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EXPERIMENTAL ALLERGY AND IMMUNOLOGY

Galectin-9 induced by dietary synbiotics is involved in suppression of allergic symptoms in mice and humans

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Abstract:

Background: Prebiotic galacto- and fructo-oligosaccharides (scGOS/lcFOS) resembling non-digestible oligosaccharides in human milk reduce the development of atopic disorders. However, the underlying mechanisms are still unclear. Galectins are soluble-type lectins recognizing β -galactoside containing glycans. Galectin-9 has been shown to regulate mast cell degranulation and T-cell differentiation. In this study, the involvement of galectin-9 as a mechanism by which scGOS/lcFOS in combination with *Bifidobacterium breve* M-16V protects against acute allergic symptoms was investigated.

Methods: Mice were sensitized orally to whey, while being fed with a diet containing scGOS/lcFOS and *Bifidobacterium breve* M-16V (GF/Bb) or a control diet. Galectin-9 expression was determined by immunohistochemistry in the intestine and measured in the serum by ELISA. T-cell differentiation was investigated in the mesenteric lymph nodes (MLN) as well as in galectin-9-exposed peripheral blood mononuclear cells (PBMC) cultures. Sera of the mice were evaluated for the capacity to suppress mast cell degranulation using a RBL-2H3 degranulation assay. In addition, in a double-blind, placebo-controlled multicenter trial, galectin-9 levels were measured in the sera of 90 infants with atopic dermatitis who received hydrolyzed formulae with or without GF/Bb.

Results: Galectin-9 expression by intestinal epithelial cells and serum galectin-9 levels were increased in mice and humans following dietary intervention with GF/Bb and correlated with reduced acute allergic skin reaction and mast cell degranulation. In addition, GF/Bb enhanced T_H1 - and T_{reg} -cell differentiation in MLN and in PBMC cultures exposed to galectin-9.

Conclusions: Dietary supplementation with GF/Bb enhances serum galectin-9 levels, which associates with the prevention of allergic symptoms.

The gastrointestinal immune system is the largest and most complex immunological organ of the human body. It effectively maintains homeostasis to harmless food antigens. Loss of tolerance toward food antigens results in food allergy (FA) (1), which is in majority characterized by induction of a T_H2 polarized immune response during the sensitization phase resulting in the production of allergen-

specific IgE. Subsequent exposure to the allergen causes diarrhea and ultimately anaphylactic shock through IgE-mediated mast cell degranulation. Intestinal epithelial cells (IEC) provide a first-line barrier between luminal contents and immune cells. However, emerging evidence points out that IEC are important regulators involved in modulating immune responses (2, 3).

In a murine model for cow's milk allergy (CMA), dietary supplementation of a specific prebiotic 9 : 1 mixture of short-chain galacto-oligosaccharides (scGOS; [Gal β 1-4] $_3$ -8Glc; Gal, galactose; Glc, glucose) and long-chain fructo-oligosaccharides (lcFOS, ([Frc β 2-1] $_{>20}$ Frc β 2-1Glc; Frc, fructose) (scGOS/lcFOS) reduces the acute hypersensitivity response (AHR) to whey – a major allergen in CMA (4). scGOS/lcFOS structurally and functionally resembles non-digestible oligosaccharides present in human milk, which are involved in the maturation of immune responses of young infants and oral-tolerance induction (5, 6). A synbiotic diet containing scGOS/lcFOS and *Bifidobacterium breve* M-16V (GF/*Bb*) reduced AHR in the CMA model even more pronounced (7) and reduced the atopic dermatitis score in infants suffering from IgE-mediated eczema after 12 weeks treatment. Asthma-like symptoms and the prevalence of asthma medication 1 year later were declined as well (8, 9). The underlying mechanisms by which scGOS/lcFOS exerts its protective effect on AHR are unknown; however, scGOS/lcFOS may interfere with mast cell degranulation and may induce T_H1- and T_{reg}-cell polarization.

Galectins are soluble-type lectins expressed by IEC exhibiting binding specificity for β -galactosides (10, 11). IEC express galectin-2, -3, -4, and -9 (12, 13), which are localized in the cytoplasm, but are secreted through yet unknown mechanisms. Galectins are involved in the regulation of immune responses and tolerance induction by inducing signaling

through the formation of galectin-glycoprotein lattices on cell surfaces (14, 15). Recently, galectin-9 was shown to neutralize IgE and to induce T_{reg} type immune responses (16, 17). We hypothesized that galectin-9 is involved in the suppression of allergic disease.

Material and methods

Murine model for CMA

Animal use was performed in accordance with guidelines of the Dutch Committee of Animal Experiments. The CMA model was performed as described (7). Three-week-old specific pathogen-free C3H/HeOuJ mice (Charles River Laboratories, Maastricht, The Netherlands) were fed a control diet, *Bifidobacterium breve* M-16V (2% wt : wt 2×10^9 CFU/g; probiotic), scGOS/lcFOS (1% wt:wt, 9 : 1 scGOS : lcFOS; prebiotic) or a combination of both (GF/*Bb*) as described (4). Prebiotics were exchanged for the same amount of total carbohydrates in the diet. Acute hypersensitivity response was measured after 1 h after i.d. challenge with whey in the ear pinnae (10 μ g/100 μ l PBS). Ear thickness was measured in duplicate using a digital micrometer (Mitutoyo, Veenendaal, The Netherlands). Ear swelling was calculated by subtracting basal ear thickness from the ear thickness after 1 h. Oral challenge was performed using 100 mg whey/0.5 ml PBS (Fig. 1A).

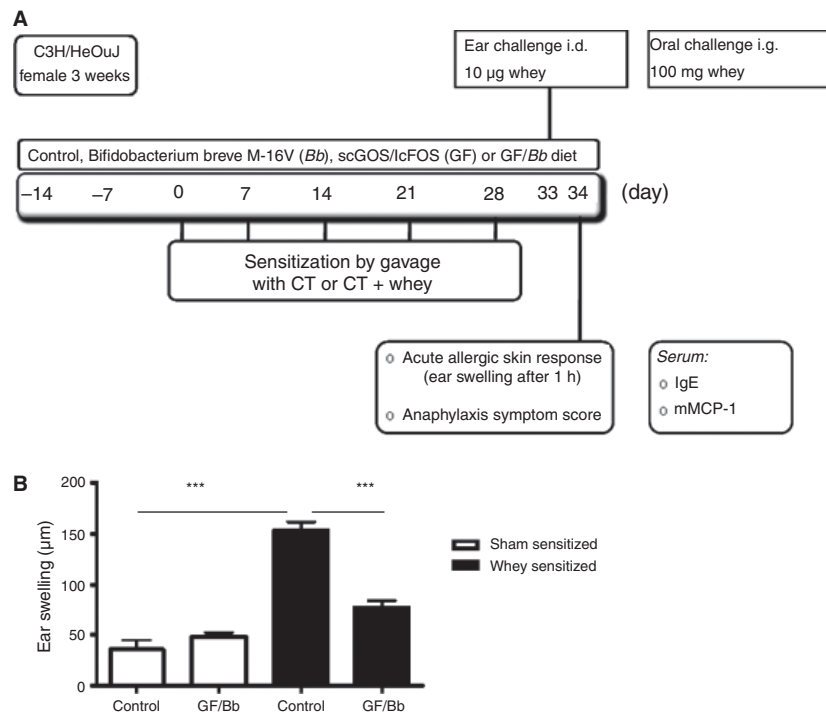


Figure 1 scGOS/lcFOS in combination with *Bifidobacterium breve* M-16V (GF/*Bb*) suppress acute hypersensitivity response (AHR) in mice. (A) Schematic overview of the murine cow's milk allergy model. Mice were fed a control diet or a diet supplemented with either *Bifidobacterium breve* M-16V, scGOS/lcFOS or a combination of both (GF/*Bb*) starting 2 weeks before and during five consecutive

weeks of sensitization to whey, using cholera toxin (CT) as adjuvant. (B) One hour after intradermal challenge with whey in the ear, AHR was reduced in whey-sensitized mice treated with GF/*Bb* compared with whey-sensitized mice. Data represent mean $\pm$ SEM of six mice, *** $P < 0.001$.

Immunohistochemistry

Mouse swiss rolls from proximal ileum were fixed in neutral 10% formalin for 24 h and paraffin embedded. Five-micrometre sections were mounted on coated slides, dewaxed, microwaved in 0.01 M sodium citrate for 10 min and cooled down for 30 min. Aspecific background was blocked with PBS/5% rabbit serum/1% BSA for 30 min and incubated overnight at 4°C with polyclonal goat-anti-mouse galectin-9 antibody (R&D Systems, Minneapolis, MN, USA) in PBS/1% BSA. Galectin-9 was detected with biotinylated donkey-anti-goat antibody (Dako, Glostrup, Denmark) in PBS/1% BSA for 1 h. Slides were incubated in 3% H₂O₂/PBS for 30 min, followed by ABC-HRP complex (Vector Laboratories, Burlingame, CA, USA) for 1 h. Staining was visualized using DAB for 10 min and counterstained with Mayer hematoxylin.

ELISA

IL-10, IFN- γ (Biosource CytoSetsTM, Nivelles, Belgium), IL-17A (Arcus Biologicals, Modena, Italy) and mouse mast cell protease (mMCP)-1 (Moredun Scientific, Midlothian, UK) were measured according to manufacturer's protocol. Specific serum immunoglobulins were measured as described (7). For galectin-9, high-binding EIA/RIA 96-well plate (Costar, Lowell, MA, USA) were coated with polyclonal goat-anti-human or anti-mouse galectin-9 antibodies (R&D Systems) in PBS overnight at 4°C. Plates were blocked 1 h with PBS/1% BSA. Samples (1 : 2, 1 : 5 or 1 : 10 dilutions) were added for 2 h and incubated 1 h with biotinylated polyclonal goat-anti-human galectin-9 antibodies (R&D Systems) in PBS/1% BSA. Plates were incubated 1 h with streptavidin-HRP (R&D Systems) followed by development with tetramethylbenzidine (TMB; Thermo Scientific, Rockford, IL, USA). The reaction was stopped with 2 M H₂SO₄ and optical density (OD) was measured at 450 nm. The 1 : 5 dilution was most optimal for the mice sera, with OD values ranging between 0.05 and 2.416 after correcting for the OD value of the negative control (0.054). Samples were added in a single plate, allowing direct comparisons of the OD values.

RBL-2H3 assay

The release of β -hexosaminidase by the rat basophil leukemic RBL-2H3 cell line was measured as a model for mast cell degranulation. RBL-2H3 cells were cultured in minimal essential medium supplemented with L-glutamine, 10% RPMI1640, 10% FCS, and penicillin (100 U/ml)/streptomycin (100 μ g/ml) (Sigma, Zwijndrecht, The Netherlands). RBL-2H3 cells (1×10^5) were cultured in 96-well culture plates for 1 h at 37°C/5%CO₂. RBL-2H3 cells were incubated with 15%v/v anti-DNP-OVA IgE and washed in Tyrode buffer. Cells were stimulated with DNP-HSA (25 ng/ml) (Sigma) for 1 h. Incubation with Triton X-100 was used as 100% degranulation. Supernatants were collected and incubated for 1 h with 4-methylumbelliferyl-N-acetyl- β -D-glucosaminide. The reaction was stopped with glycine (pH = 10.7)

and β -hexosaminidase release measured by fluorescence (excitation: 360 nm, emission: 460 nm). Lactose (100 mM, sucrose as negative control) or murine T-cell immunoglobulin and mucin (TIM) domain-3-Ig fusion protein (R&D Systems) were used to neutralize serum galectin-9.

cDNA synthesis and real-time PCR

Mesenteric lymph nodes (MLN) were collected in 200 μ l RNAlaterTM (Qiagen GmbH, Hilden, Germany), 1 cm proximal ileum was snap frozen in liquid nitrogen. Samples were stored at -20°C until cDNA synthesis. mRNA was isolated using the mRNA capture kit (Roche, Mannheim, Germany). Real-time PCR was performed as described (18). *GAPDH* was used as reference gene. Relative target mRNA abundance was calculated by: relative mRNA abundance = $100 \times 2^{Ct[GAPDH] - Ct[target\ mRNA]}$. Primers were commercially purchased (SA Biosciences–Qiagen GmbH, Hilden, Germany).

Purification of PBMC

Human peripheral blood mononuclear cells (PBMC) from healthy donors were isolated from buffy coats (Sanquin, Amsterdam, The Netherlands) and purified using Ficoll-Paque Plus (GE Healthcare Life Sciences, Uppsala, Sweden) gradient centrifugation (1000 g, 20 min). Peripheral blood mononuclear cells were collected and washed in PBS/2% FCS. Remaining erythrocytes were removed using lysis buffer (4.14 g NH₄Cl, 0.5 g KHCO₃, 18.6 mg Na₂EDTA in 500 ml water, pH = 7.4 and filter sterilized) for 5 min on ice. Peripheral blood mononuclear cells were resuspended in RPMI1640 (Lonza, Verviers, Belgium) supplemented with 2.5% FCS, penicillin (100 U/ml)/streptomycin (100 μ g/ml) and sodium pyruvate (1 mM; Sigma).

PBMC stimulation

Peripheral blood mononuclear cells were stimulated with anti-CD3 (CLB-T3/2) and anti-CD28 antibodies (CLB-CD28; both 1 : 10 000; Sanquin) in the presence of recombinant galectin-9 (0.04–1.0 mM, kindly provided by Dr. L.G. Baum, UCLA School of Medicine, Los Angeles, USA) (19) for 24 h.

Flow cytometry

Cells were stained with CD4-PerCP-Cy5.5 (OKT-4), CD69-PE (FN50), CD25-AlexaFluor488 (BC96), Foxp3-PE (236A/E7) (eBioscience, San Diego, CA, USA) and CD183 (CXCR3)-AlexaFluor488 (1C6, BD Biosciences, San Jose, CA, USA). Cells were fixed with 0.5% paraformaldehyde or permeabilized using the FoxP3 Staining Set according to the manufacturer's protocol (eBioscience). Flow cytometric analysis was performed using a FACSCantoII (BD Biosciences).

Human serum samples

In a double-blind, placebo-controlled multicentre trial, 90 infants with atopic dermatitis received a hydrolyzed formula

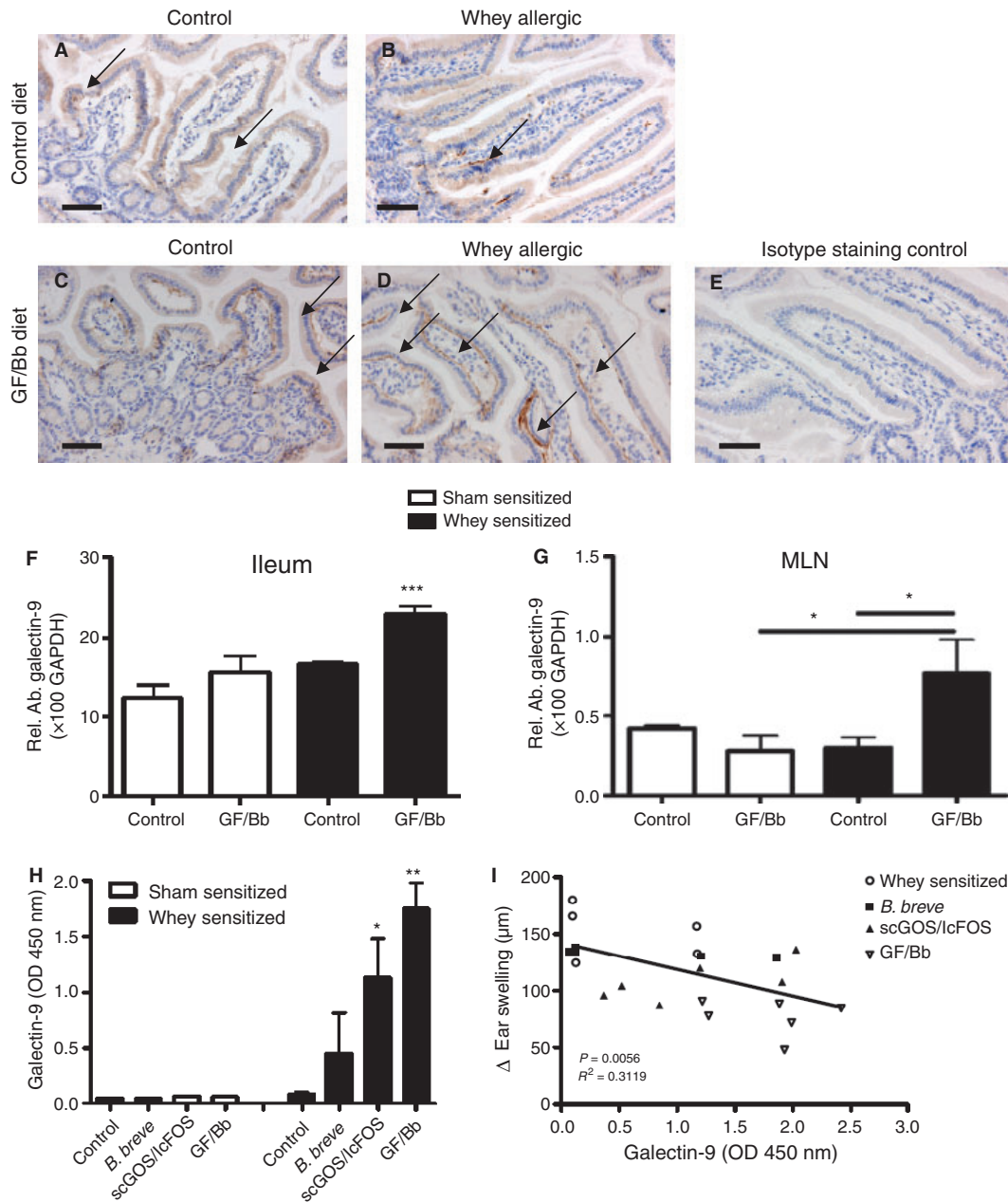


Figure 2 Galectin-9 is basolaterally expressed by intestinal epithelial cells and increased in serum in whey allergic mice fed GF/Bb to protect against allergy. Immunohistochemical analysis of galectin-9 expression in the proximal ileum (A–E). Diffuse galectin-9 staining was observed in the epithelium of sham- or whey-sensitized mice fed the control diet (A,B) as well as in sham-sensitized mice fed GF/Bb (C). In whey-sensitized mice fed GF/Bb, specific basolateral galectin-9 expression was observed (D). Isotype staining control

showed no background (E). In whey-sensitized mice – but not sham-sensitized mice – fed GF/Bb, galectin-9 expression in ileum (F) and mesenteric lymph nodes (MLN) (G) was increased, which was reflected by increased serum galectin-9 levels (H). Increased serum galectin-9 levels correlated with a reduction in acute hypersensitivity response in whey-sensitized mice (I). Data represent $n = 5$ –6 mice per group, mean $\pm$ SEM, * $P < 0.05$, ** $P < 0.01$.

with or without GF/Bb for 12 weeks as described (8). The hydrolyzed formula contained 1.3×10^9 CFU/100 ml *Bifidobacterium breve* M-16V and 0.8 g/100 ml scGOS/lcFOS (9 : 1). At baseline and week 12, a 2–3-ml blood sample was collected.

Statistics

Data obtained from the CMA model and clinical study were tested using unpaired Student's *t*-test or one-way ANOVA. Data obtained from PBMC cultures were analyzed using a

one-way ANOVA. Correlation was tested by Pearson's correlation coefficient. Analyses were performed using Graphpad Prism 4.0 (La Jolla, CA, USA). $P < 0.05$ was considered statistically significant.

Results

GF/Bb prevents AHR in mice

To study the contribution of GF/Bb in the prevention of AHR, a murine model for CMA was used in which mice were orally sensitized and challenged with whey (Fig. 1A) (7). Whey-sensitized mice showed an increased AHR, and upon dietary supplementation with GF/Bb AHR was reduced, showing a preventive effect of the diet on the development of AHR (Fig. 1B). However, reduction of allergy was not associated with decreased levels of whey-specific IgE (416 ± 139.7 vs 633.6 ± 106.2 arbitrary units in allergic mice compared with whey-sensitized mice fed GF/Bb).

Allergic mice fed GF/Bb show specific basolateral galectin-9 expression by IEC

Galectin-9 has been described to neutralize IgE and to induce a T_{reg} -mediated immune response (16, 17). To assess whether galectin-9 is involved in the suppression of allergic symptoms induced by GF/Bb *in vivo*, galectin-9 expression in the intestine was investigated. Immunohistochemical staining revealed expression of galectin-9 by IEC (Fig. 2A–E). Localization of expression of galectin-9 was not changed in whey-sensitized and challenged mice or control mice fed GF/Bb (Fig. 2A–C). However, whey-sensitized mice fed GF/Bb show specific galectin-9 staining at the basolateral side of IEC (Fig. 2D), suggesting that GF/Bb induces basolateral epithelial secretion of galectin-9. Galectin-9 expression in

ileum and MLN and serum galectin-9 levels were increased in whey-sensitized mice fed GF/Bb (Fig. 2F–H). The separate components of GF/Bb enhanced serum galectin-9 levels as well, albeit less effectively, suggesting a possible interaction between *Bifidobacterium breve* M-16V-induced TLR signaling and scGOS/lcFOS. Increased serum galectin-9 levels correlated with reduced AHR (Fig. 2I), which depended on sensitization with whey as sham sensitization did not increase serum galectin-9 levels despite oral challenge to whey. Galectin-4 expression was not modulated by the diet, indicating a galectin-9-specific effect (data not shown).

Galectin-9 enhances T_{H1} - and T_{reg} -cell development

To examine whether T-cell polarization in whey-sensitized mice fed GF/Bb is modulated, we evaluated the expression of the transcription factors T-bet, GATA-3, ROR γ T, and Foxp3 as a reflection of respectively T_{H1} , T_{H2} , T_{H17} , and T_{reg} cells in draining MLN as galectin-9 is known for its capacity to induce T_{H1} cell apoptosis (20). T-bet and Foxp3 expression tended to increase in MLN, while GATA-3 expression was not modulated by GF/Bb (Fig. 3A–C). Expression of ROR γ T was expressed at low levels in MLN (Fig. 3D). Unexpectedly, the T_{H1}/T_{H2} and T_{reg}/T_{H2} ratio were significantly increased in whey-sensitized mice fed GF/Bb, indicating induction of T_{H1} and T_{reg} immunity (Fig. 3E,F). The T_{reg}/T_{H1} ratio was not modulated (Fig. 3G). Furthermore, whey-sensitized mice showed a decrease in the $T_{reg}/(T_{H1} + T_{H2})$ ratio, indicating that T_{reg} -cell development over effector T-cell induction is suppressed upon whey sensitization compared with controls, while dietary supplementation with GF/Bb reversed the $T_{reg}/(T_{H1} + T_{H2})$ ratio in favor of T_{reg} -cell development (Fig. 3H).

To study the involvement of galectin-9 in the induction of T_{H1}/T_{reg} differentiation of human cells, CD3/CD28-activated

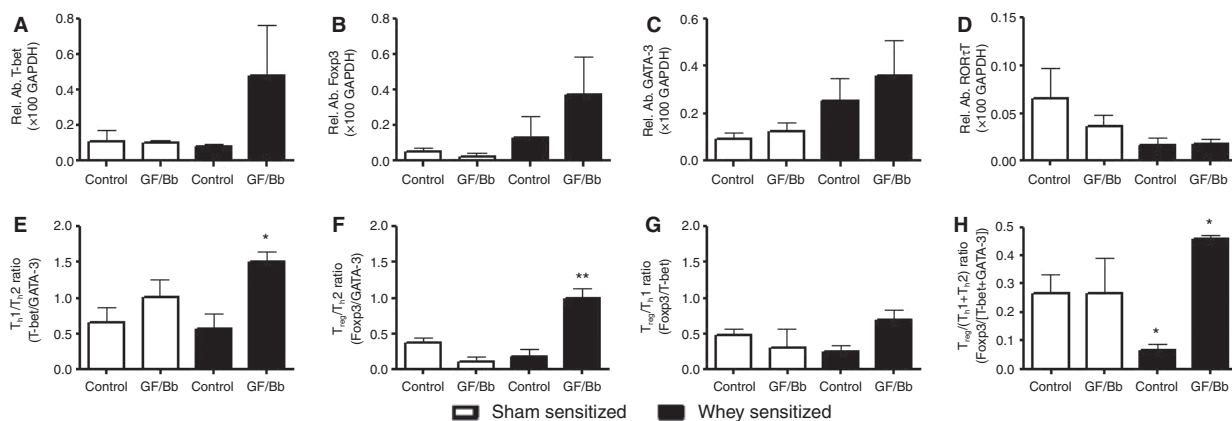


Figure 3 Whey-allergic mice fed GF/Bb show enhanced T_{H1} - and T_{reg} -cell development in mesenteric lymph nodes (MLN). T-cell polarization in MLN was evaluated by analyzing the expression of T-bet (T_{H1} , A), Foxp3 (T_{reg} , B), GATA-3 (T_{H2} , C) and ROR γ T (T_{H17} , D). The expression of T-bet and Foxp3 tended to increase in whey-sensitized mice fed GF/Bb, while GATA-3 expression was not changed. Expression of ROR γ T was hardly detected.

Whey-sensitized mice fed GF/Bb showed an increased T_{H1}/T_{H2} (E) and T_{reg}/T_{H2} ratio (F), but the T_{reg}/T_{H1} ratio remained unaltered (G). The ratio between T_{reg} cells and effector T_{H1} and T_{H2} cells (H) was decreased in whey-allergic mice and increased in whey-allergic mice fed GF/Bb. Data represent $n = 4$ mice per group, mean $\pm$ SEM, * $P < 0.05$, ** $P < 0.01$.

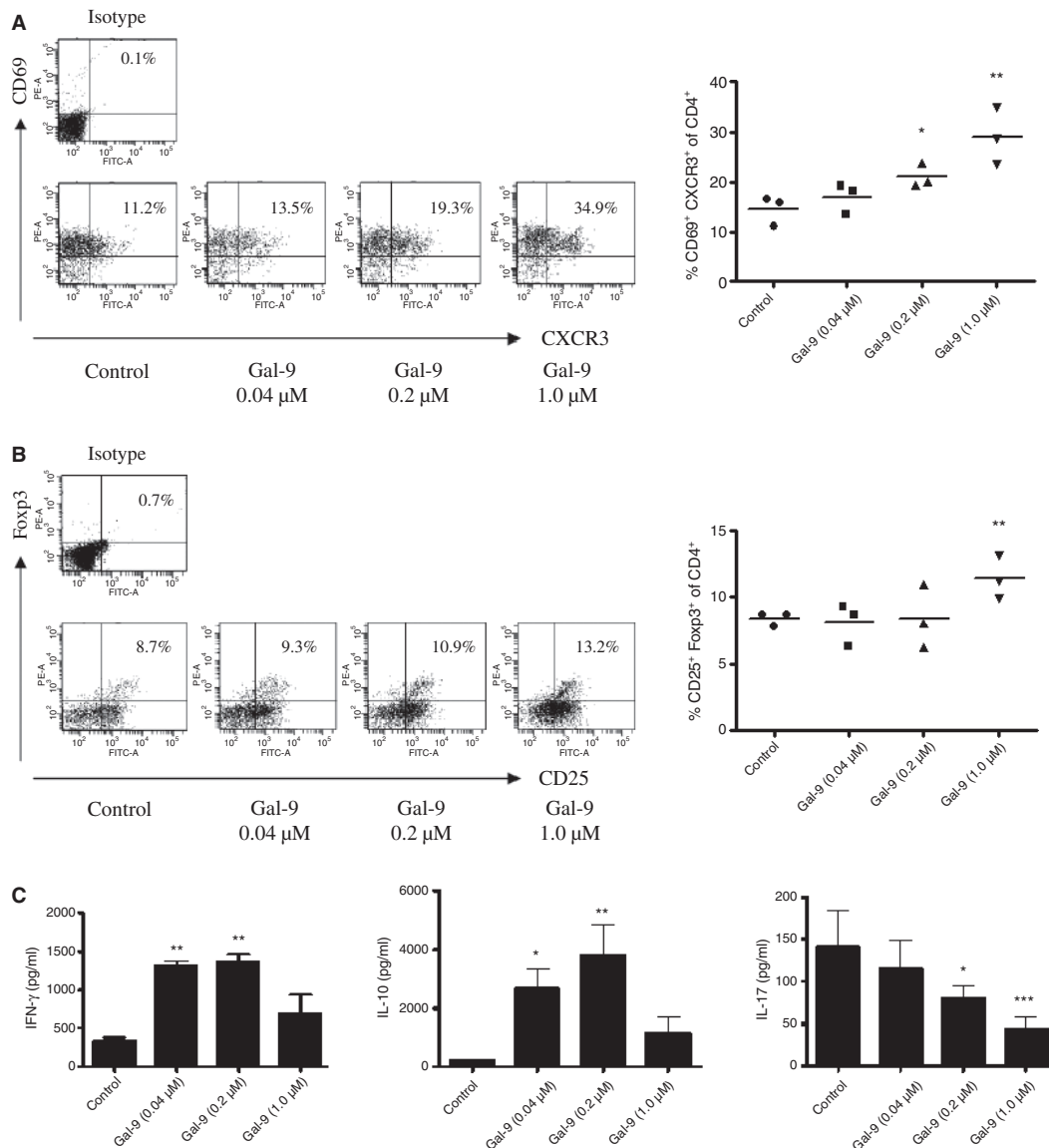


Figure 4 Recombinant galectin-9 induces the development of a T_H1 and T_{reg} effector response. CD3/CD28-activated peripheral blood mononuclear cells (PBMC) were incubated with either medium or increasing concentrations of recombinant galectin-9 for 24 h. The number of activated T_H1 cells ($CD4^+CD69^+CXCR3^+$; A) and T_{reg} cells

($CD4^+CD25^+Foxp3^+$; B) were determined by FACS. Galectin-9 enhanced the percentage of T_H1 and T_{reg} cells dose dependently. (C) Cytokines produced by galectin-9 exposed PBMC were measured in the supernatant by ELISA. Data represent $n = 3$ independent PBMC donors, mean $\pm$ SEM, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

PBMC were exposed to increasing concentrations of recombinant galectin-9. Galectin-9 induced the development of T_H1 and T_{reg} cells as analyzed within the live $CD4^+$ T-cell population (Fig. 4A,B), resulting in increased secretion of IFN- γ and IL-10 and suppressed IL-17 production (Fig. 4C).

Sera of GF/Bb-treated whey-sensitized mice suppress mast cell degranulation

To investigate the involvement of mucosal mast cells as a target of GF/Bb, serum mMCP-1 concentrations were

measured. Suppression of AHR was mast cell-associated as serum mMCP-1 concentrations were reduced, indicating that intestinal mast cell degranulation was inhibited (Fig. 5A). In whey-sensitized mice fed GF/Bb, enhanced serum galectin-9 levels correlated with reduced serum mMCP-1 levels (Fig. 5B).

To evaluate whether galectin-9-containing sera from GF/Bb-treated whey-sensitized mice were able to reduce mast cell degranulation, a RBL-2H3 degranulation assay was performed. Before IgE cross-linking by the hapten TNP-OVA, RBL-2H3 cells were sensitized with anti-TNP-OVA IgE in

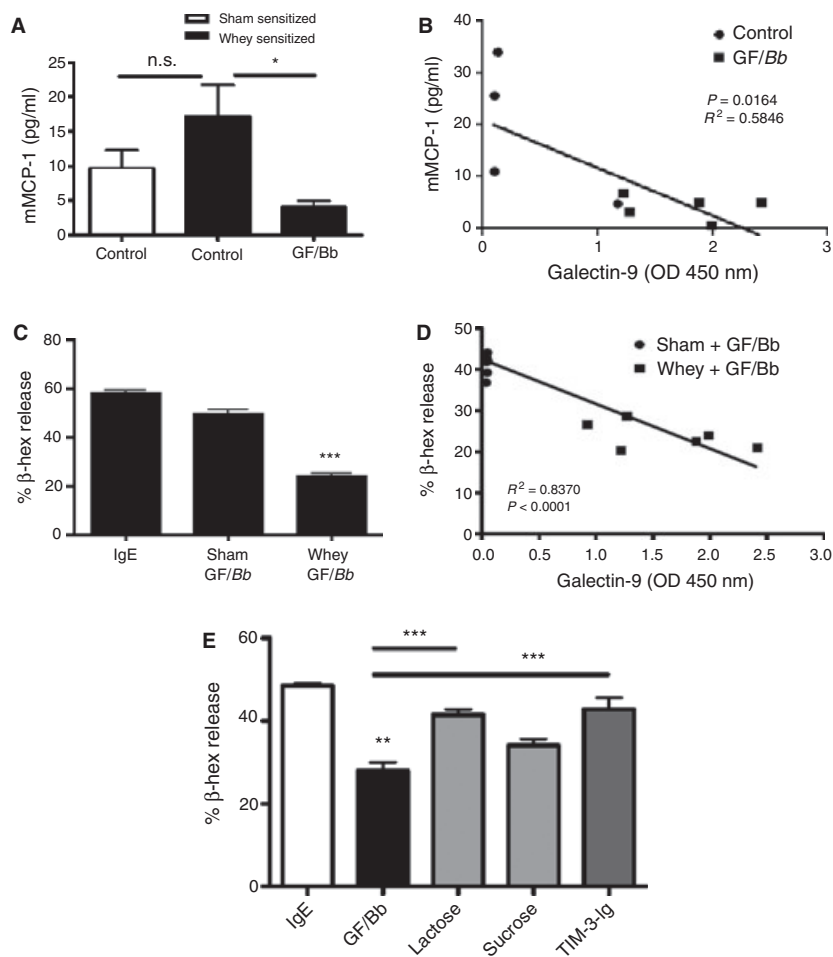


Figure 5 Dietary intervention with GF/Bb suppresses mast cell degranulation. *In vivo* mucosal mast cell degranulation was evaluated by measuring serum mouse mast cell protease (mMCP)-1. Whey-sensitized mice showed a tendency toward increased serum mMCP-1 concentrations after challenge with whey, which was abrogated when mice were fed GF/Bb during sensitization (A). The suppression in serum mMCP-1 concentrations negatively correlated with serum galectin-9 levels (B). *In vitro*, mast cell degranulation was evaluated by β-hexosaminidase release by RBL-2H3 cells upon IgE cross-linking. RBL-2H3 cells were primed with anti-TNP-OVA IgE in the presence of sera derived from sham or whey-

sensitized mice fed GF/Bb. Sera from whey-sensitized mice fed GF/Bb suppressed mast cell degranulation after stimulation with TNP-OVA (C). Anti-TNP-OVA in the presence of fetal calf serum was used as control (IgE). The reduced β-hexosaminidase release correlated with increased serum galectin-9 levels (D). Neutralization of galectin-9 by lactose or T-cell immunoglobulin and mucin (TIM)-3-Ig fusion protein partially abrogated the protective effects of sera from whey-sensitized GF/Bb-fed mice (E). Sucrose was used as control for lactose intervention. Data represent $n = 4-6$ mice per group, mean $\pm$ SEM, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, n.s. not significant.

the presence of sera from GF/Bb-treated animals either sham or whey sensitized. Only serum derived from whey-sensitized GF/Bb-treated mice was able to suppress IgE-mediated degranulation (Fig. 5C). Indeed, increased serum galectin-9 levels from whey-sensitized mice fed GF/Bb correlated with a reduction of RBL-2H3 cell degranulation (Fig. 5D). To prove the role of galectin-9 in suppression of RBL-2H3 cell degranulation, we incubated serum of whey-sensitized mice fed GF/Bb with lactose or TIM-3-Ig fusion protein. Neutralization of galectin-9 by lactose or TIM-3-Ig partially abrogated the protective effect of sera derived from whey-sensitized mice fed GF/Bb (Fig. 5E).

Increased serum galectin-9 levels are associated with GF/Bb-induced suppression allergic symptoms in humans

Recently, it was reported that children suffering from IgE-mediated atopic dermatitis showed reduced allergic symptoms upon treatment with GF/Bb for 12 weeks (8). To determine the link between serum galectin-9 and reduction of allergic symptoms, we investigated whether galectin-9 levels in the serum of these infants were elevated upon treatment with GF/Bb. Serum galectin-9 levels were not different at baseline of intervention. However, after 12 weeks of GF/Bb treatment, serum galectin-9 levels were increased compared with

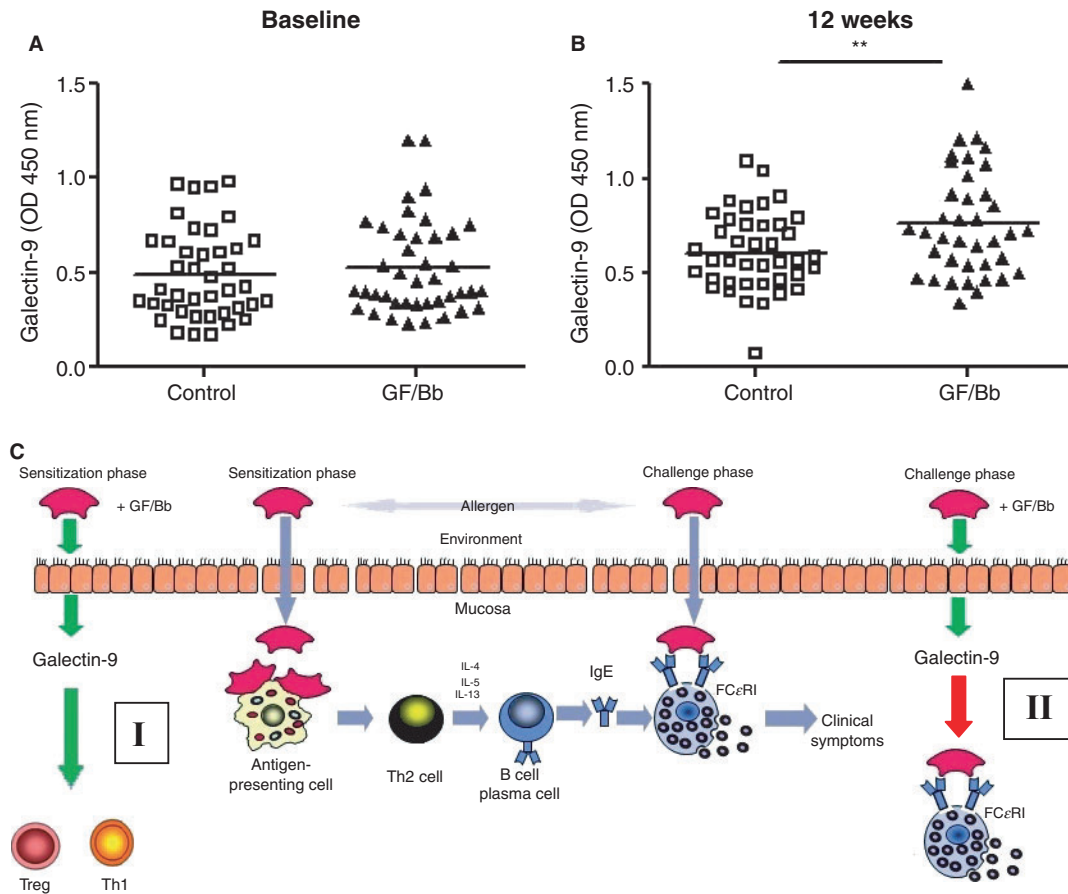


Figure 6 Serum galectin-9 is enhanced in infants that were effectively treated with GF/Bb. ELISA was performed on serum collected at baseline (A) or at 12 weeks (B) after treatment with the GF/Bb diet of infants suffering from atopic dermatitis. Each patient represents one symbol. Treatment with GF/Bb resulted in enhanced serum galectin-9 levels. The horizontal bar represents mean, $**P < 0.01$. (C) A schematic view of the mechanisms by which GF/Bb suppresses IgE-mediated allergic reactions. During allergic sensitization, a T_H2 -type effector response is raised

against an allergen, which results in IgE production by B cells. Upon repeated exposure to the allergen, IgE-primed mast cells degranulate, resulting in clinical symptoms (blue arrows). Supplementation of GF/Bb to the diet 2 weeks before and during sensitization to whey induces galectin-9 secretion by the intestinal epithelium driving T_H1 and T_{reg} cell development (I). During the challenge phase, galectin-9 induced by GF/Bb directly inhibits mast cell degranulation (II). Green arrows, stimulation; red arrows, suppression.

placebo-treated patients (Fig. 6A,B). IgE levels were not affected upon treatment with GF/Bb (8).

Discussion

scGOS/lcFOS mimic structural and functional properties of non-digestible oligosaccharides present in human milk, which are involved in the maturation of immune responses and reduce the risk of developing allergic disease in humans (5, 6, 8, 21). Combination of scGOS/lcFOS with *Bifidobacterium breve* M-16V most effectively reduced AHR in whey-allergic mice (7). Here, we demonstrate a novel mechanism by which GF/Bb suppresses allergic symptoms, involving suppression of both mast cell degranulation as well as generating a T_H1 - and T_{reg} -driven immune response through galectin-9.

Classical IgE-mediated allergy is characterized by a T_H2 -driven immune response against an allergen, resulting in

allergen-specific IgE-mediated mast cell degranulation. Hence, GF/Bb-induced protection against allergy may affect T-cell polarization and mast cell degranulation. Dietary supplementation with scGOS/lcFOS supports a T_H1 -type effector response and prevents CMA in mice through T_{reg} cells (4, 7). We demonstrated that whey-sensitized mice fed GF/Bb showed increased basolateral galectin-9 expression by IEC and increased serum galectin-9 levels. Galectin-9 is a ligand for the TIM-3 receptor expressed by T_H1 , T_H17 , and dendritic cells (DC). Activation of TIM-3 induces apoptosis of T_H1 and T_H17 cells (20). We surprisingly found that galectin-9 was able to drive T_H1 - and T_{reg} -cell development, resulting in increased IFN- γ and IL-10 secretion, but also suppressing IL-17 secretion by activated PBMC. However, a high concentration of galectin-9 abolished the T_H1/T_{reg} effector response, possibly by inducing T-cell apoptosis. *In vivo* studies, however, have shown that galectin-9 is also involved in the suppression of

T_H17-mediated immune responses, while promoting T_{reg}-cell induction (17). Furthermore, it has been described that galectin-9 interacting with TIM-3 results in the activation of macrophages (22). Hence, galectin-9 may induce immunity, while preventing T_H1-driven pathology through T_H1-cell apoptosis, resulting in a self-regulating immune response.

Galectin-9 has been described to activate DC, which elicit the secretion of IFN- γ , but not IL-4 and IL-5, by CD4⁺ T cells, promoting a T_H1-type immune response (23). We observed increased T-bet and Foxp3 expression in MLN, reflecting T_H1 and T_{reg} polarization. Upon oral challenge, DC present in the intestinal lamina propria may have been exposed to IEC-derived galectin-9 and migrated toward MLN to induce a T_H1 and T_{reg} immune response. Collectively, our data indicate that GF/Bb treatment suppresses AHR through galectin-9-induced T_H1- and T_{reg}-cell skewing. Although it is uncertain by which mechanisms galectin-9 is involved in the induction of adaptive immunity via DC, future experiments are necessary to investigate whether galectin-9 secreted by IEC directly targets T cells or whether DC are conditioned by galectin-9 to instruct T_H1- and T_{reg}-cell development.

Despite the observation that GF/Bb largely prevented AHR, IgE levels in serum remained elevated. This was also observed in clinical allergy prevention studies in infants at risk, evaluating the effect of probiotics or scGOS/lcFOS on allergy prevention (24–27). Using a murine model for CMA, increased serum galectin-9 levels were found to negatively correlate with AHR and serum mMCP-1 levels. Furthermore, sera of whey-sensitized mice fed GF/Bb suppressed IgE-mediated RBL-2H3 cell degranulation, which was partially prevented by neutralizing serum galectin-9 by lactose or TIM-3-Ig. Galectin-9 binds to IgE with high affinity, preventing antigen–IgE complex formation to reduce mast cell degranulation (16). Likewise, serum galectin-9 levels were increased in patients affected with atopic dermatitis treated

with a hydrolyzed formula containing GF/Bb. Although it was recently shown that galectin-9 expressed by IEC of the duodenum sustained FA via mast cell-derived tryptase in FA patients, and in an OVA-induced murine model for FA (28), our data implicate a protective role for galectin-9 in the prevention of allergic symptoms.

In summary, dietary intervention with GF/Bb induces T_H1- and T_{reg}-cell polarization through the induction of galectin-9 secreted by IEC. In addition, galectin-9 induced by GF/Bb directly suppresses mast cell degranulation (Fig. 6C). This study shows that GF/Bb may serve as an effective and safe strategy to prevent IgE-mediated allergy.

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Author contributions

S.d.K. designed and performed the experiments and wrote the manuscript; E.S., A.K., L.K., J.G., Y.v.K. and L.W. provided supervision during the study and manuscript preparation, B. S. and B.v.E. assisted with the *in vivo* cow's milk allergy (CMA) model, H.v.d.K. performed the immunohistochemical staining of galectin-9 in mouse swiss rolls; J.K. provided *Bifidobacterium breve* M-16V used in the dietary intervention study in the murine model for CMA; A.S. and L.v.d.A. provided serum samples from the Synbad study/clinical trial.

Conflict of interest

The authors declare that there was no conflict of interest.

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