netherlands) and 0.26 g/L medetomidine (Pfizer Animal Health, Exton, PA) in NaCl solution (B Braun Melsungen AG, Melsungen, Germany),

⁸ Abbreviations used: ASR, allergic skin response; BAL, bronchoalveolar lavage; lcFOS, long-chain fructo-oligosaccharide; OVA, ovalbumin; scGOS, short-chain galacto-oligosaccharide; Th1, T helper 1; Treg, regulatory T cell.

using 50 $\mu\text{L}/10$ mg body weight. When sedated, the mice underwent tracheotomy, and a cannula was inserted into the trachea. The cannula was connected to a ventilator. The mice were mechanically ventilated under the following conditions: air composition 0.5 O_2 and 1.0 compressed air at a frequency of 150 beats/min with a tidal volume of 0.3 mL of H_2O .

Mice were exposed to increasing doses of aerosolized methacholine (0.39–25 g/L; Sigma-Aldrich) in NaCl solution. The response to methacholine is expressed in lung resistance. Pulmonary resistance was determined by dividing transpulmonary pressure by airflow at isovolume points. Data are presented as average R_L (in $\text{cm H}_2\text{O} \cdot \text{mL}^{-1} \cdot \text{s}^{-1}$). During the measurements the vital signs were monitored. After the last measurement, the mice were killed by cardiac puncture. The *in vivo* airway hyperresponsiveness to methacholine from an experiment performed separately from the study described here was otherwise performed with the same experimental procedures described for the sham group and the control group (Supplemental Figure 1). In all experimental groups, some mice had to be excluded from the analyses, because they died before the measurement could be finished.

Determination of cellular composition and cytokine concentrations in BAL fluid. Bronchoalveolar lavage (BAL) fluid collection was performed immediately after the mice were killed as described earlier (22). Briefly, the lungs were lavaged through a tracheal cannula with 1 mL of NaCl solution supplemented with protease inhibitor (complete mini tablet; Roche Diagnostics, GmbH, Mannheim, Germany) warmed to 37°C. Three subsequent lavages were performed with 1 mL of NaCl solution warmed to 37°C. Cells in the first milliliter of recovered lavage fluid were centrifuged at 500 g for 5 min and the supernatant was stored at -20°C until further analysis. The other 3 mL of recovered lavage fluid was spun down, supernatants were discarded, and all pelleted cells were pooled. For differential BAL fluid cell counts, cytopsin preparations were made and stained with DiffQuick (Medion Diagnostics AG, Düringen, Germany) as described earlier (22). Numbers of eosinophils, macrophages, neutrophils, and lymphocytes were scored by standard morphology and staining characteristics. The investigator counting the cells (ST) was blinded to the treatment groups. In the control group, 1 mouse was excluded from analysis because of insufficient retrieval of the lavage fluid, which could lead to skewed observations.

Determination of cytokine concentrations in the BAL fluid was performed in the first milliliter of recovered lavage fluid as described previously (23) by using a Bio-Rad mouse cytokine 8-plex (Bio-Rad Laboratories, Veenendaal, The Netherlands) according to manufacturer's instructions. The 8-plex consisted of the following cytokines: IFN- γ , IL-4, IL-5, IL-10, IL-12(p70), IL-13, IL-17, and TNF- α .

Whole-blood assay. Whole-blood stimulations were performed with blood samples as previously described (24). Briefly, blood samples taken by cardiac puncture were stimulated with 10 $\mu\text{g}/\text{mL}$ OVA in RPMI-1640 (Lonza, Basel, Switzerland) as a final concentration, or the wells were coated with 50 $\mu\text{g}/\text{mL}$ anti-CD3 antibody (eBioscience, San Diego, CA, USA) in PBS. Concentrations of IFN- γ , IL-4, IL-5, IL-10, IL-12(p70), IL-13, IL-17, and TNF- α were determined as described previously (6, 23).

Antigen-specific and total antibody measurements. OVA-specific immunoglobulins were measured as described earlier (22). Results were analyzed with MicroplateManager PC software (Bio-Rad Laboratories). Concentrations in test sera were calculated in arbitrary units for each individual isotype, relative to a standard curve of pooled plasma.

Flow cytometric analysis. Single-cell suspensions of the spleen were prepared, and the cells were stained as described previously (6). The following monoclonal antibodies were used: rat anti-mouse CD3–peridinin chlorophyll protein complex cyanine 5.5, rat anti-mouse CD4–fluorescein isothiocyanate, rat anti-mouse CD25–phycoerythrin, rat anti-mouse forkhead box 3 (FoxP3)–allophycocyanin. Isotype controls were included for all fluorochromes. All antibodies were obtained from BD Biosciences (Breda, The Netherlands). Fix/Perm and permeabilization buffers were obtained from eBiosciences (San Diego, CA). Flow cytometric analysis was performed on a Becton Dickinson FACSCanto II flow cytometer, and data were analyzed

with BD FACSDiva Software. Gating strategy is depicted in Supplemental Figure 2. In the sham group, 1 mouse was excluded from analyses because of technical difficulties, leading to loss of the sample.

Statistical analysis. To calculate group sizes, power analysis was performed with SampleSize software (Columbia Basin Research). Because some mice died during the measurement of the lung hyperreactivity, it was not possible to include all the mice for the analysis of lung hyperreactivity or to determine cellular composition and cytokine concentrations in BAL fluid. One mouse in the scGOS/lcFOS group was excluded from the analysis of the acute ASR because of a wound on its ear which made it impossible to calculate ear swelling. Statistical analyses were performed with IBM SPSS Statistics 20. Levene's test for equality of variance was used to estimate the probability that treatment groups had different variances. One-factor ANOVA was used to detect differences between multiple treatment groups if all conditions were met, and Tukey's HSD test was used to identify significant differences between the means; otherwise the nonparametric Dunnett's C test was performed. Differences between the means were considered significant at $P < 0.05$. All data are expressed as means $\pm$ SEMs.

Results

Acute ASR. Ear swelling in mice of the control group and the scGOS/lcFOS group was significantly greater than ear swelling measured in the mice of the sham group ($P < 0.001$ and $P = 0.009$, respectively; Figure 1), showing that our current sensitization protocol was sufficient to induce an acute ASR. Interestingly, the ear swelling in the scGOS/lcFOS group was significantly less than the ear swelling observed in mice in the control group ($P = 0.01$), indicating that maternal supplementation with scGOS/lcFOS can help reduce acute ASR.

Airway responsiveness. Airway responsiveness, as measured by lung resistance at 25 g/L methacholine, was greater in the control group than in the sham group. Supplementing dams with scGOS/lcFOS during pregnancy resulted in a suppressed development of OVA-induced airway hyperresponsiveness in the offspring compared with the control group (Figure 2). Statistical significance of differences between the mean lung resistance cannot be calculated within this experiment because of the low

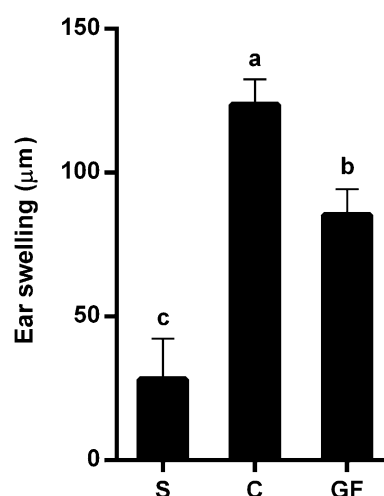


FIGURE 1 Ear swelling after intradermal challenge with ovalbumin in male offspring of dams fed a control or scGOS/lcFOS-supplemented diet during pregnancy. Values are means $\pm$ SEMs. Means without a common letter differ, $P < 0.05$ (Tukey's HSD test). C, control ($n = 4$); GF, short-chain galacto-oligosaccharide/long-chain fructo-oligosaccharide ($n = 5$); S, sham ($n = 4$).

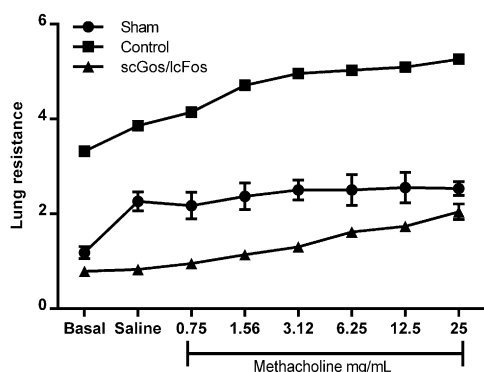


FIGURE 2 Lung resistance in response to increased doses of methacholine in male offspring of dams fed a control or scGOS/lcFOS-supplemented diet during pregnancy. Values are means or means $\pm$ SEMs. C, control ($n = 2$); scGOS/lcFOS, short-chain galacto-oligosaccharide/long-chain fructo-oligosaccharide ($n = 4$); S, sham ($n = 3$).

number of control mice in this trial. However, other parallel studies reported similar levels, indicating the biological relevance and reliability of the data (Supplemental Figure 1).

BAL cells. The percentage of macrophages found in the control group and the scGOS/lcFOS group was less than the percentage found in the sham group ($P = 0.001$ and $P = 0.003$, respectively; Figure 3). The lower percentage of macrophages is most likely caused by the influx of inflammatory cells. The percentage of eosinophils in the control group and the scGOS/lcFOS group was greater than the percentage found in the sham group ($P = 0.009$ and $P = 0.014$, respectively; Figure 3). The control group and the scGOS/lcFOS group showed a significantly higher percentage of lymphocytes in the BAL fluid than in the sham group ($P = 0.001$ and $P = 0.024$, respectively; Figure 3). The percentage of lymphocytes in the scGOS/lcFOS group was lower than in the control group although not significantly so ($P = 0.067$). The percentage of neutrophils in BAL fluid in the control group and the scGOS/lcFOS group was higher than in the sham group ($P = 0.002$ and $P = 0.01$, respectively; Figure 3).

OVA-specific IgE, IgG1, and IgG2a. OVA-specific IgE concentrations in the control group and the scGOS/lcFOS group were significantly greater than the concentration of OVA-specific IgE in the sham group ($P = 0.001$ and $P = 0.002$, respectively; Figure 4). Higher concentrations of OVA-specific IgG1 were found in the control group than in the sham group ($P = 0.011$;

Figure 4). OVA-specific IgG1 concentrations in the scGOS/lcFOS group were higher than in the sham group but not significantly so ($P = 0.081$). Finally, in the scGOS/lcFOS group higher concentrations of OVA-specific IgG2a were found than in the sham group and the control group ($P = 0.001$; Figure 4). The concentrations of total IgG2a in the scGOS/lcFOS group were significantly lower than in the control group and the sham group ($P = 0.036$ and $P = 0.011$, respectively; Figure 4). No significant differences were found between the sham, the control, and the scGOS/lcFOS groups in the concentrations of total IgE (data not shown). Similarly, concentrations of total IgG1 in plasma also were not statistically significantly different between the sham group, the control group, and the scGOS/lcFOS group (data not shown).

Cytokine concentration in BAL fluid and whole-blood assay. Cytokine concentrations of IFN- γ , IL-4, IL-5, IL-10, IL-12(p70), IL-13, IL-17, and TNF- α in BAL fluid and plasma were not statistically significantly different (data not shown).

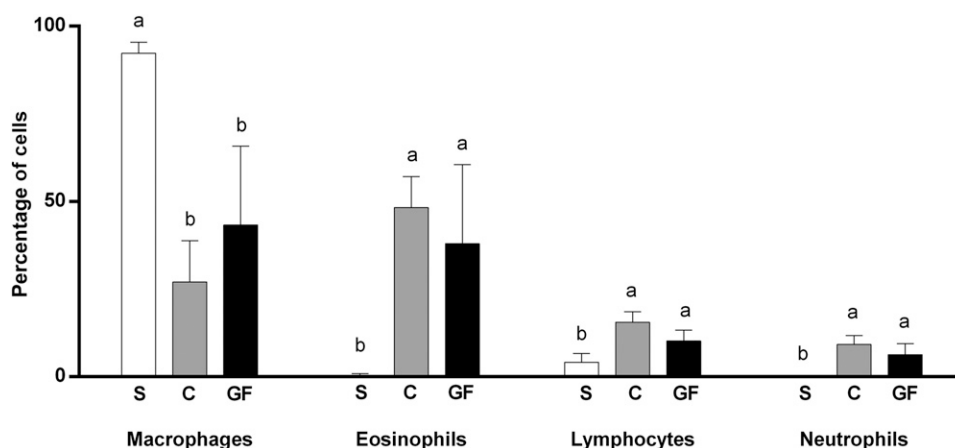
Flow cytometry. No differences in percentages of CD4 $^{+}$ cells were observed (data not shown). In the scGOS/lcFOS group, a significantly higher percentage of the CD4 $^{+}$ CD25 $^{+}$ FoxP3 $^{+}$ (forkhead box 3) Treg population was observed than in both the sham group and the control group ($P < 0.001$; Supplemental Figure 2, Figure 5).

Discussion

The early stages of development are of great importance to the capacity of an organism to thrive later in life. Factors such as maternal diet that affect the developmental program in utero may influence the way in which an adult organism adapts to its environment (14). In previous studies we demonstrated that maternal supplementation with scGOS/lcFOS led to a more tolerogenic immune milieu during pregnancy (20). We hypothesized that this could affect long-term immune development in the offspring. Therefore, in the present study we investigated the effects of maternal scGOS/lcFOS supplementation on the development of allergic asthma in the adult offspring of Balb/c females and C57BL/6 males.

We chose this experimental setup first because an allogeneic pregnancy more closely resembles the human situation. Second, Tregs are shown to be involved in the induction of the observed beneficial effects of supplementation with nondigestible oligosaccharides (13, 14), but these cells are also thought to have a

FIGURE 3 Differential cell count in bronchoalveolar lavage in male offspring of dams fed a control or scGOS/lcFOS-supplemented diet during pregnancy. Values are means $\pm$ SEMs. Means without a common letter differ per cell type, $P < 0.05$ (Tukey's HSD test). C, control ($n = 3$); GF, short-chain galacto-oligosaccharide/long-chain fructo-oligosaccharide ($n = 6$); S, sham ($n = 4$).



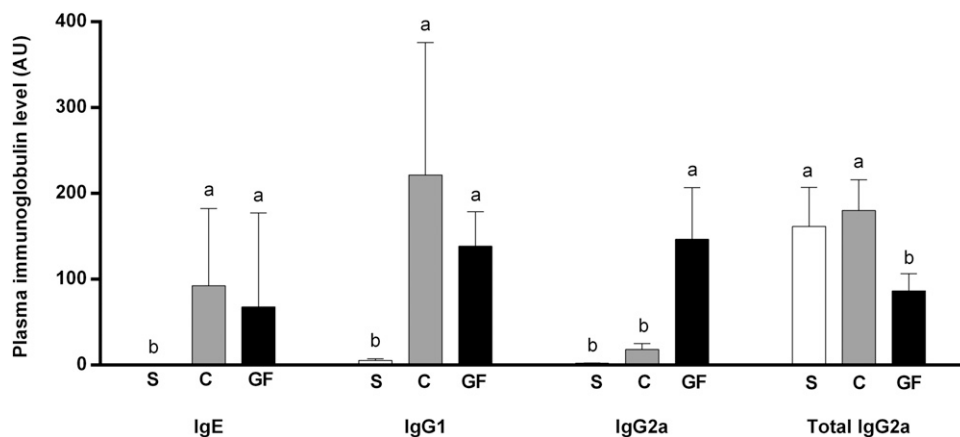


FIGURE 4 Concentrations of OVA-specific IgE, IgG1, IgG2a, and total IgG2a in plasma in male offspring of dams fed a control or scGOS/lcFOS-supplemented diet during pregnancy. Values are means $\pm$ SEMs. Means without a common letter differ per immunoglobulin, $P < 0.05$ (Dunnett's C test). AU, arbitrary unit; C, control ($n = 4$); GF, short-chain galacto-oligosaccharide/long-chain fructo-oligosaccharide ($n = 6$); OVA, ovalbumin; S, sham ($n = 4$).

profound effect on the maintenance of the maternal-fetal tolerance (25). A syngeneic pregnancy may not necessarily challenge the maternal immune system, because paternal antigens will, for the largest part, be identical to maternal antigens. Thus, we reasoned that the putative effects of supplementation with scGOS/lcFOS on the induction of maternal tolerance toward the fetus and that possible subsequent effects on the immune development in the fetus itself may be more outspoken during an allogeneic pregnancy.

We now demonstrate that maternal supplementation with scGOS/lcFOS during pregnancy can affect OVA-induced allergic responses in the adult offspring. A statistically significantly attenuated ASR was observed in the scGOS/lcFOS group. Furthermore, lung hyperresponsiveness to methacholine after OVA challenge was lowered in the scGOS/lcFOS group compared with the control group. It should however be realized that these proof-of-concept data within these experiments included only 2 control mice which cannot lead to definite statistical conclusions about in vivo lung function. However, in another experiment (A Hogenkamp, personal communication) that was recently finalized by using the same model in which the control group was treated similarly as described here, we have observed similar responses to methacholine and included 5 mice. For this reason our data in connection to the 5 mice from this parallel study seem to be representative for the lung resistance as a result of methacholine challenge in allergic offspring of dams fed a control diet (Supplemental Figure 1). Altogether, these results show that maternal supplementation with scGOS/lcFOS can affect in vivo immune responses in the adult offspring.

We did not detect any significant differences in BAL fluid cell populations of the control group and the scGOS/lcFOS group. When the results of the lung hyperreactivity measurement are considered, the absence of significantly lower amount of infiltration of inflammatory cells is a striking finding, indicating that lung function changes and inflammation are not by definition linked as shown in other models for respiratory allergy (26). Note that the lymphocytes present in the BAL were not further examined, and the presence of Tregs in the lung would be interesting to measure. Tregs in the lung limit the expression of CD80, CD86, and major histocompatibility complex class II and increase the IL-12 expression in pulmonary dendritic cells, which prevents T-cell proliferation and Th2 cytokine production (27), and an absence of Tregs is found in lungs that developed airway hyperresponsiveness (27).

In line with this latter hypothesis that Tregs might be involved, a significantly higher percentage of Tregs was found in the spleens of the scGOS/lcFOS group than in the other groups.

The higher population of Tregs in the spleen in offspring of dams supplemented with scGOS/lcFOS during pregnancy could affect the availability of Tregs to migrate into the lungs.

This hypothesis requires further investigation, because differences in Treg populations in the lungs would also be expected to generate different IL-10 concentrations in the BAL fluid, which were not observed in this study. Alternatively, there is a possibility that lung tissue macrophages may be involved in the reduction of the lung hyperreactivity in the scGOS/lcFOS group. These cells recently were shown to be able to generate inducible Tregs, which are important in maintaining airway tolerance (28). IL-10 does not appear to play a crucial role in this process (28–30), whereas TGF- β and retinaldehyde dehydrogenase 1 and 2 expressed by lung tissue macrophages were found to be essential (28). We did not include the analysis of TGF- β and retinaldehyde dehydrogenase 1 and 2 expression in this study, but it would be interesting to further examine the possible involvement of these lung tissue macrophages in future studies.

Significantly higher concentrations of OVA-specific IgG2a were found in blood plasma in the scGOS/lcFOS group, which also may reflect increased regulatory responses. In another study, direct supplementation of whey-sensitized mice with oligosaccharides led to a slight reduction in Th2-related IgE and IgG1

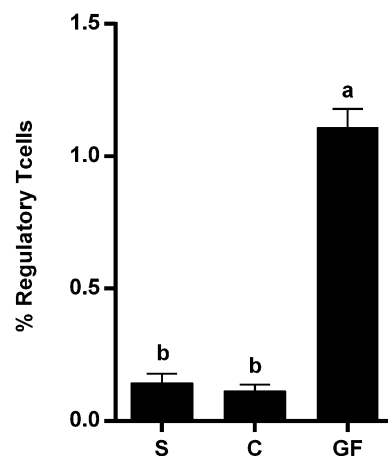


FIGURE 5 Mean percentages of CD3⁺CD4⁺CD25⁺FoxP3⁺ in the spleens of male offspring of dams fed a control or scGOS/lcFOS-supplemented diet during pregnancy. Values are means $\pm$ SEMs. Means without a common letter differ, $P < 0.05$ (Tukey's HSD test). C, control ($n = 4$); FoxP3, forkhead box 3; GF, short-chain galacto-oligosaccharide/long-chain fructo-oligosaccharide ($n = 6$); S, sham ($n = 3$).

concentrations, whereas Th1-related IgG2a concentrations remained high, suggesting that an improved balance of Th1/Th2 responses could underlie the observed beneficial effects (13). Allergen immunotherapy in a murine asthma model also results in an increase of allergen-specific IgG2a in combination with complete inhibition of OVA-induced airway hyperresponsiveness, reduced influx of eosinophils to the lungs, and decreased OVA-specific IgE concentrations in the plasma (31). However, in the present study OVA-specific IgE concentrations were not significantly lower in the scGOS/lcFOS group than in the control group. Furthermore, because determination of cytokine concentrations in whole-blood samples and BAL fluid did not reveal any significant differences, it is difficult to speculate in further detail about differences in Th1, Th2, or Treg responses.

Although the results do not correspond completely with earlier studies in which mice were supplemented with oligosaccharides, it should be stressed that in the present study the dams were supplemented, and the effects observed in the adult offspring were not an effect of direct (oral) exposure to scGOS/lcFOS. It will be a challenging but interesting task to further elucidate the underlying mechanism leading to this immune-programming effect. It is possible that maternal supplementation with scGOS/lcFOS affected the maternal microbiome (16, 17), thereby affecting the availability of short-chain FAs, which are thought to be able to elicit epigenetic changes (32, 33). It is possible that through mechanisms, such as histone modification and/or DNA methylation, alterations to the epigenome of the fetus were induced, leading to these long-term effects. We are currently setting up experiments that will further explore these possibilities. In conclusion, the results from this study demonstrate that maternal scGOS/lcFOS supplementation during pregnancy can have a long-term effect on immune development in the offspring.

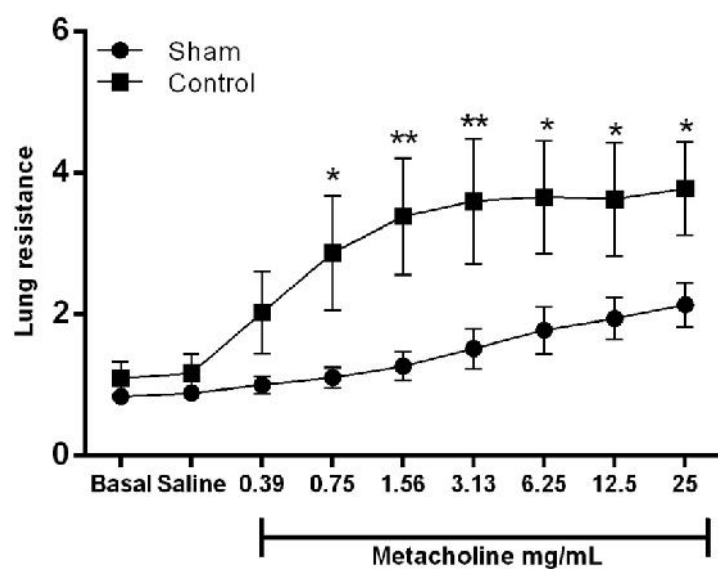
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AH, ST, and NvV designed the research; AH and ST conducted the research and wrote the paper; JG primarily had responsibility for the final content. All authors read and approved the final manuscript.

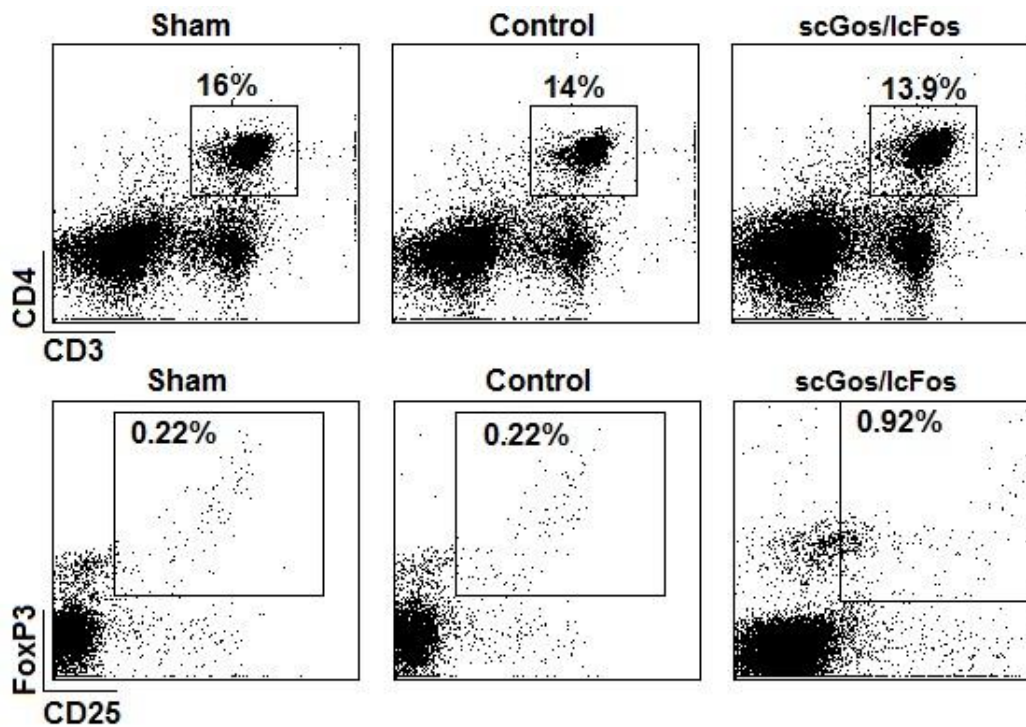
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Supplemental Figure 1. Lung resistance in response to increased doses of metacholine, taken from a parallel study. Values are means $\pm$ SEMs. *: $P < 0.05$, **: $P < 0.01$ (Tukey's HSD test) Sham: $n=7$; Control: $n=5$



Supplemental Figure 2. Gating strategy for splenic Treg populations in the spleen. The upper panels depicts representative dot-plots of a staining for Thelper cells. Cells positive for both CD3 and CD4 were selected in the gate and used as the parent population for the lower panels. The lower panels thus only show CD3⁺CD4⁺ cells, which were analyzed for their expression of CD25 and FoxP3. Percentages of Tregs (CD3⁺CD4⁺CD25⁺FoxP3⁺ cells) are indicated in the gates