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Dietary, nondigestible oligosaccharides and *Bifidobacterium breve* M-16V suppress allergic inflammation in intestine via targeting dendritic cell maturation

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ABSTRACT

Dietary intervention with short-chain galacto-oligosaccharides (scGOS), long-chain fructo-oligosaccharides (lcFOS) and *Bifidobacterium breve* M-16V (*Bb*) (GF/*Bb*) suppresses food allergic symptoms in mice, potentially via intestinal epithelial cell (IEC)–derived galectin-9. Furthermore, in vitro studies showed galacto- and fructo-oligosaccharides (GF) to enhance the immunomodulatory capacity of a TLR9 ligand representing bacterial CpG DNA when exposed to IEC. In this study, we investigated whether GF/*Bb* modulates dendritic cells (DCs) and subsequent Th2 and regulatory T cell (T_{reg}) frequency in the small intestinal lamina propria (SI-LP). BALB/c mice were fed GF/*Bb* during oral OVA sensitization. DC and T cell phenotype were determined in SI-LP mononuclear cells using flow cytometry. Murine bone marrow-derived DCs (BMDCs) were exposed to recombinant galectin-9 or human monocyte-derived DCs (moDCs) and were cultured in IEC-conditioned medium from GF and TLR9 ligand-exposed HT-29 cells. GF/*Bb* reduced allergic symptoms and enhanced serum galectin-9 levels, while suppressing activation, restoring phagocytic capacity, and normalizing CD103 expression of SI-LP DCs of OVA-allergic mice. In vitro, galectin-9 suppressed LPS-induced activation markers and cytokine secretion by BMDCs, and IEC-conditioned medium suppressed moDC activation in a galectin-9–dependent manner. Besides suppression of SI-LP DC activation, dietary GF/*Bb* also lowered the frequency of activated Th2 cells, while enhancing T_{reg} in the SI-LP of OVA-allergic mice compared to the control diet. Dietary intervention with GF/*Bb* enhances galectin-9 and suppresses allergic symptoms of OVA-allergic mice in association with reduced intestinal DC and Th2 activation and increased T_{reg} frequency in these mice. *J. Leukoc. Biol.* 102: 000–000; 2017.

Abbreviations: Bb = *Bifidobacterium breve* M-16V, BMDC = bone marrow-derived dendritic cell, DC = dendritic cell, GF = galacto- and fructo-oligosaccharide, IEC = intestinal epithelial cell, i.g. = intragastrically, lcFOS = long-chain fructo-oligosaccharide, MLN = mesenteric lymph node, moDC = monocyte-derived dendritic cell, PP = Peyer's patch, scGOS = short-chain galacto-oligosaccharide, SIGN = specific intercellular adhesion molecule-3-grabbing nonintegrin, SI-LP = small intestinal lamina propria, T_{reg} = regulatory T cell

The online version of this paper, found at www.jleukbio.org, contains supplemental information.

Introduction

The prevalence of food allergies is rising in developed countries. Hypersensitivity reactions toward hen's eggs, cow's milk, and peanuts are most predominant [1]. Food allergy results from a break in tolerance toward harmless, food-derived Ags. Changes in DCs have been observed in a murine model for food allergy, and those changes include a reduced frequency of $CD103^+$ migratory DCs with a regulatory phenotype, which are known for their potential to induce T_{regs} [2]. PPs or lamina propria DCs take up orally provided Ags and migrate to the MLNs [3–6]. T cells primed in the MLN return home to the intestinal lamina propria, where they exert their effector function. In case of allergic sensitization, matured DCs induce a strong Th2 response toward the allergen [7, 8]. OVA, a major allergen associated with the hen's egg allergy, is taken up by DCs via the mannose receptor, leading to DC maturation, which can contribute to the intrinsic capacity of OVA to trigger allergic sensitization [7, 9]. Furthermore, suppression of costimulatory molecule expression on those DCs has beneficial effects in allergic disease [10].

The intestinal epithelium provides a first line of defense against luminal Ags present in the gut. IECs regulate DC function and were found to suppress costimulatory molecule expression

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by moDCs [11, 12]. We have recently identified the intestinal epithelium as a source of galectin-9 [13, 14]. In a coculture model of IECs and activated PBMCs, synthetic CpG DNA and DNA derived from *Bb* enhanced Th1 and T_{reg} polarization. That effect was further enhanced by GF and mediated by galectin-9 released by IECs [13]. Furthermore, a synbiotic mixture of nondigestible oligosaccharides and *B. breve* M-16V (GF/*Bb*) was found to increase serum galectin-9 levels in a murine model for cow's milk allergy, which inversely correlated with allergic symptoms [14]. Other studies showed that galectin-9 suppresses airway hypersensitivity responses in mice [15]. Dietary GF/*Bb* protects against development of allergies in mice and reduces the incidence of atopic dermatitis in young infants [16–19]; the mechanisms of action need to be further unraveled. In this study, the effects of GF/*Bb* on allergic inflammation were investigated by studying innate and adaptive immune cells of the SI-LP in a murine model for OVA-induced food hypersensitivity.

MATERIALS AND METHODS

Mice

Five to 6-wk-old, female, specific pathogen-free BALB/c mice (Charles River Laboratories, Maastricht, The Netherlands) were sensitized intragastrically [20] with 20 mg OVA (grade V; Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands) in 0.5 ml PBS containing 10 μg cholera toxin (DMV International, Veghel, The Netherlands) as an adjuvant. Sham-sensitized control mice received cholera toxin alone. Mice were sensitized once a week for 5 wk consecutively. Two weeks before and during oral sensitization and challenge with OVA, mice were fed a control (AIN93G) diet or a GF/*Bb* diet supplemented with *Bb* (2% wt:wt, 2×10^9 CFU/g) (Morinaga Milk Industry, Tokyo, Japan) and scGOS [Vivinal GOS (syrup containing 45% scGOS, 15% lactose, 15% galactose, 25% water), FrieslandCampina, Borculo, The Netherlands]/lcFOS [Raftiline HP (powder containing 99% lcFOS); Orafti, Wijchen, The Netherlands] (total 2% wt:wt, consisting of 1% wt:wt GF 9:1, 0.9% wt:wt scGOS, and 0.1% wt:wt lcFOS), a similar diet was previously used by Schouten et al. [18]. scGOS, lcFOS, and carbohydrates were compensated isocalorically by exchanging against cellulose (for scGOS, lcFOS, and galactose) or lactose (for lactose) of the control diet (**Fig. 1A**). The acute allergic skin response (ear swelling 1 h upon i.d. OVA challenge, 12.5 μg OVA/25 μl PBS) was measured [20], and the fecal water percentage was determined in feces collected 18 h after oral OVA challenge (50 mg OVA/500 μl PBS), when mice were sacrificed. All animal procedures were approved by, and conducted in accordance with, the guidelines of the Animal Ethics Committee of Utrecht University (DEC approval 2012. II.02.038, 2011.II.08.121).

ELISA for OVA-specific Igs

OVA-specific Ig levels were measured in serum, according to a previously described protocol adapted by coating ELISA plates with 20 μg/ml OVA [18].

Cell isolation from murine tissue

From OVA-sensitized and i.g.-challenged mice PPs, MLNs, and small intestinal tissues were collected. PPs and MLNs were crushed, and cells were resuspended in RPMI-1640 medium, supplemented with 5% FCS and penicillin (100 U/ml) and streptomycin (100 μg/ml) (mouse complete cell medium). SI-LP mononuclear cells were collected from 12-cm, proximal small intestines. In short, intestines were opened longitudinally after PPs were excised, minced into 1-cm fragments, and washed briefly in HBSS (Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 15 mM HEPES (pH = 7.2). Next, intestinal fragments were incubated for 15 min in HBSS. supplemented with 15 mM HEPES, 5 mM sodium EDTA, 10% FCS, and penicillin (100 U/ml) and streptomycin (100 μg/ml) (pH = 7.2) 4 times. After incubation, fragments were vortexed for 10 s to remove IECs. The EDTA-containing solution was washed by a 5 min incubation of the fragments in RPMI-1640 medium, supplemented with 10% FCS and penicillin (100 U/ml) and streptomycin (100 μg/ml), followed by incubation 2 times in mouse complete cell medium (RPMI-1640 medium, supplemented with 5% FCS and penicillin (100 U/ml) and streptomycin (100 μg/ml) with 0.25 mg/ml collagenase type VIII (Sigma-Aldrich) for 45 min. SI-LP cells were collected by vortexing intestinal fragments for 10 s after each 45-min incubation to completely disrupt the intestinal tissue. SI-LP cells were subjected to Percoll gradient centrifugation (850 *g* for 20 min) and collected from the interphase. Cells were washed by adding an excess amount of mouse complete cell medium.

Receptor-mediated phagocytosis assay and OVA restimulation of SI-LP cells

Phagocytosis of 40 kDa FITC-dextran (1 mg/ml; Sigma-Aldrich) by 1×10^5/200 μl SI-LP cells in mouse complete cell medium was studied in a U-bottom plate (CoStar Group, Washington DC, USA) at 37°C, as previously described [21]. Incubation at 4°C served as a negative control. Phagocytosis was stopped with ice-cold PBS/0.01% sodium azide after 30 or 60 min, and cells were resuspended in PBS and 2% FCS, stained with CD11c-PerCP-Cy5.5, and analyzed by flow cytometry. The difference in the FITC mean fluorescence intensity between $CD11c^+$ cells maintained at 37°C and 4°C was calculated. Furthermore, 1×10^5 SI-LP cells in 100 μl mouse complete cell medium were restimulated for 5 d with medium, with or without 50 μg/ml OVA, in the presence or absence of recombinant mouse galectin-9 (1 μg/ml; R&D Systems, Minneapolis, MN, USA). Cell culture supernatants were collected and stored at −20°C.

Culture of murine BMDCs

BMDCs were cultured in mouse complete cell medium, with 1% nonessential amino acids (Thermo Fisher Scientific) and 50 μM β-mercaptoethanol. BMDCs were generated as described by Lutz et al. [22] in the presence or absence of 1 μg/ml recombinant mouse galectin-9 (R&D Systems) added from d 3 until d 7 of culture. At d 7, BMDCs were spun and washed to remove galectin-9 and were matured with 1 μg/ml LPS for 48 h. Supernatants were collected and stored at −20°C.

Culture of human HT-29 cells and HT-29 cell-conditioned medium

HT-29 cells were grown confluently in 6-well, 0.4-μm transwell insert filters, as previously described [23]. HT-29 monolayers were basolaterally exposed to recombinant human TNF-α and IFN-γ (both 10 ng/ml; Thermo Fisher Scientific) in RPMI 1640 (Thermo Fisher Scientific) with 10% heat-inactivated FCS and penicillin (100 U/ml) and streptomycin (100 μg/ml). After 6–12 h, the monolayers were washed, and the TNF-α/IFN-γ–primed HT-29 cells were apically exposed to 5 μM TLR9 ligand (M362 CpG oligonucleotide type C; Thermo Fisher Scientific) in the presence or absence of 0.5% w/v of a 9:1 mixture of scGOS/lcFOS for 24 h. Previous studies have shown that scGOS/lcFOS enhance TLR9 ligand-induced epithelial galectin-9 secretion [13]; therefore, both were added to the activated epithelial cells. Basolateral HT-29-conditioned medium was collected and stored at −20°C until further use.

Culture of human moDCs

Healthy donor PBMCs from buffy coats prepared <24 h before PBMC isolation (Sanquin Reagents, Amsterdam, The Netherlands) were isolated, as previously described [23]. $CD14^+$ cells were collected by negative selection using magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). moDC differentiation (see Supplemental Materials and Methods) was performed in the presence or absence of 50% IEC-conditioned medium (as described above), with or without TIM-3-Fc fusion proteins (1 μg/ml) (R&D Systems), according to the manufacturer's instructions. Purity was assessed by flow cytometry and was generally 90–95%. moDCs were generated by culturing

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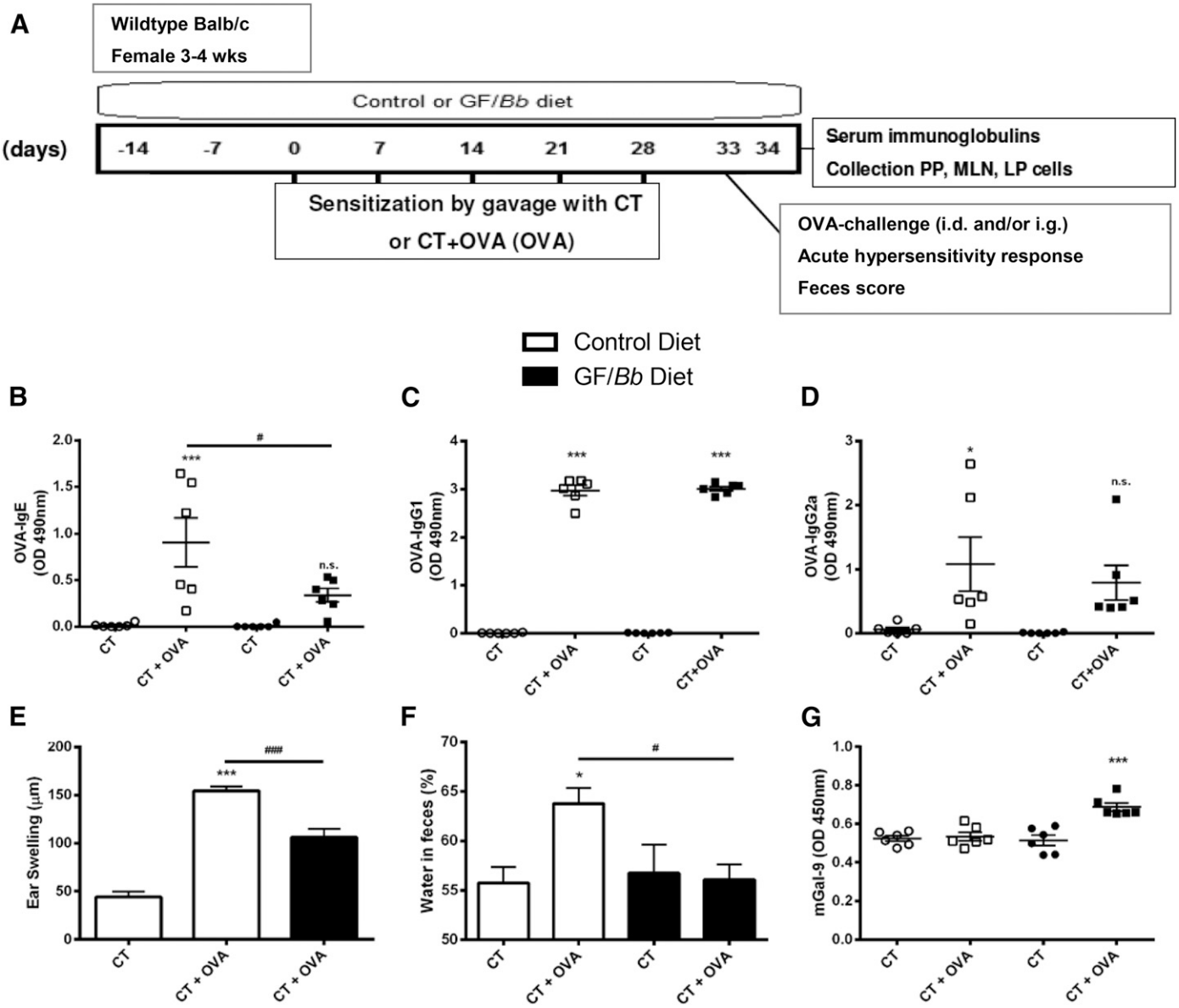

Figure 1. The GF/*Bb* diet suppresses serum OVA-specific IgE levels and acute food-allergic symptoms in an OVA-induced, murine model of food allergy. (A) Schematic overview of the murine food-allergy model and the dietary interventions performed. (B–D) Serum OVA-specific IgE, IgG_1, and IgG_{2a} levels were significantly increased in OVA-allergic mice fed a control diet compared with sham-sensitized animals fed a control diet. Dietary intervention with GF/*Bb* in OVA-allergic animals reduced the OVA-specific serum IgE but not OVA-specific IgG_1 and IgG_{2a} levels compared with OVA-allergic animals fed a control diet. n.s., not significant. (E) In parallel, OVA-sensitized mice fed GF/*Bb* showed a reduced acute allergic-hypersensitivity response upon i.d. allergen challenge in the ear compared with OVA-sensitized mice fed a control diet. (F) In addition, the GF/*Bb* diet prevented diarrhea-like symptoms upon oral OVA challenge in OVA-sensitized mice. (G) Murine galectin-9 (mGal-9) levels in serum were increased in OVA-allergic mice fed GF/*Bb* during allergic sensitization. Data are representative of $n = 2$ independent experiments, $n = 6$ mice/group. Means ± SEM. $^{*}P < 0.05$; $^{***}P < 0.001$ compared with sham-sensitized mice fed a control diet. $^{\#}P < 0.05$; $^{\#\#\#}P < 0.001$ compared with OVA-allergic mice fed a control diet.

10^6 $CD14^+$ cells in complete medium supplemented with recombinant human IL-4 and GM-CSF (30 and 15 ng/ml, respectively; ProSpec, Rehovot, Israel). Medium was refreshed on d 2, 3, and 5, and moDCs were collected on d 7 in PBS with 2% FCS and analyzed by flow cytometry. Immature moDCs were characterized as $CD14^-$DC-SIGN$^+$ cells on d 7 of culture.

Flow cytometry

MoDCs and murine cells isolated from PPs, MLNs, and SI-LP were stained and either fixed in 0.5% paraformaldehyde or permeabilized for intracellular staining. MoDCs were characterized using DC-SIGN-FITC(120507), CD14-PerCp-Cy5(61D3), CD40-FITC(5C3), CD80-PE(2D10.4), CD83-PE(HB15e), CD86-PE(IT2.2), HLA-DR-PE(LN3), and isotype controls, FITC-mouse IgG2b, PE-mouse IgG2b, and PerCP-Cy5.5-mouse IgG1 Abs. After staining, cells were taken up in PBS with 2% FCS and fixed with 0.5% paraformaldehyde.

Fcγ-receptors of murine cells isolated from PPs, MLNs, and SI-LP in PBS with 2% FCS were blocked using 10 µg/ml CD16/CD32 Abs. Cells were stained using CD11c-PerCP-Cy5.5(N418), CD40-FITC(HM40-3), CD80-APC (16-10A1), CD83-FITC(Michel-17), CD86-APC(GL1), CD103-APC(B-Ly7),

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CD103-APC(2E7), I-A/I-E-APC-Cy7(M5/114.15.2), CD4-PerCP-Cy5.5(OKT-4), CD4-PerCP-Cy5.5(RM4-5), and CD69-FITC(H1.2F3) and either fixed in 0.5% paraformaldehyde or permeabilized for intracellular staining using Foxp3-PE (PCH101), Foxp3-APC(FJK-16s), GATA-3-PE(TWAJ) (eBioscience, San Diego, CA, USA). Flow cytometric analysis was performed using FACSCantoII and FACSDiva software (BD Biosciences, San Jose, CA, USA).

Cytometric bead assay for murine cytokines

Murine cytokines produced by BMDCs and SI-LP cells were measured in supernatants using a cytometric bead assay (BD Biosciences) the FACSCantoII and FCAP software (BD Biosciences).

Statistics

For in vitro moDC and ex vivo BMDC experiments, data were analyzed using 1-way ANOVA for repeated measurements. Data derived from the murine food-allergy model were analyzed by 1-way ANOVA. Both were followed by Bonferroni's post hoc test. $P < 0.05$ was considered statistically significant. Data were analyzed using GraphPad Prism software (version 5.0; GraphPad Software, La Jolla, CA, USA).

RESULTS

GF/*Bb* reduces allergy-associated DC maturation in SI-LP DCs in vivo

Control diet fed, i.g.-challenged, OVA-allergic mice showed increased OVA-specific IgE, IgG_1, and IgG_{2a} serum levels compared with sham-sensitized mice, whereas GF/*Bb*-fed, OVA-allergic mice had significantly lower OVA-specific IgE, but not OVA-specific IgG_1 or IgG_{2a} (Fig. 1B–D). The reduced serum OVA-IgE levels were paralleled by a reduction in acute, allergic skin response and diarrhea (Fig. 1E and F). As demonstrated previously, in a murine model for cow's milk allergy [14], serum galectin-9 levels were increased in OVA-allergic mice fed GF/*Bb* compared with the control diet (Fig. 1G).

The percentages of $CD11c^+MHC\text{-}II^+CD11b^-CD103^+$, $CD11c^+MHC\text{-}II^+CD11b^+CD103^-$, and $CD11c^+MHC\text{-}II^+CD11b^+CD103^+$ DCs remained unaffected, whereas $CD11c^+CD40^+CD80^+$ or $CD11c^+CD83^+$ cells in the SI-LP were increased in i.g.-challenged OVA-allergic mice compared with sham-sensitized mice fed the control diet (**Fig. 2A** and **B**). GF/*Bb* lowered the percentage of the activated DCs in OVA-allergic mice (Fig. 2B). CD86 expression was not affected (data not shown). OVA-allergic mice had an increased percentage of $CD11c^+CD83^+$ and $CD11c^+CD86^+$ cells in MLNs, whereas no changes were found in $CD11c^+CD40^+$ $CD80^+$ cells (Supplemental Fig. 1A). In PPs, no effects of OVA allergy were observed on the expression of activation markers by $CD11c^+$ cells. GF/*Bb* did not affect DC activation in the MLNs or PPs (Supplemental Figs. 1A and B and 2).

Because matured DCs have a lower phagocytic capacity [21, 24], the phagocytic capacity of SI-LP $CD11c^+$ DC was analyzed ex vivo. In OVA-allergic mice fed the control diet, SI-LP $CD11c^+$ DC showed reduced FITC-dextran uptake as compared with sham-sensitized mice. This was restored in the $CD11c^+$ cells of OVA-allergic mice fed GF/*Bb* (Fig. 2C).

Galectin-9 suppresses proinflammatory cytokine production by DC. Because OVA-allergic mice fed GF/*Bb* showed increased serum galectin-9 levels (Fig. 1G), we assessed whether in vitro exposure of BMDCs from untreated BALB/c mice to galectin-9 modulated the LPS-induced maturation. LPS up-regulated CD40 and CD80 expression of BMDCs along with an increased production of the inflammatory cytokines IL-1β, IL-6, TNF-α, and the Th2-associated cytokine IL-13 (**Fig. 3**). Preexposure of BMDCs to galectin-9 suppressed the induction of CD40 and CD80 expression upon consecutive LPS stimulation (Fig. 3A), which was also reflected by reduced production of IL-1β, IL-6, TNF-α, and IL-13 (Fig. 3B–E). The production of IL-10 or IL-12p70 by BMDCs remained below the detection limit, irrespective of stimulation (data not shown).

TLR9 and scGOS/lcFOS-induced galectin-9 release by IECs suppresses DC maturation under inflammatory conditions

IECs are an important source of galectin-9, and TLR9 activation of IECs is known to contribute to tolerance induction in the gut mucosa [23–25]. Previously, it has been shown that the TLR9 ligand CpG ODN, as a representative for bacterial DNA, enhanced epithelial galectin-9 secretion by intestinal epithelial cells, which was further increased in the presence of scGOS/lcFOS in an in vitro coculture model of HT-29 and activated PBMCs [13]. In the current study, apical exposure of activated HT-29 cells, which were primed with TNF-α and IFN-γ to create an inflammatory environment, to CpG ODN with scGOS/lcFOS (T9GF) for 24 h, also resulted in enhanced galectin-9 concentrations in the basolateral medium (**Fig. 4A**). Exposure of immature moDCs to the basolateral-obtained T9GF IEC-conditioned medium suppressed CD40, CD80, CD83, and HLA-DR expression by human moDCs, whereas control IEC-conditioned medium had no effect (Fig. 4B–F). Finally, neutralization of galectin-9 in the medium of T9GF-exposed IEC, using a TIM-3-Fc fusion protein, abrogated the reduction in costimulatory molecule expression of moDCs (Fig. 4B–F). Taken together, these data show that combined GF and TLR9 (which may be triggered by *Bb*) exposure of IECs induces galectin-9 secretion by activated IECs, which, in turn, down-regulates DC maturation.

The effect of the GF/*Bb* diet on DCs and the T cell phenotype was further studied in vivo in the OVA-allergic mice. Although the frequency of migratory $CD103^+$ DC in OVA-sensitized mice was unaltered compared with sham-sensitized mice (Fig. 2A), CD103 expression on $CD11c^+MHC\text{-}II^{mid}$ DCs, as well as the frequency of $CD4^+Foxp3^+$ T_{reg} cells, was reduced in OVA-allergic mice fed the control diet (**Fig. 5A–D**). These effects were partially restored by the GF/*Bb* diet (Fig. 5A and D). In parallel, an increased frequency of activated Th2 cells ($CD4^+CD69^+GATA\text{-}3^+$) was observed in the SI-LP of the OVA-allergic mice fed the control diet (Fig. 5E and F), which was normalized by the GF/*Bb* diet.

Galectin-9 suppresses proinflammatory and Th2-associated cytokine production by SI-LP cells ex vivo. To evaluate whether galectin-9 could suppress an established Th2-associated cytokine response, ex vivo OVA restimulations of SI-LP cells of control diet–fed mice were performed. SI-LP cells from OVA-allergic mice showed increased OVA-induced secretion of the Th2-associated cytokines IL-4, IL-5, and IL-13, as well as the proinflammatory cytokine IL-6. TNF-α and IFN-γ secretion tended to increase. The presence of galectin-9 reduced IL-4, IL-5, IL-6, and TNF-α, but not IL-13 or IFN-γ concentrations (**Fig. 6**).

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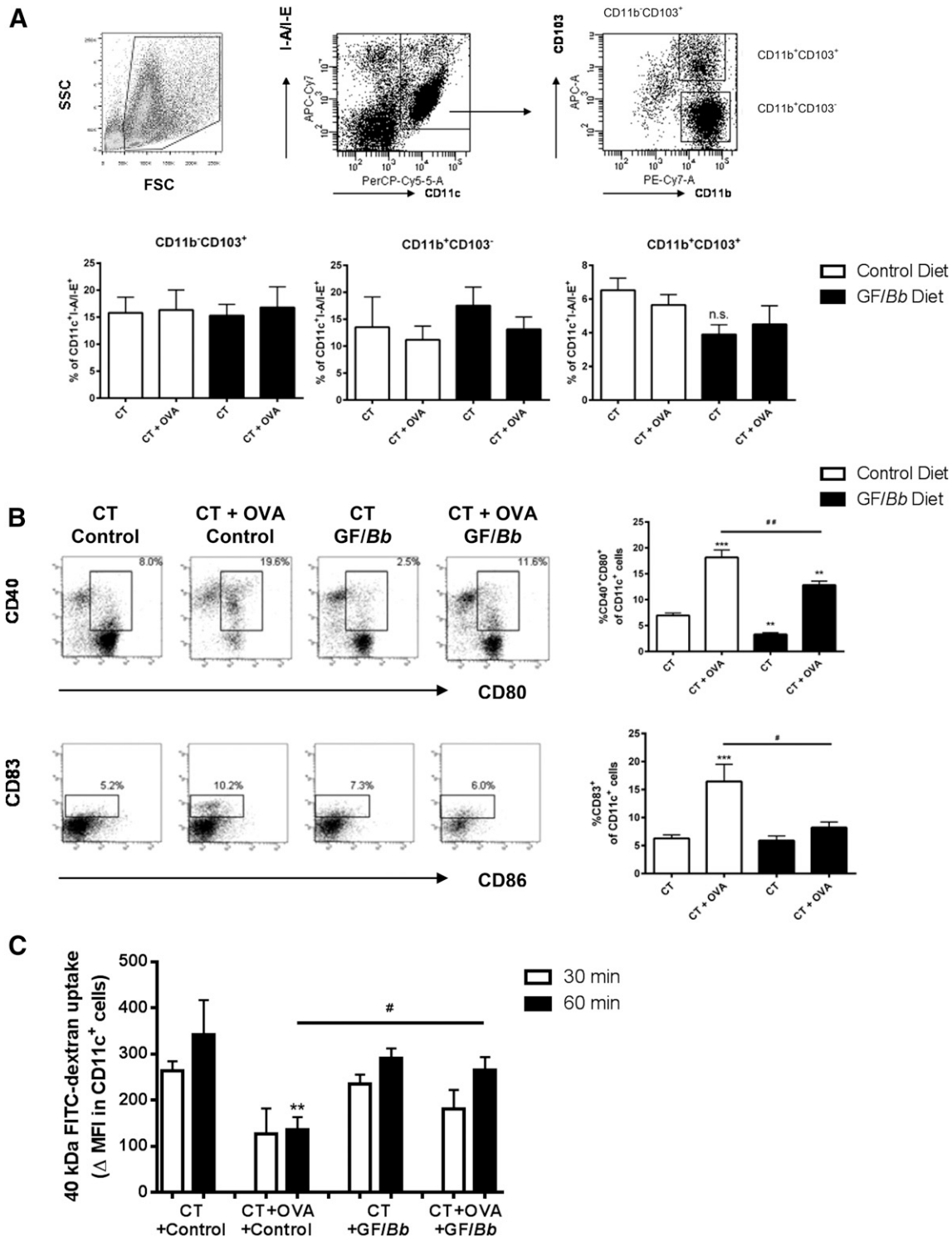

Figure 2. GF/*Bb* suppresses activation and restores phagocytic capacity of DC in the SI-LP of OVA-allergic mice. (A) Upon oral OVA challenge, SI-LP cells were collected, and DCs were characterized by the expression of CD11c, I-A/I-E (MHC-II), CD11b, and CD103. The frequency of CD11c$^+$MHC-II$^+$CD103+DC remained unaffected. SSC, side scatter of light; FSC, forward scatter of light, n.s., not significant. (B) CD11c$^+$ DCs from OVA-allergic mice fed a control diet expressed increased levels of costimulatory molecules, which were reduced upon GF/*Bb* supplementation to the diet. (C) In OVA-allergic animals fed a control diet, the phagocytic capacity of CD11c$^+$ DC was significantly reduced compared with sham-sensitized animals fed a control diet after 60 min of uptake. In OVA-allergic animals fed the GF/*Bb* diet, the phagocytic capacity of CD11c$^+$ DC was restored to control levels. Data are representative of n = 4–6 mice/group. Means ± SEM, $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared with sham-sensitized animals fed a control diet. $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ compared with OVA-allergic mice fed a control diet.

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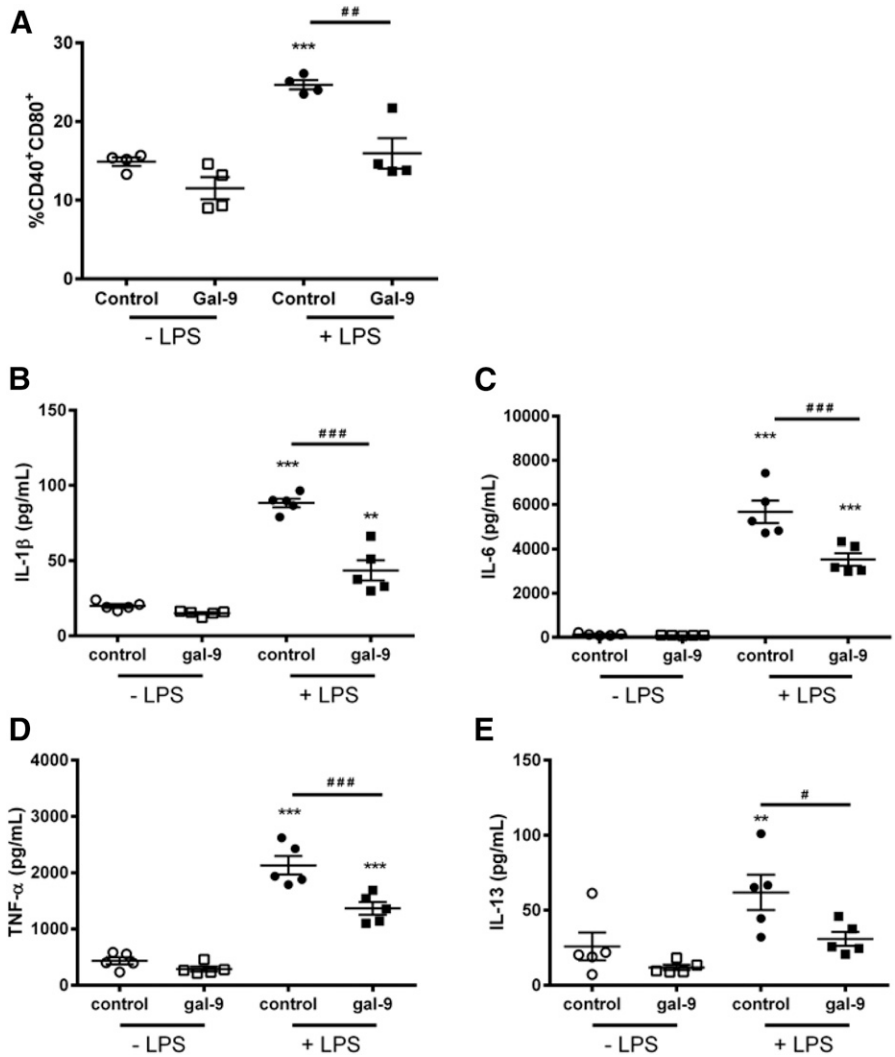

Figure 3. Galectin-9 suppresses the activation and production of proinflammatory cytokines production by murine BMDC upon LPS stimulation. (A) Untreated BALB/c BMDCs were conditioned with recombinant galectin-9 (Gal-9) and stimulated with LPS for 48 h. Galectin-9–conditioned BMDCs were less responsive to LPS stimulation because expansion of $CD40^+CD80^+$ BMDCs was suppressed compared with BMDCs stimulated with LPS in the absence of galectin-9. In parallel, galectin-9–conditioned BMDCs showed reduced production of IL-1β (B), IL-6 (C), TNF-α (D), and IL-13 (E) upon LPS stimulation. Data represent $n = 5$ independent BMDC cultures. Means ± SEM, $^{\#}P < 0.05$; $^{**}P < 0.01$; $^{\#\#\#}$,$^{***}P < 0.001$.

DISCUSSION

As previously shown in a murine model for cow's milk allergy using C3H/HeOuJ, dietary intervention with GF/*Bb* during oral ovalbumin (OVA) sensitization and challenge suppressed allergic symptoms in BALB/c mice. This was in association with enhanced serum galectin-9 levels. In addition, GF/*Bb* partially suppressed allergic sensitization because OVA-IgE levels were reduced; however, specific IgG_1 levels—also known to correlate with the acute allergic reactions in mice [20]—remained unaffected. In this study, the allergy-inhibitory effects of GF/*Bb* were associated with reduced SI-LP DC maturation. Likewise, in vivo suppression of food allergy by soybean isoflavones was related to the suppression of DC activation [25], indicating the relevance of targeting DC in silencing food allergies. The GF/*Bb* diet reduced costimulatory molecule expression and restored the phagocytic capacity of SI-LP $CD11c^+$ DCs to a level comparable to sham-sensitized mice. Uptake of 40-kDa FITC-dextran, similar to OVA, occurs via the mannose receptor [9, 26], implying that in addition to suppression of DC maturation, GF/*Bb* may also interfere with allergen uptake and presentation by SI-LP DCs. Although the frequency of SI-LP $CD11c^+CD103^+$ DC in the OVA-allergic mice was not reduced, as was previously shown in a murine model for peanut allergy [2], we observed that CD103 expression was reduced on $CD11c^+MHCII^{mid}$ DCs, which was prevented by the GF/*Bb* diet. $CD103^+$ DCs may instruct tolerance via induction of T_{reg} cells [27–29]. Indeed, dietary intervention with GF/*Bb* normalized the frequency of $Foxp3^+$ T_{reg} cells in the SI-LP, in association with the restored expression of CD103 on $CD11c^+MHCII^{mid}$ DCs. In parallel, the increased frequency of activated Th2 cells in the SI-LP of OVA-allergic mice was normalized by the GF/*Bb* diet.

Commensals like *Bb*, which belong to the commensal Actinobacteria species, contain DNA enriched with immunosuppressive GTCGTT CpG-rich sequences, which serve as TLR9 ligands and can be tolerogenic, although CpG motifs can also be found in pathogenic species and contribute to inflammation [30–32]. Epithelial TLR9 ligation is known to contribute to intestinal homeostasis and has recently been shown to suppress an allergic effector response in vitro [23, 33–35]. An oligosaccharide mixture scGOS/lcFOS was previously studied for its

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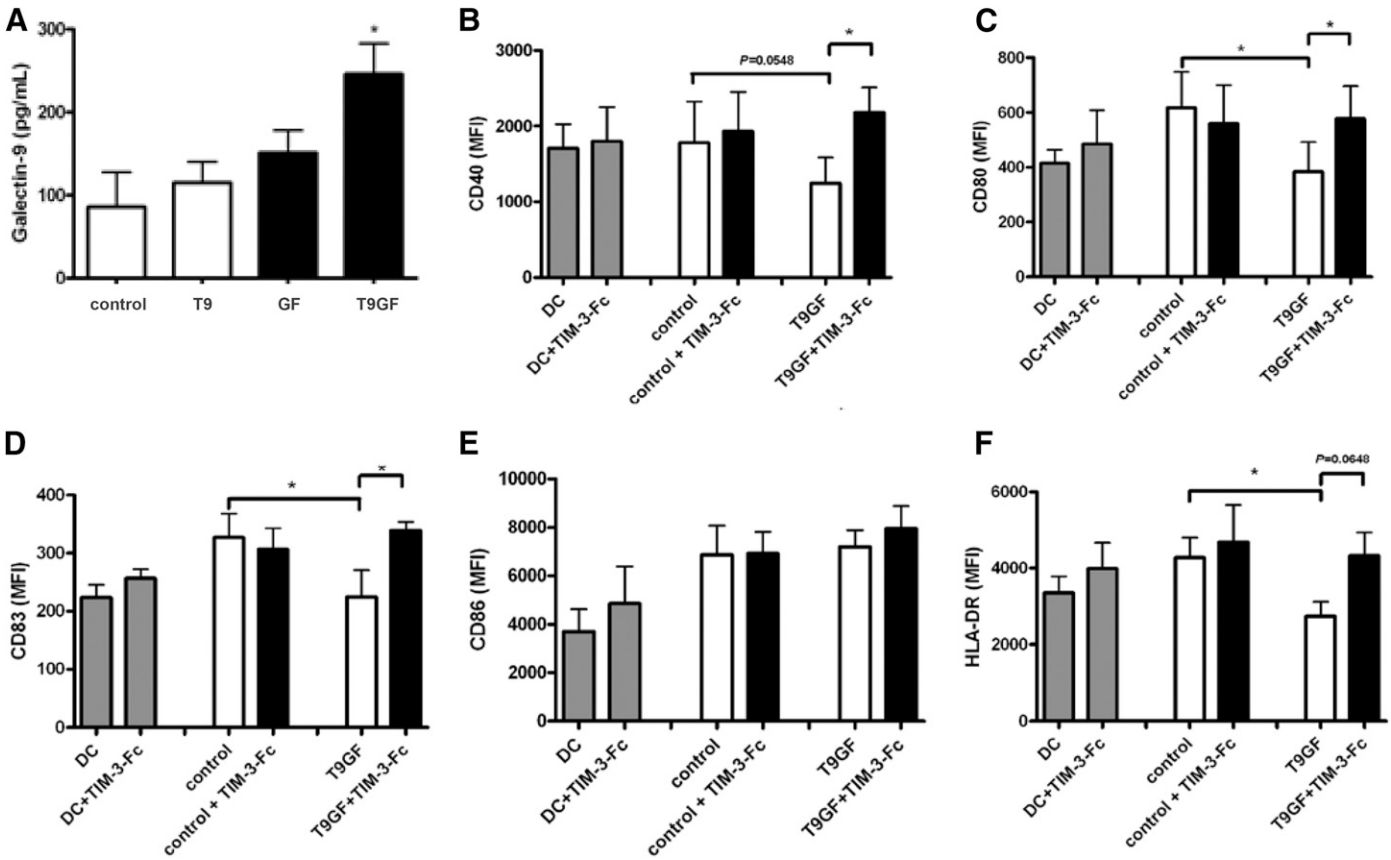

Figure 4. Galectin-9 derived from scGOS/lcFOS and TLR9 ligand-exposed IEC contributes to suppression of moDC activation. (A) Apical exposure of TNF-α/IFN-γ–primed HT-29 cells to CpG DNA (TLR9 ligand) in the presence of scGOS/lcFOS leads to increased secretion of galectin-9 by HT-29 cells. (B–F) Neutralization of galectin-9 by TIM-3-Fc in the IEC-conditioned medium derived from CpG DNA and scGOS/lcFOS (T9GF) exposed, TNF-α/IFN-γ–primed HT-29 cells abrogated the suppression of the costimulatory molecule and HLA-DR expression on moDCs. Direct exposure of moDC (DC) to TIM-3-Fc did not modulate costimulatory molecule and HLA-DR expression. Mean fluorescence intensity (MFI) was corrected for isotype background staining. Data represent $n = 4$ independent PBMC donors. Means ± SEM, $*P < 0.05$.

mechanism of allergy prevention and was found most effective in enhancing galectin-9, modifying the mucosal immune response, and reducing allergic symptoms in whey-allergic mice, when combined with *Bb* [14, 18]. When studying the immunomodulatory effects of UV-killed, whole *Bb* in a coculture of human intestinal epithelial cells and activated blood mononuclear cells, no immunomodulatory effects were observed; however, synthetic CpG oligonucleotides (CpG ODN), as representatives for bacterial CpG DNA that bind TLR9, as well as *Bb* DNA, were able to induce a Th1 response, whereas regulatory IL-10 remained high [23, 35]. Human intestinal epithelial cells were shown to increasingly express and secrete galectin-9 upon ligation with CpG ODN, this was further enhanced by scGOS/lcFOS and causally involved in a regulatory type Th1 polarization [13]. Similar effects were observed in the OVA-allergic mice in the current study and previously in the MLNs of cow's milk–allergic mice [14]. This may indicate that *Bb* acts via TLR9 in enhancing galectin-9 expression, which is further supported by scGOS/lcFOS. However, because whole *Bb* contains multiple other ligands for pattern-recognition receptors, it cannot be excluded that beyond TLR9 these are also involved. Future studies are needed to further study the molecular mechanisms by which *Bb* and its pattern-recognition receptor ligands may be capable of modifying epithelial galectin-9 expression in presence and absence of nondigestible oligosaccharides. Apical exposure of IECs to synthetic CpG DNA and scGOS/lcFOS under inflammatory conditions increased galectin-9 release into the basolateral medium. Similar to the IEC-conditioned medium used in the current study, in addition to the in vitro transwell coculture model—enabling the crosstalk between IECs and activated PBMCs—it was shown that galectin-9 expression increased upon exposure to CpG DNA and GF, which was reflected by increased galectin-9 secretion by IECs [13]. Hence, the oligosaccharides and TLR9 ligand act together in immune modulation. From other studies, galacto-oligosaccharides are known to affect epithelial cells because they may protect against toxin-induced epithelial IL-33 and IL-8 release and break in barrier integrity [36, 37]. Currently, it is unknown via which receptor the oligosaccharides may exert their effects. C-type lectins are glycan-binding receptors and, within that family, galectins are soluble-type lectins that can be expressed and secreted by epithelial cells [13, 38, 39]. Typically galectin-9 is known to bind β-galactoside bonds, which are present in the galacto-oligosaccharides; hence, galectin-9 expression and secretion may not only be modified by the oligosaccharides, it may be involved in its binding as well [38].

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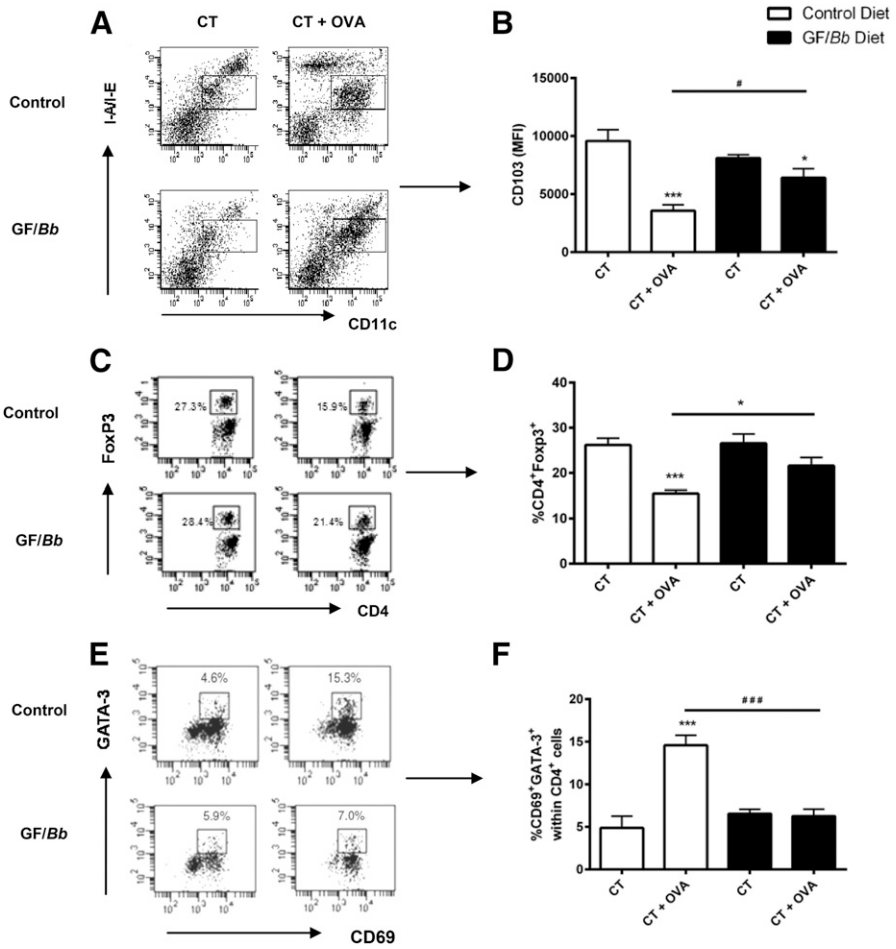

Figure 5. GF/*Bb* normalized T_{reg} frequency and reduced activated Th2 in the SI-LP. The percentage of CD11cMHCII(I-A/I-E)^{mid} DCs increased in OVA-allergic mice compared with sham-sensitized mice (A and B); however, reduced CD103 expression on these cells was observed in control diet–fed, OVA-allergic mice, which was partially restored by the GF/*Bb* diet (B). (C and D) Furthermore, the frequency of SI-LP CD4+Foxp3$^+$ T_{reg} cells was decreased in the OVA-allergic mice fed the control diet, which was restored by the GF/*Bb* diet to levels of sham-sensitized mice. (E and F) In parallel, an increased frequency of activated Th2 cells (CD4$^+$CD69$^+$GATA-3$^+$ cells) was observed in the SI-LP of OVA-allergic mice fed a control diet but not in the SI-LP of OVA-allergic mice fed GF/*Bb*. Data are representative of $n = 6$ mice/group. Means ± SEM, $^*P < 0.05$; $^{***}P < 0.001$ compared with sham-sensitized animals fed a control diet. $^{\#}P < 0.05$; $^{\#\#\#}P < 0.001$ compared with OVA-allergic animals fed a control diet.

Other receptors that may be targeted by the oligosaccharides include TLR2 and TLR4 receptors, which are also present on the HT-29 cells [23, 40, 41]. Future studies are needed to identify which receptor is involved in the additive effect of scGOS/lcFOS on TLR9-induced epithelial galectin-9 secretion.

In the current study, the IEC-conditioned medium reduced DC maturation in a galectin-9–dependent manner, as was shown by using a TIM-3–Fc fusion protein, which selectively binds and neutralizes galectin-9 in the supernatant. In addition, in previous studies using a transwell coculture model, the TIM-3–Fc fusion protein was capable of fully blocking galectin-9–induced immune modulation [13].

Indeed, we have previously shown that galectin-9 is expressed in the intestinal epithelial lining in food-allergic mice fed the GF/*Bb* diet [14]. In those studies, *Bb*, as well as GF, was also fed separately; however, the combination of GF and *Bb* was most effective in increasing serum galectin-9 and suppressing food allergic symptoms [14, 18]. GF is known to modulate the intestinal microbiota, for example, by enhancing bifidobacteria counts in mice and human [42–45]. Hence, GF may have induced its effect via microbiota adaptations. Bifidobacteria are butyric acid–producing bacteria, whereas they have been shown to increase acetate and lactate levels in infants [45, 46]. These short-chain fatty acids, produced upon oligosaccharide fermentation, are important in controlling intestinal homeostasis and may protect against food allergies [47]. They are known to support barrier integrity [48–51], enhance the mucosal T_{reg} and effector response [52–55], and instruct intestinal epithelial cells to induce tolerogenic DCs [56]. In contrast, in the current study, and in previous in vitro studies using the transwell coculture model, we have shown that GF enhances the immunomodulatory effect of synthetic CpG ODN and/or DNA isolated from *Bb* [13]. Therefore, besides its prebiotic capacities, GF may also be directly capable of modulating the intestinal immune response by instructing intestinal epithelial cells to enhance Th1 polarization and the regulatory immune response [13] and by strengthening the intestinal barrier function [57, 58].

DCs of patients suffering from food allergy have been reported to produce increased amounts of proinflammatory cytokines, including IL-6 and TNF-α [59]. IL-6 can promote Th2 cell responses by directing CD4$^+$ T cells to produce IL-4 and by inhibiting Th1 type immune responses [60, 61]. Our results show that galectin-9 is able to inhibit that Th2 cell-promoting effect of DCs. Preexposure of murine-derived BMDCs with recombinant galectin-9 suppressed secretion of IL-6 and TNF-α as well as Th2-associated cytokine IL-13 upon LPS activation. IL-10 and IL-12p70 concentrations remained below the detection limit; however, secretion of those cytokines is generally low compared with IL-6, TNF-α, and IL1-β upon LPS stimulation [62]. In addition to dampening allergic symptoms, galectin-9 has been shown to enhance Th1 and T_{reg} responses [14, 63–65]. However, galectin-9 has also been shown to induce Th1 cell apoptosis and sustain a food

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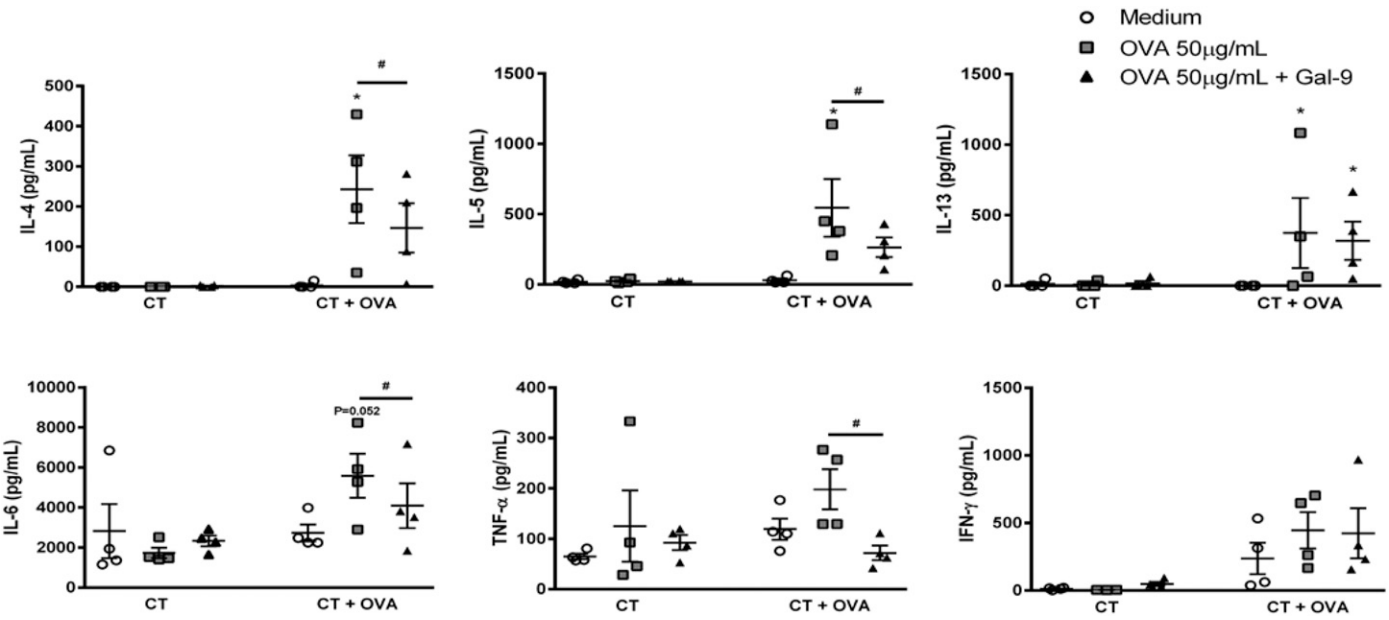

Figure 6. Galectin-9 suppresses OVA-specific, Th2-associated, and proinflammatory cytokine production by SI-LP cells from OVA-allergic mice. (A–F) SI-LP cells from sham and OVA-allergic mice fed a control diet were isolated and ex vivo restimulated with OVA for 5 d in the absence or presence of recombinant galectin-9 (Gal-9). An OVA-specific response was detected only in SI-LP cells of OVA-allergic animals. Galectin-9 inhibited the secretion of IL-4, IL-5, IL-6, and TNF-α production by SI-LP cells from OVA-allergic mice upon OVA restimulation, whereas the secretion of IL-13 and IFN-γ was not affected. Data represent $n = 4$ mice/group. Means ± SEM, $^{*}P < 0.05$ compared with medium controls; $^{\#}P < 0.05$ compared with OVA-stimulated SI-LP cells.

allergic response [66, 67]. In addition, galectin-9 was previously shown to activate DCs [68, 69], whereas, in the current study, galectin-9 preincubation suppressed DC maturation and activation in vitro. These discrepancies need to be further investigated and probably depend on the conditions in which galectin-9 exerts its immune regulatory function. Interestingly, in the present study, recombinant galectin-9 not only suppressed BMDC activation but also reduced Th2-associated and inflammatory cytokine secretion upon ex vivo OVA restimulation of SI-LP cells of OVA-allergic mice. This is in line with previous studies indicating the antiallergic properties of galectin-9 on allergic inflammation [13, 68, 70, 71]. Although we cannot exclude the possibility that galectin-9 has bound free OVA and thereby reduced its allergenicity.

In conclusion, the beneficial effects of dietary GF/*Bb* in food allergies are associated with decreased maturation and activation of DCs. In addition, normalized CD103 expression by DCs and restored T_{reg} cell frequency in association with suppressed Th2 activation in the SI-LP was observed. Galectin-9, a soluble mediator among others secreted by the intestinal epithelium, reduced DC maturation and activation in vitro, and suppressed Th2-associated cytokine responses upon ex vivo exposure. These data may help to further understand the underlying mechanisms by which nondigestible oligosaccharides and *Bb* may have beneficial effects in allergic disease.

AUTHORSHIP

S.d.K. designed and performed the experiments and wrote the manuscript. A.I.K. helped out with the in vitro studies and data discussion. J.K. and M.E.M. assisted with the lamina propria mononuclear cell isolation and data discussion. V.A.M. and G.A.H. assisted with in vitro and in vivo studies, respectively. L.M.J.K. and J.G. supervised the studies. A.D.K. and L.E.M.W. supervised the studies, discussed the data, and assisted in writing the manuscript.

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DISCLOSURES

J.G. and L.M.J.K. are employed at Nutricia Research. The other authors declare no conflicts of interest.

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KEY WORDS:
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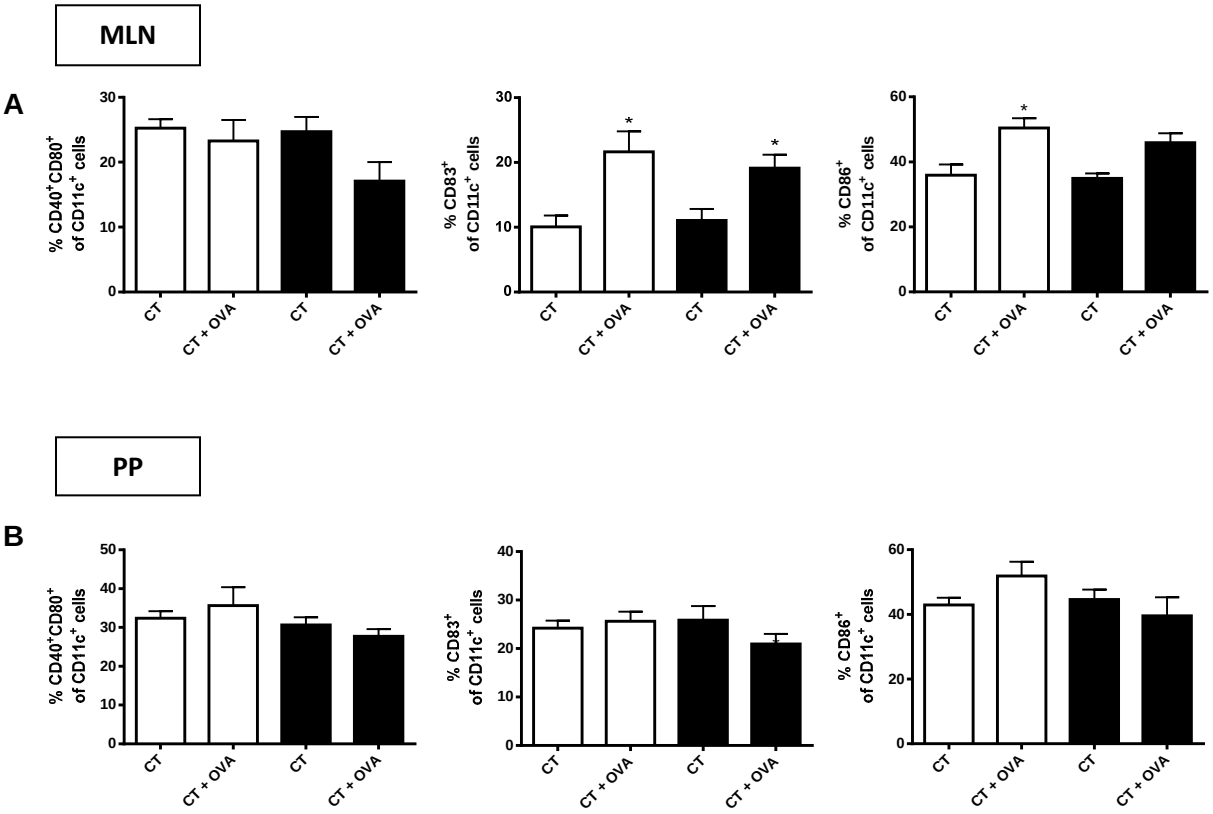
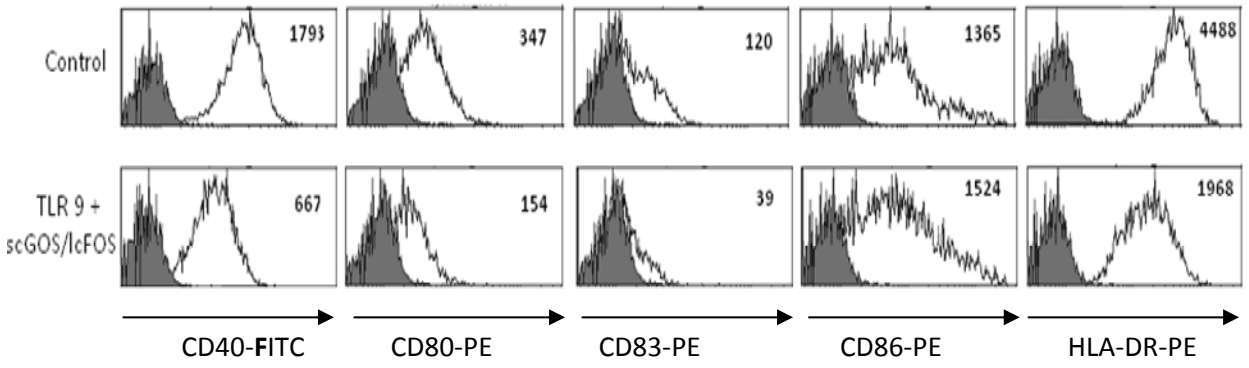
Supplementary Figure 1



Supplementary Figure 2