Combined Exposure of Activated Intestinal Epithelial Cells to Nondigestible Oligosaccharides and CpG-ODN Suppresses Th2-Associated CCL22 Release While Enhancing Galectin-9, TGFβ, and Th1 Polarization

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Background. Short-chain galacto- and long-chain fructo-oligosaccharides (scGOS/lcFOS) and CpG-ODN affect intestinal epithelial cells (IEC). Epithelial IL1α may contribute to allergic sensitization via autocrine mediator release affecting dendritic cells (DC). We studied whether IL1α contributes to Th2-associated mediator release by activated IEC and IEC/DC cocultures and possible modulation by scGOS/lcFOS±CpG-ODN. Methods. Solid phase or transwell cultured IEC were preincubated with IL1α and/or IFNγ/TNFα for 6 h. The transwell IEC were also apically exposed to scGOS/lcFOS±CpG-ODN for 6 h, washed, and re-exposed, while cocultured with immature moDC (ccDC) for 48 h. These ccDC were subsequently added to allogeneic naïve T cells (MLR). IEC- and/or DC-derived mediators and T cell cytokines were measured. Results. IL1α tended to enhance IL25 and enhanced IL33 and CCL20 release by IEC, while IL1α or TNFα or IFNγ enhanced CCL22. These were all further increased upon combined exposure of IFNγ/TNFα±IL1α coinciding with increased IL33 secretion in the solid phase culture. In the transwell, IL25 and IL33 remained undetected, while CCL20 and CCL22 were induced by IL1α or IFNγ/TNFα, respectively, and a synergistic increase was observed upon combined exposure of IFNγ/TNFα and IL1α. Furthermore, IFNγ was found to enhance galectin-9 secretion, which was more pronounced in IFNγ/TNFα±IL1α-exposed IEC and coincided with TGFβ increase. Epithelial CpG-ODN exposure further increased CCL20, while reducing CCL22 release by IFNγ/TNFα±IL1α-activated IEC; however, scGOS/lcFOS suppressed both. Combined scGOS/lcFOS and CpG-ODN reduced CCL22, while CCL20 and regulatory galectin-9 and TGFβ remained high in the supernatant of IFNγ/TNFα/IL1α-activated IEC and the following IEC/DC coculture. ccDC of scGOS/lcFOS- and CpG-ODN-exposed IFNγ/TNFα/IL1α-activated IEC increased IFNγ, IL10, TGFβ, and galectin-9 secretion in the MLR compared to ccDC exposed to control-activated IEC. Conclusion. IL1α enhanced CCL20 and Th2-associated CCL22 release by IFNγ/TNFα-activated IEC. Combined scGOS/lcFOS and CpG-ODN exposure suppressed CCL22, while maintaining high CCL20, TGFβ, and galectin-9 concentrations. In addition, ccDC derived from this IEC/DC coculture enhanced Th1 and regulatory mediator secretion mimicking known in vivo effects.
1. Introduction

The mucosal surface of the gastrointestinal tract is covered by a monolayer of intestinal epithelial cells (IEC). These form a protective barrier between the outside environment and the mucosal immune system of the host, keeping antigenic proteins and bacteria in the lumen, while selectively allowing the transport of nutrients and water [1, 2]. Intestinal antigen-presenting cells, such as dendritic cells (DC), are integral components of the mucosal immune system and control mucosal homeostasis [3]. Intestinal DC can orchestrate durable tolerance to the microbiota and food proteins [3, 4]. IEC are known to support the tolerogenic DC phenotype [5]. However, the process of oral tolerance induction can be disrupted, leading to an inappropriate response towards, for example, a food antigen resulting in food allergies which can provoke gastrointestinal symptoms, atopic dermatitis and/or respiratory symptoms, or even anaphylactic shock [1, 4, 6]. Therefore, strategies to prevent allergy development and/or respiratory symptoms, or even anaphylactic shock can provoke gastrointestinal symptoms, atopic dermatitis [7, 19]. Here, we set out to study whether IL1α may be a factor contributing to Th2-polarizing cytokine and associated chemokine release by activated IEC. Moreover, it was studied whether this affects DC function and can be modulated by epithelial exposure to scGOS/lcFOS and CpG-ODN.

2. Materials and Methods

2.1. Culture of Intestinal Epithelial Cells (IEC). Human colon adenocarcinoma HT29 cells (ATCC, HTB-38; passages 142-148) were cultured in 75 cm² culture flasks (Greiner, Frickenhausen, Germany) in McCoy’s 5A medium (Gibco, Life Technologies, Breda, the Netherlands) supplemented with 10% heat-inactivated FCS (Gibco, Life Technologies, Breda, the Netherlands) and penicillin (100 IU/mL)/streptomycin (100 µg/mL) (both Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands). HT29 cells were kept in an incubator at 37°C and 5% CO₂. Medium was refreshed every 2–3 days and cells were passaged once a week.

2.2. Solid Phase Model. HT29 cells were cultured in 24-well flat bottom plates (Thermo Fisher Scientific-Nunc, Waltham, USA). After reaching confluence, the cells were incubated with IL1α (R&D Systems Europe Ltd., Abingdon, UK), IFNγ (Gibco, Life Technologies, Breda, the Netherlands), and/or TNFα (Gibco, Life Technologies, Breda, the Netherlands) and penicillin (100 IU/mL)/streptomycin (100 µg/mL) (both Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands). HT29 cells were kept in an incubator at 37°C and 5% CO₂. Medium was refreshed every 2–3 days and cells were passaged once a week.

2.3. Isolation of Monocytes from Healthy Donors. Human peripheral blood mononuclear cells (PBMC) from healthy donors were isolated from buffy coats (Sanquin, Amsterdam, the Netherlands). PBMC were obtained by centrifugation on Ficoll-Paque™ PLUS (GE Healthcare Life Sciences, Uppsala, Sweden; density: 1.077 g/mL). PBMC were collected and washed in PBS (Lonza Westburg BV, Leusden, the Netherlands) + 2% heat-inactivated FCS, followed by hypotonic lysis of erythrocytes with sterile lysis buffer (0.15 M NH₄Cl, 0.01 M KHCO₃, and 0.1 mM EDTA pH at 4°C is 7.4, all from Merck, Darmstadt, Germany). After lysis, the PBMC were
resuspended in PBS, supplemented with 0.5% BSA (Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands) and 2 mM EDTA (pH at 4°C is 7.2). Monocytes were isolated from this PBMC fraction by negative selection using MACS beads and a magnetic cell separator (Monocyte Isolation Kit II, Miltenyi Biotec, Bergisch Gladbach, Germany).

2.4. Culture of Monocyte-Derived Dendritic Cells (DC). Monocytes were cultured at a concentration of $7.5 \times 10^5$ cells/mL in a 6-well plate in RPMI 1640 supplemented with 10% heat-inactivated FCS, penicillin (100 U/mL)/streptomycin (100 µg/mL), IL4 (10 ng/mL; ProSpec-Tany TechnoGene Ltd., Ness Ziona, Israel) and GM-CSF (5 ng/mL; ProSpec-Tany TechnoGene Ltd., Ness Ziona, Israel). The cells were kept for 7 days in an incubator at 37°C and 5% CO$_2$. At days 2, 3, and 6, 1 mL medium was refreshed. At day 7, the imDC were suitable to use in the transwell IEC-DC coculture assay.

2.5. Transwell IEC and IEC-DC Coculture Model. HT29 cells were cultured on transwell inserts (12-well plates, 0.4 µm polyester membrane, Corning, USA). After reaching confluence, the cells were preincubated basolaterally with IFNγ and TNFα in the presence or absence of IL1α (all 10 ng/mL) and apically exposed to scGOS/lcFOS ± synthetic CpG-ODN for 6 h, washed, and basolaterally exposed to either medium for 24 h (b) or immature DC for 48 h (c), while apically reexposed to medium, scGOS/lcFOS±synthetic CpG-ODN. After 48 h of IEC-DC coculture, ccDC were added to allogeneic naïve T cells for 6 days (MLR) (d) and immune mediators were measured. scGOS/lcFOS: short-chain galacto- and long-chain fructo-oligosaccharides; CpG-ODN: synthetic CpG-ODN type C (TLR9 ligand); imDC: immature DC; ccDC: coculture DC; MLR: mixed lymphocyte reaction.

2.6. Isolation of Naïve CD4$^+$ T Cells. Naïve CD4$^+$ CD45RO$^-$ T cells were purified from the PBMC fraction by negative selection using CD4$^+$ T cell isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany), together with CD45RO Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Naïve T cells were used as responder T cells in the mixed lymphocyte reaction (MLR).
2.7. Mixed Lymphocyte Reaction (MLR). After 48 hours of coculture with IEC, ccDC were used to stimulate allogeneic naïve CD4+ CD45RO+ T cells. 10^5 ccDC were incubated in 24-well flat bottom plates with 10^6 naïve T cells in IMDM medium (Gibco, Life Technologies, Breda, the Netherlands) supplemented with 10% heat-inactivated FCS, apo-transferrin (20 μg/mL, Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands), 2-mercaptoethanol (50 μM, Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands), and penicillin (100 U/mL)/streptomycin (100 μg/mL). After 6 days in an incubator at 37°C and 5% CO₂, the supernatants were harvested and mediators were measured (Figure 1(d)).

2.8. Cytokine Production of HT29 Cells, DC, and MLR. Supernatants of HT29 cells (solid phase or transwell) or the IEC-DC coculture or ccDC/naïve T cell MLR were collected as described above. And CCL20, CCL22, IL25, IL33, TGFβ, and galectin-9 were measured by conducting an in-house developed and validated multiplex immunoassay based on Luminex technology (xMAP, Luminex, Austin, TX USA) as extensively described previously [27]. Acquisition was conducted with the Bio-Rad FlexMAP 3D (Bio-Rad Laboratories, Hercules, USA) in combination with xPONENT software version 4.2 (Luminex). Data was analyzed by 5-parametric curve fitting using Bio-Plex Manager software, version 6.1.1 (Bio-Rad).

In the supernatants of the MLR, the cytokines IFNγ, IL10, IL13, TGFβ, and galectin-9 were measured by conducting the aforementioned Luminexin.

2.9. Statistical Analysis. Data are expressed as mean ± SEM. The statistical significance of the data was analyzed using GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA, USA).

2.9.1. IEC Exposure Experiments. Solid phase: normalized data were analyzed using one-way repeated measures ANOVA followed by Bonferroni’s post hoc analysis; transwell: data (normally distributed) were analyzed using two-way repeated measures ANOVA followed by Tukey post hoc analysis.

2.9.2. IEC-DC Coculture Model. Normalized data were analyzed using one-way repeated measures ANOVA followed by Tukey post hoc analysis.

2.9.3. MLR. Data (normally distributed) were analyzed using one-way repeated measures ANOVA followed by Tukey post hoc analysis. Data were considered significant at p < 0.05.

3. Results

3.1. Combined IFNγ and TNFα Exposure with or without IL1α Enhances the Release of Th2-Driving Cytokines and Chemokines by IEC. To study the effect of IEC cytokines on Th2 polarization, solid phase grown HT29 cells were exposed to IL1α, TNFα, or IFNγ alone or in combination (Figure 1(a)). In solid phase cultures, IL1α tended to enhance IL25 and enhanced IL33, and IL1α, TNFα, or IFNγ enhanced CCL22 release by HT29 cells. Furthermore, combined exposure to IFNγ and TNFα±IL1α significantly increased the release of IL25, IL33, and CCL22 (Figures 2(a), 2(b), and 2(d)). In addition, TNFα, IL1α, or IFNγ and TNFα±IL1α activation induced the release of CCL20 (Figure 2(c)). Combined IFNγ and TNFα±IL1α exposure also induced regulatory mediators TGFβ (Figure 2(e)) and galectin-9 (Figure 2(f)).

Pilot studies showed that established Th2-driving cytokines IL4 and IL13 failed to induce a significant mediator secretion by the HT29 cells, and therefore, these were not used in these studies.

3.2. IFNγ and TNFα±IL1α Exposure Provokes Th2-Associated Chemokine CCL22 Release by Transwell IEC, Which Is Suppressed by Incubation with CpG-ODN and scGOS/lcFOS While CCL20 and Regulatory Mediators Remain High. To test the effects of CpG-ODN and scGOS/lcFOS exposure to IFNγ/TNFα±IL1α-activated IEC, a transwell model was used (Figure 1(b)). Th2-driving cytokines IL25 and IL33 remained below detection in the HT29 transwell model. However, combined IFNγ and TNFα activation did induce the release of CCL22 by HT29 cells, which was further enhanced by IL1α (Figure 3(b)). CCL20, a strong chemoattractant for DC, was released after activation with IL1α. This was further enhanced by IFNγ and TNFα exposure, although IFNγ and TNFα activation alone did not lead to a significant release of CCL20 (Figure 3(a)). Apical IEC exposure to scGOS/lcFOS and/or CpG-ODN reduced the release of Th2-associated chemokine CCL20 by IFNγ- and TNFα±IL1α-activated IEC (Figure 3(b)), while CCL20 remained high (Figure 3(a)). Single IFNγ exposure and exposure to IFNγ in combination with TNFα±IL1α increased the release of TGFβ and galectin-9. In addition, while CpG-ODN reduced the concentration of regulatory mediators TGFβ and galectin-9, TGFβ and galectin-9 remained high when IEC were exposed to the combination of CpG-ODN and scGOS/lcFOS (Figures 3(c) and 3(d)).

3.3. ScGOS/lcFOS±CpG-ODN Exposure of IFNγ/TNFα±IL1α-Activated IEC Increases CCL20, TGFβ, and Galectin-9 Concentrations in Subsequent IEC-DC Coculture While Decreasing CCL22. To investigate whether apical exposure to dietary nondigestible oligosaccharides and bacterial CpG-ODN influences the IEC-DC crosstalk, a coculture experiment with activated IEC and imDC was conducted (Figure 1(c)). After 48 hours of coculture, CCL20, CCL22, TGFβ, and galectin-9 concentrations were determined in the supernatant of IEC-DC cocultures (Figure 4). CpG-ODN was found to enhance CCL20 in the supernatant of IEC-DC cocultures of IFNγ/TNFα/IL1α IEC-activated IEC cells in the presence or absence of scGOS/lcFOS compared to the intrinsic controls (Figure 4(a)). However, CpG-ODN decreased CCL22 in the supernatant of IEC-DC cocultures of IFNγ/TNFα/IL1α IEC-activated and nonactivated IEC cells in the presence or absence of scGOS/lcFOS compared to the intrinsic controls (Figure 4(b)). Moreover, scGOS-/lcFOS was found to enhance galectin-9 in the supernatant.
of IEC/DC cocultures of activated and nonactivated IEC cells in the presence or absence of CpG-ODN compared to the intrinsic controls. This significant increase in galectin-9 concentrations after 48 hours of coculture was accompanied by a significantly higher release of TGFβ after 24 hours of IEC-DC coculture (Figures 4(c) and 4(d)).
Figure 3: Continued.
Figure 3: CpG-ODN plus scGOS/lcFOS reduces the release of Th2-associated chemokine CCL22 by IFNγ/TNFα±IL1α-activated IEC, while CCL20 and regulatory mediators TGFβ and galectin-9 remain high. HT29 cells cultured in transwells were preincubated basolaterally with IFNγ, TNFα, or IL1α (all 10 ng/mL) alone or in combinations and apically exposed to synthetic CpG-ODN (5 μM) in the presence or absence of scGOS/lcFOS (0.5% w/v) for 6h. Subsequently, the cells were washed and apically reexposed to CpG-ODN in the presence or absence of scGOS/lcFOS, after which (a) CCL20, (b) CCL22, (c) TGFβ, and (d) galectin-9 were measured in the basolateral compartment after 24h; apical exposure to medium (white bars; 0), CpG-ODN (light grey bars; CpG), scGOS/lcFOS (dark grey bars; GF), or CpG-ODN +scGOS/lcFOS (black bars; CpG+GF); N = 3. Two-way ANOVA (p value interaction for CCL22 <0.05; all other mediators <0.001), post hoc test Tukey; *p < 0.05; **p < 0.01; and ***p < 0.001.
3.4. ccDC Exposed to IFNγ/TNFα/IL1α-Activated IEC Ligated with scGOS/lcFOS and CpG-ODN Significantly Enhance IFNγ, IL10, TGFβ, and Galectin-9 in an Allogeneic MLR.

The functionality of the ccDC (DC from the IEC-DC coculture) was tested in an MLR with allogeneic naïve CD4+ T cells. After two days of IEC-DC coculture, ccDC were harvested and washed (Figure 1(c)). Subsequently, ccDC were incubated with allogeneic CD4+ naïve T cells (Figure 1(d)). The supernatant was harvested after 6 days of culture and IFNγ, IL10, IL13, TGFβ, and galectin-9 concentrations were measured. It was observed that IFNγ/TNFα/IL1α-activated IEC did not significantly affect IFNγ, IL10, IL13, TGFβ, or galectin-9 concentrations in the MLR with ccDC originating from the IEC-DC coculture. However, ccDC derived from IEC-DC cocultures with combined scGOS/lcFOS and CpG-ODN exposure of IFNγ/TNFα/IL1α-activated IEC enhanced the IFNγ, IL10, TGFβ, and galectin-9 concentrations compared to ccDC derived from cocultures with medium exposed IEC (Figures 5(a) and 5(c)–5(e)). By contrast, this exposure did not lead to a significant IL13 production (Figure 5(b)). A positive correlation was observed between galectin-9 and TGFβ in MLR samples derived from ccDC obtained from cocultures of IFNγ/TNFα/IL1α-activated IEC with DC (Figure 5(f)).

4. Discussion

In the current study, it was investigated whether IL1α may be a factor contributing to Th2-polarizing cytokine and associated chemokine release by activated IEC in vitro. Upon exposure to allergens and/or inflammatory insults, IEC may contribute to allergic sensitization since they can produce sensitizing mediators like IL-33, IL-25, and TSLP that are known to contribute to allergic sensitization amongst others via priming of Th2-driving DC. One of the first steps in this cascade may be allergen-induced IL1α release by IEC, as was
Figure 5: ccDC derived from IEC/DC of IFNγ/TNFα/IL1α-activated IEC ligated with scGOS/lcFOS and CpG-ODN significantly enhance IFNγ, IL10, TGFβ, and galectin-9 in an allogeneic MLR. After 2 days of coculture, ccDC were harvested and washed and ccDC were incubated in a 1:10 ratio with allogeneic CD4⁺ naïve T cells. The supernatant was harvested after 6 days of culture and (a) IFNγ, (b) IL13, (c) IL10, (d) TGFβ, and (e) galectin-9 concentrations were measured; N = 5. One-way ANOVA, post hoc test Tukey; *p < 0.05; **p < 0.01; and ***p < 0.001. (f) Correlation of TGFβ and galectin-9. Correlation was analyzed using Spearman’s correlation test.
shown for lung epithelial cells, which leads to autocrine stimulation and production of the sensitizing mediators. IL1α caused the release of DC chemoattractant CCL20. Moreover, upon exposure to IL1α plus inflammatory cytokines, IEC secreted Th2-driving IL25, IL33, and CCL22. CCL22 is also known to be produced by DC that instruct Th2 polarization and allergic sensitization. Interestingly, in the solid phase studies, increased IEC-derived CCL22 release was associated with enhanced IL25 and IL33 secretion, while these mediators were often below detection limits in the transwell studies. CCL22 therefore may be an interesting biomarker when studying factors that activate IEC to release sensitizing mediators. By contrast, IEC are also known to contribute to mucosal homeostasis and tolerance. IEC produce regulatory mediators like galectin-9 and TGFβ which may be able to modify the priming of the DC and consequent T cell development. Nondigestible oligosaccharides may be able to modulate the mucosal immune response and hereby help to prevent or treat allergies [20]. In the current study, exposure of IEC to scGOS/lcFOS and CpG-ODN (as substitute for bacterial DNA) resulted in decreased CCL22 release by IEC, while supporting the secretion of regulatory mediators like IL-10, TGFβ, and galectin-9 also by immune cells. In addition, Th1 type IFNγ secretion was increased, which is known to dampen Th2 cell activation. This may help to reduce the risk of allergic sensitization for example to food proteins (see Fig. S2).

In literature, it has been described that IL1α released by IEC contributes to intestinal inflammation in mice [15], which may contribute to allergic sensitization or local intestinal inflammation hampering the process of natural oral tolerance induction or the efficacy of OIT. Indeed, in a murine asthma model, it was shown that IL1α release by pulmonary epithelial cells induces allergic sensitization to inhaled house dust mite via autocrine release of Th2-driving cytokines IL25, IL33, and TSLP [11]. These cytokines are known to prime DC that produce amongst others CCL22 and induce Th2 cells that secrete IL4, IL5, and IL13 which are crucial for allergic sensitization [12, 28–32]. In addition to the release of Th2-driving cytokines, epithelial cells release chemokines associated with DC chemotaxis and allergic sensitization: CCL20 [33–35] and CCL22 [13, 36]. The current study shows that single exposure to IL1α, IFNγ, or TNFα does not result in significant release of IEC-derived IL-25. However, IL1α exposure induced IL33, CCL20, and CCL22 release by IEC, confirming the ability of IL1α to promote sensitizing mediator release by IEC. Furthermore, exposure to inflammatory cytokines IFNγ or TNFα induced the release of CCL22 or CCL20 and CCL22, respectively. However, combined exposure of solid phase grown IEC to inflammatory cytokines IFNγ and TNFα in the presence or absence of IL1α did induce IL25 and IL33 as well as CCL22. In the transwell model of these mediators, only CCL22 was detected, which was further increased by IL1α when combined with IFNγ and TNFα. Interestingly, CCL20 release by IEC was not increased by IFNγ and TNFα and solely depended on IL1α exposure. However, IFNγ and TNFα further enhanced IL1α-induced CCL20 release by IEC. Overall, these data show that combined exposure to IL1α, IFNγ, and TNFα has a strong capacity to activate HT29 cells to release Th2-associated mediators. IL1α alone tends to induce epithelial CCL22 release and synergizes with inflammatory mediators IFNγ and TNFα in activation of epithelial cells. In addition, IL1α alone caused CCL20 release, a chemokine known to attract immature DC [37]. This indicates that Th2-polarizing mediators can be released by IEC upon inflammatory insult concomitantly with a DC chemoattractant, which may lead to the development of Th2-driving DC.

Beyond these mediators, IEC also secreted galectin-9 and TGFβ. Recent reports show that soluble type lectin galectin-9 may be involved in the suppression of allergic symptoms. It was described that galectin-9 can specifically bind IgE, thereby preventing IgE-antigen complex formation leading to reduced degranulation of mast cells and basophils [23, 38]. Moreover, it was suggested that galectin-9 supports Treg development and acts synergistically with TGFβ to further enforce induced Treg differentiation and maintenance [23–26]. A diet containing scGOS/lcFOS and Bifidobacterium breve M16V was shown to partially protect mice from developing food allergic symptoms in association with increased intestinal galectin-9 expression and galectin-9 serum levels [7, 39]. A similar diet also increased galectin-9 levels in serum of infants affected with atopic dermatitis, in association with reduced skin symptom scores [7, 19]. In the current study, IEC secreted galectin-9 after incubation with inflammatory cytokine IFNγ. This was further enhanced by TNFα in the presence or absence of IL1α. In previous studies, epithelial release of galectin-9 was shown to be enhanced by CpG-ODN when IEC were cultured in the presence of activated peripheral blood mononuclear cells and further increased by scGOS/lcFOS [21]. The current study identifies IFNγ as an initiator of galectin-9 release by epithelial cells in parallel with regulatory TGFβ secretion. CpG-ODN ligation in this case suppressed galectin-9 release. However, when combined with scGOS/lcFOS, the suppression of galectin-9 and TGFβ by CpG-ODN was abrogated. This shows the relevance of studying IEC-derived mediator release which may contribute to the maintenance of mucosal homeostasis.

The modulatory effect of scGOS/lcFOS and/or CpG-ODN on Th2-associated cytokine and chemokine secretion by IFNγ/TNFα/IL1α-activated HT29 cells was also studied. Interestingly, apical exposure of IEC to CpG-ODN reduced the release of Th2-associated chemokine CCL22 in the presence or absence of scGOS/lcFOS, while CCL20 remained high. DC arrive at the site of inflammation via CCL20–CCR6 binding where they are activated and instructed by locally secreted mediators. Upon concomitant epithelial exposure to scGOS/lcFOS, these DC will be exposed to lower levels of CCL22 and high levels of galectin-9 and TGFβ released by IEC. This may differentially impact the DC phenotype and maturation and consequent instruction of T cell responses upon migration to the lymph nodes. Hence, in addition to reducing mast cell degranulation mediated by galectin-9 [7] and inducing Th1- and Treg-cell polarization [7, 20, 21], IEC exposure to CpG-ODN and scGOS/lcFOS may affect Th2 polarization by decreasing the release of cytokines and chemokines contributing to allergic sensitization.
by activated IEC, while maintaining high galectin-9 and TGFβ concentrations. In mice fed a diet containing scGOS/lcFOS and *Bifidobacterium breve* M16V during oral sensitization for hen’s egg protein ovalbumin, galectin-9 levels increased while in the lamina propria regulatory T cells were maintained and the Th2 cell frequency was reduced compared to allergic mice fed a control diet [39].

To investigate whether apical exposure of IEC to scGOS/lcFOS and CpG-ODN influences the IEC-DC cross-talk, a coculture experiment with activated IEC and iDC was conducted. IEC pretreated with IFNγ and TNFα in the presence or absence of IL1α did not influence DC maturation. However, scGOS/lcFOS and CpG-ODN ligation of IFNγ- and TNFα-activated IEC resulted in a significant increase in percentages of more matured CD14⁺DC-SIGN⁺⁺⁺ HLA-DR⁺ ccDC. A similar pattern was shown for the percentage of CD14⁺CD40⁺⁺⁺CD80⁺⁺⁺ cells when compared to medium-activated IEC. These effects were lost in the presence of IL1α (Fig. S1). Hence, depending on the type of inflammatory mediators, scGOS/lcFOS and CpG-ODN ligation of IEC may differentially affect the phenotype of the DC exposed to soluble mediators produced by these IEC. In parallel with the studies with activated IEC, also in the IEC-DC cocultures, scGOS/lcFOS reduced CCL20 whereas CpG-ODN in the presence or absence of scGOS/lcFOS enhanced the secretion of CCL20 while reducing CCL22. scGOS/lcFOS enhanced the simultaneous secretion of both galectin-9 and TGFβ not only in the presence but also in the absence of CpG-ODN either when DC were exposed to control or activated IEC. Therefore, also in the IEC-DC coculture, scGOS/lcFOS and CpG-ODN may modify (tolerogenic) mediator release during inflammatory conditions. This may affect DC maturation and as a consequence its phenotype and function. The functionality of the IEC/DC coculture-derived ccDC was tested in a MLR with allogeneic naïve T cells. It was observed that DC derived from an IEC/DC coculture with IFNγ/TNFα/IL1α-activated IEC did not induce ccDC capable of instructing the release of Th1 cytokine IFNγ, Th2 cytokine IL13, and regulatory mediators IL10, TGFβ, and galectin-9 when compared to medium controls. However, ccDC from IFNγ/TNFα/IL1α-activated IEC/DC cocultures exposed to scGOS/lcFOS and CpG-ODN did enhance the IFNγ and IL10 concentrations in this allogeneic MLR compared to medium exposed IEC controls, whereas this exposure did not lead to a significant IL13 production. This may suggest that scGOS/lcFOS and CpG-ODN can skew T cell polarization towards a regulatory Th1 phenotype under these conditions. This may protect against the possible sensitizing capacity of IL1α under inflammatory conditions. Indeed, previous *in vitro* studies and studies in mice affected with cow’s milk allergy have shown that nondigestible oligosaccharides can support Th1 and regulatory T cell responses [20, 21, 40, 41]. Strikingly, both galectin-9 and TGFβ concentrations in the MLR using ccDC derived from this coculture were also only increased in the MLR using ccDC from CpG-ODN- and scGOS/lcFOS-exposed IFNγ/TNFα/IL1α-activated HT29 cells. Hence, the current study shows that scGOS/lcFOS adapts the outcome of CpG-ODN exposure on IEC as was shown previously [21], by supporting combined galectin-9 and TGFβ release. This combined galectin-9 and TGFβ release did not only occur in the IEC model but also in the IEC/DC coculture and the MLR using ccDC derived from this coculture.

### 5. Conclusions

In conclusion, in these newly developed models in which IL1α is used as an additional trigger to provide sensitizing conditions, it has been shown that IL1α, TNFα, IFNγ, and/or a combination of these enhances Th2-associated cytokine and chemokine release by IEC which may contribute to allergic sensitization. In the transwell model, IL1α synergized with TNFα and IFNγ in increasing CCL20 and CCL22 release by IEC. IEC exposure to CpG-ODN and/or dietary scGOS/lcFOS suppressed CCL22 release, while CpG-ODN enhanced CCL20 in the presence or absence of scGOS/lcFOS. Furthermore, IEC exposure to CpG-ODN suppressed galectin-9 and TGFβ release, while scGOS/lcFOS enhanced these regulatory mediators in the presence or absence of CpG-ODN. DC cocultured with CpG-ODN- and scGOS/lcFOS-exposed IEC instruct Th1 and increase regulatory IL-10, galectin-9, and TGFβ secretion in a MLR with naive T cells representing immune priming (see Fig. S2). This may contribute to the suppression of allergic sensitization.

### Data Availability

The data used to support the findings of this study are included within the article and the supplementary information file.

### Ethical Approval

Utrecht University is licensed to isolate PBMC from buffy coats provided by Sanquin Blood Bank, Amsterdam. Data related to human samples were all analyzed anonymously.

### Consent

Written informed consent for the use of buffy coats for research purposes was obtained from all blood donors.

### Conflicts of Interest

None of the authors have a competing financial interest in relation to the presented work; JG is head of the Division of Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science at the Utrecht University, and partly employed by Nutricia Research. LK and BL are employed by Nutricia Research. BL is leading a strategic alliance between University Medical Centre Utrecht/Wilhelmina Children’s Hospital and Nutricia Research.

### Authors’ Contributions

SO and LW designed the experiments; SO, AK, MB, SH, and WJ conducted experimental procedures; SO and LW analyzed data and drafted the manuscript; BL, LK, JG,
and LW supervised the program. All authors contributed to manuscript revision and read and approved the submitted version.

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Supplementary Materials

Figure S1: ScGOS/lcFOS and/or CpG-ODN ligation of IEC and the DC phenotype in IEC-DC crosstalk after initial IEC activation. HT29 cells, cultured on transwell filters, were pre-incubated basolaterally with IFNγ and TNFα± IL1α (all 10 ng/mL) and apically exposed to synthetic CpG-ODN (5 μM) in the presence or absence of scGOS/lcFOS (0.5% w/v) for 6h. Subsequently, the cells were washed and the HT29 cells were again apically exposed to scGOS/lcFOS±CpG-ODN. To study IEC-DC crosstalk, imDC were added basolaterally for 48 hours of coculture (ccDC). Subsequently, the ccDC were collected and incubated with a panel of antibodies (CD14, CD40, CD80, and HLA-DR (all eBioscience, San Diego, CA, USA) and DC-SIGN (R&D Systems Europe Ltd., Abingdon, UK)) and fluorescence was measured by flow cytometry (FACS Canto™ II; BD Biosciences, Franklin Lakes, NJ, USA). The gating technique of fluorescence minus one (FMO) controls was used to interpret the flow cytometry data using FlowLogic software (Inivai Technologies, Mentone, VIC, Australia). Data were normalized by dividing respective percentages by the percentage of the control for every donor. (A) Gating strategy for CD14 population, (B) gating strategy for CD14+ CD40− CD80+ population, (C) % CD40− CD80+ of the living CD14+ population, (D) gating strategy for CD14+ DC-SIGN+ HLA-DR+ population, and (E) % DC-SIGN+ HLA-DR+ of the living CD14+ population; exposure to medium (white bars; 0), CpG-ODN (light grey bars; CpG), scGOS/lcFOS (dark grey bars; GF), or CpG-ODN+scGOS/lcFOS (black bars; CpG+GF); N = 3. One-way ANOVA on normalized nonparametric data, post hoc test Dunn’s; *p < 0.05. FMO: fluorescence minus one. Figure S2: cartoon of proposed mechanism of action. Structural cells such as intestinal epithelial cells (IEC) may contribute to tolerance induction or allergic sensitization when exposed to allergens and/or inflammatory insults (danger signal). Mucosal allergen exposure may cause release of epithelial-derived autocrine inflammatory IL1α and danger signals may provoke release of inflammatory cytokines like TNFα and IFNγ, produced by mucosal immune cells which may contribute to further increase of epithelial-derived mediator release. IL1α-induced release of CCL20 (and CCL22) by IEC may attract immune cells such as DC. Moreover, IL1α plus inflammatory cytokines enhances secretion of Th2-driving CCL22 in parallel with IL25 and IL33 secretion by IEC. These mediators may prime Th2-polarizing DC that produce CCL22, leading to Th2 polarization and allergic sensitization (see left site Figure S2). However, other epithelial mediators such as regulatory galectin-9 and TGFβ may be able to modify the priming of the DC. These mediators may contribute to the production of regulatory mediators such as IL-10, TGFβ, and galectin-9 by DC and/or T cells and generate Th1 cells to produce IFNγ in the presence of regulatory IL-10. Addition of scGOS/lcFOS and bacterial CpG DNA, for example, from bacteria with DNA rich in CpG islands, before or during IEC activation, may decrease CCL22 release by IEC and/or DC (model IEC, model IEC-DC) and support the production of regulatory mediators like IL-10, TGFβ, and galectin-9 also by DC and/or T cells (model IEC-DC and model ccDC/T-cell), which may modify T cell priming at the inductive site (for example, the mesenteric lymph nodes) and support a more regulatory Th1 response over Th2 polarization (see right site of Figure S2). This may help to reduce the risk of allergic sensitization and allergies to, for example, food proteins. (Supplementary Materials)

References

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