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Intestinal Epithelium-Derived Galectin-9 Is Involved in the Immunomodulating Effects of Nondigestible Oligosaccharides

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Key Words
Galectin-9 · Intestinal epithelial cells · Nondigestible oligosaccharides · Regulatory T cells · T_h1 cells · TLR9

Abstract
Dietary intervention using nondigestible oligosaccharides, short-chain galacto-oligosaccharides (scGOS)/long-chain fructo-oligosaccharides (lcFOS), in combination with *Bifidobacterium breve* M-16V prevents allergic disease involving galectin-9. In addition, apical TLR9 signaling contributes to intestinal homeostasis. We studied the contribution of galectin-9 secreted by intestinal epithelial cells (IEC; HT-29 and T84) in T_h1 and regulatory T-cell (T_{reg}) polarization in vitro. IEC were grown in transwell filters, cocultured with CD3/CD28-activated human peripheral blood mononuclear cells (PBMC) and apically exposed to genomic DNA derived from *B. breve* M-16V or synthetic TLR9 ligand in the absence or presence of scGOS/lcFOS. Cytokine production and T-cell phenotype were determined and galectin expression by IEC was assessed. Galectin-9 was neutralized using lactose or a TIM-3-Fc fusion protein. IEC exposed to DNA from *B. breve* M-16V or TLR9 ligand in the presence of scGOS/lcFOS enhanced IFN-γ secretion by PBMC and increased the percentage of T_h1 and T_{reg} cells. Expression and secretion of galectin-9 by IEC was increased and neutralization of galectin-9 prevented the induction of IFN-γ secretion and also suppressed the production of IL-10 by PBMC. Furthermore, we show that galectin-9 induces T_{reg} and T_h1 polarization through interaction with antigen-presenting cells. Our findings show that galectin-9 secreted by IEC apically exposed to TLR9 ligand in the presence of scGOS/lcFOS is involved in T_h1 and T_{reg} polarization and may be a promising target to prevent or treat allergic disease.

Introduction

The gastrointestinal immune system is the largest immunological compartment present in the human body which constantly faces both harmful and harmless antigens present in the intestinal lumen. The intestinal mucosa has therefore the task to discriminate between inducing an immune response in case of pathogen invasion and maintaining tolerance to harmless food antigens or the commensal microbiota. Loss of tolerance towards food antigens may result in the development of food allergy [1]. A monolayer of intestinal epithelial cells (IEC) provides an important barrier between the intestinal lu-

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men and the lamina propria, but it is also known to be involved in the modulation of innate and adaptive immune responses [2, 3].

IEC are known to contribute to effector immune responses and have the capacity to express pathogen recognition receptors including Toll-like receptors (TLR). TLR recognize fragments of bacteria for example. Human IEC were found to express TLR at the surface, and TLR expression is increased under inflammatory conditions, whereas under homeostasis IEC are unresponsive toward TLR ligands [4–9]. Epithelial TLR9 ligation by unmethylated bacterial CpG DNA was found to induce tolerance to subsequent TLR stimuli, illustrating its involvement in the maintenance of intestinal homeostasis [10]. In vitro, IEC modulate effector immune responses in a polarized fashion as apical, but not basolateral TLR9 ligand exposure enhances IFN-γ and IL-10 secretion, while suppressing IL-13 production by peripheral blood mononuclear cells (PBMC) [11]. In a murine model of peanut allergy, oral administration of TLR9 agonists prevented allergy, linking TLR9 activation to a reduction in allergic diseases [12]. Upon activation, IEC secrete mediators that shape the phenotype of dendritic cells (DC) to induce regulatory T-cell (T_{reg}) differentiation and to suppress the development of both T_h1 and T_h17 cells [3, 13].

As IEC play an important role in the recognition of microbial stimuli, dietary intervention using specific bacterial strains may be beneficial in modulating effector immune responses in the intestine. Previously, the effects of oral administration of *Bifidobacterium breve* M-16V, *B. infantis, B. animalis*, *Lactobacillus plantarum* and *L. rhamnosus* on the suppression of airway responses, pulmonary inflammation, ovalbumin-specific immunoglobulins and cytokines present in serum were assessed in a murine model of asthma. In this study, *B. breve* M-16V was identified as the most potent bacterial strain to suppress allergic responses upon allergen challenge [14]. Follow-up in vitro studies using UV-killed whole bacteria showed that in contrast to *Lactobacillus* GG, *B. breve* M-16V did not modulate the effector immune response in IEC/PBMC cocultures [15]. Recently, it has been reported that both DNA from commensals as well as probiotic bacterial strains contain immunosuppressive CpG motifs, thereby facilitating T_{reg} conversion and anti-inflammatory cytokine production [16, 17]. Hence, DNA derived from *B. breve* M-16V, but not membrane components, may play a role in modulating effector immune responses through activation of TLR9 on IEC. In addition, a combination of *B. breve* M-16 V with a specific prebiotic 9:1 mixture of short-chain galacto-oligosaccharides (scGOS; $[Gal\beta1\text{-}4]_{3\text{-}8}Glc$; Gal, galactose; Glc, glucose) and long-chain fructo-oligosaccharides (lcFOS, $[Fr c\beta2\text{-}1]_{>20}Fr c\beta2\text{-}1Glc$; Frc, fructose) (scGOS/lcFOS) most effectively protects against the development of allergic symptoms in a murine model of cow's milk allergy as well as in infants at risk [18, 19]. Although human milk nondigestible oligosaccharides as well as scGOS/lcFOS are able to shape the intestinal microflora and may induce mucosal tolerance [20, 21], it is not known whether these oligosaccharides directly contribute to the modulation of effector immune responses in the intestine.

Receptors involved in the recognition of carbohydrate structures, lectins, may be involved in the recognition of nondigestible oligosaccharides. One family of soluble-type lectins expressed by IEC that contain carbohydrate recognition domains are galectins, which exhibit binding specificity for β-galactosides [22, 23]. IEC were found to express galectin-2, -3, -4 and -9 [24, 25]. Galectins are localized in the cytoplasm, but can also be secreted through yet unknown mechanisms. Upon secretion, galectins can bind to glycosylated proteins thus forming galectin-glycoprotein lattices on the cell surface, thereby regulating immune responses and potentially inducing immunological tolerance [26, 27]. We have previously shown that dietary intervention using scGOS/lcFOS in combination with *B. breve* M-16V enhances serum galectin-9 levels. To this end, we studied the effects of IEC exposure to scGOS/lcFOS and TLR9 ligand on human PBMC in an in vitro coculture system [11]. We demonstrate that galectin-9 is expressed and secreted by IEC upon apical exposure to TLR9 ligand and scGOS/lcFOS, which drives a T_h1/T_{reg} response.

Materials and Methods

Transwell Cocultures

Culture of human IEC lines (HT-29 and T84 cells), isolation of human PBMC and transwell cocultures were performed as previously described [11]. In short, HT-29 or T84 cells were grown till confluence on transwell insert filters (Corning, N.Y., USA). Confluence was examined by light microscopy or transepithelial electrical resistance (125 $\Omega \times cm^2$ for HT-29 cells and 1,500 $\Omega \times cm^2$ for polarized T84 cell monolayers). IEC were cocultured with 3×10^6 CD3/CD28-activated PBMC for 24 h. IEC were apically exposed to either TLR9 ligand (M362 type C, 5.0 μM; InvivoGen, San Diego, Calif., USA) alone or in combination with 0.5% w/v of a 9:1 mixture of scGOS (Vivinal GOS; Borculo Domo) and lcFOS (Raftiline HP; Orafti). For the determination of galectin secretion by IEC, HT-29 cells were placed on fresh medium for another 24 h after coculture with CD3/CD28-activated PBMC. To study the involvement of galectins in immune modulation, lactose (100 mM; Sigma, Zwijndrecht, The Netherlands) was added to the ba-

solateral compartment during coculture. Sucrose (100 mM; Sigma) was used as negative control. To specifically block galectin-9, a TIM-3-Fc fusion protein (1.0 μg/ml; R&D Systems, Minneapolis, Minn., USA) was added to IEC/PBMC cocultures. In addition, HT-29 cells were basolaterally exposed to medium containing recombinant TNF-α and IFN-γ (both 10 ng/ml; Invitrogen, Carlsbad, Calif., USA) to study whether inflammatory conditions are essential for HT-29 cells to produce galectin-9 upon apical exposure to TLR9 ligand and scGOS/lcFOS.

Generation of Monocyte-Derived DC

CD14+ monocytes were isolated from PBMC by negative selection using MACS beads (monocyte isolation kit II; Miltenyi Biotec, Bergisch Gladbach, Germany) and cultured in RPMI 1640 supplemented with 10% FCS and penicillin (100 U/ml)/streptomycin [100 μg/ml; monocyte-derived DC (moDC) medium] with IL-4 and GM-CSF (both Prospec, East Brunswick, N.J., USA) at a concentration of 30 and 15 ng/ml, respectively, and incubated for 6 days at 37°C and 5% CO_2. After 2, 3 and 6 days, 1 ml medium was refreshed. For moDC conditioning experiments, monocytes were differentiated into moDC with IL-4 and GM-CSF in the presence of recombinant human galectin-9 (1.0 μg/ml; R&D Systems). On day 7, the phenotype of moDC was confirmed by FACS.

In vitro T-Cell Differentiation

In mixed lymphocyte cultures, 2×10^4 moDC were seeded per well and cultured with naive allogeneic CD4+ T cells (10^5). For T_{reg}-cell differentiation, 1 ng/ml recombinant human TGF-β (Prospec) was added to the cultures. Cultures were incubated at 37°C and 5% CO_2 for 5 days. Before FACS analysis of intracellular cytokines, cells were re-stimulated with phorbol-12-myristate-13-acetate (50 ng/ml; Sigma) and ionomycin (750 ng/ml; Sigma) for 6 h. After 1 h of restimulation, GolgiPlug (BD Biosciences, San Jose, Calif., USA) was added according to the manufacturer's protocol. CD4+ cells producing IL-10 and IFN-γ were analyzed by flow cytometry.

Culture and Isolation of Genomic DNA of B. breve M-16V

B. breve M-16V was cultivated in Mann Rogosa Sharp broth (Oxoid) for 48 h at 37°C under anaerobic conditions. Genomic DNA was extracted and purified by CTAB extraction as described [28] with additional lysozyme treatment of the cell suspension (2 mg/ml, 5 min at 20°C).

ELISA

Concentrations of IL-6, IL-10, IL-12, IFN-γ, TNF-α (CytoSets™; Biosource, Nivelles, Belgium), IL-13 (R&D Systems) and IL-17A (Arcus Biologicals, Modena, Italy) were measured according to the manufacturer's protocol. For galectin-4 and galectin-9 ELISA, high-binding EIA/RIA 96-well plates (Costar; Corning Inc., Corning, N.Y., USA) were coated with 0.75 μg/ml primary antibodies (R&D Systems) dissolved in PBS overnight at 4°C. Plates were blocked for 1 h with 1% BSA in PBS, samples were then added for 2 h, followed by incubation with 0.75 μg/ml biotinylated secondary antibody (R&D Systems) in 1% BSA in PBS for 1 h. Plates were incubated with streptavidin-HRP (R&D Systems) for 1 h followed by development with tetramethylbenzidine (Thermo Scientific, Rockford, Ill., USA) for 10 min. The reaction was stopped with 2 M H_2SO_4 and optical density was measured at 450 nm [29].

Flow Cytometry

T_h1 cells were stained with CD4-PE-Cy5 (eBioscience, San Diego, Calif., USA), CD69-PE (eBioscience) and CXCR3-Alexa Fluor 488 (BD Biosciences) and fixed with 0.5% paraformaldehyde. T_{reg} cells were stained with CD4-PE-Cy5 and CD25-Alexa Fluor 488 (eBioscience), followed by intracellular staining with Foxp3-PE using the Foxp3 staining set according to manufacturer's protocol (eBioscience). For intracellular cytokine staining, PBMC were restimulated with phorbol-12-myristate-13-acetate (50 ng/ml; Sigma) and ionomycin (750 ng/ml; Sigma) for 6 h in the presence of a Golgi transport inhibitor according to the manufacturer's instructions (GolgiPlug) followed by intracellular staining using IFN-γ-antigen-presenting cells (APC) and IL-10-PE (BD Biosciences) as described above. Flow-cytometric analysis was performed using a FACSCanto II (BD Biosciences).

cDNA Synthesis and Real-Time PCR

HT-29 cells were washed once in PBS 2 h after coculture with CD3/CD28-activated PBMC and taken up in 200 μl RNAlater™ (Qiagen GmbH, Hilden, Germany). Samples were stored at –20°C until cDNA synthesis. mRNA was isolated using the mRNA capture kit (Roche, Mannheim, Germany) and real-time PCR reactions were performed as previously described [30]. *GAPDH* was used as reference gene. Relative target mRNA abundance was calculated by applying the formula: relative mRNA abundance = $100 \times 2^{Ct[GAPDH] - Ct[\text{target mRNA}]}$. Primers for all galectins were designed using the computer software Primer Express 2.0 (Applied Biosystems, Carlsbad, Calif., USA).

Fluorescence Microscopy Staining of Galectin-9 in IEC

IEC were fixed using 4% formalin in PBS for 10 min, permeabilized in 0.1% Triton X-100 (Sigma) and 1% BSA in PBS for 15 min and incubated with anti-human galectin-4 or galectin-9 antibodies or normal goat IgG as isotype control (all 0.75 μg/ml; R&D Systems) in 0.1% Triton X-100 and 1% BSA in PBS for 1 h. IEC were incubated with secondary Alexa Fluor 546 donkey anti-goat IgG (Invitrogen) in 0.1% Triton X-100 and 1% BSA for 30 min and embedded in Hoechst. Object glasses were stored at 4°C until microscopic examination.

Statistics

Statistical analyses were performed using paired Student's t test or one-way ANOVA for repeated measurements followed by Bonferroni's post hoc test. Analyses were performed using GraphPad Prism 5.0. $p < 0.05$ was considered statistically significant.

Results

The scGOS/lcFOS Mixture Specifically Enhances a TLR9-Induced IFN-γ Response via IEC

To investigate the molecular mechanisms by which scGOS/lcFOS exerts its immunomodulatory effects, we used an in vitro coculture model with human IEC and PBMC to study IEC-immune cell crosstalk. We hypothesized that DNA of *B. breve* M-16V may activate TLR9 on

IEC as apical TLR9 ligation of IEC can induce a regulatory-type T_h1 response [11]. IEC apically exposed to purified DNA from *B. breve* M-16V or a synthetic TLR9 ligand enhanced IFN-γ secretion by activated PBMC, which was potentiated by scGOS/lcFOS (fig. 1a). Direct stimulation of CD3/CD28-activated PBMC with CpG DNA in the presence of scGOS/lcFOS did not enhance IFN-γ secretion (fig. 1d). Furthermore, it was observed that scGOS/lcFOS did not increase IFN-γ production on their own, indicating that the presence of both scGOS/lcFOS and the bacterial DNA is essential for the observed additional effect. In addition, both in the presence and absence of IEC, the production of the T_h2-associated cytokine IL-13 was significantly reduced upon apical exposure of HT-29 to synthetic CpG DNA. The presence of scGOS/lcFOS did not modulate IL-13 secretion by PBMC in the presence of HT-29 cells; in the absence of IEC IL-13 secretion was reduced. The combination of both CpG DNA and scGOS/lcFOS suppressed IL-13 in the presence and absence of HT-29 cells. However, exposure of HT-29 cells to the genomic DNA derived from *B. breve* M-16V did not result in decreased IL-13 secretion in the presence or absence of scGOS/lcFOS (fig. 1b, e).

In addition to the induction of a T_h1-polarized effector response, we studied whether IEC apically exposed to TLR9 ligand in the presence of scGOS/lcFOS enhances anti-inflammatory IL-10 production as well. TLR9 ligation on IEC, as well as exposure of IEC to DNA from *B. breve* M-16V, resulted in increased IL-10 secretion. TLR9-induced IL-10 secretion by CD3/CD28-activated PBMC was not affected by scGOS/lcFOS (fig. 1c). In the absence of IEC, the TLR9 ligand enhanced IL-10 secretion as well, and a similar tendency was observed in the presence of scGOS/lcFOS (fig. 1f).

T_h1 and T_{reg} Cells Are Increased by IEC Exposed to TLR9 Ligand in the Presence of scGOS/lcFOS

To confirm whether scGOS/lcFOS in combination with CpG DNA modulates T-cell polarization, T-cell phenotype was assessed. T_h1 cells characterized as either CD4+CD69+CXCR3+ cells and IFN-γ-expressing CD4+ T cells were analyzed. An increased frequency of CD4+CD69+CXCR3+ T_h1 cells was observed when IEC were apically exposed to TLR9 ligand, which was further increased by scGOS/lcFOS (fig. 2a, b). In the absence of IEC, the percentage of activated T_h1 cells remained unaltered (fig. 2c). To confirm the induction of T_h1 cells, intracellular IFN-γ staining was performed. Similarly, only in the presence of IEC, the percentage of IFN-γ-producing CD4+ T cells was increased upon apical exposure of IEC to TLR9 ligand, which was boosted by scGOS/lcFOS (fig. 2d, e).

Since CpG DNA and genomic DNA derived from *B. breve* M-16V enhanced anti-inflammatory IL-10 secretion, the frequency of CD4+CD25+Foxp3+ T_{reg} cells was analyzed, too. The percentage of T_{reg} cells was only increased when IEC were apically exposed to TLR9 ligand in the presence of scGOS/lcFOS (7.7 ± 1.2% for medium-exposed IEC vs. 10.4 ± 1.5% for IEC exposed to CpG DNA in the presence of scGOS/lcFOS; fig. 3a–c). Likewise, intracellular IL-10 expression by CD4+ T cells was increased upon TLR9 ligation in the presence and absence of IEC, however, scGOS/lcFOS did not enhance IL-10 production by CD4+ T cells (fig. 3d, e).

Galectin-9 Expression by IEC Is Increased upon Exposure to TLR9 Ligand and scGOS/lcFOS

Galectins expressed by IEC may be involved in the induction of T_h1- and T_{reg}-cell development. To address this possibility, we evaluated whether apical exposure of HT-29 cells to TLR9 ligand and scGOS/lcFOS modulates epithelial galectin expression. First, it was assessed which galectins are expressed by HT-29 cells when cocultured with CD3/CD28-activated PBMC (fig. 4a). Galectin-4 and the long isoform of galectin-9 were highly expressed by HT-29 cells. Apical TLR9 ligation of IEC increased galectin-9 mRNA expression by HT-29, but not galectin-4, while in the presence of scGOS/lcFOS galectin-9 expression was further enhanced (fig. 4b, c). Furthermore, enhanced basolateral secretion of galectin-9 was observed upon epithelial exposure to TLR9 ligand, which was significantly potentiated by the combination with scGOS/lcFOS (fig. 4d). Galectin-4 expression by HT-29 cells was not modulated upon apical exposure of HT-29 cells to TLR9 ligand or scGOS/lcFOS (fig. 4c, f). Hence, TLR9 ligand and scGOS/lcFOS specifically enhanced galectin-9 expression and secretion by IEC.

In addition, galectin-9 secretion by HT-29 upon apical exposure to TLR9 ligand in the presence of scGOS/lcFOS did not occur under noninflammatory conditions. Unstimulated HT-29 cells secreted low levels of galectin-9 (55.6 ± 3.7 vs. 56.9 ± 10.3 pg/ml for medium vs. TLR9 ligand and scGOS/lcFOS-exposed HT-29, n = 4). However, exposure of pre-incubated HT-29 cells with proinflammatory cytokines TNF-α and IFN-γ to TLR9 ligand and scGOS/lcFOS resulted in enhanced galectin-9 secretion (114.0 ± 43.6 vs. 246.0 ± 36.6 pg/ml for medium vs. TLR9 ligand and scGOS/lcFOS-exposed HT-29, n = 4, $p < 0.05$). These data indicate that IEC respond to TLR9 ligand in the presence of scGOS/lcFOS only under inflammatory conditions.

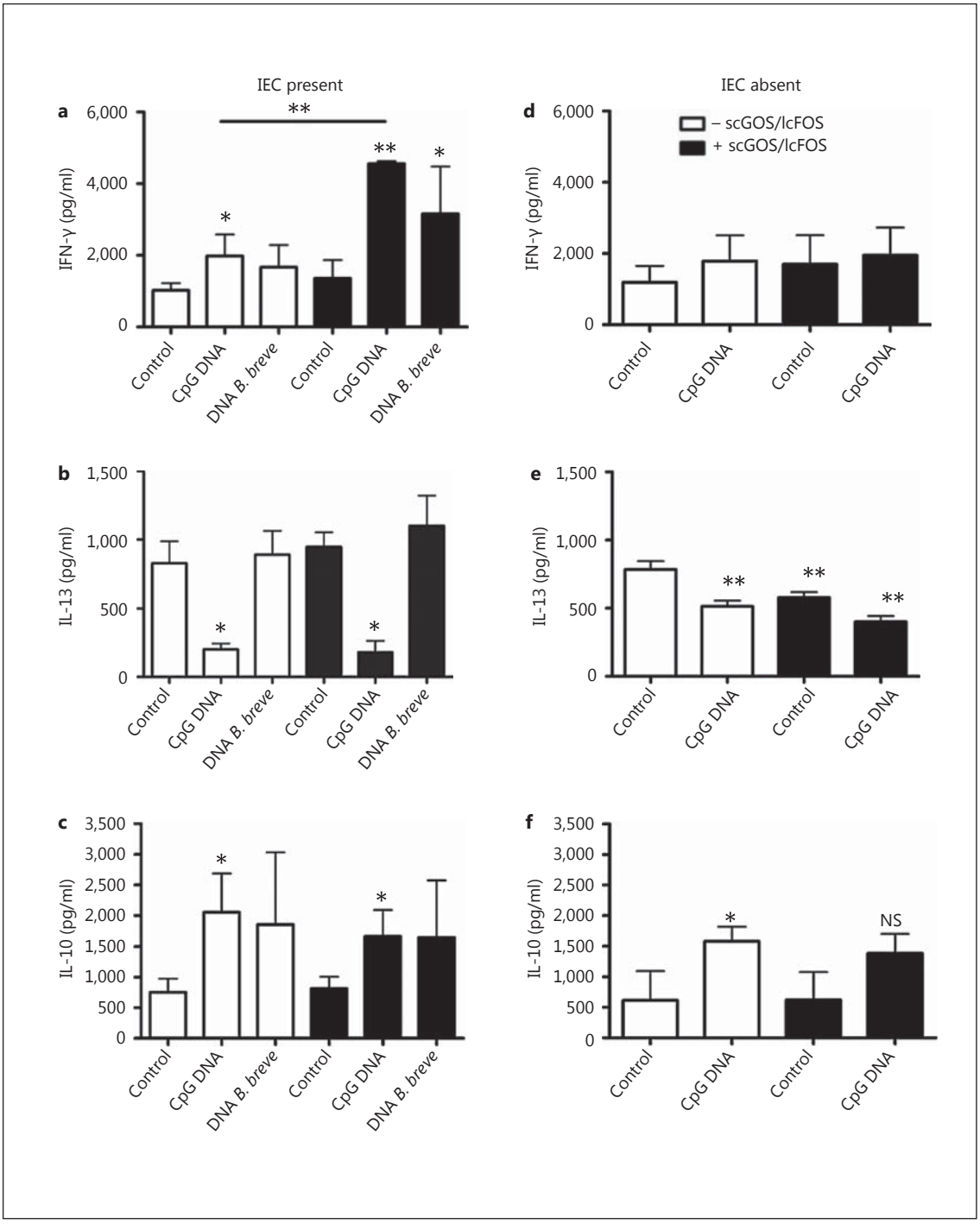


Fig. 1. TLR9 ligation of IEC in the presence of scGOS/lcFOS modulates cytokine secretion by PBMC. HT-29 cells were apically exposed to TLR9 ligand or DNA from *B. breve* M-16V in the presence or absence of scGOS/lcFOS and cocultured for 24 h with CD3/CD28-activated PBMC. In addition, PBMC were directly stimulated with TLR9 ligand in the presence of scGOS/lcFOS. Exposure of HT-29 cells to TLR9 ligand or DNA from *B. breve* M-16V increased IFN-γ secretion by PBMC, which was further enhanced in the presence of scGOS/lcFOS (**a**). This was not observed in the absence of HT-29 cells (**d**). The secretion of IL-10 was enhanced upon apical exposure of HT-29 cells to TLR9 ligand or DNA from *B. breve* M-16V, while IL-13 production was reduced. The presence of scGOS/lcFOS did not affect IL-13 (**b**) and IL-10 secretion (**c**). The effects on IL-10 and IL-13 secretion by PBMC were also observed in the absence of HT-29 cells (**e**, **f**). Means ± SEM of 3–4 independent PBMC donors. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

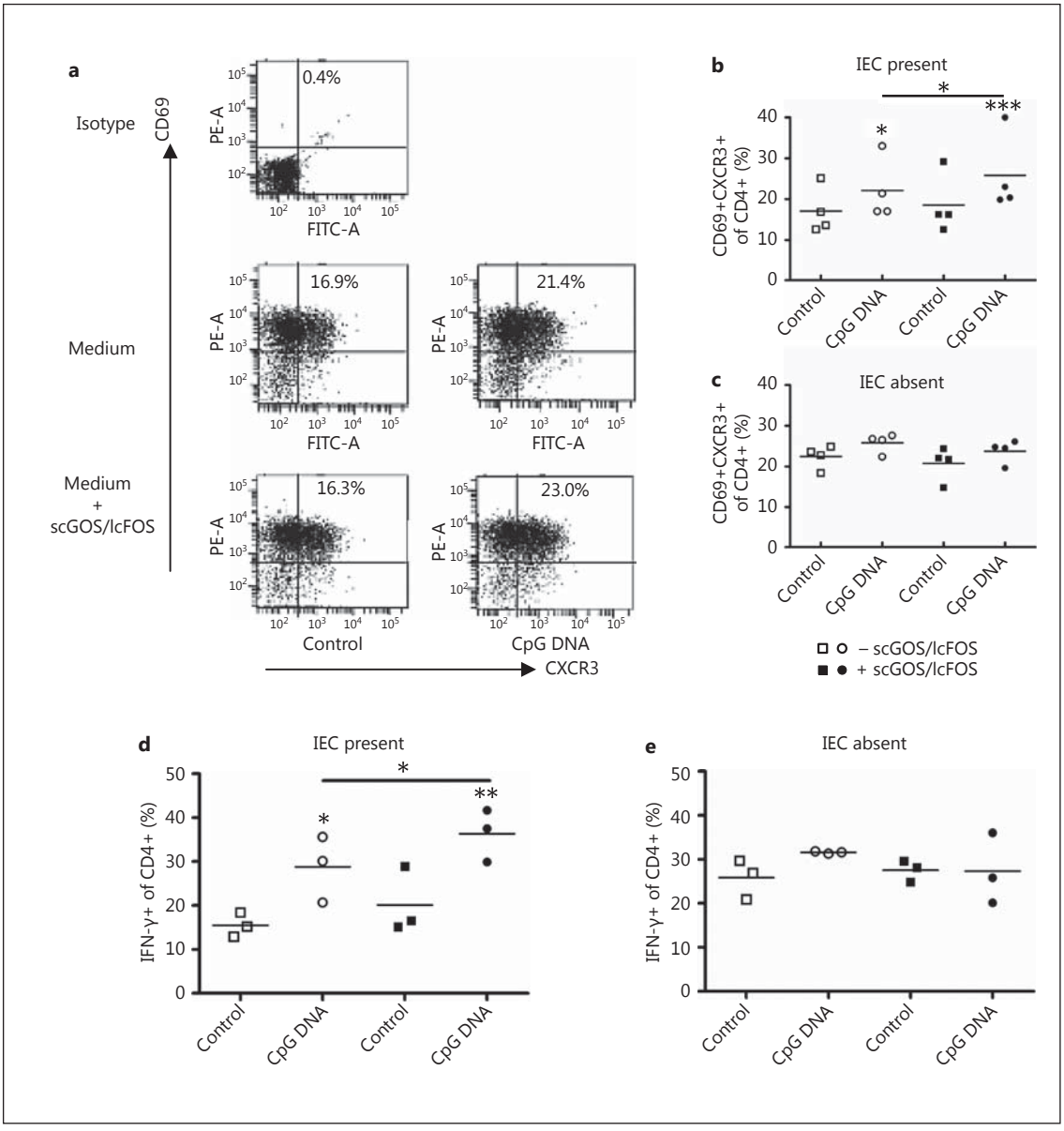


Fig. 2. TLR9 ligation of IEC in the presence of scGOS/lcFOS results in an enhanced T_h1-type effector response. HT-29 cells were apically exposed to TLR9 ligand in the presence or absence of scGOS/lcFOS and cocultured for 24 h with CD3/CD28-activated PBMC. In addition, PBMC were directly stimulated with TLR9 ligand in the presence of scGOS/lcFOS. The frequency of activated T_h1 cells (CD4+CD69+CXCR3+) and CD4+IFN-γ+ cells was only increased by apical TLR9 ligation of IEC, which was further enhanced by the presence of scGOS/lcFOS (**a**, **b**, **d**). Direct stimulation of PBMC with TLR9 ligand in the absence or presence of scGOS/lcFOS did not alter the percentage of T_h1 cells (**c**, **e**). Means ± SEM of 3–4 independent PBMC donors. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

IEC-Derived Galectin-9 Secretion Is Involved in the Induction of IFN-γ and IL-10 Polarized-Type Effector Responses

In order to study the involvement of galectins in the induction of T_h1 and T_{reg} differentiation, galectin function was inhibited by the addition of lactose to the basolateral compartment of HT-29/PBMC cocultures. Lactose binds galectins through their carbohydrate recognition domain and thereby prevents binding of galectins to glycan structures expressed on the cell surface. To deter-

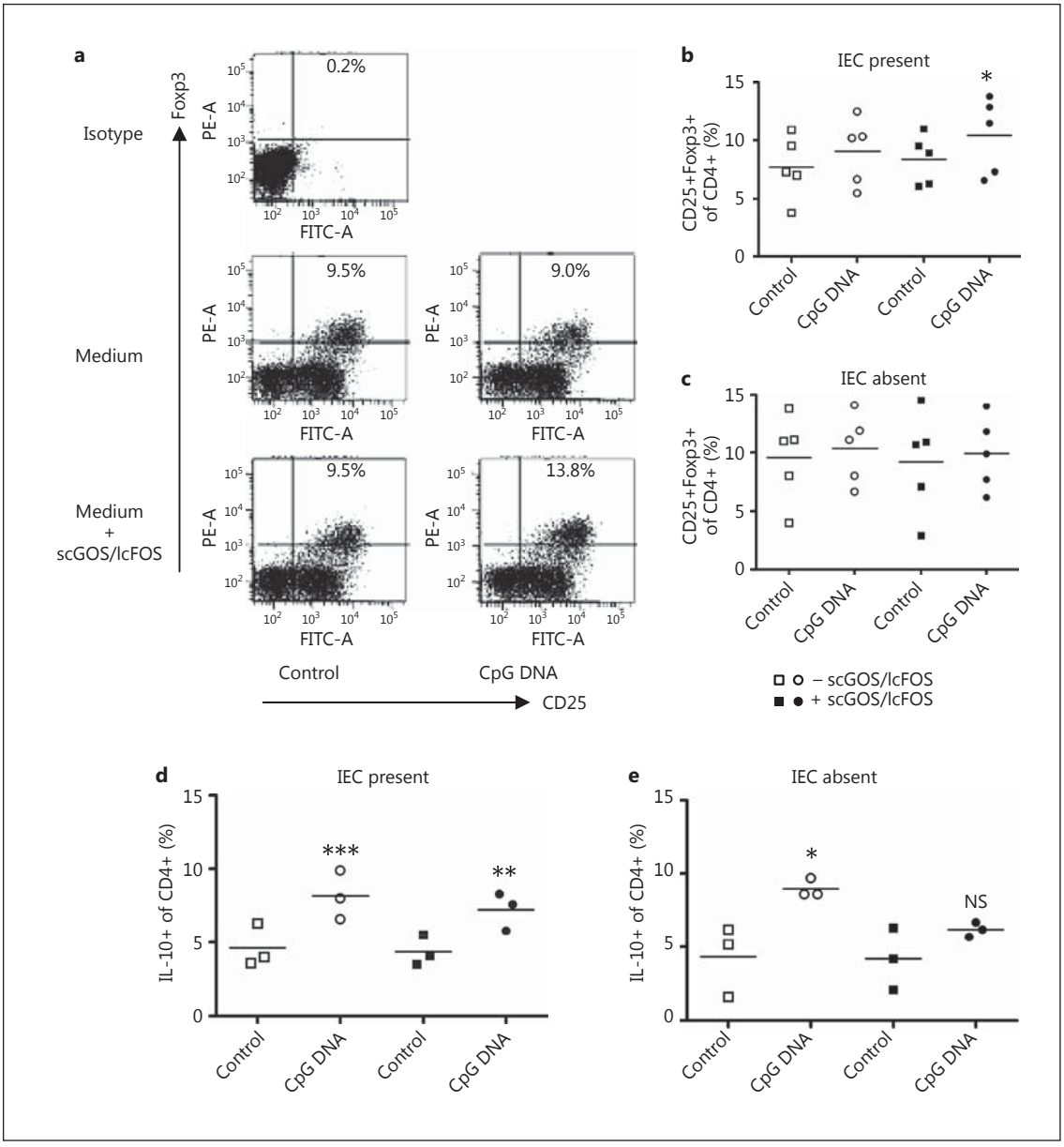


Fig. 3. TLR9 ligation of IEC in the presence of scGOS/lcFOS results in enhanced T_{reg}-type effector responses. HT-29 cells were apically exposed to TLR9 ligand in the presence or absence of scGOS/lcFOS and cocultured for 24 h with CD3/CD28-activated PBMC. In addition, PBMC were directly stimulated with TLR9 ligand in presence of scGOS/lcFOS. The frequency of T_{reg} cells (CD4+CD25+Foxp3+) was only increased by apical TLR9 ligation of IEC in the presence of scGOS/lcFOS (**a**, **b**). Direct stimulation of PBMC with TLR9 ligand in the absence or presence of scGOS/lcFOS did not modulate the number of T_{reg} cells (**c**). Intracellular IL-10 expression by CD4+ T cells was increased by TLR9 ligand and remained unaltered in the presence of scGOS/lcFOS (**d**). Similar results were observed in the absence of IEC (**e**). Means ± SEM of 3–5 independent PBMC donors, * $p < 0.05$.

mine the specificity of the intervention, sucrose was used as a negative control. Neutralization of galectins by lactose in the basolateral compartment in HT-29/PBMC cocultures exposed to TLR9 ligand and scGOS/lcFOS resulted in decreased IFN-γ and IL-10 secretion (fig. 5a, b), while the production of IL-13 was increased (fig. 5c). In addition, IL-6, IL-12, IL-17 and TNF-α secretion were increased by adding lactose to the basolateral compartment during coculture (fig. 5d–g). Thus, inhibition of galectins induced by IEC exposed to TLR9 ligand and scGOS/

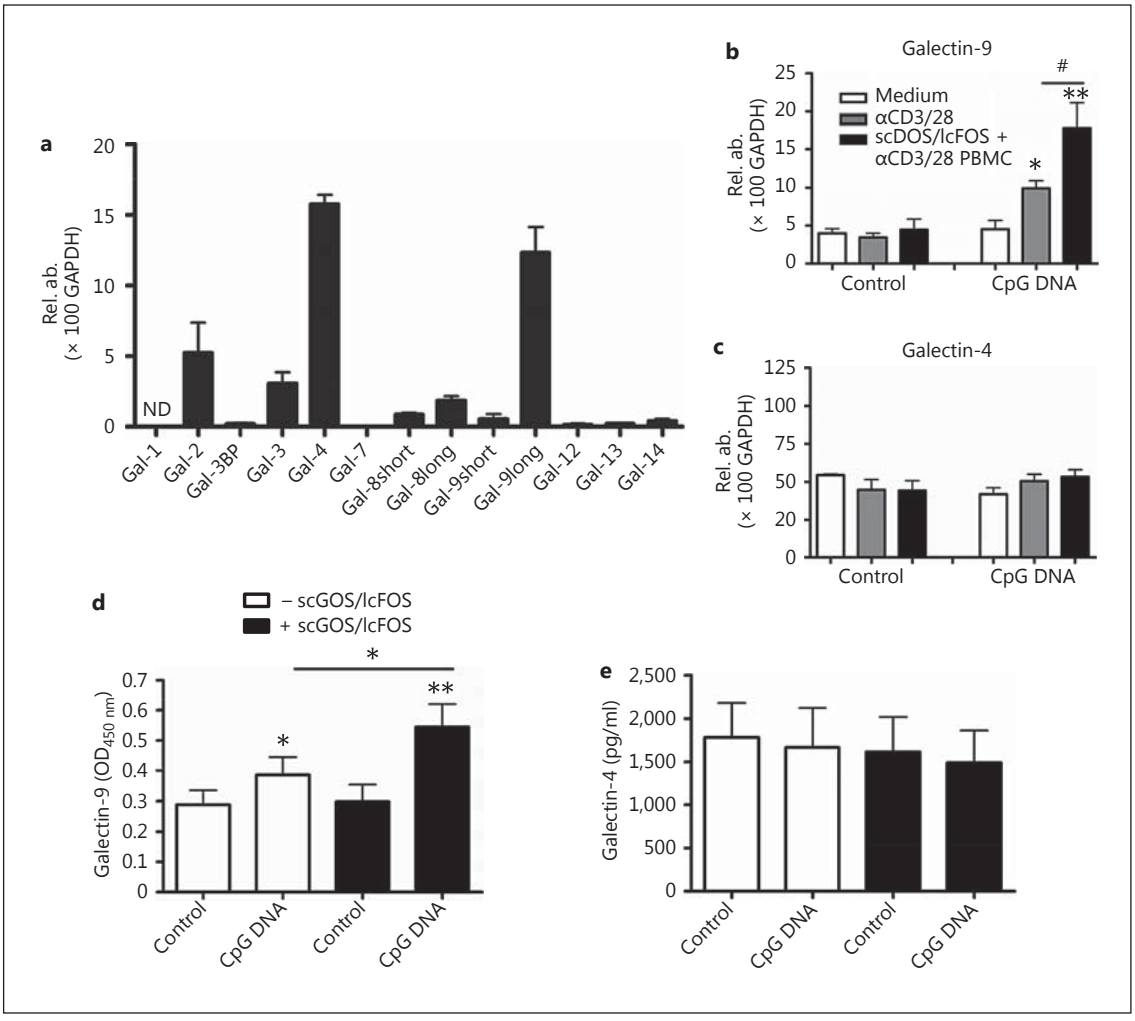


Fig. 4. Expression and secretion of galectin-9 in HT-29 cells is regulated by TLR9 ligation and scGOS/lcFOS. **a–c** Analysis of galectin (Gal) mRNA expression by HT-29 cells by quantitative PCR analysis. Evaluation of the expression profile of galectins by IEC (**a**). Apical TLR9 ligation of HT-29 cells, in the absence or presence of scGOS/lcFOS, specifically increases galectin-9 expression (**b**, **c**). Protein expression of galectin-4 and galectin-9 and its modulation upon TLR9 ligation and scGOS/lcFOS was confirmed by immunofluorescence microscopic staining of HT-29 monolayers (**d**). ELISA was performed in the basolateral supernatant of HT-29 monolayers (**e**). TLR9 ligation of HT-29 cells in the presence of scGOS/lcFOS enhanced galectin-9, but not galectin-4 secretion. n = 3 (**a–d**) or n = 6 (**e**) independent PBMC donors, means ± SEM, * $p < 0.05$, # $p < 0.05$, ** $p < 0.01$.

lcFOS abrogated the induction of IFN-γ and IL-10 as well as the reduction in IL-13 secretion by PBMC upon apical exposure of IEC to TLR9 ligand in the presence of scGOS/lcFOS. In contrast, galectin neutralization enhanced a proinflammatory response.

To prove that galectin-9 is involved in inducing IFN-γ- and IL-10-mediated effector responses, a TIM-3-Fc fusion protein was used to specifically neutralize secreted galectin-9. TIM-3-Fc prevented increased IFN-γ and IL-10 secretion by activated PBMC in HT-29/PBMC cocultures, but did not modulate IL-17 production (fig. 6a–c). Similar results were obtained using the polarized T84 cell line (fig. 6e–g). These data collectively suggest that IEC contribute to enhanced IFN-γ- and IL-10-type effector responses upon apical exposure to TLR9 ligand and scGOS/lcFOS through secretion of galectin-9. Apical exposure of HT-29 as well as T84 cells to CpG DNA in the presence of scGOS/lcFOS resulted in suppression of IL-13 secretion. Furthermore, neutralization of galectin-9 abrogated the immunomodulating effect of apical epithelial exposure to

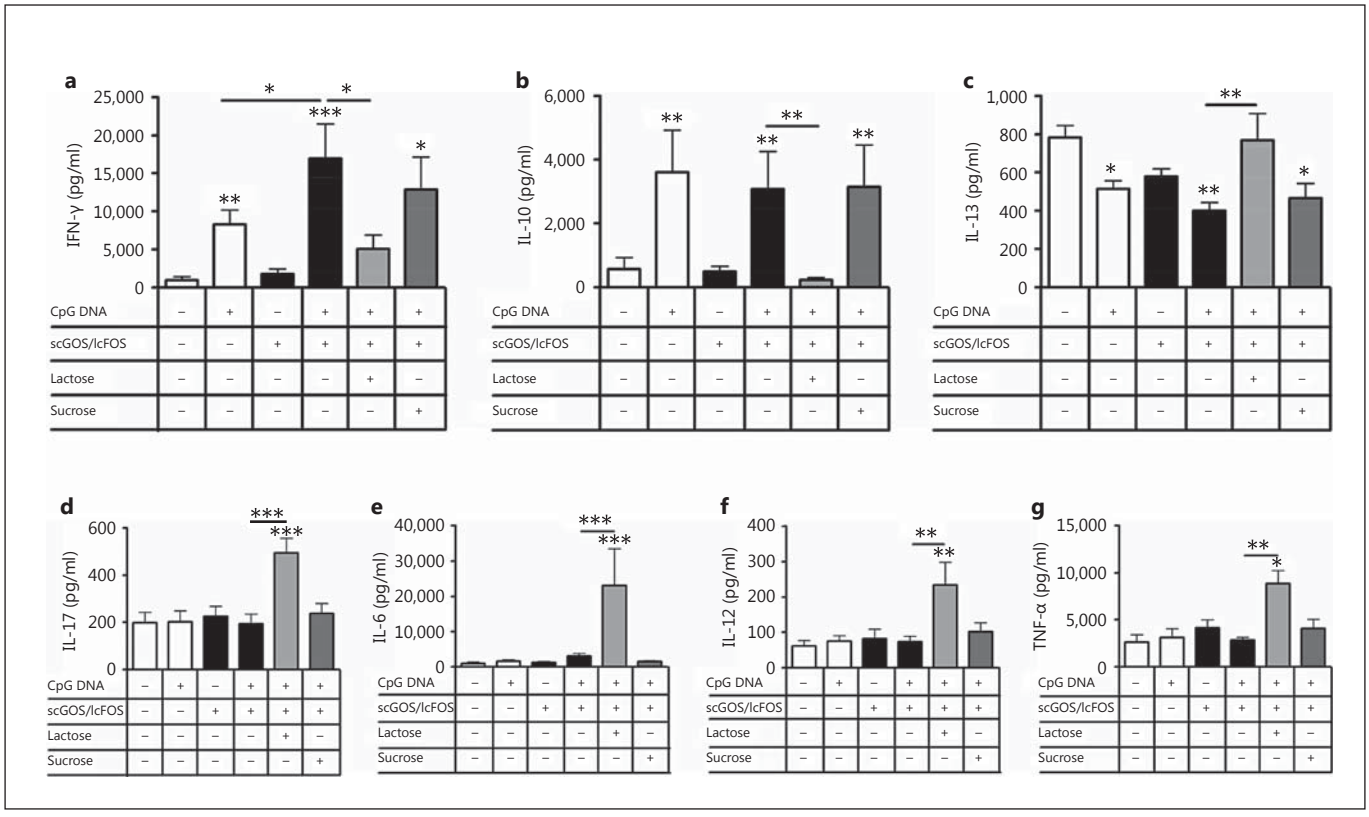
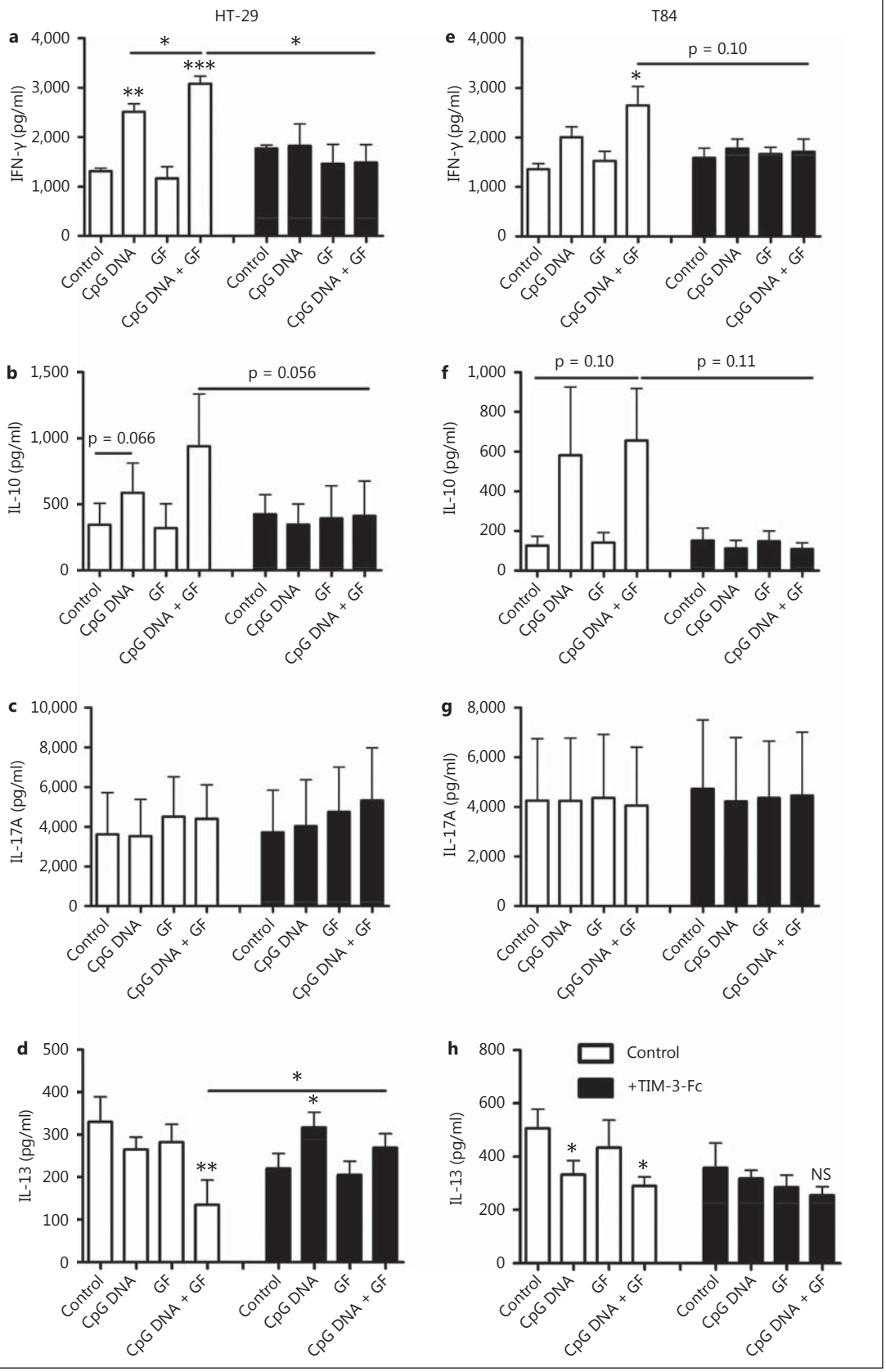


Fig. 5. Neutralization of galectins by lactose redirects the TLR9 and scGOS/lcFOS-induced IFN-γ/IL-10-mediated response to a pro-inflammatory response. HT-29/PBMC cocultures were performed in the presence of lactose or sucrose in the basolateral compartment. Sucrose served as negative control. Presence of lactose, but not sucrose, suppressed IFN-γ and IL-10 secretion induced by CpG DNA and scGOS/lcFOS, and abrogated the reduction in IL-13 production (**a–c**). The secretion of IL-17, IL-6, IL-12 and TNF-α was increased upon neutralization of galectins by lactose but not sucrose (**d–g**). n = 6 independent PBMC donors, means ± SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 6. Galectin-9 neutralization in IEC/PBMC cocultures abrogates IFN-γ and IL-10 secretion by PBMC. HT-29 (unpolarized) and T84 (polarized) IEC were cocultured with CD3/CD28-activated PBMC for 24 h and apically exposed to TLR9 ligand in the presence or absence of scGOS/lcFOS. Basolateral galectin-9 was neutralized using TIM-3-Fc fusion protein. Galectin-9 neutralization abrogated the induction of IFN-γ (**a**, **e**) and IL-10 (**b**, **f**) by PBMC, but did not modulate IL-17A secretion by PBMC (**c**, **g**). Furthermore, the immunomodulating effects on IL-13 secretion induced by exposure of HT-29 or T84 cells to TLR9 ligand in the absence or presence of scGOS/lcFOS was abolished upon neutralization of galectin-9 (**d**, **h**). Means ± SEM of 3 independent PBMC donors. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. GF = scGOS/lcFOS.

Fig. 7. Functional responses of galectin-9-stimulated naïve CD4+CD45RA+ T cells and moDC. To study whether galectin-9 acts on moDC, T cells or via modulation of the interaction between moDC and T cells, galectin-9 was added during the differentiation of monocytes to moDC or during coculture of moDC with naïve CD4+ T cells (**a**). Naïve CD4+CD45RA+ T cells, moDC or moDC-T-cell cocultures were exposed to recombinant human galectin-9. Mixed lymphocyte cultures were maintained for 5 days. For T_{reg}-cell differentiation, exogenous TGF-β was added to the culture. For intracellular cytokines, T cells were restimulated as described in the Materials and Methods. Stimulation of naïve CD4+ T cells with galectin-9 did not induce CD25+Foxp3+ T_{reg} cells (**b**, **d**), IL-10 (**b**, **e**) or IFN-γ expression by CD4+ T cells (**b**, **f**). However, moDC generated in the presence of galectin-9 (Gal-9 DC) or exogenous galectin-9 added to mixed lymphocyte cultures with unconditioned (control) moDC resulted in increased differentiation of CD25+Foxp3+ T_{reg} cells (**c**, **d**) and IL-10-expressing CD4+ cells (**c**, **e**). Only Gal-9 DC had the capacity to induce IFN-γ expression by CD4+ T cells (**c**, **f**). n = 3 independent PBMC donors. * $p < 0.05$, ** $p < 0.01$ vs. control DC.

(For figures see next pages.)


HT-29
T84
a
IFN-γ (pg/ml)
4,000
3,000
2,000
1,000
0
*
*
***
**
e
p = 0.10
*
b
IL-10 (pg/ml)
1,500
1,000
500
p = 0.056
p = 0.066
f
1,000
800
600
400
200
p = 0.10
p = 0.11
c
IL-17A (pg/ml)
10,000
8,000
6,000
4,000
2,000
g
8,000
6,000
4,000
2,000
d
IL-13 (pg/ml)
500
400
300
200
100
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h
800
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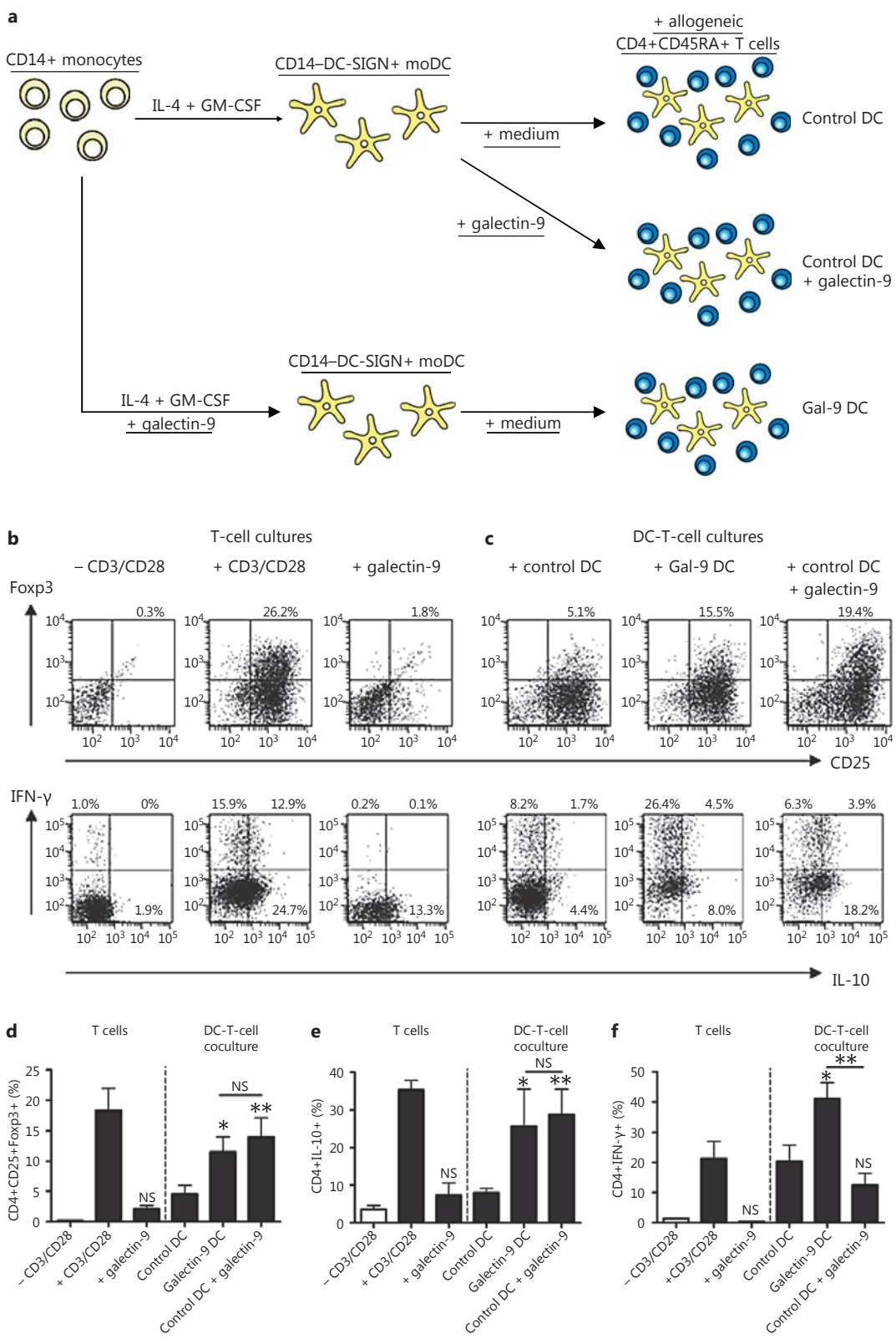
6



7

CpG DNA and scGOS/lcFOS regarding IL-13 secretion by PBMC (fig. 6d, h). TIM-3-Fc did not affect the viability of CD3/CD28-activated PBMC (data not shown).

Galectin-9 Induces T_{reg} and T_h1 Polarization through APC

Finally, we investigated whether galectin-9 induces T-cell polarization through a direct effect on T cells or via activation of APC. Under inflammatory conditions, monocytes migrate from the circulation into the intestinal lamina propria [31], where these cells are conditioned by mediators present in the mucosal microenvironment. Therefore, moDC were generated in the absence or presence of galectin-9 prior to mixed lymphocyte cultures. In addition, galectin-9 was added to CD4+CD45RA+ naïve T cells. To evaluate whether galectin-9 plays a role in the interaction between APC and T cells, moDC were cocultured with naïve CD4+ T cells in mixed lymphocyte cultures in the presence of galectin-9 (fig. 7a). Exposure of naïve CD4+CD45RA+ T cells to galectin-9 did not induce T_{reg} development or cytokine expression (fig. 7b, d–f). However, exposure of moDC to galectin-9 prior to interaction with naïve T cells enhanced T_{reg} development compared to unconditioned moDC. When galectin-9 was added to moDC-T-cell cultures using control DC, T_{reg} polarization was also enhanced (fig. 7c, d). In addition to induction of T_{reg} cells, galectin-9-conditioned moDC increased IL-10 and IFN-γ production by CD4+ T cells (fig. 7c, e, f). These data indicate that galectin-9 conditions APC that have increased capacity to induce T_{reg} cells as well as IFN-γ- and IL-10-mediated effector responses in vitro.

Discussion

The intestinal mucosa is constantly facing a high load of antigens derived from the diet and microorganisms for example. The intestinal epithelium serves as the first line of defense against foreign antigens. A single monolayer of IEC forms a tight barrier between the intestinal lumen in the lamina propria. In addition, IEC have been shown to condition DC to induce T_{reg} cells [13], indicating that besides providing a barrier, IEC take actively part in innate immune responses and shape adaptive immunity. As IEC are at the interface between the intestinal lumen and the lamina propria, IEC may serve as an interesting target for intervention strategies to modulate immune responses in the gut.

Dietary intervention using *B. breve* M-16V and scGOS/lcFOS has been shown to prevent the development of acute allergic symptoms [18]. However, the underlying mechanisms are not yet known. Data presented in this study indicate that the genomic DNA from *B. breve* M-16V enhances IFN-γ and IL-10 responses in an IEC-dependent manner. Recently, it was described that oral administration of DNA from *B. breve* enhances IL-10 and TGF-β production in the colon, indicating an anti-inflammatory role of *B. breve* DNA [17]. We elaborate on these data by showing that the anti-inflammatory role of *B. breve* M-16V is similar to the effects exerted by a synthetic CpG DNA. However, the synthetic CpG DNA but not *B. breve* M-16V DNA was capable of suppressing IL-13 as well. This may relate to intrinsic immunomodulatory capacities of synthetic CpG DNA, however we cannot exclude the possibility that the purity of the bacterial DNA was suboptimal [17]. Although the combination of synthetic CpG DNA in the presence of scGOS/lcFOS did not consistently further decrease the T_h2 response, the overall balance of the effector response is in favor of a T_h1- and T_{reg}-polarized response. Furthermore, we have identified galectin-9 as a soluble mediator released by IEC upon apical TLR9 ligation, which supports IFN-γ- and IL-10-polarized effector responses. Regulation of galectin expression is largely unknown, but the data presented in the current study indicate that IEC only secrete galectin-9 upon apical exposure to CpG DNA and scGOS/lcFOS under inflammatory conditions. In endothelial cells, galectin-9 expression is regulated by the proinflammatory cytokine IFN-γ in a PI3K/IRF3-signaling pathway, involving HDAC3 [32, 33].

Our data suggest a potential role for TLR9 signaling in the secretion of galectin-9. The presence of scGOS/lcFOS potentiated the effects of apical TLR9 ligation. Interestingly, scGOS/lcFOS on their own did not modulate the effector response in the IEC/PBMC coculture model. Since galectins create galectin-glycoprotein lattices on cell surfaces, the presence of scGOS/lcFOS may stabilize these lattices [26]. In addition, the extracellular domain of TLR is heavily glycosylated, which is important for proper TLR function [34]. Hence, galectin-9 may facilitate TLR9 signaling at the apical membrane of IEC and scGOS/lcFOS may contribute to effective TLR9 signaling. Further investigation on the underlying signaling pathways resulting in the release of galectin-9 and the interactions of galectins with surface glycoproteins expressed at the membrane of IEC may provide more insight into the regulation of adaptive immune responses by IEC.

Galectin-9 is a ligand for the TIM-3 receptor expressed by T_h1, T_h17 and DC. Activation of TIM-3 has been described to induce apoptosis of T_h1 and T_h17 cells, but also

to activate DC at high concentrations, which instruct naïve CD4+ T cells to produce IFN-γ, but not IL-4 or IL-5 [35, 36]. The present data demonstrate that galectin-9 at 1.0 μg/ml induces IFN-γ production by CD4+ T cells only when galectin-9 was added during moDC differentiation, whereas induction of Foxp3 expression and IL-10 production by CD4+ T cells occurred both when galectin-9 is present during DC differentiation and during DC-T-cell interaction. Interestingly, when galectin-9 is added during moDC differentiation, these DC have the capacity to induce a T_h1 response paralleled by a T_{reg} response, which may prevent exaggerated T_h1 inflammation. Galectin-9 is, in addition to the induction of T-cell apoptosis, more often associated with increased induction and expansion of T_{reg} cells in both allergic and inflammatory diseases [37–39]. In IEC/PBMC cocultures, IFN-γ and IL-10 responses were abolished upon neutralization of galectin-9. Dose-response experiments using recombinant galectin-9 added to CD3/CD28-activated PBMC indeed showed that galectin-9 can induce IFN-γ and IL-10 production and Foxp3 expression in CD4+ T cells [29]. Furthermore, galectin-9 secretion by IEC upon apical TLR9 ligation in the presence of scGOS/lcFOS only occurred under inflammatory conditions. This may be the consequence of low TLR9 expression by IEC in the absence of inflammatory conditions [12].

In an allergic setting, galectin-9 secretion by IEC may play an important role in suppressing T_h2-associated cytokine production. It currently remains unclear whether the reduction in IL-13 secretion by CpG DNA and scGOS/lcFOS depends on galectin-9 as well. However, the current data implicate that galectin-9 is involved in regulating immune responses by TLR9-exposed IEC in the presence or absence of scGOS/lcFOS. Furthermore, suppression of IL-13 secretion also occurred in the absence of IEC. This may suggest that, besides IEC, PBMC may also produce galectin-9. In fact, IEC may secrete galectin-9 and thereby instruct immune cells to produce galectins as well. This phenomenon has been described for intestinal epithelium-derived thymic stromal lymphopoietin, too [40]. Future studies are warranted to investigate the involvement of galectin-9 in regulating T_h2-polarized immune responses.

In summary, the present in vitro experiments show a potential role for apical TLR9 ligation of IEC in the presence scGOS/lcFOS in supporting IFN-γ- and IL-10-mediated effector responses. Furthermore, T_h2-associated cytokine production was also reduced, implicating the involvement of nondigestible oligosaccharides in suppressing allergy. Galectin-9 secreted by IEC under inflammatory conditions supported IFN-γ and IL-10 production acting on APC. Regulation of galectins expressed in the intestine may be a worthwhile approach to prevent or treat allergic and inflammatory diseases of the gastrointestinal tract.

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